Deep Brain Stimulation Mediates Neurotrophin Signaling in an Animal Model of Antidepressant Resistance

by

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B.A. (Hons)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Psychology)

Deakin University

May 2016
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Acknowledgements
The author extends deepest and sincerest gratitude and appreciation to Dr. Susannah Tye for her support and guidance while undertaking this thesis. Utmost thanks also to Professor Jane McGillivray, Dr. Nicky Konstantopoulos and Mrs. Shari Sutor for your support and expertise. Special thanks to the Metabolic Research Unit, especially Andrew Sanigorski for conducting RNA extractions, microarray procedures and preparation of the microarray dataset for further data mining. Kind regards to the Mental Health Research Institute and respective faculty and researchers for assistance throughout western blotting procedures and data collection. Many thanks are also expressed to friends and family, especially Adam Walker and Kyoko Hasebe. Lastly, and in the utmost of unorthodoxy, thanks to myself, without whom this would literally have been impossible.
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Abstract

Major depressive disorder (MDD) is a heterogeneous affective mental health disorder that contributes significantly to burden of disease worldwide. Much of this can be attributed to ineffective or incompletely effective antidepressant treatments, leaving approximately one-third of patients suffering with treatment resistant depression (TRD). Revised theories of MDD, notably the neurotrophic hypothesis and the network model, are based on neurophysiological data indicating MDD is the net result of maladapted neural plasticity which is corrected by neurotrophic mechanisms downstream of monoamine activity. Individuals who do not respond to available antidepressants may therefore require more direct target engagement to alleviate symptoms. Deep brain stimulation (DBS) of the subcallosal cingulate has proven effective in this task. This research aimed to elucidate the role of brain derived and glial cell line derived neurotrophic factors (BDNF and GDNF respectively) in mediating antidepressant response to DBS in an animal model of adrenocorticotrophic hormone (ACTH) induced antidepressant resistance. GDNF, BDNF and its receptor tropomyosin receptor kinase B (TrkB) proteins were measured in the hippocampus of ACTH pre-treated rats. GDNF was also measured in ACTH pre-treated rats treated with DBS of the infralimbic cortex (IL), the homologous area targeted in humans with TRD receiving DBS. Microarray was used to assess mRNA expression of intracellular signalling mechanisms of the neurotrophin family, to which BDNF belongs, in the IL of antidepressant resistant rats with and without IL-DBS. This data was then used to guide the selection of key intracellular signalling proteins to be assessed by western blot. Mnemonic abilities were also assessed, addressing lingering ethical issues facing the use of DBS for TRD. Results are interpreted under the neurotrophic and network paradigms. Future directions and limitations are also addressed.
Chapter I: A Role for Neurotrophic Factors in Mediating the Antidepressant Effects of Deep Brain Stimulation in a Pre-Clinical Model of Antidepressant Resistance

Major depressive disorder (MDD) is classified by The Diagnostic and Statistical Manual of Mental Disorders, (5th ed.; DSM-5; American Psychiatric Association, 2013) as an affective mood disorder. Diagnosis requires two successive weeks of depressed mood and/or anhedonia accompanied by any combination of significant weight change, sleep disturbances, psychomotor changes, fatigue, worthlessness or excessive guilt, diminished cognition and/or preoccupation with death or suicidal ideation or attempt. These symptoms must not be resultant of schizoaffective disorder, nor accompanied by mania and may be considered recurrent if two or more episodes are experienced within two months. According to the World Health Organization’s Global Burden of Disease project, MDD is currently the second highest ranking disease associated with time lived with disability in developed nations and is ranked as the third highest contributing factor to disease burden worldwide (Murray & Lopez, 1997; Ressler & Mayberg, 2007) projected to be the first by 2030 (Holtzheimer & Mayberg, 2011). The Australian Bureau of Statistics (ABS) * National Survey of Mental Health and Wellbeing (2007, Cat. no. 4326.0) found 20% of those surveyed were suffering with, or had experienced, a 12-month mental health disorder, 62% of which were affective disorders. Symptoms of this condition have far reaching effects on the global community, for example, in Australtalia, each employee with depression will take an average of three to four days off work per month, the equivalent of more than six million days of productivity lost each year (Korten & Henderson, 2000).

* Public information available from ABS website:  
The extensive incidence of MDD can be largely attributed to its chronically remitting and relapsing nature (Tye, Frye, & Lee, 2009). This is in part due to the diathetic and neuroprogressive profile of depression, which has a reciprocal relationship with kindling, such that one prone to MDD and having experienced a depressive episode is then more vulnerable to subsequent depressive episodes (Berton & Nestler, 2006; Maletic et al., 2007). Without continued antidepressant maintenance therapy, more than 50% of sufferers having recovered from a first episode of depression will experience a relapse within 6 months (Gold & Chrousos, 2002 as cited from Frank et al., 1990). Moreover, antidepressant treatments often fail to bring relief. Analyses prepared for the Agency for Healthcare Research and Quality, U.S. Department of Health and Human Services (2007) concluded from 187 studies of good or fair quality that overall, 38 percent of patients did not respond to second-generation antidepressants over 6-12 weeks and 54 percent failed to attain remission. These conclusions are supported by findings from The Sequenced Treatment Alternatives to Relieve Depression (STAR-D) study which found a comparable remission rate (54 percent) at step 2 (Warden, Rush, Trivedi, Fava, & Wisniewski, 2007). Those failing to attain or maintain remission with successive treatment trials are often referred to as non-responders, and may represent an as yet clinically unrecognized form of depressive disorder referred to as treatment resistant depression (TRD) (Ressler & Mayberg, 2007; Tye, Frye, & Lee, 2009). As a testament to the complexity underlying TRD, there is as yet no clear consensus regarding its clinical diagnostic criteria (Holtzheimer & Mayberg, 2010). A working definition presumes clinically effective doses and durations of multiple antidepressant medications (at least two antidepressant trials) have failed to have therapeutic effect (Fava & Davidson, 1996). Pharmaceutical interventions may or may not have been augmented with behavioural therapy or counselling (Tye, Frye, & Lee, 2009). While it is known that an individual's risk for developing TRD significantly increases if initial
antidepressant treatments are met with non-response or incomplete response, likely the direct result of kindling, this cannot be considered a diagnostic tool.

Due to the prevalence and impact of this disorder, ongoing research aimed at understanding the physiological basis of disease onset, progression and treatment are critical. This review will outline how previous research has impacted the current understanding of MDD and led to contemporary theories of depression, notably the neurotrophic hypothesis (Duman & Monteggia, 2006) and the network model of depression (H. Mayberg, 2003; Helen S. Mayberg, 1997), the latter theory being credited with leading to the successful application of deep brain stimulation (DBS) to treat refractory depression (Mayberg et al., 2011). These theories rely on the innate plasticity of neural networks to change in response to stimuli, and suggest aberrancy in adaptive remodelling may contribute to MDD development and maintenance. The focus of the work presented within this thesis is on antidepressant like-effects of DBS in an animal model of TRD induced via chronic administration of an acute dose of adrenocorticotropic hormone (ACTH) at circadian nadir (Kitamura et al., 2002, 2008, Walker et al., 2013). Concurrent with this, the work herein has investigated the role of brain and glial-cell line derived neurotrophic factors (BDNF and GDNF respectively), as well as neurotrophic signaling pathway activation in mediating the antidepressant action of DBS in this model. The impact of ACTH treatment and DBS on hippocampal memory was also explored. Although no significant changed in BDNF nor GDNF were observed in the hippocampus, consistent with no memory-related behavioural deficits, this work identifies an important role of neurotrophic signaling in the prefrontal cortex as a mediatour of antidepressant resistance and response to DBS. Proteins of interest within the neurotrophic factor intracellular signaling cascade included RAC serine/threonine-protein kinase (AKT) and nuclear factor NF-kappa-B, subunit p65 (NF-κB). The serine/threonine-protein kinase mTOR (mTOR) was also quantified given its putative role in the antidepressant actions of ketamine, which modulate similar
mechanisms as IL-DBS (Jimenez-Sanchez, Castane, et al., 2016). This work serves as a platform on which future antidepressant treatments can be built and assessed.

1.1 A Paradigm Shift

The underlying pathophysiology of depression is believed to have been discovered serendipitously, while investigating the anti-hypertensive drug, reserpine, which was found to induce a state indistinguishable from depression (Nestler et al., 2001). Further investigation revealed the drug caused a depletion of norepinephrine, serotonin and dopamine which were then deduced to underlie the depressed state (Nestler et al., 2001). This discovery heralded the age of the monoamine theory of depression which has been guiding interpretation and development of research and treatments for depressive disorders for over half a century.

The first antidepressants, monoamine oxidase inhibitors (MAOIs) and tricyclics, increased the bioavailability of these neurotransmitters and consequently provided relief from depressive symptoms. Unfortunately, this was at the expense of quite severe negative cardiovascular and gastrointestinal side effects as well as common and serious drug interactions (Nestler et al., 2001; Hindmarch, 2001). More selective monoaminergic antidepressants were subsequently developed in an attempt to minimise these negative side effects. These drugs include selective serotonin reuptake inhibitors (SSRIs), selective noradrenaline reuptake inhibitors (NRIs) and selective serotonin and norepinephrine reuptake inhibitors (SNRIs), with the former documented to reduce discontinuation by 4% compared to tricyclics (Hindmarch, 2001). When pharmaceutical treatments fail, somatic interventions such as electroconvulsive therapy (ECT) or transcranial magnetic stimulation (TMS) may be administered. However, monoamine modulators remain the preferred treatment modality of patients and prescribers (Hindmarch, 2001)
The monoamine hypothesis and resulting monoamine antidepressant treatments have provided invaluable information on depression onset and recovery; however, the non-response of such a large proportion of depressed individuals to this antidepressant drug class clearly indicates a role for other aetiological mechanisms that remain incompletely understood. Given the heterogeneous profile of MDD, understanding how and why approximately one-third of sufferers do not respond to current treatment modalities will require a multi-pronged approach. One area in need of re-examination is the animal models used to understand the vast mechanisms of neurobiology at work in MDD and antidepressant treatments.

Current research models of depression aimed at clarifying these mechanisms are best viewed as having pharmacological predictive validity, with some models also displaying varying levels of face and aetiological validity. However, this research must be interpreted cautiously, bearing in mind two principle caveats. Firstly, data from human studies are convoluted by individual risk, which plays a complicated part in depression, while secondly, the use of healthy wild-type animal strains may lead to inaccurate animal models (Sahay & Hen, 2008). For instance, the drug-induced transcript profiles for the cingulate cortex, recently discovered to have a critical role in depression, of chronically mildly stressed animals varies greatly to profiles of control animals, meaning the molecular correlates of treatment appear to be state-dependent, and therefore data of drug treatment in normal animals is likely not precisely relevant to the disease and therefore interpretations less applicable to generalisation (Surget et al., 2009).

Thus far, the chronic mild stress (CMS) research model most closely resembles the human condition of depression, consisting of mildly irritating stimuli such as cage tilt or food/water deprivation and is considered an extremely valid and reliable model (Willner, 1997). Critically, this model requires chronic antidepressant drug treatment to elicit a behavioural response. Taken together, this is a robust animal model with high levels of face
and aetiological validity, however due to its antidepressant response, the CMS paradigm is not a reliable model of TRD. The behaviours of interest elicited by the CMS model have been replicated using exogenous stress-hormone administration models, which supplement animals with increased levels of the primary stress hormone, corticosterone, either through injection or slow-release pump (Zhao et al., 2009; Ulloa et al., 2010; Murray et al., 2008) or oral administration (Shannon Leigh Gourley, 2009; Ardayfio & Kim, 2006). These models allow researchers to gain a more controlled understanding of the known role neuroendocrine mediated stress plays in depression; however, the extreme variation in the methodology between these models precludes confident generalization of the findings. Furthermore, many of these models have been shown to respond to antidepressant treatments. In light of this, there is a critical need for new clinical and pre-clinical research models of depression that exhibit face and aetiological validity while simultaneously encompassing all subtypes of depression, not merely those which respond to the most popular therapies.

Elucidating mechanisms of TRD utilizing a model that is resistant to current mainstream antidepressants has the potential to supplement our understanding of the biological basis of stress induced depressive illness, with insight into the biological mechanisms underlying antidepressant-resistance. This understanding, in turn, can help facilitate translation of novel antidepressant treatments for refractory depression, as well as aid development of diagnostic tools for personalised psychiatric treatments for affective disorders. The first step to achieving this relies on expanding the paradigm of depression research to include thorough characterization of neurobiological mechanisms implicated in failed monoaminergic antidepressant treatments.
1.1.1 Technology Unveils a Surprising Link: From the Bench to the Bedside

There are two long-standing anomalies of the monoamine paradigm. One is the diverse range of symptomatology expressed in the MDD population, ranging from the relatively non-specific 'fatigue' to the defining characteristic of the condition, anhedonia. The diversity of symptom expression has resulted in a delineation of subtypes of depression since the 1950s, but not major improvement in subtype-specific biomarkers or treatments. In fact, findings from meta-analyses and the STAR-D report indicate a modest effect of drug-drug comparisons with no clinically significant impact of drug choice (Warden, Rush, Trivedi, Fava, & Wisniewski, 2007). The second inconsistency is therapeutic lag time. Neurotransmitter levels are increased within 1-2 days, but behavioural improvement is not observed for an average of 2-3 weeks after continuous drug exposure (Hindmarch, 2001), with some estimates as high as 6-8 weeks (Lanni, Govoni, Lucchelli, & Boselli, 2009). A commonly held theory regarding the delay in therapeutic benefit of monoaminergic antidepressants purports that it is not the increase in monoamines themselves, but the structural and synaptic responses to these increases that mediate the therapeutic antidepressant effects (Hindmarch, 2001; Lanni, Govoni, Lucchelli, & Boselli, 2009). This plasticity theory is supported by the knowledge that the delay associated with antidepressant effects of these medications matches the amount of time required for neurons to mature into the surrounding network (Balu & Lucki, 2008). Furthermore, ECT, which remains one of the most potent antidepressant treatments for refractory depression into the twenty-first century, is known to increase monoamine levels as well as plasticity more than any other treatment (Duman & Monteggia, 2006).

Our understanding of the pathophysiology of depression and mechanisms mediating antidepressant response has been significantly advanced by imaging technology such as positron emission tomography (PET), single photon emission computed tomography, functional magnetic resonance imaging (fMRI), magnetic resonance spectroscopy, evoked
potentials, magnetoencephalography, and optical imaging (Helen S. Mayberg, 2006). Such techniques have culminated in the *dysregulated networks model* (Helen S. Mayberg, 1997) which has greatly advanced the understanding of MDD and treatment options for TRD (see figure 1.1). This model defines a dysfunctional limbic-cortical pathway involved in mediating depressive states, characterised by opposing systems of hypo-active “positive motivational dorsal area” and a hyper-active “negative stress ventral area” (Stone *et al.*, 2008). These networks are linked by the cingulate structure, comprised of Brodmann areas 24 and 25, which have since been found to exhibit a distinct hyperactive profile in TRD patients (Helen S. Mayberg, 1997). This model has proven to be quite robust, with consistent findings from imaging studies revealing critical roles for the frontal lobe, cingulate structures and basal ganglia in depression (Mayberg & Holtzheimer, 2011). The frontal lobe often displays atrophy, as does the hippocampus, in turn, these neurodegenerative changes are thought to contribute to the mood and cognitive symptoms of MDD outlined in the DSM-V (Liotti & Mayberg, 2001; Pariante, 2009; Duman & Monteggia, 2006; Castrén & Rantamäki, 2010; Thakker-Varia & Alder, 2009).

This model is enhanced by innervation, cytoarchitecture and function of the structures, which readily lend themselves to the delineation of individual, but interconnected networks. The prefrontal cortex can be divided into the dorsolateral area and a ventromedial area. The dorsolateral prefrontal cortex (dIPFC) is comprised of middle and superior frontal gyri on the lateral surface of the frontal lobes and receives input from sensory cortices; it is also densely interconnected with premotor areas, the frontal eye fields and the lateral parietal cortex (Koenigs & Grafman, 2009). The ventromedial prefrontal cortex (vmPFC) includes the area below the genu of the corpus callosum, and the medial section of the orbital surface; targets include the hypothalamus and periaqueductal gray, as well as the ventral striatum while dense reciprocal connections exist with the amygdala (Koenigs & Grafman, 2009).
Figure 1.1. Schematic of the dysregulated limbic-cortical network model of depression from PET (Helen S. Mayberg, 1997). Motivational dorsal components (red) exhibit a decrease in activation, while stress ventral components (blue) show a relative increase in those suffering with depression and in healthy individuals experiencing sadness. Successful treatment with fluoxetine, which increases serotonin from the dorsal raphe, indicated by dashed lines from mb-p, attenuates hyperfunctioning of ventral components and hypofunctioning of dorsal components, which is in part achieved through reciprocal and bi-directional connections of both dorsal and ventral areas with the rostral anterior cingulated (yellow), indicated by solid black lines and - / +.

Red: dFr dorsolateral prefrontal; inf Par inferior parietal; dCg dorsal anterior cingulate; pCg posterior cingulate; lines indicate reciprocal connections. Blue: Cg 25 subgenual cingulate (infralimbic cortex); vIns ventral anterior insula; Hc hippocampus; vFr ventral frontal; Hth hypothalamus; lines indicate reciprocal connections. Yellow: rCg rostral anterior cingulate. White: Mb-p midbrain-pons; BG basal ganglia; Th thalamus; Am amygdala. Numbers indicate Broadmann designations.
Functional imaging studies have consistently revealed that resting state brain activity of depressed versus non-depressed people confirm significantly higher levels of vmPFC and lower levels of dlPFC activity in depressed brains, and recovery is associated with a reversal of this activation pattern (Helen S. Mayberg, 1997; Koenigs & Grafman, 2009). Specifically these findings highlight the reciprocal relationship between ventral limbic regions and dorsal cortical regions (Mayberg & Holtzheimer, 2011) as well as the inverse relationship between prefrontal activity and depression severity (Helen S. Mayberg, 1997, 2006).

Elevated activity within the amygdala and anterior cingulate cortex are particularly associated with dysphoric mood. Activity in these regions increases in healthy people upon the induction of transient sadness while chronic elevations are observed in people suffering depression (Krishnan & Nestler, 2008). The subgenual cingulate, Brodmann designation 25 (BA25), has proven to be an acutely crucial structure in this model. It is an anterior continuation of the anterior cingulate cortex, a deep structure optimally situated and innervated to influence mood. BA25 maintains reciprocal innervations with orbitofrontal, medial prefrontal and anterior and posterior cingulate cortex regions to influence learning, memory, motivation and reward, while sleep, appetite, libido and neuroendocrine function are influenced by connections with the brainstem, hypothalamus and insula (Mayberg et al., 2005). The anterior cingulate has been shown to be both metabolically hyperactive and hypoactive in chronically depressed individuals, although the latter has been linked to non-response TRD based on reduced adaptive potential (Helen S. Mayberg, 1997; Mayberg et al., 2005). Neuroanatomical, cytoarchitectural and functional imaging pinpoint BA25 as a node in the network model of depression and antidepressant response (Holtzheimer & Mayberg, 2010).

The network hypothesis of depression is in keeping with the plasticity theory, suggesting that recruitment and integration of new neurons may act to promote dysregulated networks underlying the symptoms of depressive disorders by enhancing plasticity within this
maladaptive network, although it is not yet clear how. Armed with these new insights, it was hypothesised that applying the techniques of deep brain stimulation (DBS) to BA25, a procedure already effectively applied to other brain areas to slow the symptoms of the progressive and degenerative neurological disorder, Parkinson’s disease, could alter the disrupted limbic-cortical networks in TRD patients. Helen Mayberg, together with neurosurgeon Andres Lozano, developed a pilot study to take these findings from the lab and apply them to patients with chronic treatment resistant depression.

1.1.2 Evidence Based Framework of BA25 DBS: Past and Future

Neurosurgery for mental disorders as a valid treatment option for affective disorders is arguably one of the most stringently and objectively evaluated medical methods in recent history. This is largely due to the stigma associated with the frontal lobotomies of Walter Freeman and similar drastic surgical approaches to mental health disorders of the same era, which while barbaric by modern standards, were influential in the current use of DBS. The selection of the anterior cingulate for DBS in TRD from imaging data was, in a way, retrograde confirmation of previous lesion research and practices, the classification of lesion-deficit studies having now been largely confirmed with functional imaging data. These lesion findings are concurrent with the limbic-cortical model of depression and taken within this framework suggest the efficacious use of the subcaudate tractotomy lies in severing the white matter tracts of the vmPFC that connect to deeper structures while sparing the dLPFC, (Koenigs & Grafman, 2009; Tye, Frye, & Lee, 2009). Similarly, surgeries such as the anterior leukotomy, subcollosal or superior cingulotomy, may also sever the connection between malfunctioning ventral and dorsal structures thereby releasing each from the abnormal inputs of the other (Helen S. Mayberg, 1997).
Although modern neurosurgical techniques are vastly improved compared to the nefarious techniques of early psychosurgery, the underlying physiological mechanisms are believed to share many similarities. Contemporary neurosurgery was enabled with the invention of the stereotactic frame. DBS was specifically complimented by advances in understanding and technology that enabled chronic electrical stimulation to be effectively utilised clinically, such as in the first implantable pacemaker of the 1960s and in the following decade, stimulation was applied for analgesia (Schwalb & Hamani, 2008). Rapid advances in imaging were also especially important in enabling electrical stimulation to be used for the more delicate and precise purposes within the brain, most notably treating symptoms of Parkinson's disease. The success of DBS for Parkinson’s led to applying the technique to a wide array of disorders with neurological bases, including alleviating negative symptoms of Tourette's Syndrome, Epilepsy, and Obsessive Compulsive Disorder, the latter only gaining US Food and Drug Administration (FDA) approval under humanitarian device exemption in February of 2009 (Malone, 2010).

There is strong historical and modern evidence to support clinically effective application of DBS to the anterior cingulate to attenuate the dysregulated metabolism in this structure, thereby re-establishing the proper communication between the dorsal and ventral components of the limbic-cortical network. A proof of principle study (Mayberg et al., 2005) demonstrated the clinical efficacy of chronic bilateral BA25 white matter tracts stimulation for treatment resistant patients (see figure 1.2). Of the six patients, four attained antidepressant response after 6 months and half of the patients achieved remission, or near remission. These results were corroborated in a larger group of 14 patients, again achieving a 60% response rate at six months (Lozano et al., 2008). The subgenual cingulate is now the primary target for DBS application in TRD worldwide (Puigdemont et al., 2010, 2012).
Collectively, this research lead to the first double-blind multi-site clinical trial, known as BROADEN, aimed at evaluating BA25 DBS for TRD, which was unfortunately halted in early 2014 due to failing an FDA futility analysis. Neither the details of this assessment or data collected during the active study have been made publicly available, obviating worthwhile scientific evaluation in favour of speculation regarding management and methodology of the trial given the undeniable success of previous DBS applications (Berlim, McGirr, Van den Eynde, Fleck, & Giacobbe, 2014). This result also echoes the importance of thorough pre-clinical research, which will progress based on the continued promising data and anecdotal
evidence emerging regarding DBS for TRD. Pre-clinical research will utilise the homologous area to BA25 in the rat, known as the infralimbic cortex (IL), enabling increased understanding of the role structure plays in depression, treatment resistance and antidepressant action.

Pre-clinical research of IL-DBS is necessary as, despite the surge of interest and effective application of DBS techniques, relatively little is understood about the mechanisms through which the procedure modulates this particularly resistant neural circuitry to relieve depressive symptoms (see figure 1.3). Indeed, prominent theory suggests that electrical stimulation acts as a type of ablative surgery, a paradoxic opposition to the very idea of stimulating. A computational model supports the idea that DBS causes frequency dependent modulation of neuronal output variability such that attaining the critical therapeutic frequency eclipses innate neuronal firing activity, in essence creating a functional lesion (Grill, Snyder, & Miocinovic, 2004; Dzirasa & Lisanby, 2012). Currently, there are several primary theories regarding the mechanisms mediating the effects of DBS (from Eitan & Lerer, 2006):

1. DBS may correct abnormal neuronal firing, thereby increasing the accuracy of information transfer within and between networks.
2. DBS causes excitatory stimulation in nearby axon terminals which results in the release of auto-inhibitory factors.
3. DBS at clinically effective high frequency blocks neuron depolarization by blocking intrinsic voltage gated currents of a neuron.
4. DBS may produce local synaptic fatigue and transmitter depletions leading to synaptic depression.
5. DBS may induce local and distal changes in neurotrophic signaling
6. DBS may induce local inflammatory responses that facilitate neuroadaptations locally and distally within the network
It is clear that more research is needed to elucidate the means through which DBS elicits antidepressant action; however, the success of this treatment based on the dysregulated network model is a milestone for MDD research as a field and TRD patients as individuals. Despite the success of this treatment in open label trials, it is important to remember that, while BA25 is itself critical, the reciprocal innervations this structure maintains serve to reinforce
that depression is a systems wide disorder encompassing memory, attention, circadian rhythms, and the range of chemical messengers and new evidence indicates the ability of DBS to modulate many of these systems simultaneously due to afferent and efferent connectivity of the target area (Lujan et al., 2013; Insel et al., 2015; Srejic, Hamani, & Hutchison, 2015; Sun et al., 2015; Chakravarty et al., 2016). Dysregulated plasticity within and between structures of the limbic-cortical network is emerging as a critical mechanism involved in the pathophysiology of mood disorders and the underlying cause of therapeutic lag. Furthermore, the involvement of such a diffuse structural network essentially negates the reduction of depressive disorders to a single chemical or structural source. Hence, those who respond to popular antidepressant medications and psychotherapy treatments are likely experiencing restored plasticity that manifests as the behavioural improvements used as measures of recovery from depressive states, while those who do not achieve measurable improvement are unable to attain the necessary level of plasticity required to reshape the network (Srejic et al., 2015).

It is not unreasonable to hypothesize that non-response to common antidepressant treatments may indicate a greater impairment in plasticity or in mechanisms mediating the return of plasticity to basal levels. This may explain, at least in part, why chronic DBS treatment is necessary for clinical therapeutic effect based on two critical points. Firstly, anterior cingulate function can be used with high accuracy as a marker of potential plasticity on the presumption that greater hypometabolism in this region is predictive of poor outcome for depression therapy (Helen S. Mayberg, 1997), a finding that has been found across studies and with varying imaging techniques (Pizzagalli et al., 2001). Such hypoactivity does not lend itself to the necessary processes by which effective plasticity is achieved and maintained.

Secondly, an alternative postulate states that high frequency stimulation may cause neuronal injury or death, as is believed to occur in ECT. DBS is essentially a reversible localised application of ECT, so this is a viable mechanism of action (McNeely, Mayberg,
Lozano, & Kennedy, 2008). Such insults could prompt small amounts of neurogenesis or the recruitment of neural progenitors from neurogenic regions—as is documented to occur in the striatum, neocortex, corticospinal motor neurons and corno ammonis of the hippocampus (Geraerts, Krylyshkina, Debyser, & Baekelandt, 2007). Further to this, neuronal injury sustained as a consequence of ECT is thought to incite the inflammatory response, upregulating trophic neuroprotective mechanisms such as glia and growth factors in turn decreasing the effects of excitotoxicity and neural damage (Altar et al., 2004; Jansson, Wennström, Johanson, & Tingström, 2009; Wennström, Hellsten, & Tingström, 2004). The capacity of growth factors to impart these effects has been demonstrated in Parkinson disease animal models wherein brain derived neurotrophic growth factor (BDNF) application resulted in cell recruitment to the striatum (Geraerts, Krylyshkina, Debyser, & Baekelandt, 2007) indicating a reciprocal connection between growth factors and neural integration. It is very plausible that DBS calls upon the innate ability of the brain to adapt to the environment through plastic mechanisms which have been documented in similar techniques such as ECT; however, unlike ECT, DBS does not result in severe cognitive side effects, even after long term use (Kennedy et al., 2011).

Together, these data support that those attaining remission from depressive symptoms are able to re-wire the dysfunctional networks through a myriad of intracellular, neurotrophic and pre and post-synaptic alterations (Mayberg & Holtzheimer, 2011). It is thereby not the addition of antidepressants that is actively altering the maladaptive neurophysiological function of an individual, but the augmented plasticity which enables the individual to effectively adapt to the environment and cope more actively with perceived stressors. It is reasonable to investigate the possibility that similar strategies may underlie the reversal of symptoms in depression with the use of DBS. New neurons, whether local or recruited, may then be integrated into the neural network to reform emotional pathways and alleviate depressive
symptoms, or existing neuropil may undergo changes that re-direct the maladaptive connections toward a more efficient and healthy neural substrate. To better understand this, it is imperative that valid antidepressant-resistant animal models be developed to ascertain the mechanisms of action of DBS treatment (Hamani et al., 2012; Hamani & Nobrega, 2012) and, of course, antidepressant resistance itself.

1.2 Pre-Clinical Models of Antidepressant Resistance

The use of pre-clinical animal models is essential to comprehend the aetiology of TRD and mechanisms of antidepressant action of DBS. Building on the efficiency of the corticosterone model of depression, chronic subcutaneous administration of exogenous adrenocorticotropic hormone (ACTH) has established a tricyclic antidepressant resistant animal model as per the pharmacologically predictive forced swim test (FST) (Kitamura, Araki, & Gomita, 2002). In this model, adult male Wistar rats treated with 14 days of ACTH exhibited increased immobility in the FST that was not attenuated with either single or chronic doses of the tricyclics imipramine or desipramine. This model encompasses high levels of pharmacological, aetiological and face validities; the latter being satisfied by the reliance of this model on the stress system, an established trigger and maintenance mechanism for depression in many organisms, including humans. Given these attributes, a modified version of this model is the basis of the investigations presented herein.

1.2.1 Stress and Depression: Why This Model Works

A critical component implicated in the development of MDD is stress. Often an acute stressor, such as death of a loved one, will precede a depressive episode. Alternatively cumulative chronic life stress also contributes to the propensity for MDD. The stress response, often referred to as the fight or flight response, is a survival mechanism largely mediated by the
hypothalamic-pituitary-adrenal (HPA) axis, a group of neural structures that regulate
corticosteroid hormones. As the name suggests, the system is by and large incited during
stressful events that threaten the safety and survival of an organism. The essential role of the
HPA axis is to link the central nervous system to the endocrine system (Nestler et al., 2001) in
order to monitor and react to exteroceptive stimuli through coordinated physiological responses
mediated through the release of hormones, such as glucocorticoids (see figure 1.4).
Figure 1.4. See overleaf for caption
Figure 1.4. Schematic of the hypothalamic-pituitary-adrenal axis (modified from Netter, 2003). The HPA axis is well integrated with the surrounding limbic structures. The hypothalamus, the primum mobile of the stress response, is connected via the medial forebrain bundle, fornix, stria terminalis and septal nuclei which deliver excitatory input from the amygdala (solid green lines) and inhibitory input from the hippocampus (solid red lines). Net excitatory input prompts the induction of the neuroendocrine cascade with the release of the primary secretagogue, CRF. This neuropeptide is released through magnocellular neurons within the infundibulum (solid purple line) and directly into the posterior pituitary, which then emits AVP. Simultaneously, CRF is transmitted via parvocellular neurons from the hypothalamus, to the median eminence of the infundibulum, where a vascular plexus carries the chemical to the anterior pituitary (solid blue line), where secratory corticotroph cells produce and release ACTH (solid yellow line). Because the median eminence and other parts of the hypothalamic-pituitary connections are circumventricular organs, the chemicals utilised in this cascade also exert systemic effects, most notably the stimulation of glucocorticoid release from the adrenals by ACTH (solid red lines). The predominant glucocorticoid in humans is cortisol, most of which is bound to cortisol-binding globulin (CBG) making it inert, however, when cortisol release is stimulated through the stress response, CBG levels are also decreased resulting in increased free and active glucocorticoid levels. These levels act primarily through GRs in the hippocampus and pituitary (pink and brown dots) to provide negative feedback to the HPA axis, halting the stress response.
The first stage of HPA-axis response to a perceived threat is the simultaneous release of the neuropeptides, corticotropin releasing factor (CRF) and vasopressin (AVP, arginine vasopressin), from parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus (Lanni, Govoni, Lucchelli, & Boselli, 2009). This prompts the anterior pituitary to release ACTH, and the posterior pituitary to release systemic AVP as well as ACTH (Nestler et al., 2001). In the final stage of HPA-axis response, Both ACTH and AVP signal the adrenal cortex to release the major endogenous glucocorticoid, cortisol (corticosterone in the rat) and other glucocorticoids into the blood stream to act both centrally and peripherally (Nestler et al., 2001). The majority of these glucocorticoids are quickly bound to cortisol-binding-globulin (CBG) making them inert while the remaining free hormones act as inhibitory feedback through two intracellular corticosteroid receptors: type I, or mineralocorticoid receptor (MR), and type II, glucocorticoid receptor (GR) both of which are found in the hypothalamus, pituitary and hippocampus of the brain (Pariante, 2009).

While mild to moderate short term activation of this system ensures the survival of a myriad of species through increased mental acuity and physical strength, a consistently prolonged stress response is disruptive and may result in an hyperactive HPA axis and hypercortisolism or a hypoactive HPA axis and hypocortisolism both of which are linked to respective diseases. Melancholic depression has been found to be associated with hyperactivity of the HPA axis, while atypical depression has been linked to hypoactivity of the HPA axis (Ehlert, Gaab, & Heinrichs, 2001), though most diagnosed with a MDD exhibit mixed symptoms and a more severe illness (Gold & Chrousos, 2002).

Hyper-activation of the HPA axis is largely attributed to disrupted feedback from both MR and GR. However, due to its lower affinity than the MR to endogenous glucocorticoids at basal levels, GR is primarily activated when glucocorticoid levels are elevated, such as during a *fight or flight* situation, thus disruption of GR signaling is likely responsible for the
deleterious effects of hypercortisolemia (Pariante, 2009; Ferguson & Sapolsky, 2007, 2008; Sapolsky et al., 1986). In fact, reduced GR function through genetic alteration in mice has led to the establishment of an animal model with predisposition to depressive behaviours under stressful environment (Schulte-Herbrüggen et al., 2006). The failure of this receptor to properly inhibit HPA-axis activity under conditions of elevated endogenous glucocorticoids is known as glucocorticoid resistance (Pariante, 2009). Glucocorticoid resistance can therefore result in an hypofunctioning negative feedback HPA system within an hyperfunctioning centrally mediated HPA system.

The chronic activation of the HPA axis via glucocorticoid resistance results in a destructive cascade of neuroendocrine and immune physiological processes, the results of which are often present in those suffering from MDD. Approximately half of all cases of major depression involve hypercortisolism (Lee, Ogle, & Sapolsky, 2002) and conversely, clinical populations with long term corticosteroid use often develop depression and many comorbidities of depression in both populations are specific to increased circulating glucocorticoids, such as a decline in declarative and working memory (Frohman, 1959; Brown, 2009). The chronic increase of glucocorticoid levels accounts for other co-morbid endocrine abnormalities often associated with depression, such as insulin resistance, abdominal obesity, heart disease and inflammatory complications (Krishnan & Nestler, 2008). It is also an underlying cause of core MDD diagnostic criteria such as psychomotor retardation and vegetative states and is further implicated in impaired immune function, largely due to associated low-grade, chronic inflammatory response of such prolonged exposure to glucocorticoids (Munhoz, Sorrells, Caso, Sapolsky, & Scavone, 2010; Sorrells et al., 2009; Sorrells & Sapolsky, 2007; Munhoz et al., 2006).

The glucocorticoid resistance contribution to depression pathophysiology is further supported in humans by the dexamethasone/CRF suppression tests, which do not induce
glucocorticoid suppression by inhibitory feedback in many depressed patients, inferring
dysregulated inhibitory feedback (Pariante, 2009). Elevated levels of cortisol are well
documented in depressed patients via urinary, plasma and cerebrospinal fluid samples, while
at the same time, the structures responsible for producing and monitoring cortisol levels,
respectively the adrenal glands and pituitary, show increased structural volume as a result of
the high demands of cortisol production (Pariante, 2009; Sapolsky et al., 1986). Postmortem
data also concur that major depression is consistent with increased CRF mRNA and
immunoreactive neurons in the hypothalamic PVN, prefrontal and frontal cortex, locus
coeeruleus and raphe nucleus (Hauger, Risbrough, Oakley, Olivares-Reyes, & Dautzenberg,
2009).

The congruous relationship between glucocorticoids and depression strongly supports
a causative role for a centrally hyperactive glucocorticoid resistant HPA axis in the
development and maintenance of MDD and has consequently been the centre of intense
investigation by many groups, notably Dr. Robert Sapolsky and colleagues. The link between
elevated glucocorticoids and depressed behaviour has been evident since steroids were
introduced into clinical practice in the 1940s (Brown, 2009) however, the understanding of the
molecular reorganisation that occurs as a consequence of life-long extensive release of these
hormones is adding a new dimension to the aetiology of depression, most prominently
through the effects of the stress system on plasticity. This has led to an increased
understanding of how prolonged exposure to stress and resultant hormones are implicated in
the development of depressive behaviours. Within the framework of the dysregulated network
model of depression, these findings support the role of somatic, emotional and cognitive
stresses in contributing to the maladaptive regulation of the network underlying MDD through
region specific changes in plasticity, namely increased ventral plasticity and activation
combined with decreased dorsal plasticity and activation (Pittenger & Duman, 2008; Mayberg & Holtzheimer, 2011).

1.2.2 The Glucocorticoid Cascade Hypothesis, The Network Model and The Hippocampus

The dense concentration of GRs within the hippocampus make this structure simultaneously vulnerable to, and responsible for, regulating elevated levels of stress hormones and therefore a critical component of stress mediated disease states (Sapolsky et al., 1986). The important relationship between the hippocampus and cortisol is embodied in the glucocorticoid cascade hypothesis (GCH), put forth by Sapolsky, Krey and McEwen (1986). This theory holds that both ageing and chronic high levels of circulating glucocorticoids contribute to a stress response profile that is less suited to proper adaptation in the face of new stressful events through a reduction in both glucocorticoid receptors and neuropil (see figure 1.5). The healthy daily fluctuation of endogenous glucocorticoids, known as allostasis (Sterling & Eyer, 1988), is maintained through up and down regulation of MRs and GRs. Allostatic efficiency is attenuated with increasing age and under chronic activation, such as occurs with stress, culminating in insufficient concentration of glucocorticoid receptors to properly transmit inhibitory feedback information (Sapolsky et al., 1986). The result of the hypoactive negative feedback system is elevated and potentially continuous hormone release and further damage through the heightened neuroendocrine and inflammatory reactions induced via the hyperactive HPA axis.
These characteristics combine to form a “feed-forward cascade of degeneration” (Sapolsky et al., 1986) where the hippocampus is presumed to lose volume due to loss of glucocorticoid concentrating cells (Sapolsky et al., 1985). Chronic corticosteroid administration in animal studies has been demonstrated to induce extensive and persistent depletions of corticosteroid concentrating cells, especially in the CA3 region of the hippocampus, and remaining cells bind significantly less to free glucocorticoids (Sapolsky et al., 1985). This cell loss is due to insult by sustained increase in glucocorticoid levels. Hippocampal atrophy is therefore both a result of, and a contributory cause to, increased basal glucocorticoid levels (Lupien et al., 1998). The failure of the HPA-axis to maintain a homeostatic state results in allostatic overload which correlates with the aberrant cognitive and behavioural functioning as well as physiological consequences present in mood disorders.
like anxiety and depression (Dumas, Gillette, Hamilton, Ferguson, & Sapolsky, 2010; Ferguson & Sapolsky, 2008).

Despite being originally investigated as a contributing factor to accelerated ageing, this theory is readily applicable to depression due to the striking behavioural and morphological similarities they share (Sapolsky et al., 1985, 1986; Lupien et al., 1998). The similarities are, in part, due to the impact of glucocorticoids on the brain and body generally, and the hippocampus specifically. Animal studies show that chronic corticosterone treatment results in the loss of neurons of the same size as those lost in aged animals while aged and chronic corticosterone treated animals both exhibit an increase in small darkly staining cells of the CA3 hippocampal region, thought to be glial infiltration as a response to insult and injury (Sapolsky et al., 1985). In aged rats, increased glucocorticoid levels lead to hippocampal dysfunction which positively correlates with deficits in spatial learning (Shou-Sen, Shu-hong, Bang-ping, Fang, & Zun-Ling, 2010). Similarly, elderly human patients display comparable symptoms with prolonged cortisol elevations culminating in deficits in hippocampal dependent memory tasks and decreased hippocampal volume (Lupien et al., 1998).

While the gradual decline of inhibitory glucocorticoid feedback from the hippocampus is indeed a normal part of ageing, the decline may be expedited by several acute or prolonged phases of elevated glucocorticoids. This expedited ageing profile seems to contribute to the development of major depressive disorder symptomatology as well as being a consequence of depressive disorders. A meta-analysis of 12 studies found hippocampal volume of patients with major depressive disorder (MDD) to be consistently and significantly reduced, and the degree of this reduction to be in direct proportion to the number and duration of untreated depressive episodes (Maletic et al., 2007; Sahay & Hen, 2008). Furthermore, the degree of cortisol elevation over time and current basal cortisol levels in humans positively correlates with the degree of hippocampal atrophy (Lupien et al., 1998).
Other effects of hypercortisolemia in the hippocampus include dendritic atrophy, reduced neurogenesis and synaptic plasticity, and increased susceptibility to insult such as free radicals, and neurotoxicity all of which combine functionally as reduced cognition and memory performance (Lee, Ogle, & Sapolsky, 2002; McEwen & Magarinos, 2001; Sapolsky, 2000). Investigations into the mechanisms behind these glucocorticoid mediated events point to inflammation (Sorrells et al., 2009) and neuroenergetics (Bliss et al., 2004). Rather than inducing direct damage and apoptosis, glucocorticoids result in a state of extreme vulnerability such that cells are rendered susceptible to otherwise minor insults (Roy & Sapolsky, 2003; Lee, Ogle, & Sapolsky, 2002). This hypersensitive state is largely the result of altered neuroenergetics of both neurons and glia from impaired glucose uptake and energy metabolism which combine with the increased levels of synaptic glutamate and free-radical accumulation induced by glucocorticoid elevation to result in excitotoxic cell death (Roy & Sapolsky, 2003; Lee, Ogle, & Sapolsky, 2002). Furthermore, this cell death is necrotic and itself inflammatory and is therefore the direct result of injury, rather than organised apoptosis (Roy & Sapolsky, 2003). Supplementing cells with energy (Bliss et al., 2004). antioxidant enzymes (Wang et al., 2003) or increasing MR and GR (Ferguson & Sapolsky, 2007) can protect against these glucocorticoid mediated effects by altering the neuroenergetics of the cells, gene transcription and inflammation. It is also possible that the protective role of glia during allostatic overload has been previously overlooked, as post-mortem studies consistently identify glial loss in brains of depressed patients (Mayberg & Holtzheimer, 2011).

Together, chronic stress and glucocorticoid elevation suppresses DG neurogenesis and cell survival, resulting in dendritic pruning in the hippocampus, especially the CA3 region (McEwen, 2008; Lee, Ogle, & Sapolsky, 2002). This pruning is believed to impact the ability of an organism to react properly to the environment, leading to the adoption of maladaptive
responses to stressors, which set in motion a cascade of behavioural and neurobiological self-harming events and may contribute to the onset and maintenance of depression (Surget et al., 2008; Santarelli et al., 2003; Chih-Hao et al., 2004; Doi et al., 2010). Morphological changes have been demonstrated to occur not only throughout the hippocampus, including CA1, CA3 and DG, but also innervating regions such the nucleus accumbans (Morales-Medina et al., 2009) and the amygdala. In a reciprocal fashion, remodelling of neurons and connections in the amygdala caused by glucocorticoids is associated with concurrent neuronal remodelling in the hippocampus and prefrontal cortex (Roozendaal et al., 2009). The synergistic relationship between the amygdala and the hippocampus has no doubt enhanced primate survival via strengthened recall of dangerous and pleasurable stimuli, yet it may also be linked to post traumatic stress disorder, anxiety and depression. The neuroadaptations that occur with stress, within and between these brain areas, result in localised structure-function deficits as well as dramatic global effects on behaviour that perpetuate depressive states.

1.2.3 The Importance of Learning: A Cognitive Perspective of Plasticity in Depression

The role of cognition is believed to be a primary maintenance factor of MDD, and the target of many effective treatments, such as cognitive behavioural therapy. These ideas are largely based on Beck’s cognitive model of depression which developed from clinical observation and experimental testing. This theory purported MDD is built on the schemas, cognitive errors and the cognitive triad of self, world and future of the individual (Beck, 1967). Negative schemas are thought to be activated by a stressor, which then act to guide information processing toward negatively biased attention, processing and memory, which perpetuate into themselves resulting in the depressed ruminative state (Disner et al., 2011).

The recall of past events through memory has the capacity to greatly affect how any given stimuli will be interpreted by an organism. These memories are consolidated and
recalled by unique form of neural plasticity known as long term potentiation (LTP), whereby synaptic connections are strengthened under repeated stimulation, therefore repetition of a process, behaviour or mood state increases the neural substrates that execute these processes making them less effortful to replicate in the future. This allows an organism to perform necessary tasks without consistently taxing attentional processes and energy resources. This LTP plasticity is the neural foundation of rodent depression paradigms such as learned helplessness. A human under chronic stress is engaged in a similar paradigm; learning to associate aversive stimuli with negative affect throughout life, reinforcing the ventral control of emotional and attentional processing, increasing the strength of synapses and aborizations in this region, while concomitantly decreasing the influence of logical thought on ameliorating these negative states through the reverse process. This then becomes the normal state of functioning for that individual.

In line with the dysregulated network model of depression, the processes of Beck’s model have since been found to be underscored by overactive ventral emotional processing, which is uninhibited by underactive dorsal cognitive processing (see figure 1.6). As research and technology have evolved, the anterior cingulate again emerges as a key regulator in this cognitive model of depression; the dorsal sector inefficiently relays top-down cognitive signals, the ventral sector increases the emotional valence of incoming information and the rostral sector amplifies the relation of this incoming information to the self (Disner et al., 2011). In an amended dysregulated network model, it becomes apparent that mid-line structures, that is, areas with reciprocal connections to both dorsal and ventral circuits, can act to sway the brain towards negative bias, attention and memory. This negative memory bias is one of the core cognitive risks and maintenance factors for MDD and remains present even in a state of remission (Gerritsen et al., 2011; Schlosser et al., 2012).
Figure 1.6. Amended neural circuit model of depression modified from Helen Mayberg and Paul Holtzheimer (2011). The neurophysiological underpinnings of the cognitive model are readily apparent in this schematic. Areas associated with imparting hyper-emotive salience to stimuli, outlined in yellow boxes, are able to sway the circuit toward a negative bias served by the ventral system. At the same time, these mediating structures fail to execute inhibiting cognitive process that would normalize these aberrant thought processes.

Red: PF prefrontal; PM premotor; Par Parietal; MCC mid-cingulate; dpHc dorsal-posterior hippocampus; PCC posterior cingulate. Yellow: mF medial frontal; pACC pregenual anterior cingulate; mOF medial orbital frontal; vst-cd ventral striatum-caudate; dm-thal dorsomedial thalamus; amyg amygdala; mb-vta midbrain-ventral tegmental area. Blue: SCC subcallosal cingulate; va-hc ventral-anterior hippocampus; a-ins anterior insula; hth hypothamus; bstem brainstem. Numbers Brodmann areas. Small arrows relevant anatomical connections. Large solid arrow putative connections between compartments mediating a specific treatment. CBT cognitive behavioral therapy; meds SSRI pharmacotherapy; DBS subcallosal cingulate white matter deep brain stimulation.
The HPA axis has been strongly linked to the pathology underlying cognitive impairment in MDD, with one review suggesting 70% of studies found a significant correlation between cortisol levels and visual/verbal and working memories as well as executive function in those with MDD (Schlosser et al., 2011). Cortisol levels have been shown to exhibit an inverted U-shaped dose-response curve with cognition as both low and elevated levels impede memory formation. As previously noted, high cortisol levels are associated with a decrease in hippocampal arborisation and consequently detriments in LTP and may be involved in vulnerability and maintenance towards negative shift.

The amygdala, which exhibits hyperactivation in depression, is pivotal in both the dysregulated network model and cognitive model and is greatly affected by HPA axis hyper-functionality. The structure mediates identification of emotional significance and, in concert with efferent connections to the anterior cingulate, results in the formulation of affective states and emotional behaviours proportional to the heightened emotional valence of identified stimuli (Phillips et al., 2003). Interaction between the hippocampus and the basolateral complex of the amygdala (BLA) may be one factor mediating the increase in emotional sensitivity and decrease in working memory associated with stress and depression. Noradrenergic stimulation of the BLA causes memory enhancement and increased levels of activity-regulated cytoskeletal-associated protein- a marker of increased hippocampal plasticity- while inactivation of the BLA results in impaired memory consolidation and a down-regulation of plasticity (Roozendaal et al., 2009). The reverse is also true in that hippocampal initiated memory formation requires an intact BLA (Roozendaal et al., 2009). Amygdala hyper-activation, as measured by fMRI of depressed individuals, has been shown to interfere with hippocampal based memory processing of affectively neutral stimuli, indicating a potential inherent vulnerability for MDD to perpetuate maladaptive self-referential ruminative thoughts (van Eijndhoven et al., 2011). Furthermore, larger amygdala to
hippocampal volume ratio was found to be associated with negative memory bias in non-depressed individuals (Gerritsen et al., 2012). These findings indicate a clear ability for the amygdala to contribute to remodelling network connections under stress conditions which influence the efficiency of the mnemonic system, consequently shifting behaviour toward a negatively focused and maladaptive strategy.

These amplified affective processes are then left uninhibited by hypofunctioning hippocampus, basal ganglia and dorso-medial and lateral cortex which no longer efficiently perform the effortful executive control of attention shift and healthy combative memory recall which would serve to attenuate the impact of negative stimuli (Phillips et al., 2003; Disner et al., 2011). Compounding this disinhibition is the recruitment of other emotionally salient structures, such as the insula, thalamus and retrosplenial cortex for effective performance of neutral episodic memory formation (van Eijndhoven et al., 2011). The hippocampus, amygdala and prefrontal cortex exhibit dendritic pruning and reduced spine density under repeated stress conditions while increased innervations are established from consolidating emotional memories (Roozendaal et al., 2009).

Several studies using human and animal models have demonstrated the role of glucocorticoids in hampering mnemonic ability, though large variation does exist. Hippocampal-dependent memory has been found to be more sensitive to glucocorticoids than any other form of learning and this is amplified by individual exposure to cumulative cortisol (Abercrombie et al., 2011). Enhanced fear acquisition, as measured by classic differential fear conditioning paradigm, and reduced declarative memory consolidation, as measured by word-pair and texture discrimination recall, were present in treated depressed individuals (Nissen et al., 2010). In an associative learning paradigm imaged with fMRI, medicated depressed patients did not differ from control in hippocampal activity during encoding or retrieval, but differences occurred in other memory related regions; for depressed individuals the left
parahippocampal gyrus showed an increase, while frontal and parietal regions exhibited decreases during encoding with the latter also being seen during retrieval (Werner et al., 2009). Other work has found that a group of depressed individuals \( n = 8, 2 \) non-mediacted did display a dysregulation in hippocampal activation and successful encoding, but again without a deficit in performance (Fairhall et al., 2010). Finally in a pre-clinical neonatal clomipramine model of endogenous depression, decreased CA1-LTP was associated with decreased monoamine levels and acetylcholinesterase activity, impaired learning in radial arm amaze and the presence of anhedonia which were reversed by chronic SSRI (escitalopram) treatment (Bhagya et al., 2011).

Taken together, the hippocampus emerges as a critical structure in the network, glucocorticoid and cognitive models of depressive disorders. In the wake of metabolic and tractography data, these models are clearly complementary, being linked by neuroplasticity. As our ability to measure the influence of this unique adaptability is refined, its contribution to depressive states and symptoms can be elucidated. Equally relevant is the role of plastic changes in mediating antidepressant response which, when combined with aetiopathology, may reveal the challenges to be overcome in treating in antidepressant resistant patients (see figure 1.7). Presently neurotrophic factors are known mediators of plasticity and may be fundamental mechanisms of neuroplasticity in mental health disorders. These proteins exhibit changes with exposure to stress and in MDD diagnosed populations which are often reversed with antidepressant response. These changes are furthermore predicted by the network model, displaying a region specific profile. As such, these proteins may prove critical in our understanding of MDD. The pre-clinical ACTH model combined with IL DBS can be instrumental in characterising the role that neurotrophic factors and plasticity play in non-responsive neural networks and contribute to the development of novel antidepressant therapies.
Figure 1.7. Schematic of plasticity of a hippocampal pyramidal neuron as affected by MDD, highlighting a role for neurotrophic factors in mediating antidepressant response, from Berton & Nestler, 2006. The normal neuron receives balanced chemical input from innervating neurons (a) which becomes aberrant during MDD (b), in part due to increased glucocorticoids, resulting in reduced dendritic abortion, reduced BDNF and reduced protective gene translation of protective mechanisms like BDNF. Effective antidepressant treatment restores proper chemical signaling, dendritic innervation and gene transcription which allows an overall shift in the neural network that presents as symptom improvement (c). BDNF brain-derived neurotrophic factor; CREB cyclic AMP-response-element-binding protein
1.3 Countering the Effects of Stress and Restoring the Neural Network: A Role for Neurotrophic Factors

Despite the laudible advances in our understanding of the heterogeneous aetiopathology of MDD, no testable marker has yet been identified to definitively delineate the presence or subtype of depression and physiological markers of best treatment modality remain equally elusive. Nonetheless, aberrant plasticity in neural and synaptic connectivity is now recognized as a core contributing factor to MDD and attenuation or reversal of these maladaptations is considered critical to antidepressant response. The subgenual cingulate is a node in the depressive pathology as it mediates the interactions of the limbic cortical network and is itself affected by maladaptive plastic changes. This hippocampus is also a key neural structure in mediating the symptomatology of aberrant plasticity as it is one of two neurogenic regions of the adult brain and contains the highest concentration of neurotrophic factors of any brain region (Thakker-Varia & Alder, 2009).

Prior to their current status as novel therapeutic targets, neurotrophic factors were believed to primarily assist in the developmental stage of the central nervous system influencing growth, differentiation and the survival of neurons. While this is indeed a critical purpose of neurotrophic factors, the ability of these molecules to continue aiding neurons in adult life is proving to be just as important with reduced neurotrophic support recently pinpointed as a significant contributing factor to development and maintenance of depression-like behaviour in pre-clinical models (Duric & Duman, 2013). For the purposes of this review, focus will be limited to BDNF and glial cell line derived neurotrophic factor (GDNF) for their known association with both structures and chemicals linked to depression onset and recovery. BDNF has recently received much attention in MDD research as it is particularly associated with the serotonergic system and is also highly concentrated in the hippocampus. GDNF research as it pertains to MDD is comparably in its infancy, but because of its role in
glial function and dopaminergic signalling, it is highly probable this protein has a pivotal role in stress-induced damage reduction and anhedonia respectively.

Upregulation of BDNF is observed across antidepressants- SSRIs, NERIs, MAOIs and ECT, with the latter two and most efficacious therapies having the most robust effect (Duman & Monteggia, 2006). Conversely, pharmacological blockade of BDNF prevents the behavioural effects of monoaminergic and tricyclic treatments in mouse models (Kozisek, Middlemas, & Bylund, 2008). GDNF and its preferential receptor display a similar critical role in affective states. GDNF is particularly important for the development and maintenance of glial cells, serotonergic and dopaminergic neurons (Michel et al., 2008). Lithium, which is often co-administered with monoaminergic antidepressants to boost their effectiveness, has also been shown to increase BDNF and GDNF levels in an animal model of depression (Angelucci et al., 2003). Furthermore, levels of neurotrophic factors may predict responders from non-responders (Wolkowitz et al., 2011; Zhang et al., 2009). The underlying implication of these findings presupposes the role of these factors in plasticity and neural integration in contributing to recovery from depressive states.

1.3.1 General Profile of Brain Derived Neurotrophic Factor

One of the best defined families of neurotrophic factors is the neurotrophins, which includes nerve growth factor (NGF), neurotrophin-3,4 and 5 (NT-3,4/5) and BDNF. These factors are present in both central and peripheral nervous systems where they provide a supportive role to proper cell function. All of the neurotrophins are small proteins that act through the tropomycin kinase (Trk) group of receptors. Each neurotrophin has an affinity for selective Trk receptors: TrkA binds NGF and NT-3, TrkB binds BDNF, NT-3 and NT-4, and TrkC binds NT-3. The receptor p-75 is also a general receptor for the neurotrophin family. Within this family, BDNF has emerged as a particularly important neurotrophin regarding
MDD. Decreased levels are associated with depressive states and increased levels with antidepressant effects (Castren, Voikar, & Rantamaki, 2007). The negatively correlated relationship between low BDNF levels with depressive states, increased glucocorticoids, neuronal atrophy and cell loss is a currently under extensive study as a mediator of MDD and antidepressant response. Findings from various animal models of stress, including the forced swim test, learned helplessness and CMS paradigms, as well as human serum and post mortem measurements, support this claim.

BDNF is secreted in two active forms- proBDNF and the cleaved mature BDNF (mBDNF). ProBDNF is believed to primarily regulate cell death through the p-75 receptor. Through its high affinity receptor TrkB, mBDNF causes autophosphorylation of tyrosine residues (Castrén & Rantamäki, 2010) and activation of G-proteins such as Ras (Kristen rat sarcoma viral oncogene), Rap-1 (Ras-related protein Rap-1B) and Cdc-42-Rac-Rho (Rho family proteins) as well as phospholipase –Cγ (PLCγ), Map kinase (MAPK) and PI 3-kinase (PI3K). Through MAPK, PI3K and PLCγ, mBDNF promotes neuronal survival via phosphorylating the transcription factor, cyclic AMP response element binding protein (CREB, see figure 1.7) (Numakawa et al., 2010). The transcription of BDNF through activation of CREB can also influence synaptic plasticity and the establishment of neural networks (Hauger, Risbrough, Oakley, Olivares-Reyes, & Dautzenberg, 2009).
**Figure 1.8.** Intracellular pathways of BDNF-TrkB*. See text for description of topically relevant components.
Since its discovery, the role of BDNF has expanded beyond that of a support molecule, proving capable of influencing synaptic activity (Fiore, Angelucci, Aloe, Iannitelli, & Korf, 2003), and meeting many of the criteria set for neurotransmitters. Firstly, BDNF is anterogradely transported in the CNS, as it is found in the neostriatum, neocortex, mossy fibers of hippocampal granule neurons, medial habenula, central amygdala, lateral septum and spinal cord without coexisting mRNA implicating that the protein must have been transported there from the BDNF enriched innervating structures (Altar & DiStefano, 1998). This anterograde transport supports the role of BDNF in cell survival, differentiation and growth (Fiore, Angelucci, Aloe, Iannitelli, & Korf, 2003). Secondly BDNF is presynaptically synthesised and stored in vesicles, exhibits depolarization evoked release and has postsynaptic receptor localisation (Altar & DiStefano, 1998). Thirdly, the co-expression of BDNF and TrkB in hippocampal formation and cerebral cortex neurons indicates that BDNF has properties of axo-dendritic communication as well as paracrine and autocrine functions (Fiore, Angelucci, Aloe, Iannitelli, & Korf, 2003). Finally, BDNF also exhibits an activity dependent signaling profile as shown in mice with low levels of TrkB receptors in new neurons which exhibit decreased dendritic aborization and integration which is linked with increased newborn cell death (Bergami, Berninger, & Canossa, 2009).

Through the diffuse trophic and apoptotic pathways, BDNF is critical in mediating plasticity required for both depression onset and recovery. This is in part mediated by the susceptibility of BDNF to exteroceptive stimuli, including stress, leading to an activity dependent profile that is susceptible to endocrine levels which change in response to environmental conditions (Duman & Monteggia, 2006). This is due in part to the BDNF gene being stress responsive, as it utilizes the same transcription factor as glucorcortioids-CREB. In addition, increased BDNF levels are associated with short and long term memory formation, of both spatial (Falkenberg et al., 1992) and inhibitory avoidance tasks (Alonso et
al., 2002). Together, these support a direct relationship between stress and BDNF as well as a consequent yet independent role of BDNF in modifying the neural structures underlying both the cognitive and emotional networks through long term potentiation and depression. BDNF can therefore be important in negative (depressive) and positive (antidepressant) behaviours depending on the dose and region in which it is activated, which can be predicted by the dysregulated networks model. Moreover, the time required for BDNF synthesis and secretion is consistent with the therapeutic lag time of monoamine antidepressant therapies, and the modulation of BDNF is specific to such therapies given psychotropic, antipsychotic and psychostimulant drugs have no significant effect on hippocampal BDNF levels (Duman & Monteggia, 2006). Based on this information, the following will outline the role played by BDNF and stress in depression onset and antidepressant response, with a focus on the hippocampus due to its established role in moderating stress hormones and the finding that it contains highest concentration of BDNF in the brain, especially within the DG, CA3 and CA4 regions (Hansson et al., 2000).

1.3.2 Brain Derived Neurotrophic Factor and Depression

Studies in both humans and animals report clear evidence for a deleterious relationship between stress and BDNF/TrkB levels contributing to depression onset in a dose and region dependent manner as predicted by the dysregulated networks paradigm. This relationship is also subject to type and duration of stress as well as age of the organism undergoing stress (Shou-Sen, Shu-hong, Bang-ping, Fang, & Zun-Ling, 2010), a profile that is echoed in the glucocorticoid cascade hypothesis. As previously mentioned, BDNF and glucocorticoids share CREB mediated activation, but the relationship is far more intimate. Glucocorticoids exert transcriptional changes on BDNF via both GR and MR in the hippocampus and neocortex of the rat brain under normalised adrenalectomy conditions
These changes are evident at both mRNA and protein levels (Schaaf et al., 1999). Reciprocally, under chronic restraint stress, BDNF appears to act as a regulator of HPA activity in order to assist with adaptability (Naert, Ixart, Maurice, Tapia-Arancibia, & Givalois, 2011).

Early stress can also exert maladaptive programming effects via the BDNF/TrkB system. Maternal separation can cause decreased BDNF levels within the hippocampus of pups which later manifests as a permanently impaired BDNF response to acute stress (Kozisek, Middlemas, & Bylund, 2008). Early perturbations in the BDNF system by BDNF knockout also results in more severe hippocampal dependent memory problems in later life versus adult BDNF knockout (Monteggia et al., 2004) as well as a hyperactive phenotype (Monteggia et al., 2004; Hill et al., 2010). Adult rodent stress paradigms present a down-regulation of BDNF expression in the CA3 pyramidal and DG granule cell layers of the hippocampus (Shirayama et al., 2002; Fiore, Angelucci, Aloe, Iannitelli, & Korf, 2003; Angelucci et al., 2004; Balu & Lucki, 2009; Otsuki et al., 2008). Further work has suggested that the CMS model results in decreased BDNF protein expression and CREB activation in the DG alone, which was correlated with anhedonic behaviours as per the sucrose preference test (Grønli et al., 2006). Postmortem studies confirm reduced levels of BDNF and its TrkB receptor in the hippocampus and frontal cortex of suicide victims Lanni, Govoni, Lucchelli, & Boselli, 2009; Taliaz, Stall, Dar, & Zangen, 2010) and the expression of various factors, such as CREB, in the pathways mediating BDNF are also reduced (Shirayama et al., 2002).

Alongside BDNF/TrkB changes with stress application, direct modulation of the HPA axis and BDNF system results in reciprocal neural and behavioural profiles. Compared to wild type animals, transgenic mice with impaired GR expression show reduced levels of the activity dependent transcription of BDNF exon IV as well as decreased BDNF binding in the hippocampus, which are believed to directly impair the ability of the organism to cope with
acute environmental stressors (Albone et al., 2011). In a rather extensive study, Taliaz and colleagues (2010) were able to reduce neurogenesis and neural differentiation as well as elicit depressive markers, such as increased immobility in the forced swim test by BDNF knockdown in the dorsal DG. In addition, by knocking out TrkB receptors in hippocampal progenitor cells it was deduced that BDNF is necessary for neuronal differentiation, but not necessarily proliferation of new cells (Balu & Lucki, 2008; Taliaz, Stall, Dar, & Zangen, 2010).

Complementary to the depression-like effects of low levels of BDNF, bilateral injections of BDNF (at .25 microg and 1.0g) into the dentate gyrus and CA3 pyramidal cell layer in stress conditioned rats leads to significant antidepressant response behaviour in these animals (Shirayama et al., 2002). This treatment showed a region and dose specificity for efficacious BDNF effects, and proved to be long lasting, enduring up to 10 days post injection (Shirayama et al., 2002). Similarly, direct infusion of BDNF into the hippocampus, raphe nucleus and brain stem elicits antidepressant effects within days, mediated, in part through activation of its receptor, TrkB (Castren, Voikar, & Rantamaki, 2007; Kozisek, Middlemas, & Bylund, 2008; Castrén & Rantamäki, 2010; Shirayama et al., 2002). Using the CMS paradigm, direct hippocampal infusion of BDNF reversed core anhedonic behaviours and partially rescued astrocyte proliferation in adult male Sprague Dawley rats (Ye, Wang, Wang, & Wang, 2011).

Not only is BDNF directly linked to antidepressant behaviour, it seems necessary to antidepressant response. Using a conditional BDNF forebrain knockout mouse model, researchers were able to induce a depressive state that was also antidepressant resistant (Duman & Monteggia, 2006). Another mouse model of TrkB dominant negative over-expression in the forebrain did not induce a significant model of depression as per the forced swim test, but did display antidepressant resistance (Duman & Monteggia, 2006). BDNF
heterozygous and TrkB dominant-negative mice also do not exhibit the increased proliferation of new neurons associated with tricyclic administration (Balu & Lucki, 2008). In animal models of deficient BDNF-TrkB signaling, including adult-onset BDNF forebrain knockout mice (Kozisek, Middlemas, & Bylund, 2008), TrkB.T1 over expressing and BDNF heterozygous null mice (Saarelainen et al., 2003), neither fluoxetine, imipramine or desipramine administration elicits an antidepressant response in the forced swim test. Conversely, ECT, considered one of the most effective treatments for resistant depression causes long term and diffuse increases in BDNF protein and mRNA (Kozisek, Middlemas, & Bylund, 2008). BDNF transcriptional changes in the hippocampus are present with antidepressants fluoxetine, ECT and sleep deprivation (Conti et al., 2006) while reuptake inhibitors and MAOI antidepressants increase BDNF and TrkB mRNA (Altar, 1998). Most recently, BDNF has also been deemed critical to the antidepressant effects of low-dose intravenous ketamine, a novel therapy that has proved efficacious in TRD patients. Serum BDNF levels are positively correlated and potentially predictive of behavioural response to ketamine in TRD patients (Haile et al., 2014). Furthermore, neutralizing mPFC BDNF release during ketamine administration blocks antidepressant behaviour in the forced swim test (Lepack, Fuchikami, Dwyer, Banasr, & Duman, 2014).

The importance of BDNF in MDD is mediated through several avenues, most importantly its potent neurotrophic properties. In line with the dysregulated networks model of depression, this characteristic becomes even more compelling, as increased and decreased levels of BDNF in depressive-anxiolytic structures mediate those behaviours; for example, when administered to the ventral tegmental area or nucleus accumbens, BDNF produces depressive behaviours (Castrén & Rantamäki, 2010). Increasing plasticity via BDNF in hypoactive areas, such as the hippocampus, elicits symptomatology relief; while administration to those areas noted to act in limbic shift, such as the ventral tegmental area or
amygdala (see figure 1.6) seemingly reinforce the ventral rule of the emotive network dimorphism. Perhaps most astonishingly, a single nucleotide polymorphism (SNP) of the BDNF gene, known as Val66Met wherein a methionine is substituted for a valine at codon 66, impairs the activity dependent release of BDNF, and consequently reduces the trophic support available in the system, and has been linked to depression susceptibility and poor memory and hippocampal function in humans (Egan et al., 2003; Autry & Monteggia, 2012). This SNP has also been associated with reduced response to ketamine in TRD patients (Laje et al., 2012). This work was built upon pre-clinical research using knock-in mice that found the presence of Met was related to impaired synaptogenesis, with Met+/+ displaying severe impairments as well as no antidepressant response to ketamine in the forced swim test (Liu et al., 2012). These findings suggest the activity dependent release of BDNF is critical to antidepressant response due to its role in repairing the dysfunctional and pathological neural network present in MDD.

The other critical role of the BDNF/TrkB system in regards to MDD is the symbiotic relationship shared with serotonin. As previously mentioned, reducing BDNF/TrkB function effectively blocks the antidepressant response of established medications such as fluoxetine and ketamine. Serotonin release in response to stress is dysregulated in MDD, hence the efficacious use of SSRIs, however, in a serotonin depleted model coupled with repeated stress, GR receptor increase was inhibited and BDNF mRNA was decreased along with increased anhedonia, negative learning bias and decreased spatial memory (Zhou et al., 2008). This relationship may be based upon transportation of serotonin. Removal of BDNF function through heterozygous phenotype results in altered function of serotonin transport in the hippocampus, and may be linked to SSRI non-response (Guiard et al., 2008).

Taken together, these findings indicate that functional BDNF and TrkB are not simply neural support, but necessary and sufficient mediators of antidepressant effects, in part due to
the modification of gene expression by the BDNF-TrkB intracellular transduction pathways, which ultimately effect cell migration and survival (Angelucci et al., 2004; Kozisek, Middlemas, & Bylund, 2008). New neurons have a lower threshold for long term potentiation, presumably to expedite their integration into the neural network and thus increase the rate of survival. When BDNF levels are low, as occurs with high levels of stress, facilitation of this activity-dependent maturation will be limited and neural integration will be depressed or inappropriate (Balu & Lucki, 2008). Decreased or maladaptive integration of neurons between structures results in the establishment and maintenance of miscommunication and consequent inappropriate behaviours both in evaluating and responding to stimuli, lending to the development of mood disorders. There is also a clear indication for hippocampal BDNF-serotonin co-activation in restoring proper dorsal network function and attenuation of depression symptoms, as without proper BDNF function, many antidepressants do not have the desired effect. Through these various pathways, BDNF proves to be a critical mediator in the onset and recovery of depression and as such, exogenous modulation of this protein and its pathways presents a novel target for the development of new pharmaceutical interventions for affective disorders.

1.3.3 General Profile of Glial Cell Line Derived Neurotrophic Factor

Glial cell line derived neurotrophic factor is a member of the glial cell line derived neurotrophic factor family of ligands (GFLs), part of the transforming growth factor-β superfamily. It is a glycosylated protein characterised from the rat B49 glial cell line for its dopaminergic properties and clinical applications in Parkinson's disease (Lin, Doherty, Lile, Bektesh, & Collins, 1993). This family of ligands function by binding to respective high affinity α co-receptors (GFRα 1-4) and activating Ret transmembrane receptor tyrosine kinase via calcium influx (Airaksine & Saarma, 2002). GDNF can also function using Ret
independent pathways, via the 140kDa isoform of neural cell adhesion molecule (NCAM) co-adaptor and use of Src-family tyrosine kinase Fyn and Fak (Paratcha, Ledda, & F. Ibáñez, 2003). Like many neurotrophic factors, GFLs are secreted in a pro form known as preproGFL while the mature GFLs are known to induce transcription regulation and neuronal survival and integration via Akt (v-akt murine thymoma viral oncogene homolog 1), MAPK, PI3K, and PLCγ intracellular pathways (see figure 1.8) (Airaksinen & Saarma, 2002).

In the periphery, the role of GDNF is quite well understood. It has been found to be critical in cell migration during embryonic development in the retina, sensory and motor neurons and perhaps most notably during development of the enteric nervous system, malformation of which results in Hirschprung's disease (Newgreen & Young, 2002 as cited in Pahnke et al., 2002), while heterozygous null GDNF animal models are not viable due to drastic kidney malformation. In the adult, GDNF continues this role in the ganglia. In dorsal root ganglion neuron, cultured from young adult mice, incubation with GDNF showed an increase in lamellipodia formation, axon initiation and elongation via Src-family kinases and the preferred GDNF GFRα-1 co-receptor (Paveliev, Airaksinen, & Saarma, 2004). Similarly, application of GDNF to rat dorsal root ganglion neurons in vitro (Reid et al., 2011) and in vivo (Fine et al., 2002) were able to repair large nerve gaps. The role of GDNF in peripheral nervous system development and repair may be due, at least in part, to regulating Schwann proliferation and migration as well as myelin formation (Iwase, Jung, Bae, Zhang, & Soliven, 2005).
**Figure 1.9.** Intracellular pathways of GDNF-GFRα (circled); © 2009 QIAGEN, all rights reserved, the original image may be found at:

Within the brain, GDNF is released by both glia and neurons to perform roles similar to those within the periphery, including neural migration, activity dependent synaptogenesis, neurite outgrowth and protection from oxidative-stress. Although originally refined because of potent dopaminergic properties and subsequent therapeutic application to movement disorders, GDNF has been found to exert extensive trophic support during development and is important in the maintenance of glia, catecholaminergic and serotoninergic neurons as well as regulating noradrenergic and GABA (γ-Aminobutyric acid)-ergic pathways while also buffering against oxidative stress (Michel et al., 2008). These effects have been demonstrated in vitro and in vivo. GDNF infusion into the brain increases substantia nigra and hippocampal progenitor cell proliferation, achieving both neuronal and glial differentiation (Chen, Ai, Slevin, Maley, & Gash, 2005). Neuronal progenitor cell cultures of ST14A cells manipulated to over-express GDNF exhibited gene up-regulation for neurite outgrowth, axonal sprouting and spine formation (Alcam [Activated Leukocyte Cell Adhesion Molecule], Nrg1[neuregulin 1], EphB2 [EPH Receptor B2] and Fst [Follistatin]) while simultaneously down-regulating genes for microtubule bundling and stabilisation thereby promoting migration and differentiation of the ST14A striatal precursor cells (Pahnke et al., 2004).

More recently, intracerebral infusions of GDNF have been found to promote striatal neurogenesis post middle cerebral artery occlusion induced stroke in adult rats (Kobayashi et al., 2006), which may have implications for purported neurogenic mediated antidepressant effects (Surget et al., 2008; David et al., 2009).

Research into the role of GDNF in mental health disorders is still in its infancy, having previously been overshadowed by the efficacious applications of GDNF in movement disorders. In spite of the relative paucity of data on the subject, findings thus far are promising and echo previous reports regarding the role of neurotrophic factors in mediating effects of onset and recovery of mental health disorders. This is largely due to the immense
influence of the protein in mediating plasticity, specifically for dopaminergic signalling throughout the mesolimbic area (Bourque & Trudeau, 2000) which is believed to play an important part in the core depressive symptom, anhedonia. GDNF is also a potential key in buffering against neurotoxicity induced by dysregulated HPA axis function.

1.3.4 Glial Cell Line Derived Neurotrophic Factor and Depression

A comparative study of medicated bipolar disorder patients showed differences in blood plasma GDNF levels between patients in euthymic states compared to both healthy controls and manic patients, where GDNF levels exhibited a negatively correlated relationship with mania severity (Barbosa et al., 2011). GDNF blood plasma levels also showed significant decreases in patients with late-life depression compared to age-matched healthy controls as well as a negative correlation with depression rating scores, however medication status of subjects was not disclosed (Diniz et al., 2012). In contrast, other data support increased peripheral GDNF levels in unmedicated late-onset depression (Wang et al., 2011) as well in post mortem tissue, significantly in the parietal cortex, from individuals suffering ante-mortem depression (Michel et al., 2008). In attempt to address these discrepancies, P.T. Tseng et al (2013) analysed serum GDNF in a group of 55 mixed (31.8% unmedicated, 26 remitted) depressed patients compared to healthy control and found an age associated decrease for depressed groups only when compared to their aged matched healthy controls, furthermore the group found a significantly decreased level of serum GDNF for TRD patients compared to other depressed subjects.

Taken in context on the glucocorticoid cascade hypothesis, these data suggest it may be prudent to assess if stress has a similar effect as ageing on GDNF levels, as human data do suggest GDNF levels are reduced in the adult brain (Saavedra, Baltazar, & Duarte, 2008). In a model of accelerated ageing, using senescence accelerated mouse strains (SAMP8 and 10),
show reduced hippocampal GDNF levels, even at a young chronological age, which is believed to mediate the increased hippocampal neuronal death and learning difficulties in these animals at a later chronological age compared to normal ageing strain (Miyazaki, Okuma, Nomura, Nagashima, & Nomura, 200). It is of note that SAMP8 exhibits impaired learning and memory while SAMP10 also displays brain atrophy and depressive behaviour associated with ageing (Miyazaki, Okuma, Nomura, Nagashima, & Nomura, 200). Similar characteristics can be found in GDNF heterozygous mice which exhibit significant impairment in the Morris water maze indicating a deficit of spatial memory function, despite any measureable changes in underlying neurophysiology of midbrain dopaminergic system (Gerlai et al., 2001). Reciprocally, intrahippocampal administration of lentiviral vectors constructed with human GDNF to aged Fisher 344 rats resulted in increased synthesis of serotonin, dopamine and acetylcholine as well as cognitive improvement in Morris water maze compared to control (Pertusa et al., 2008).

Sprague Dawley rats exposed repeatedly to neonatal isolation combined with later juvenile immobilisation stress resulted in decreased mRNA levels of hippocampal GDNF suggesting early life stress may reduce the efficiency of GDNF to buffer against later life stressors (Kawano et al., 2008). Somewhat contradictorily, chronic unpredictable stress in mice resulted in increased GDNF in the hippocampus and medial prefrontal cortex as well as increased microglia and astrocyte activation in the CA3 and pre-limbic areas which contributed to behavioural despair and memory impairment (Bian et al., 2012). With 40-days of recovery, these stress-induced responses were reversed, except the GDNF levels in the hippocampus continued to increase and astrocytes were still partially hyper-activated suggesting a role for them in stress adaptation (Bian et al., 2012).

The differences in published data thus far may be the result of differences in methods and patient demographics between studies, and are quite common in molecular neuroscience.
For example, after a single GDNF infusion, immunohistochemistry was able to detect GDNF 14 days after administration, compared to enzyme-linked immunosorbent assay and high-pressure liquid chromatography (Hadaczek, Johnston, Forsayeth, & Bankiewicz, 2010). It also indicates GDNF, like many neurophysiological features of MDD, may display region and age specific changes with disease onset and reversal. This highlights the need for further investigation into the role of GDNF in depression using tightly controlled experimental in vivo procedures as the evidence strongly suggests that GDNF is important in MDD onset and symptomatology, but it is not clear exactly how at this juncture.

The role of GDNF in antidepressant efficacy is better understood. Due to previous work on neurotrophic factors, it has long been anticipated that GDNF would be up or down regulated as needed to attenuate the dysregulated networks via its trophic properties. Patients shown to exhibit significantly lower serum GDNF levels compared to healthy control gained a significant increase in GDNF levels with 8 weeks of various prescription chemical antidepressant treatments (Zhang et al., 2009). Similarly, adult male Sprague Dawley rats exposed to chronic unpredictable stress for 2 weeks and simultaneously treated chronically with the tricyclic clomipramine for a further 3 weeks showed a reversal of downregulated GDNF mRNA and protein in the CA3 and dentate gyrus of the hippocampus alongside behavioural improvements (Liu et al., 2012).

ECT was shown to increase mRNA levels of the preferential GDNF receptor, GFRα 1, in the dentate gyrus of adult male Sprague Dawley rats and this effect was enhanced with chronic ECT treatment; however, GDNF protein levels were not seen to increase and no effects were found after chronic administration of several classes of antidepressant drugs (Andrew et al., 2001). This particular work emphasizes the potential inherent flaw in generalizing data from healthy animal models to mechanisms of disease (Surget et al., 2009), as these findings may be a consequence of upregulation of several protective mechanisms in
the face of neuronal injury known to occur with ECT therapy. For example, ECT applied to treatment resistant humans did result in serum GDNF increases; furthermore these increases were 58% greater for responders compared to non-responders (Zhang et al., 2009). While the methodologies of these studies differ significantly, it does highlight the importance of using valid disease models as well as the importance of neurotrophic mediated plasticity in antidepressant response.

Investigations are currently under way to clarify the mechanisms through which GDNF acts to promote these trophic events in vivo, though mounting evidence suggests it may be due to the various signalling types utilised by GDNF, which may allow the neurotrophic factor to play a mediating role between neurons and glia. In fact, GDNF exhibits a complex variegated signalling profile that allows for an instructive role in neuron development through the unique ability to signal using membrane bound receptors as well as through long range soluble means (Ledda, Paratcha, Sandoval-Guzmán, & Ibáñez, 2007). This allows GDNF to induce trans-homophilic interactions between GFRα1 receptors resulting in a novel cell-cell interaction that promotes the formation of synapses between GFRα1 expressing neurons using both soluble and membrane bound particles (Ledda, Bieraugel, Fard, Vilar, & Paratcha, 2008; Ledda, Paratcha, Sandoval-Guzmán, & Ibáñez, 2007). Furthermore, via the NCAM mechanism, GDNF can mediate intercellular communication across short and long ranges (Paratcha et al., 2003). This is also important as disruption of trans-signaling has been implicated in cognitive disorders such as autism and schizophrenia (Dalva, McClelland, & Kayser, 2007) and may underlie some of the symptoms of depression, particularly decreased cognitive function and hippocampal atrophy.

The intimate relationship between GDNF, glia and neurons, combined with the unique signalling properties of GDNF reveals a potential protective role in buffering against the excitotoxic and neuroenergetic crisis that arise with stress. Substantia nigra neuron-glia
mixed cell cultures exposed to mild oxidative stress injury exhibited a concentration dependent up-regulation of GDNF triggered by soluble signaling of neurons to astrocytes implicating a powerful role for neuron-glia crosstalk (Saavedra, Graça, Paulo, Caetana, & Emília, 2006). Similarly, organotypic hippocampal slices exposed to NMDA (N-methyl-D-aspartate) induced excitotoxicity in concert with GDNF exhibited neuroprotection in CA1 and more extensively CA3 regions through increased Ret phosphorylation in microglial cells (Boscia et al., 2009).

The culmination of these signals is ultimately the transcription of GDNF, which has been shown to be induced by several antidepressant therapies utilizing multiple intracellular pathways, including MAPK, and several transcription factors, such as CREB and NK-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), and activation of specific GDNF promoter regions (Golan, Schreiber, & Avissar, 2011; Hisaoka et al., 2001, 2007; Kim et al., 2011). Perhaps most exceptionally is the responsiveness of GDNF to neuronal injury induced by oxidative stress, excitotoxicity and inflammatory responses (Tanabe, Nishimura, Dohi, & Kozawa, 2009) which utilize the same pathways as antidepressants to buffer against neuronal damage. The novel role this protein plays in cell migration, neurite outgrowth, synaptic strengthening and neural protection allows for both permissive and instructive influence on plasticity warranting extensive research into the function of GDNF in the MDD. Principally, the relationship between GDNF and dopamine in mediating mesolimbic dopamine levels that are responsible for reward and pleasure has the potential to provide keen insight into the mechanisms underlying anhedonia, while simultaneously, possibly shedding light on the negative bias of cognition and attentional shift exhibited in depression.
**1.4 Summary and Specific Aims**

Major depressive disorder is a debilitating and widespread mental health disorder with a complex heterogeneous aetiology and pathophysiology that remains incompletely understood. The paucity of insight into the physiology of MDD is due, in part, to the dominance of the monoamine paradigm, which governed both modelling and treatment of the disease state, creating a tautological closed loop of knowledge. The long standing inconsistencies of therapeutic lag time and non-response by a considerable number of patients coupled with advances in real-time *in vivo* technologies were the primary influences prompting the dysregulated networks and neurotrophic hypotheses of depression. Together, these hypotheses extend the monoamine hypothesis into a more complete systems wide model of depression, wherein the cumulative output of hypo-active “positive motivational dorsal area” and hyper-active “negative stress ventral area” (Stone, Lin, & Quartermain, 2008) can culminate in a negative shift in the neural network in pro-depressive situations. This shift disrupts the adaptive mechanisms aimed at maintaining allostasis through neuroendocrine and cognitive processes leading to maladaptive and depressive behaviours in susceptible individuals. This shift may be subserved by plasticity in that reduced plasticity could both prohibit an individual from adapting and coping to depressogenic stimuli and prevent an individual executing the necessary remodelling that exerts therapeutic benefit of monoamine therapies.

The work presented here will build on the existing literature and contribute to the current knowledge of mechanisms mediating antidepressant treatment resistance in depression as well as the role of HPA-axis dysfunction in contributing to antidepressant resistance. All studies have used a modified version of the 14 day ACTH paradigm, established to block behavioral responses to tricyclic antidepressant compounds which act predominantly via serotonergic and noradrenergic mechanisms (Kitamura, Araki, & Gomita,
This work herein will serve to confirm the replicability of this model as well as investigate related phenotypic features of depression, including memory function. Peripheral corticosterone levels were also measured in order to quantify the effect of the ACTH administration on overall HPA axis reactivity. Also, to the best of our knowledge, this work is the first to use IL-DBS as a mode of antidepressant treatment in this ACTH model.

The first study will assess the effects of the ACTH paradigm on levels of BDNF in the dorsal and ventral hippocampus. As hippocampal BDNF is largely believed to decrease as a result of depression, this will both speak to the validity of the model as one of depression and evaluate the role of hippocampal BDNF in mediating antidepressant resistance. The second study will assess the effect of the ACTH paradigm and DBS on hippocampal memory in the rat. This will again inform on the validity of this animal model as one of depression, as diminished memory function is a core diagnostic criteria of MDD while also addressing lingering ethical issues associated with the potential of DBS to diminish or enhance cognitive function. The third study will be the first known assessment of GDNF levels in the dorsal and ventral hippocampus for both the ACTH paradigm and DBS application. This work will expand the neurotrophic hypothesis of depression and potentially identify a novel mechanism of DBS antidepressant efficacy. As the second and third studies were conducted using the same cohort of animals, GDNF levels can also be correlated with memory performance and FST behaviours. The fourth study will assess the effects of the ACTH model and DBS on mRNA gene expression within the neurotrophic signalling pathway of the prefrontal cortex. Genes of interest will be confirmed at the protein level via western blot in the fifth and final study which will use the ACTH pre-treatment paradigm in combination with DBS, sham, ACTH only and untreated (saline) animals. Limitations and future directions are also discussed.


hippocampus is associated with improved spatial memory and enriched environment. *Neuroscience Letters, 138*(1), 153-156. doi: 10.1016/0304-3940(92)90494-r


Coupling in Treatment Resistant Depression. Brain Stimul, 8(6), 1033-1042. doi: 10.1016/j.brs.2015.06.010


Chapter II: Methodology

2.1 Animals

Adult male Sprague Dawley rats were used for 3 experiments and adult male Wistar rats were used for one experiment. All animals were aged 5-10 weeks throughout the course of experimental protocols.

2.2 Stereotaxic Surgery

The basic principles for executing stereotaxic surgery in animals are similar to those utilised in human neurosurgery. 3-D coordinates obtained from a neuroanatomical atlas (Paxinos & Watson, 2007) allow for electrodes to be positioned accurately in distinct brain nuclei. Animals allocated to sham and high frequency DBS experimental groups received identical procedures for electrode implantation into the infralimbic cortex, the homologue of structure BA25 (Drevets et al., 2008; Ongur et al., 2003) targeted in human DBS trials for TRD.

Throughout surgery, the animal was situated on a thermal heating pad and core temperature was monitored using a rectal thermometer. Isotonic saline, sterile gauze and swabs were used to clean the surgical area as needed. Firstly, animals received a pre-anaesthetic sedation of buprenorphine (0.3mg/ml 1:10) and acepromazine (2mg/kg) injected subcutaneously. The animal was then placed into an isoflurane induction chamber until surgical depth of anaesthesia was attained, as verified using pinch reflexes.

Once fully anaesthetised, hair was removed from the surgical site using stock standard clippers, and the area sterilised with iodine. The animal was then positioned into the Kopf Stereotaxic instrument (David Kopf Instruments, Tujunga, CA USA) such that the surgical plane was immobile and level. Once secured in the stereotax, a small animal mask (Kopf
anaesthesia mask, model 906) was fixed to the stereotax delivering oxygen and isoflurane directly to the snout of the animal to maintain a surgical depth of anaesthesia. Lubricant was applied to the eyes and the rectal thermometer was inserted (Bat-10 thermometer, Physite mp). Core body temperature was assessed at 5-minute intervals throughout the surgical procedure.

The initial incision was made beginning slightly behind the eyes and continued posterior to a line level with the back of the ears (see figure 2.1, A). Skin was pulled aside and restrained using expansion clips, leaving a small area of skull exposed (see figure 2.1, B). To minimize the potential for undue tissue damage during subsequent drilling, subcutaneous tissue was gently removed from the skull surface using the scalpel. Bleeding was allowed to subside before surgery was continued; a cauterizer was used to assist this if needed.

Once the site had been cleaned, the location of the electrode (PlasticsOne Roanoke, VA, USA) and four 2mm long screws was determined. The site of electrode implantation was determined first from Bregma using coordinates AP: +/- 3.0, ML: +/- 0.4, DV:-5.6 (Paxinos & Watson, 2007) and marked using a cauterizer or sterile marker. Placement of screws was determined secondly, so as not to interfere with the placement of the electrode. Screws were 0.5mm in diameter and served to act as scaffolding for the dental cement that would hold the electrode in place.

Two screws were placed anterior to the position of the electrode, and two posterior. Each screw was inserted by pre-drilling a shallow groove into the skull, and then firmly manually inserted without touching the dura of the brain. Once the 4 scaffolding screws were in place, a 1.0mm hole was drilled into the skull at the pre-determined site for the electrode, such that the piece of bone was easily removed and the dura was not disrupted. Dura was carefully punctured using a sterile needle or other appropriate surgical instrument, such as sharp tweezers. This method greatly reduced the propensity of undue damage or bleeding,
optimizing the function of the electrode and safety of the animal. The electrode was positioned and slowly lowered to the site of the infralimbic cortex (IL) at a depth of 5.6mm ventral to the surface of the exposed brain (see figure 2.1, C).

The electrode and 4 scaffolding screws were then secured into place using 2 part acrylic cement. The cement was applied such that a smooth contour was created covering the screws and leaving approximately 1mm of the electrode exposed to allow for attachment to the pulse generator. This ensured a stable mount for the electrode and also prohibits the removal of the electrode and screws by the animal and its cage mate. Once the cement cured, approximately 5 minutes, the incision was closed around the fibreglass implant using simple interrupted sutures. The animal was then removed from the stereotax and fluids and pain relief (carprofen, 5mg/kg) administered. Animals received 5 days of post-operative care with additional analgesic administered as needed. Inclusion in study protocol was thereafter assessed on body condition, mobility, appetite and responsiveness. Animals were not permitted to begin study protocol unless good health was achieved. Stimulation was conducted using the AMPI / Master-9 system, which was attached via the ISO-Flex Stimulus (A.M.P.I., Jeruselum, Israel) to the implanted electrode of the singly housed animal for time dictated by specific experimental protocol.
Figure 2.1. Schematic of surgery for electrode implantation into the infralimbic cortex.

A: The incision is made using a sterile scalpel, beginning approximately 2-3mm behind the most posterior level of the eyes, continuing posteriorly to a level approximately equal with the base of the ears. B: Tissue is gently pulled back and held in place with expansion clips. The area is cleaned and cleared of subcutaneous tissue that. C: An illustration of scaffolding screws and electrode in place, image not to scale (Paxinos & Watson, 2007). D. Image of bilateral electrode placement, N.B. area is non-specific, but close to lateral habenula.
2.3 Behavioural Testing

The use of animal models in neuropsychological research allows for the assessment of particular symptoms of a disease within a controlled environment. While it is impossible for a rat to display a comprehensive profile of MDD symptoms simultaneously, it is possible to induce distinct depressive-like behaviours reflective of particular symptoms present in the human condition. Several behavioural assays have been developed to measure a particular depressive-like behaviour. The assays utilised for the following work included: the Porsolt forced swim test (FST), T-maze test (T-maze), open field test (OFT) and novel object preference test (NOP).

2.3.1 Forced Swim Test

The Forced Swim Test, also known as the Porsolt test and behavioural despair test, was developed specifically to address the lack of animal models available to evaluate clinically effective antidepressants (Porsolt et al., 1977). The model, though often criticized as being overly anthropomorphic, displays pharmacological predictive validity making it the primary screening tool in use for the evaluation of clinical applications of novel antidepressant agents (Castagné et al., 2011) and it has been extensively validated (Porsolt et al., 1977; Kitamura, Araki, & Gomita, 2002). As a testament to its validity, the development of this test was based in behaviour rather than biochemistry (Porsolt et al., 1977). The behavioural underpinnings of this test have since been shown to correlate with the neurological structures and biochemistry underlying depressive behaviour and antidepressant efficacy.

The crux of this experimental procedure relies on exposing the animal to 2 inescapable swim sessions. The first habituation session acquaints the animal to a novel and moderately stressful situation from which it learns there is no escape. The second test session
occurs 24 hours after habituation and allows for the evaluation of therapeutic agents in this moderately stressful condition, without the confounding factor of novelty. It is thought that the animal learns to conserve energy in the first swim session by adapting a passive coping technique of immobility, similar to the lethargy exhibited by individuals suffering from depression. Therefore animals exhibiting increased immobility may be said to be more passive in their behavioural coping strategy, a condition often interpreted as behavioural despair. This is in contrast to those who exhibit more active behaviours, indicative of their continued attempt to escape (i.e. swimming, climbing or diving).

For the habituation session of the studies presented herein, animals were placed in the testing room for a minimum of one hour prior to commencement of the testing protocol. Two tall clear plexiglass cylinders (60.5cm in height, 20cm diameter on a base measuring 30 x 30cm) were filled to a depth of 35cm with 25°C +/- 1°C clean tap water. As image capturing software was used (CleverSys Forced Swim Scan; Reston, VA USA) cylinders were situated in front of a blue background with a divider allowing two animals to undergo the sessions simultaneously (see figure 2.2). This set-up ensured optimal colour contrast would be readily visible to the analysis software and also allowed for animal groups to be counterbalanced throughout testing. Animals were placed gently in the water, hind quarters first, and allowed to swim for 10 minutes after which they were removed, dried with a clean cloth towel and placed under a warming lamp.
Figure 2.2. Forced swim test apparatus still (left) and as seen through Cleversys analysis software (right). The blue board allowed for optimal colour contrast recognition by the software program, thereby increasing accuracy. This set-up also provided the ability to run two animals through the test at one time, increasing the counterbalance of groups.

The test phase of the FST occurred 24 hours after habituation. In this study, the antidepressant effects of the chemical tricyclic antidepressant imipramine and high frequency DBS were evaluated by using immobility time as a measure of behavioural despair. To assess the role of the tricyclic antidepressant, control and ACTH animals received 10mg/kg imipramine hydrochloride (Sigma-Aldrich, NSW, AUS) i.p. 30 minutes prior to the test swim session. This ensured that the agent was biologically active during the test. Similarly, to examine the role of stimulation, animals receiving DBS underwent the test swim within 5 minutes of ceasing stimulation. Animals in the SDBS group received no extra treatment prior to the test session. The test sessions were conducted using identical parameters as the habituation session, except animals swam for only 6 minutes.
2.3.2 T-Maze Test

The T-maze test is used to investigate various aspects of memory in rodents, including spatial working memory, reference memory and spontaneous alternation (Wenk, 1998). For the purposes of this study, the rewarded alternation protocol was used to assess spatial memory. While this test is robust for the assessment of spatial working memory (Wenk, 1998; Deacon & Rawlins, 2006; Hock & Bunsey, 1998), it must be stated that the complexity of assessing spatial working memory inevitably results in the use of reference memory as well. However, it can be presumed that the reference memory is incorporated into the more critical spatial working memory to attain the reward for this task and thus achieve the goal of assessing spatial working memory (Brito & Thomas, 1981). Due to the necessity of learning inherent in this test, all forms of memory utilized represent long-term memory and thus rely on the proper encoding of information by the hippocampus for storage and recall.

Rats employ three strategies simultaneously and to varying degrees to navigate spatial tasks: 1) place, which utilizes remote sensory information; 2) cue, which utilizes proximal sensory information and; 3) response wherein the organism relies on motor sequences unrelated to sensory information (Wenk, 1998). Bearing this in mind, care was taken to ensure that each trial was conducted with identical distal and proximal cues, and the maze was oriented in the same position in relation to these cues for all animals. The total protocol for the T-maze was 5 days. Animals underwent food rationing during the wake cycle prior to beginning training for the T-maze and throughout the 5 days of T-maze training and testing. Water remained available *ad libitum*. To avoid hyperneophagia, animals were introduced to the reward (Kellog’s Froot Loops™) 5 and 2 days prior to beginning the T-maze by adding a few pieces of the cereal to the home cages before the wake cycle.

The apparatus (see figure 2.3) was based on previously published protocol (Deacon & Rawlins, 2006), composed of medium-density fibreboard. To encourage the animals to feel
comfortable while running the task, the apparatus was painted a dark grey and the testing room was dimly lit to resemble the preferred low light environment of the rodent. Small saucers were placed at the end of each goal arm, where the food reward was to be consumed. The saucers were held in place with tape.

Prior to commencing learning on day 1, animals were allowed to freely explore the maze in cage pairs for 3 ten minute sessions to reduce anxiety of the unfamiliar environment. Reward food was placed randomly throughout the maze for the first 10 minute session, then only in the goal arms for the second free exploration and finally only in the reward saucers for the third exploration, being replenished when emptied. Once animals were acclimatized to the maze environment, learning trials began. Over 5 days, each animal ran 25 learning sessions, 5 sessions per day. Each learning session consisted of 3 left and 3 right turns for a total of 6 turns. The first turn changed for each trial so the animal did not learn a pattern of turns. During learning, both saucers contained a reward at all times to control for olfactory senses. The maze was cleared of urine and faeces, but not sanitized between animals as olfaction has not been found to be a confounding factor (Deacon & Rawlins, 2006).

At the beginning of a learning session, the desired goal arm was left open by removing the guillotine door, and the other goal arm was left blocked, hence forcing the animal to choose an arm. The animal was placed in the start arm and given no more than 2 minutes to complete the task. This time was shortened to 30 seconds by day 5. The animal was always allowed to consume the reward if it completed the task. If the animal did not complete the task, it was returned to the home cage and allowed to rest, as this failure is most often considered a consequence of anxiety.

As the animals learned the task, the time between trials decreased, all practicable efforts were made to keep inter-trial interval similar between animals. This was achieved by running the animals through the maze randomly; e.g. slower animals running between faster
animals, or several fast animals running successively, such that there was the same amount of
time between trials for all animals, ideally a minimum of 10 minutes.

On the test day, day 5, two additional turns were added to each run to assess whether
the animal had learned the task. The animal ran the appropriate 6 right and left turns, then
was replaced in the start arm for a seventh forced choice turn, which was the same as the 6th
turn. If the animal completed this run, all guillotine doors were removed, but only the correct
choice arm, the opposite of turns 6 and 7, was baited. To control for olfactory senses, a
reward was placed outside of the maze at the other goal arm. The animal was placed back in
the start arm for turn 8; if the animal had learned the task, it would know that the opposite
turn from the previous two turns would garner a food reward. If the correct choice was made,
the animal was allowed to consume the reward, if the incorrect choice was made, the
guillotine door was carefully replaced, and the animal was allowed to explore the empty
saucer.
Figure 2.3. Schematic of T-maze apparatus and protocol for a left-start test run. Blue boxes denote reward saucer at the end of each goal arm, the start arm is perpendicular and centre to the goal arms. Blue lines represent placement of guillotine doors. All measurements are in centimeters. Image not to scale.

2.3.3 Open Field Test

The open field test served to examine locomotor activity as well as exploratory and anxiety behaviours in test animals. This test was conducted using a large plexiglass box (50cm x 50cm x 40cm) the sides of which were painted black. Animals were acclimatised to the testing room for a minimum of one hour prior to testing. The floor of the open field was
comprised of clear plexiglass and was demarcated into 16 equally sized squares using a black permanent marker (see figure 2.4). To assist with analysis, the bottom of the apparatus was covered with white paper on the external side which also served to provide a consistent colour and texture across testing sessions. Animals were permitted to freely explore the apparatus for 10 minutes, during which behaviour was recorded using a video camera, positioned midway between corners C and D of side 3. The simplicity of the design, having a space that is easily divisible into equally measureable dimensions and comprising both open and closed areas, combined with the novelty of exposure to this environment makes this test ideal for measuring movement, exploration and anxiety in rodents.

To measure the behaviours of interest, animals were placed into the periphery of the arena, with the head facing towards side 3, midway between corners C and D and allowed to explore the apparatus. The apparatus was cleaned using an 80% ethanol solution between each animal to avoid biasing activity with olfactory cues. Locomotor activity was quantified by measuring ambulation, operationally defined as the animal deliberately moving 2 or more paws into an adjacent square. Propensity toward exploration was evaluated by counting the number of rears the animal performed. A rear was considered any action where the animal was supporting its weight on the hind legs, and the body was elevated at an angle of at least 45 degrees above the arena floor. Behaviour where the animal was parallel to an outside wall to engage with the environment beyond the arena; i.e. the test room, was also counted as exploratory. Anxiety was measured as the amount of time, in seconds, spent in the central 4 squares of the arena (see red area, figure 2.4) as opposed to the 12 peripheral squares. For analysis, videos were de-identified, and the first 6 minutes coded manually in 2-second intervals.
2.3.4 Novel Object Preference Test

The novel object preference (NOP) test is widely used to assess recognition memory, a type of declarative memory that is believed to rely on the hippocampal formation. The test takes advantage of the innate desire of the rodent to explore unfamiliar items, and is sometimes referred to as spontaneous recognition (Mumby, 2001) and therefore requires essentially no training or aversive stimuli for effective assessment. The protocol used here was a one-trial non-matching-to-sample task measuring visual object recognition (Bevins & Besheer, 2006).

The test was carried out using the apparatus described in section 2.3.3, the open field test, and various children’s toys (see figure 2.5). All animals underwent the open field test 24-hours prior to the novel object preference test which served to familiarise subjects to the
testing apparatus and conditions. On the day of the NOP, animals were placed in the testing room for a minimum of one hour prior to implementing protocol. The test was conducted in low light for the comfort of the animal. The apparatus and all objects were sanitised between animals.

**Figure 2.5.** Novel object preference test objects. Left: All objects. Right: Samples of object combinations used as a novel objects in phase 2 of the test.

The first phase of this test required the animal freely explore two identical objects within the apparatus for 10 minutes (see figure 2.6, left). The animal was placed midway between the corners of the apparatus on the side opposite the objects and facing away from the objects. Objects were therefore positioned in the back corners of the apparatus with respect to the placement of the animal. Objects were unfixed to allow the animals to fully interact with them. Every practicable effort was made to counterbalance these sample objects for texture, complexity and colour between all animals. After the animal completed this phase of the test, they were replaced in the home cage for the inter-trial interval of one hour. The home cage remained in the testing room for the duration of this interval. The apparatus was cleaned using 80% ethanol, and objects were soaked in warm soapy water, then rinsed with distilled water and dried prior to being used again to avoid olfactory influences.
The second phase of this test (see figure 2.6, right) commenced at the end of the one hour inter-trial interval. The animal was returned to the apparatus as in the first phase and permitted to freely explore a familiar object from phase one and a novel object for 5 minutes. Placement of the novel object was equally distributed between left and right to avoid the introduction of a side preference and every practicable effort was made to counterbalance the novel objects for texture, complexity and colour between all animals. Object recognition was evaluated by comparing the time, in seconds, the animal spent with the familiar object versus time spent with the novel object.

**Figure 2.6.** Schematic of novel object preference test. Left: In phase 1, the animal was placed in the apparatus facing away from two identical objects and allowed to explore uninterrupted for 10 minutes, Right: In phase 2, one hour after phase 1, the animal was placed in the apparatus facing away from objects, one of which was encountered in phase 1 and one unfamiliar object, and allowed to explore uninterrupted for 5 minutes.
2.4 Brain Dissection

Animals were humanely euthanized and tissue samples collected at time of death and frozen in a shallow bath of liquid nitrogen after which all tissues were kept at -80°C storage until used for analysis. To attain desired structures, tissue samples were removed from -80°C storage, and kept on dry ice, until ready for dissection, at which time the brain was slightly thawed on regular ice. By allowing the tissue to slightly reduce in temperature, more precise cuts were able to be made, and the risk of fracturing the whole brain was reduced. Dissection was completed on a sterilised metal plate over ice to ensure a sufficiently cold work surface.

2.4.1 InfraLimbic, Nucleus Accumbens and Hippocampus Removal

The first cut was to remove the most anterior 2mm of prefrontal cortex and olfactory bulbs using a sterilised straight razor to make a clean coronal slice. This tissue was discarded. Thereafter a 1mm coronal slice was taken and divided into left and right hemispheres (see figure 2.8, A and C top). The pre and infra limbic cortex were demarcated as being medial to the forceps minor of the corpus colosum (fmi) and dorsal to the beginning of the anterior commissure, which present as whitish bundles. The left and right infralimbic cortex (IL) were cut out, being careful to delineate the IL as medial to the most ventral third approximation of the fmi, then placed in an eppendorf tube and stored in dry ice for the remainder of the dissection, after which they were stored at -20°C overnight and analysed the next day.

Another 1mm slice was taken exposing the nucleus accumbens. This structure was removed by cutting a square sample of brain from around, and including, the anterior commissure which was easily visible as a circular white bundle (see figure 2.7, B and C bottom). Any remaining cortex was removed from this square cut, and samples were stored by hemisphere in an eppendorf tube on dry ice during continued dissection, after which they were stored at -20°C overnight and analysed the next day.
Figure 2.7. Schematic of tissue removal. A: Photograph of brain extracted from adult male Sprague Dawley rat. Area anterior to solid blue lines denote location of coronal slice for waste tissue; dashed black lines denote location of coronal slice for structure collection; markings are not to scale. From anterior to posterior, the first dashed line is a representation of the location of the prelimbic and infralimbic cortex, the second dashed line represents the location of the nucleus accumbens. B: Representative slice showing the right hemisphere nucleus accumbens, denoted by the black box. C: Top: Schematic illustrations of the location of the prelimbic (light blue) and infralimbic cortices (purple) Bottom: The nucleus accumbens (pink) surrounding the anterior commissure (yellow) (Paxinos & Watson, 2007).
At this point the brain was placed on the dorsal side and the hypothalamus was removed by gently scooping the entire structure away from the ventral surface of the brain with curved tweezers. Then the temporal lobes were gently pushed aside by using curved tweezers. Once the temporal lobes were disarticulated from the deeper structures, they were securely held with tweezers and simultaneously, curved tweezers were also placed around the hindbrain and gently pulled in a posterior direction allowing for the removal of the rhombencephalon (see figure 2.8A). The hindbrain was then wrapped in aluminium foil and replaced on dry ice. Excess white matter was gently removed, and the hippocampal structure teased up and away from the temporal surface to be separated (see figure 2.8, B-D).

Once separated, the hippocampal structure was laid out in a horizontal line, divided evenly into left and right hemisphere sections, which were again evenly divided into dorsal (medial) and ventral (lateral) sections (see figure 2.8E). Dorsal and ventral structures were stored by hemisphere on dry ice; the remaining temporal cortex was placed in an eppendorf tube and replaced on dry ice. All unused tissue sections were stored at -80°C, and the hippocampal sections were stored at -20°C overnight and analysed the next day.
Figure 2.8. Hippocampus removal for tissue analysis. A: Pictorial schematic of lateral lobes and hind brain disarticulated from whole brain to allow access to hippocampus. B-D: Photographs of hippocampal removal; B: The hippocampus (outlined in red) prior to being gently rolled away from underlying white matter. C: The hippocampus, completely removed from underlying white matter. D: The complete structure of the hippocampus, prior to separation into left and right hemisphere and dorsal and ventral structures. E: Schematic of hippocampus in whole brain; top: lateral sagittal perspective where vertical line represents cut made to separate left and right hemispheres of hippocampal structure, horizontal line represents cut made to separate dorsal and ventral hippocampus. bottom: coronal perspective of hippocampus in intact brain; vertical line represents cut to separate left and right hemispheres of hippocampal structure, slanted lines represents cut to separate dorsal and ventral hippocampus.
Within the dysregulated networks model of depression the hippocampus is functionally segregated into dorsal and ventral components. Many studies in animals have shown the dorsal hippocampus is utilised for spatial tasks, containing a larger number of place cells in comparison to the ventral hippocampus (Jung et al., 1994; Hock & Bunsey, 1998). Historically, delineations of the hippocampus into dorsal-cognitive and ventral-emotive were based on anatomical proximity to structures of similar function, and this has since gained support from behavioural data, functional neuroscience and genetics. Gene expression has by far supported the preferential role of the septal area in learning and memory of navigation, locomotion and exploration, while the ventral is wired for emotional and motivational behaviour, having established reciprocal connections with neuroendocrine, fear and reward circuitry (Fanselow & Dong, 2010). Perhaps most astonishing is the recently identified intermediate zone of the hippocampus, which as the name suggests, lies between the dorsal and ventral poles acting to translate cognitive and spatial information into motivation and action befitting organism survival (Bast et al., 2009 as cited in Fanselow & Dong, 2010).

As dissections were done by hand and eye, care was taken to maintain consistency between samples. Animals that received DBS, sham or active, were discluded from sample analysis if the target was visibly off mark, which is easily visible as shown in Figure 2.1, D.

2.5 Biochemical Protein Analyses

2.5.1 Western Blot Procedure

Protein immunoblotting is the process of using an antibody to detect and then visualise its antigen in tissue. For the brain tissue utilised in these studies, the technique first required that proteins be released from the cells through homogenization. This homogenized sample is then forced through a polyacrylamide gel by an electric current which causes the
proteins to become stuck within the gel, with larger proteins separating first and smallest proteins last. These proteins are then blotted onto a nitrocellulose or polyvinylidene membrane, again by applying an electric current, so that enzyme labeled antibodies can be applied that will attach to the protein of interest. This antibody can be tagged with a secondary antibody that reacts to a visualisation process, most commonly chemiluminescent application or x-ray. For these visualization techniques, specifically developed software is used to quantify the protein level based on absorbency output of the wavelength or pixel density.

2.5.2 Plasma Corticosterone and GDNF ELISA

Enzyme Linked Immunosorbent Assay (ELISA) utilises a similar principle to that of the antibody detection and absorbency output quantification of the western blot, but was developed from earlier radioimmunoassay principles (Yalow & Berson, 1960; Lequin, 2005). ELISAs offer a more streamlined process of protein quantitation through direct, indirect, sandwich, competitive and multiple and portable techniques. In this work, a competitive ELISA was used to assess corticosterone levels, while a sandwich ELISA was used for GDNF.

The competitive corticosterone ELISA used in the present work (Assay Max Corticosterone ELISA Kit AssayPro, Saint Charles, MI USA) utilised a 96-well assay plate that is pre-coated with a polyclonal antibody specific to corticosterone. Biotinylated corticosterone is combined with the sample, so that the primary unlabeled antibody is incubated with the sample antigen, this is the antibody-antigen complex. This complex is then added to the plate, which is pre-coated with the same antigen. The antibody competes to bind with the sample antigen and the pre-coated antigen, such that the more antigen in the sample, the fewer antibodies available to bind with the antibody in the well. Unbound antibody is then
washed away, and remaining antibody is bound to an enzyme linked secondary antibody which is detected by adding a chromogenic developer that is subsequently measured in a specially developed absorbency output reader to give the quantity of corticosterone in each sample as compared to a standard curve.

The GDNF sandwich ELISA utilised in the present work (EK0363, Insight Genomics, Falls Church, VA, USA) also used a 96-well plate pre-coated with a known quantity of GDNF antibodies polyclonal to rat, which in turn served as the “capture” antibody. The sample was then added, and the antigen, GDNF, became bound to this capture antibody. Another antibody was then added to the sample to bind the antigen. An enzyme linked secondary antibody was used for chromogenic absorbency output detection to determine the amount of GDNF in each sample as compared to a standard curve.

2.6 Data Analysis

Data were analysed and plotted using Statistical Package for the Social Sciences (SPSS) 20.0 (IBM Corp., Armonk, NY USA) and GraphPad Prism 5.04 (La Jolla, CA USA). Statistical techniques were chosen based on the nature of the data and the question investigated, as well as the specific requirements and assumptions of each particular test. Details of specific tests used are outlined in each chapter. Results were considered significant if the alpha level was found to be equal to or less than 0.05 \( (p \leq .05) \), unless otherwise stated.
Works Cited


Wenk, G. L. (2001). Assessment of spatial memory using the T maze. *Current Protocols In Neuroscience / Editorial Board, Jacqueline N. Crawley ... [Et Al.], Chapter 8, 8.5B.*
Chapter III: Effect of Adrenocorticotropic Hormone on Brain Derived Neurotrophic Factor Levels in the Dorsal and Ventral Hippocampus
Abstract

Major depressive disorder (MDD) is well established as a stress-related psychiatric illness. Many aspects of the physiological stress response are mediated through the hippocampus, and this often manifests as decreased cognitive capacity in MDD. Recently, the neurotrophic hypothesis of depression supports a role for increased neuroprotective effects mediated by brain derived neurotrophic factor (BDNF) in buffering against stress and thereby preventing the onset of MDD. BDNF has also been shown to moderate many antidepressant therapies suggesting a potential role for this neurotrophic factor in antidepressant resistance. To assess this, Sprague-Dawley rats were treated with daily injections of either saline (0.9%) or ACTH (100μg) for 14 days. On the fifteenth day, animals were euthanized with pentobarbitone and brains were immediately removed, snap frozen in liquid nitrogen and stored at -80°C. The dorsal and ventral hippocampus were later dissected over ice and BDNF and its receptor tropomyosin kinase B (TrkB) protein levels quantified by western blot. Significant differences in BDNF and TrkB were not observed between chronically ACTH-treated relative to saline treated animals.
Treatment resistant depression (TRD) is a distressing affective mood disorder that arises in approximately 30% of those suffering from major depressive disorder (Hindmarch, 2001; Ressler & Mayberg, 2007). Activation of the stress response, both acutely and chronically, is a known predecessor to the onset and maintenance of depressive disorders (Sapolsky et al., 1986; Hauger, Risbrough, Oakley, Olivares-Reyes, & Dautzenberg, 2009). This response is mediated by glucocorticoid release from the hypothalamic pituitary adrenal (HPA) axis which redirects energy and blood flow to the muscles of the body, enabling an organism to evade or subdue a potentially life threatening situation. The same chemical cascade can simultaneously reduce levels of neurotrophic factors (Duman & Monteggia, 2006) and while this relationship is not completely understood, a role for neurotrophic factors in stress, depression and the antidepressant response has been demonstrated (Duman & Monteggia, 2006; Kozisek, Middlemas, & Bylund, 2008; Saarelainen et al., 2003).

This is especially true of brain derived neurotrophic factor (BDNF), which has an intricate relationship with the HPA axis, being both influenced by and able to influence this stress system (Hansson et al., 2000; Schaaf et al., 1999; Naert, Ixart, Maurice, Tapia-Arancibia, & Givalois, 2011). BDNF is released in pro-form, and preferentially binds to the full length tropomycin kinase receptor B (FL_TrkB) which becomes phosphorylated (pTrkb) and activates an intracellular cascade for neuronal survival and cell proliferation. BDNF seems to be necessary to attain antidepressant effects for several therapeutic agents. In line with this, intrahippocampal administration of BDNF is able to elicit an antidepressant response (Shirayama et al., 2002), while BDNF- knockout mice are antidepressant resistant (Autry & Monteggia, 2012). Theory suggests the BDNF-TrkB system may be mediating these antidepressant effects by aiding in structural plasticity as well as neuronal and glial cell survival and integration (Pittenger & Duman, 2008; Helen S. Mayberg, 1997; Holtzheimer & Mayberg, 2011).
The hippocampus is the primary neurogenic seat of the adult brain, a regulator of neurotrophic factor mediated plasticity and the primary source of inhibitory feedback to the HPA axis. BDNF is highly concentrated in this brain structure, and exhibits a decreased profile with depression that is recovered with antidepressant response (Shirayama et al., 2002; Fiore, Angelucci, Aloe, Iannitelli, & Korf, 2003; Angelucci et al., 2004). The hippocampal structure consistently shows reduced volume with stress (Bremner et al., 2000) as well as in active depression (Shou-Sen, Shu-hong, Bang-ping, Fang, & Zun-Ling, 2010; Duman, 2004). This is believed to result from chronically elevated levels of circulating glucocorticoids and the implications of this are evidenced by the observed decline in cognitive function that is common to chronic mood disorders (McEwen, 1995). Furthermore, as a component of the limbic system, the hippocampus is strongly correlated with mood states, being functionally segmented, with the ventral subregion implicated in regulation of motivation and emotion, and the dorsal sector governing navigation and locomotion (Fanselow & Dong, 2009).

These data suggest that hippocampal levels of BDNF and its receptor TrkB may be critical in mediating depression and antidepressant response under chronic or severe acute stress conditions which increase circulating glucocorticoids. To determine the effects of elevated stress hormones on hippocampal BDNF and TrkB levels, we have investigated changes in these proteins using an acute ACTH exposure, chronically administered during circadian nadir over 14 days. ACTH acts to stimulate HPA axis activation, hereby mimicking a state of repeated exposure to an acute stressor. This model has been shown to result in an antidepressant resistant phenotype (Kitamura, Araki, & Gomita, 2002a, 2002b; Walker et al, 2013). The changes were measured in a behaviourally naïve animal model to avoid the influence of exogenous environmental stress on the neurotrophic factor levels and assess effects of the model on BDNF-TrkB directly. As such, this work will also serve to further...
assess the validity of the chronic ACTH model as an animal model of depression (Kitamura, Araki, & Gomita, 2002).

Methods

3.2.1 Animals

Male Sprague Dawley rats, aged 6-8 through the duration of experimental protocol, were communally housed 2 per cage under a 12 hour light/dark cycle (lights on at 7:00am) with food and water available ad libitum. Animals were permitted to acclimatise to the facility for seven days prior to initiation of the experimental protocol, during which time all animals were randomly allocated to either the control or ACTH-treated experimental groups. All procedures were approved by Deakin University Animal Ethics Committee.

Animals received one injection per day for 14 days of 100μg ACTH-(1-24) (Sigma, AnaSpec, California USA) dissolved in biological saline or 0.09% saline vehicle as control (Walker et al, 2013; Kitamura, Araki, & Gomita, 2002). Injections were administered intraperitoneally (i.p.) at approximately 10:00 am, at a point of circadian trough for this species. Study protocol is outlined in Appendix A.

On day 15 of the protocol, animals were humanely killed via pentobarbitone (Virbac, NSW, Australia) overdose and brain tissue extracted. The brain was immediately rinsed with ice cold distilled water, wrapped in foil and placed in liquid nitrogen. All tissue was stored at -80°C until prepared for analysis. A blood sample was also collected at this time through cardiac puncture, centrifuged at 3000g for 15 minutes at 4°C, supernatant collected and stored at -80°C for later corticosterone analysis using the commercially available corticosterone ELISA kit (Assaypro St. Charles, MO USA).
3.2.2 Tissue Dissection

The hippocampus was dissected on ice into dorsal and ventral regions which were snap frozen in dry ice and stored at -80°C for later protein extraction (see section 2.4.1).

3.2.3 Western Blot Procedure

3.2.3.1 Homogenisation

Samples were homogenised in 250 μl RIPA buffer [with proteinase (1:200) and phosphotase (1:50) (Merck, Kilsyth, Victoria, Australia)] using a hand-held electric pestle (pellet pestle, Sigma-Aldrich) fitted with a blue polypropylese 1.5ml pestle (Sigma-Aldrich) and set on ice for 10 minutes. Once all tissue was homogenised, samples were solubilised on a shaker at 4°C for 45-60 minutes and finally centrifuged at 4°C, 14000g for 15 minutes. Supernatant was removed and stored at -80°C until prepared for analysis.

3.2.3.2 Bicinchoninic Acid Protein Assay

To accurately quantitate the amount of BDNF and TrkB present in each sample, protein concentration was analysed using the commercially available bicinchoninic acid kit (Pierce® BCA protein assay kit, Thermo Scientific, Rockford, IL USA). Standards, prepared according to manufacturer instructions, and supernatant, prepared at 1:10 dilution with milliQ water, were loaded in duplicate at 10μL into a 96-well plate (Greiner Bio, Germany). Then 190μL of reagent mixture was added to each well, the plate covered in plastic wrap and incubated at 35°C for 30 minutes. Following incubation, the plate was read for absorbency at 562nm wavelength for protein concentration.

The absorbency output for standards and samples were averaged. Standards were corrected for background and then used to plot a standard curve. Samples were compared against the standard curve to achieve the desired protein concentration, here 40μg. The final
protein content of 40μg protein concentration was aliquotted and stored at -20°C until used in western blot analysis. Any unused supernatant was replaced at -80°C for future use.

3.2.3.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the technique of applying an electric current to an acrylamide gel, forcing proteins to separate from a sample according to molecular weight (kDa). Larger proteins will separate first at the top of the gel, and smallest proteins last at the bottom, thus the size of the protein of interest determines the thickness of the gel to be used. BDNF has a very low molecular weight, approximately 14kDa requiring the use of a thicker 15% gel, while its receptor, TrkB, at approximately 90kDa, was run using an 8% gel.

Gels were loaded into glass casts, two at a time, (Mini PROTEAN®3 System, 1.5mm spacers) then covered with a thin layer of 70% ethanol to prevent drying, and left to set for approximately 30 minutes. Ethanol was poured off and gels rinsed twice with double distilled water. Then stacking gel was loaded into the glass casts directly on top of the acrylamide gel and a 15 well comb was immediately inserted. Gels were allowed to set at room temperature (approximately 15-25 minutes) before being loaded into the SDS-PAGE running cassettes. Cassettes which were placed in the running tank, fitted with electrical leads, and filled with running buffer.

Aliquotted samples were prepared for loading into the wells of the SDS-PAGE by mixing with equal volume of loading buffer (9 mL lysis buffer, 1 mL β-mercaptoethanol, 4 μg bromophenol blue) and denatured on a heat block at 95°C for 10 minutes. Protein marker was loaded into the left-most well of the stacking gel in 5μL volume followed by samples. Proteins were separated with electrophoresis at 120V, 3A, 300W using a Bio-Rad Power Pac HC at room temperature for approximately 2 hours.
Electroblotting is the process by which an electric current is applied to transfer proteins from the SDS-PAGE gel to a membrane that can be treated and visualised for quantitation. Gels were removed from the running tank, stacking gel discarded, and acrylamide gel containing the proteins was placed in transfer buffer to prevent drying out. The gel and nitrocellulose membrane (Santa Cruz Biotechnology, Dallas, Texas, USA) were assembled into a “sandwich” of padding, filter paper, gel, nitrocellulose membrane, filter paper and finally padding. All layers were pre-soaked in transfer buffer and rolled on top of each other to ensure clean contact. The nitrocellulose-gel sandwich was secured into the transfer tank, which was filled with transfer buffer and electric leads were applied. Electroblotting was conducted in a bath of ice at 4°C. BDNF transferred for 2 hours at 120 volts, while TrkB transferred overnight at 30 volts, followed by 1 hour at 70 volts. Following transfer, the nitrocellulose membrane was ponceau stained to ensure proper transfer of proteins and check for degradation (see figure 3.1).

![Image](image.png)

**Figure 3.1.** Ponceau stain on a nitrocellulose membrane. Each column represents a different sample and each row a different protein, with the largest proteins at the top, and the smallest proteins at the bottom, indicated by decreasing arrow. Dashed line represents cut to the membrane in order to separate the protein of interest from the control protein.
3.2.3.5 Immunodetection

If staining confirmed proper transfer of proteins, probing with selective antibodies was conducted. The membrane was cut in two such that the protein of interest and the control protein (Actin) were separated to eliminate the need to strip the membrane (see figure 3.1). Membranes were rinsed of transfer buffer and ponceau for 1-2 minutes in TBST (20mM Tris-base 150mM NaCl, 0.1% vol/vol TWEEN 20, pH 7.5) then blocked overnight in 5% skim milk in 10 mL TBST.

Following overnight blocking, membranes were rinsed twice with TBST for 15 minutes at room temperature on a slow shaker. This was followed by another period of incubation with the primary antibody probe for 24 hours on a slow shaker at 4°C. For BDNF, primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA polyclonal to rabbit) was diluted 1:200 in 5% bovine serum albumin (BSA) in 7mL TBST. For TrkB, primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA polyclonal to rabbit) was diluted 1:500 in 5% BSA in 7mL TBST, while primary antibody for phosphorylated TrkB (Signalway Antibody, Pearland, TX, USA polyclonal to rabbit) was diluted 1:1000 in 5% BSA in 7mL TBST. Actin control was simultaneously incubated with the primary antibody (anti-β-actin, Santa Cruz Biotechnology, Santa Cruz, CA, polyclonal to mouse) diluted 1:1000 in TBST.

Following incubation with primary antibody, membranes were again rinsed twice with TBST as before. The secondary antibody probe for either rabbit or mouse (IgG HRP-linked, Cell Signaling, Danvers, MA, USA) was applied for 90 minutes at a dilution of 1:2000 in 5% skim milk in 7 mL TBST on a shaker at room temperature.

3.2.3.6 Chemiluminescence and Imaging

Following secondary probing, membranes were rinsed 3 times in TBST as before. Blots were detected using 1mL of chemiluminescence (LumiGLO® 2.25mL distilled water,
125mL 20x LumiGLO and 125mL Peroxide), applied for one minute prior to exposure and imaging (GE chemiluminescent imager). Quantification of images was completed using TotalLab Quant (TotalLab Ltd.).

3.2.4 Analysis

Western blots were repeated 2-3 times for analysis of the proteins BDNF, FL_TrkB and pTrkB. Data are presented as the average of output from all western blots and displayed as the mean +/- the standard error of mean. Unpaired t-tests were conducted to assess differences in means of protein levels between treatment modality and structure. Two-way analysis of variance (ANOVA) was conducted to assess significant main effect of either region or treatment or interaction thereof on protein levels. Results were considered significant if \( p \leq 0.05 \), unless otherwise stated.

Results

3.3.1 Plasma Corticosterone

Plasma corticosterone data for this group were normally distributed, and Levene’s test for equality of variances was not violated. An independent-samples t-test was conducted to compare the plasma corticosterone levels at time of death for control and ACTH-treated animals (see figure 3.2). There was no significant difference in scores for control \((M = 13.4, SD = 8.05)\) and ACTH \((M = 15.42, SD = 8.14)\); \( t(8.6) = -.411, p = .690 \) (two-tailed). The magnitude of the differences in the means (mean difference = -2.01, 95% CI: -13.03 to 9.06) was small (eta squared = .02). These data indicate that 14 days of ACTH administered i.p. does not result in increased plasma corticosterone levels on day 15, one hour after conducting a forced swim test in adult male Sprague Dawley rats.
3.3.2 Brain Derived Neurotrophic Factor

A two-way between-groups ANOVA was conducted to explore the impact of treatment (control and ACTH) and hippocampal structure (dorsal and ventral) on BDNF levels as measured by western blot (see figure 3.3). The interaction effect between treatment and hippocampal structure was not statistically significant, $F(1,24) = .393, p = .54$. The effect size for this interaction was small (partial eta squared = .02). There was no significant main effect for treatment, $F(1, 24) = .003, p = .96$, partial eta squared = .000 nor structure $F(1, 24) = 3.72, p = .07$, partial eta squared = .134. As no significant differences were found further univariate between and within groups analyses were not computed. However, the large effect size for the main effect of structure indicates that data may attain significance with larger n (see figure 3.3). Overall, there appears to be no influence of chronic ACTH treatment on BDNF levels of the dorsal and ventral hippocampus in behaviourally naïve animals.
3.3.3 Phosphorylated TrkB

A two-way between-groups ANOVA was conducted to explore the impact of treatment (control and ACTH) and hippocampal structure (dorsal and ventral) on levels of the pTrkB as measured by western blot (see figure 3.4 A). The interaction effect between treatment and hippocampal structure was not statistically significant, $F(1, 23) = 0.0, p = .986$. The effect size for this interaction was very small (partial eta squared = 0.0). There was a statistically significant main effect for treatment, $F(1, 23) = 5.37, p = .03$; the effect size for this main effect was large (partial eta squared = .2). Further independent-samples t-tests (see figure 3.4 B) did not confirm this effect when total control pTrkB volumes ($M = .70, SD = .10$) were assessed against total ACTH pTrkB volumes ($M = .89, SD = .25$); $t(12) = -1.852, p = .102$ (two-tailed). These findings indicate that while there is a main effect of treatment on pTrkB levels in these animals, it does not reach statistical significance in this sample; however, the large effect size indicates that significance may be attained with increased power.

Figure 3.3. BDNF levels as measured by western blot in the hippocampus of animals treated with 14 days ACTH and control. Data are presented as mean ± SEM.
**Figure 3.4.** Total phosphorylated TrkB levels as measured by western blot for control and chronic ACTH treated animals. **A:** Levels represented by treatment and region for two-way between-groups analysis. **B:** Levels represented by treatment for t-test. All values are expressed as mean +/- SEM of average of 2 western blot analyses.

### 3.3.4 Full Length TrkB

A two-way between-groups ANOVA was conducted to explore the impact of treatment (control and ACTH) and hippocampal structure (dorsal and ventral) on levels of the full length TrkB as measured by western blot (see figure 3.5). There were no statistically significant effects calculated for these data, therefore further univariate between and within groups analyses were not computed.
3.3.5 Proportion of Phosphorylated TrkB to Full Length TrkB

A two-way between-groups ANOVA was conducted to explore the impact of treatment (control and ACTH) and hippocampal structure (dorsal and ventral) on levels of the proportion of pTrkB to full length TrkB as measured by western blot (see figure 3.6 A). Levene’s test for equality of variance was violated; therefore the alpha level was reduced to $p \leq .01$. The interaction effect between treatment and hippocampal structure was not statistically significant, $F(1, 22) = .492, p = .491$. The effect size for this interaction was small (partial eta squared = .02). There was a trend toward a significant main effect for structure, $F(1, 22) = 6.58, p = .018$; the effect size for this main effect was large (partial eta squared = .23). Given this trend, a further independent-samples t-test was conducted evaluate the effect of structure (see figure 3.6 B). The trend toward significance continued with ventral hippocampus showing a higher proportion ($M = 1.31, SD = .46$) of pTrkB to full length TrkB as compared to dorsal hippocampus ($M = .928, SD = .22$); $t(17.17) = -2.674, p = .016$ (two-tailed). These findings indicate a higher proportion of phosphorylated to full length BDNF
receptors in the ventral hippocampus between all treatment groups in this study. Consistent with the BDNF data (see figure 3.3) these findings support that BDNF/TrkB levels and activity may be innately higher in the ventral as compared to the dorsal hippocampus.

**Figure 3.6.** Proportion of pTrkB to FL_TrkB in adult male Sprague Dawley rats treated with 14-days ACTH or control. **A:** Levels represented by treatment and region for two-way between-groups analysis, there was a trend toward significant main effect of hippocampal region. **B:** Levels represented by region for t-test. Protein levels differed significantly, however, due to inequality of variance, this must be interpreted with caution. Data presented as mean +/- SEM of average of 2 western blot analyses. *$P \leq .05$

**Discussion**

The work presented here builds on previous work undertaken by Kitamura *et al.* (2002) and Walker *et al.* (2013) who have shown that acute (daily) ACTH administration to the Sprague Dawley rat using a chronic time course (14 days) presents as an animal model of HPA axis induced antidepressant resistance. Unlike other models of depression that use behavioural or chemical means to chronically challenge the HPA axis, such as chronic mild stress, slow pump corticosterone release or drinking water corticosterone administration, the model used here presents a chemical means to acutely challenge the HPA axis chronically at the period of circadian nadir. Thus while elevated plasma corticosterone levels would be
anticipated in a model of stress induced illness, there is a significant methodological
difference in paradigms. Walker et al. (2013) have shown that plasma corticosterone levels
are elevated following each ACTH injection, attaining significant differences to control at
days 4 and 8, consistent with work supporting that the 14-day ACTH protocol has
measureable behavioural effects by day 7 (Kitamura, Araki, & Gomita, 2002a, 2002b). The
data presented here indicate that these responses are attenuated over the course of the
treatment, indicating an effect of inhibitory feedback of the HPA system. Despite
corticosterone levels being comparably normal by day 15, the daily ACTH administration
protocol maintains antidepressant resistance, indicating that circulating glucocorticoid levels
may not be a trait marker of treatment resistant depression (Carroll et al., 2007).

As hippocampal BDNF and TrkB levels have been shown to decrease with stress and
depression (Duman et al., 1997; Duman & Monteggia, 2006; Jacobsen & Mørk, 2006; Zhou
et al., 2008; (Shou-Sen, Shu-hong, Bang-ping, Fang, & Zun-Ling, 2010) the impact of
chronic acute HPA axis ACTH challenge on levels of these proteins was measured by
western blot. There was no significant effect of treatment on BDNF levels in either dorsal or
ventral hippocampus at the end of the ACTH protocol (figure 3.3). While this result is not
anticipated based on the literature, similar results have been found under a 7 week stress
paradigm that assessed BDNF mRNA (Allaman et al., 2008). Furthermore, these findings are
consistent with results of Li et al. (2006) who found no difference in hippocampal BDNF
levels after 14-day ACTH administration. However, given that increased BDNF mRNA
levels have been found using a 28 day stress paradigm (Larsen et al., 2010) the trend for
increased BDNF in the ventral hippocampus may indicate the initial phase of increased
BDNF translation, often interpreted as a compensatory reaction to increased stress. Under
such conditions, it would be anticipated that the dorsal hippocampus would exhibit a similar
compensatory profile; however this substructure has been shown to exhibit a stress induced
adaptive plasticity in advance of the ventral substructure, potentially as a means of executing cognitive control over stressful stimuli (Hawley & Leasure, 2012). Alternatively these data may simply indicate that BDNF levels are innately higher in the ventral hippocampus of this species as there was no influence of treatment. Although speculative, this could indicate the ventral hippocampus is primed to adapt to emotionally weighted environmental stimuli.

While the mechanism mediating the increased BDNF levels in the ventral hippocampus of these animals cannot be confidently delineated at this time, it is likely to underlie the increased proportion of phosphorylated to full length TrkB seen in the ventral as compared to the dorsal hippocampus (see figure 3.6). This increase could potentially be attributable to ACTH treatment as, although not significant, phosphorylated TrkB levels show a trend toward increasing in ACTH treated animals (figure 3.4 B), suggesting a potential compensatory action. These data support no effect of treatment on full length TrkB, suggesting all animals were functioning at a similar level regarding their total number of available receptors for BDNF at the end of 14-day ACTH administration.

While these data are by themselves interesting, hippocampal BDNF per se has not been found to correlate with the onset of depression. Deletion of the functional mature protein from region CA1 and the dentate gyrus does not result in a depressive phenotype (Adachi et al., 2008). However, by and large, low levels of BDNF are known to exist in many clinically depressed individuals as well as in many animal models of depression (Autry & Monteggia, 2012). Evidence suggests that while BDNF levels may not directly influence the onset of depressive symptomatology, changes in the brain, including BDNF and TrkB levels and actions in response to chronic exposure to stress hormones, may infer susceptibility to environmental stress. Thus, to further ascertain the role of BDNF and TrkB in mediating risk, the work presented here should be expanded beyond the behaviourally naïve protocol to include both stress and antidepressant challenges.
The relationship between hippocampal BDNF expression and behavioural test performance has been found to be reliant on previous exposure to chronic stress (Taliaz et al., 2011). Furthermore there appears to be a programming effect of BDNF-TrkB activity that is evident in maternal separation models (Kozisek, Middlemas, & Bylund, 2008) which may rely on differential transcript changes to BDNF exon expression in young versus adult animals (Nair et al., 2007). This indicates that perhaps altered levels or function of BDNF-TrkB could be a measureable risk factor for MDD development under conditions of added stress or HPA axis activation. Furthermore, the BDNF-TrkB system is seemingly necessary to achieve measureable behavioural improvement from antidepressants which is attributed to its potent neurotrophic properties (Saarelainen et al., 2003; Adachi et al., 2008; Autry & Monteggia, 2012). Given the antidepressant resistance of this model, assessing how BDNF-TrkB does or does not respond with the application of antidepressants may yield insight into a mechanism of treatment resistant depression.

These data are in discrepancy with other findings on the effects stress on hippocampal BDNF levels. As discussed, chronic stress and chronic glucocorticoid exposure have both been reported to attenuate BDNF levels in the hippocampus. The lack of observed changes in BDNF following repeated daily ip injection with ACTH suggests no change has occurred in the integrity of hippocampal BDNF as is observed with other chronic forms of stress. This may related to the intermittent nature of the treatment, which reflects more of a diurnal surge of ACTH and corticosterone during the circadian nadir in response to acute ACTH treatment (Walker et al., 2013). As demonstrated here, the model is not chronically hypercortisolemic, and thus the chronic elevated glucocorticoid levels necessary for impaired hippocampal BDNF may not have been sufficient to induce such an effect. This suggests that the antidepressant treatment resistance reliably observed in this model is not the result of impaired hippocampal BDNF. Additional work must be undertaken to phenotype this model.
appropriately bearing in mind the complexities inherent to the various mechanisms mediating the effects of stress and antidepressant therapies and their consequences on pathophysiology whilst guarding against anthropomorphising characteristics that are not yet understood in the human condition of affective disorder. In expanding these findings lies the potential to better understand the neural mechanisms of antidepressant resistance, as well as how these mechanisms react to stressors and antidepressant therapy.


antidepressant drugs and is required for antidepressant-induced behavioral effects. *The Journal of Neuroscience*, 23(1), 349-357.


Chapter IV: Effect of Adrenocorticotropic Hormone and Deep Brain Stimulation on Hippocampal Memory Tasks in the Rat
Abstract

Major depressive disorder (MDD) is well established as a stress-related psychiatric illness. A core diagnostic criterion of this affective mood disorder is a diminished ability to think, concentrate or make decisions which attenuates with effective antidepressant treatment. This aberrant cognitive function may result from the deleterious effects of excessive glucocorticoids on the hippocampal structure. To assess this, Sprague-Dawley rats (n = 28) were treated with daily injections of either saline (0.9%) or adrenocorticotropic hormone (ACTH 100μg/day) for 18 days. One experimental group also received infralimbic deep brain stimulation (DBS) for 11 days at 130 Hz, 100μA, 90μSec for 6 hours per day to assess effects of this antidepressant treatment. Memory was assessed using the T-maze and novel object preference (NOP) tests, while antidepressant efficacy was assessed using the forced swim test (FST). The open field test was also conducted to confirm there were no effects of anxiety or locomotor activity in biasing results. The FST confirmed the antidepressant resistance of this model as well as the validity of DBS as an efficacious therapy in this antidepressant resistant model. There were no effects of either chronic ACTH or DBS on memory as measured by here, supporting the safety of DBS. Areas in further need of research regarding this model are also discussed.
Cognitive impairment is a core diagnostic criterion of MDD in the DSM-5 (American Psychiatric Association, 2013), manifesting as difficulty focusing, poor judgement, inability to make once easy decisions and general forgetfulness. The mechanisms mediating these cognitive changes are multifactorial, subserved by processes of memory, executive function, sensory perception and electro-chemical signalling. Of these processes, memory is perhaps the most crucial, due to its direct link to survival, in that it permits an organism to evaluate current events in light of previous experiences such that an adaptive reaction most likely to enhance survival can be executed. The memory system is subserved by many of the same structures and electro-chemical signals as cognitive processes, and while the two are intimately linked, they can be measured, and defined, as separate procedures.

The multi-store model of cognition (Atkinson & Shriiffin, 1968) highlights the unified individuality of the memory system (see figure 4.1). Here, information and recall of memories requires several areas of the brain to work in unison using both electrical and chemical processes ad infinitum; yet distinct types of memories incite different neural processes. Sensory memory, for example, the ability to recall details of a visual scene witnessed for a brief moment, is linked to both conscious and unconscious perception of stimuli through the 5 senses. Short-term memory, often referred to as working memory due to the role this process plays in storing small amounts of information for a short period of time such that it may be worked with, is an active process that builds on sensory memory through rehearsal of stimulus input. Long-term memory again builds on short-term memory through rehearsal and the phenomenon of long-term potentiation (LTP) to alter the synaptic environment through input-specificity and associativity thereby strengthening the neural structure encoding a memory (Nestler et al., 2001).
Figure 4.1. Schematic of multi-store model of human memory (adapted from Nestler et al., 2001; Mastin, 2010). The main types of memory, in bold at the top of the flow-chart, subserve distinct purposes for behavioural adaptation. Yet, as indicated by the line moving from left to right, each process builds on the previous for a more complex memory, hence a unified individual process.
LTP underlies both explicit-declarative and implicit-nondeclarative memory formation and recall. This type of memory is the most complicated and important memory process with regards to human affective states and is heavily reliant on the hippocampus. The ideal placement of the hippocampus within the medial temporal lobe allows this structure reciprocal access with both dorsal and ventral systems, with the latter having specific importance for the enhanced encoding of emotionally salient events. The cognitive enhancement of an emotional memory is an amygdala dependent hippocampal process often referred to as *flashbulb memory* (Nestler *et al.*, 2001). This type of memory is facilitated by glucocorticoids, indeed, hippocampal-dependent memory has been found to be more sensitive to glucocorticoids than any other form of learning and this is amplified by individual exposure to cumulative cortisol (Abercrombie *et al.*, 2011). As expected, chronic recall of negatively laden memories, either through rumination, repetition or reoccurrence of exogenous cue, can act to enhance that memory through rehearsal. Such recall will also cause a release of glucocorticoids in response to the emotive weight of these memories, which further functions to strengthen the memory whilst inciting the stress response.

Taken together, it becomes clear how chronic stress, either through rumination or consistent exposure, can culminate in a self-destructive cycle perpetuated by negative *emotional-ventral* cognitive shift and concurrent physiological increase in stress-response glucocorticoids. The increase in glucocorticoids can cause hippocampal atrophy through a loss of glucocortioid concentrating cells in an attempt to restore proper feedback within the HPA axis (Sapolsky *et al.*, 1986; Lupien *et al.*, 1998) which may contribute to the reduced cognitive processes presented in MDD. For example, enhanced fear acquisition, as measured by classic differential fear conditioning paradigm, and reduced declarative memory consolidation, as measured by word-pair and texture discrimination recall, were present in treated depressed individuals (Nissen *et al.*, 2010). This is in line with the consistent negative
bias that remains even in a state of remission for depressed individuals (Gerritsen et al., 2011; Schlosser et al., 2011). Other work, however, seems to support that non-sedative antidepressant therapies, including deep brain stimulation, result in an improvement of impaired memory in depressed patients, despite the persistent negative bias (Sternberg et al., 1976). An innate confound of such measures, however, is that improvement of depressive symptoms is directly proportional to improvements in cognitive function (Thompson, 1991; Elizalde et al., 2007; Rocher et al., 2004; Mayberg et al., 2005; Puigdemont et al., 2012).

In order to understand the underlying pathophysiology of this specific depressive symptom, and how it may be attenuated with effective antidepressants, many tests have been developed for use with animal models of depression. Many of these tests rely on the use of mazes and can be modified to measure short-term working memory, spatial memory, long-term memory and in essence, synaptic plasticity. These tests can be extended to assess the influence of emotion on memory by introducing positive or negative stimuli, such as food treats or dull shock respectively while increased inter-trial interval duration can assess long term memory. Lesion-function tests in animals have confirmed the role of the hippocampus in successfully acquiring and retaining spatial information in these tests, while non-spatial information seems unaffected (Jarrard, 1993; O’Keefe & Nadel. 1978) while behavioural stress paradigms have been shown to result in memory deficits over all (Elizalde et al., 2007; Rocher et al., 2004).

To assess the influence of glucocorticoid levels on spatial working and reference memory, an acute ACTH exposure, chronically administered during circadian nadir over 18 days was used in Sprague-Dawley rats. It is believed that ACTH administered acutely at circadian nadir likely acts downstream in the HPA-axis leading to homeostatic disruptions. This model induces an antidepressant-resistant state, however, its effect on memory processes has not been characterised (Kitamura et al., 2002; Walker et al, 2013). Similarly, the impact
of antidepressant therapies for treatment resistant depression (TRD), such as deep brain stimulation (DBS), require more systematic characterization. To address this, the present study quantifies the effect of ACTH treatment with and without infralimbic DBS on spatial working and reference memory. DBS was applied for 6 hours per day, beginning on day 7, alongside a sham control stimulation protocol. Stimulation of the infralimbic cortex in the murine model is homologous to stimulation of the anterior cingulate cortex currently in use for DBS of patients with TRD. As such, the use of DBS in this work also serves to address lingering ethical issues facing human DBS, such as brain doping or increased cognitive impairment as a consequence of DBS therapy (Fuchs, 2006).

Spatial memory was appraised using a rewarded forced choice T-maze protocol. This test relies on long term encoding of a spatial map and short term encoding of previous moves in order for the animal to satiate appetite. The novel object preference test was used to assess reference memory, relying on the innate curiosity of the species to forage for food in previously unexplored places. To ensure no spurious effects of treatments on ambulation or exploratory drive, an open field test was also conducted. The forced swim test was carried out to validate the antidepressant resistance of the ACTH model as well as to assess the effects of treatments on coping behaviour and reactivity to stress, behaviours which are separate from memory, but equally important in MDD.

Methods

4.2.1 Animals

Male Sprague Dawley rats, aged 9-12 weeks through the duration of experimental protocol (337-478g), were communally housed 2 per cage, under a 12 hour light/dark cycle (lights on at 7:00am), with food and water available ad libitum. Animals were permitted to acclimatise to the facility for seven days prior to initiation of experimental protocol, during
which time all animals were semi-randomly allocated by caged pairs to one of four experimental groups- control, ACHT, ACTH plus sham infralimbic deep brain stimulation (SDBS) or ACTH plus high frequency infralimbic deep brain stimulation (DBS). All procedures were approved by Deakin University Animal Ethics Committee.

All animals received 18-days of ACTH-(1-24) (AnaSpec, California, USA) 100μg/day dissolved in biological saline or 0.09% saline vehicle for control (Walker et al., 2013; Kitamura et al., 2002). All injections were administered during the circadian nadir intraperitoneally (i.p.) at approximately 10:00am (+/- 2hours) over the 18 days. Also beginning on day 1 of the experimental protocol, and once every 72 hours thereafter, for a total of 6, a small sample of approximately 500μL of tail blood was collected into a heparinised tube for corticosterone analysis. These samples were stored on ice during collection, centrifuged at 3000g for 15 minutes, supernatant collected and stored in -80°C for corticosterone analysis using the commercially available corticosterone ELISA kit (Assaypro St. Charles, MO USA). All tail blood was collected prior to ACTH administration.

On day 18 of the protocol, animals were humanely killed via pentobarbitone (Virbac, NSW, Australia) overdose and brain tissue extracted. The brain was immediately rinsed with ice cold distilled water, wrapped in foil and placed in liquid nitrogen. All tissue was stored at -80°C until prepared for analysis. A blood sample was also collected at this time through cardiac puncture, centrifuged at 3000g for 15 minutes at 4°C, supernatant collected and stored in -80°C for corticosterone analysis using the commercially available corticosterone ELISA kit (Assaypro St. Charles, MO USA). Study protocol is outlined in Appendix B.

4.2.2 Stereotaxic Surgery

After 1 week of acclimatisation, rats were induced to anaesthesia using 3% isoflurane and maintained with 1-3% isoflurane throughout surgery. Polymide-insulated stainless steel
monopolar electrodes (250 μm in diameter with 0.6 mm of surface exposed, Plastics One Roanoke, VA, USA) were implanted unilaterally into the left infralimbic area (AP: +/- 3.0, ML: +/- 0.4, DV: -5.6 (Watson and Paxinos, 2007). Small jewellers’ screws of approximately 2mm in length were fixed into the skull to act as scaffolding for dental cement that was placed around the stimulating electrode to prevent displacement or damage. All animals were designated SDBS or DBS post-operatively ensuring surgical procedures remained the same across experimental groups. Stimulation was conducted using the AMPI / Master-9 system, which was attached via the ISO-Flex Stimulus Isolator (A.M.P.I., Jeruselum, Israel) to the implanted electrode of the singly housed animal at 130 Hz, 100μA, 90μSec for 6 hours per day, beginning on day 7 of study protocol, as outlined in the appendix.

4.2.3 Behavioural Testing

For every test, animals were placed in the testing room a minimum of one hour prior to testing to allow habituation to the behavioural testing area. Care was taken to keep noise to a minimum, temperature controlled and brightness to a level amenable to the nocturnal nature of *rattus norvegicus*.

4.2.3.1 Forced Swim Test

The forced swim test is the primary screening tool in use for the evaluation of clinical applications of novel antidepressant agents (Castagné *et al*, 2011). The test has been extensively validated and previously used in this research paradigm (Porsolt *et al*, 1977; Kitamura *et al*, 2002; Walker *et al*., 2013). The apparatus consisted of a tall clear plexiglass cylinder (60.5cm in height, 20cm diameter on a base measuring 30 x 30cm), filled to a depth of 35cm with 25°C +/- 1°C clean tap water. Animals underwent a 10-minute habituation session, which was followed 24 hours later by a 6-minute test session. Control and ACTH
animals received 10mg/kg of imipramine hydrochloride (Sigma-Aldrich, NSW, Australia) i.p. 30 minutes prior to the test session to validate the antidepressant resistance of the model. DBS animals performed the test within 5-minutes of ceasing high frequency stimulation, while SDBS animals required no additional treatments (see table 4.1 for summary of FST measures by group). Image capturing software was used (CleverSys Forced Swim Scan Reston, VA, USA) to analyse active behaviours of swimming, climbing and escaping as well as passive behaviour of immobility and passive dive.
Table 4.1.

*Summary of assessments made by the forced swim test by group and treatment*

<table>
<thead>
<tr>
<th>Group</th>
<th>ACTH Induced Antidepressant Resistance</th>
<th>Tricyclic Imipramine</th>
<th>High Frequency Stimulation</th>
<th>Assessment via Immobility in FST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>✓</td>
<td></td>
<td>Measure effect of tricyclic in control animal</td>
</tr>
<tr>
<td>ACTH</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>Measure effect of tricyclic in antidepressant resistant animal</td>
</tr>
<tr>
<td>SDBS</td>
<td>✓</td>
<td></td>
<td></td>
<td>Measure effect of electrode implantation without stimulation in antidepressant resistant animal</td>
</tr>
<tr>
<td>DBS</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>Measure effect of electrode implantation with stimulation in antidepressant resistant animal</td>
</tr>
</tbody>
</table>
4.2.3.2 T-Maze Test

The T maze test is used to investigate various aspects of memory in rodents, including spatial working memory, reference memory and spontaneous alternation (Wenk, 1998). The rewarded alternation protocol was used here to assess spatial memory. The apparatus was composed of darkly painted medium-density fibreboard, fitted with guillotine doors for the arms (Deacon & Rawlins, 2006).

To habituate, animals were allowed to freely explore the maze in cage pairs for 3-ten minute sessions. Reward food was placed randomly throughout the maze for the first 10 minute session, only the goal arms for the second and finally only in the reward zone for the third. Learning trials began on the same day as habituation and continued over 5 trials per day for 5 days. Sessions consisted of an equal number of left and right turns for a total of 6 turns. The first turn changed for each trial for each animal to prevent pattern learning. Throughout learning, both reward zones contained a treat to avoid olfactory bias. The maze was cleared of urine and faeces, but not sanitized between animals as olfaction has not been found to be a confounding factor (Deacon & Rawlins, 2006). The sixth session of the fifth day acted as the test, requiring the animal to run two additional turns (a total of 8 turns), but in an unexpected pattern (see figure 4.2) which required the animal to correctly recall turns 6, 7 and 8 to receive the award. All session were video recorded and the number of correct test choices was analysed.


4.2.3.3 Open Field Test

The open field test (OFT) was used to assess locomotor, anxiety and exploratory behaviours of all animals. The apparatus was a large plexiglass box (50cm x 50cm x 40cm) the sides of which were painted black. The floor of the open field was comprised of clear plexiglass and was demarcated into 16 equally sized squares. The bottom of the apparatus was covered with white paper on the external side to provide a consistent colour and texture across testing sessions. The OFT was completed in one 10-minute free exploration session, recorded by video camera. The test room was dimly lit, with one spotlight suspended above the OFT apparatus. For analysis, videos were de-identified and the first 6 minutes manually coded in 2 second intervals to determine if any treatment imparted a significant change to locomotor, anxiety or exploratory behaviours that may influence other behavioural test outcomes.

4.2.3.4 Novel Object Preference Test

The novel object preference test (NOP) is used to assess the recognition memory of small laboratory animals. A one-trial-non-matching-to-sample learning paradigm was used (Bevins & Besheer, 2006). The apparatus consisted of a large plexiglass box (50cm x 50cm x
the sides of which were painted black and the floor covered in paper from the external side. Each animal was placed into the box with two identical objects, comprised of children’s toys, and allowed to freely explore for 10 minutes. An inter-trial interval of 60 minutes was set (Bevins & Besheer, 2006) at which time the animal was returned to the apparatus with one of the familiar objects and a new object, also a toy, and allowed to freely explore for 5 minutes to test recognition of the familiar object. All explorations were recorded by video camera, and later de-identified and manually scored for time spent with each object during test phase. All novel objects were equally distributed between left and right across trials to avoid bias and the apparatus and objects were thoroughly cleaned between subjects to preclude olfactory bias.

Results

4.3.1 Forced Swim Test

A one way ANOVA was carried out to assess the effect of treatment on immobility time in the forced swim test (see Figure 4.3). Levene’s test for equality of variance was not violated, \( W(3, 23) = 0.594, p = 0.625 \) which allowed for the use of the more robust Bonferroni post test. Results showed there was a significant effect of treatment (\( F(3, 23) = 4.357, p = 0.014 \)) on immobility time in adult male Sprague-Dawley rats. Bonferroni post hoc test revealed that immobility time was significantly higher in ACTH treated animals (\( M = 49.530, SD = 16.853 \)) compared to DBS treated animals (\( M = 20.000, SD = 15.264 \)), \( p = 0.031 \), demonstrating that DBS had antidepressant effects in ACTH treated animals which were resistant to antidepressant effects of imipramine. Post hoc also showed a significant trend for increased immobility time of ACTH treated animals compared to SDBS animals (\( M = 24.23, SD = 21.14 \)), \( p = 0.07 \) indicating an antidepressant effect of electrode insertion. Bonferroni post hoc test also revealed a trend towards a significant increase in immobility time of ACTH treated animals (\( M = 49.530, SD = 16.853 \)) relative to control animals (\( M = 23.94, SD = \))
17.4), \( p = .062 \) further supporting the antidepressant resistant effects of chronic ACTH treatment.

**Figure 4.3.** Mean Immobility time in forced swim test. All data are expressed as the mean +/- SEM (control \( n = 7 \), ACTH \( n = 8 \), SDBS \( n = 6 \) and DBS \( n = 6 \)). **\( P \leq .01 \)**

Independent samples t-tests were conducted to assess for differences in active behaviours of swimming and climbing between groups (see figure 4.4). For swimming behaviour, Levene’s test for equality of variance was not violated, \( W(3, 23) = 1.54, p = .231 \). T-tests revealed a significant difference between control (M = 28.78, SD = 15.91) and DBS (M = 80.26, SD = 55.47); \( t(11) = -2.36, \ p=.04 \) treated animals for time spent swimming (see figure 4.4, A), with DBS treated animals engaging in significantly more swimming compared to control animals. This demonstrates that DBS treated animals engaged in higher rates of the active coping mechanism, swimming, during the FST compared to controls.

Independent samples t-tests revealed a significant difference between treatment groups for time spent climbing (see figure 4.4, B). Levene’s test for equality of variance was not violated, \( W(3, 23) = .786, p = .514 \). Control animals (M = 181.19, SD = 26.89) exhibited significantly higher rates of climbing versus all other treatment groups: ACTH (M = 128.46, SD = 40.53); \( t(13) = 2.92, \ p=.01 \); SDBS (M = 133.07, SD = 48.61); \( t(11) = 2.28, \ p=.05 \); and DBS (M = 133.23, SD = 43.19); \( t(11) = 2.45, \ p=.03 \). These results indicate that control
animals engaged in the act of climbing during the FST more than any other treatment group, especially ACTH treated animals. This could be a type I error consequent of the similarity of the behaviours to the software analysis program, however software analysis was manually checked for proper coding.

![Figure 4.4](image-url)

**Figure 4.4.** Mean swimming (A) and climbing (B) times in forced swim test. All data are expressed as the mean +/- SEM (control n = 7, ACTH n = 8, SDBS n = 6 and DBS n = 6). *P ≤ .05, **P ≤ .01

4.3.2 T-Maze Test

A one way ANOVA was carried out to assess the effect of treatment on free choice test in the T-maze (see Figure 4.5). Levene’s test for equality of variance was not violated, \( W(3, 23) = .097, p = .961 \). Results showed there was no significant effect of treatment (\( F(3, 23) = .092, p = .964 \)) on correct choice in T-maze conducted using adult male Sprague-Dawley rats. These results indicate no effect of ACTH, sham or high frequency DBS on spatial memory in this test paradigm. As no significant effects were observed, further univariate and *post hoc* test were unwarranted.
4.3.3 Open Field Test

One way ANOVA was used to assess ambulation, anxiety and exploratory behaviours using the open field test. Ambulation was defined as the number of squares crossed, while anxiety was determined by the time spent in the periphery of the arena versus the centre and rearing was indicative of exploratory behaviour. Levene’s test for equality of variance was not violated, $W(3, 23) = 1.655, p = .204$. A significant effect of treatment was found for the number of rears (see Figure 4.6, A), $F(3, 23) = 4.758, p = .010$. Bonferroni post hoc test indicated this significant difference was attributable to the increased number of rears by the SDBS treated animals ($M = 43, SD = 9.612$) as compared to number of rears by control animals ($M = 23, SD = 14.83$). There was a trend towards significance for increased number of rears by SDBS animals versus DBS animals ($M = 26.83, SD = 4.08$), $p = .06$. These findings indicate an increase in exploratory behaviour by SDBS treated animals. No other behaviours measured in the OFT were found to be significantly different between groups as a result of treatment (see Figure 4.6 B-D).
Figure 4.6. Exploratory, ambulatory and anxiety behaviours as measured by the open field test. All values are expressed as the group average +/- SEM. (control n = 7, ACTH n = 8, SDBS n = 6 and DBS n = 6), **P ≤ .01

4.3.4 Novel Object Preference Test

A one-way between-groups multivariate analysis of variance was performed to investigate treatment difference in reference memory as assessed through the novel object preference test. Two dependent variables were used: seconds spent exploring a familiar object and seconds spent exploring a novel object. The independent variable was treatment. Preliminary assumption testing was conducted to check for normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices and multicollinearity with no serious violations noted, however, as violations did occur, the more stringent Pillai’s Trace was interpreted.

To control for any confounding effects of mobility in the NOP test, the relationship between ambulation, as measured by the OFT, and time spent with familiar and novel objects
was investigated using Pearson product-moment correlation coefficient. There was a small negative correlation between ambulation and time spent investigating the familiar object, $r = -0.146$, $n = 27$, $p = .47$. Ambulation therefore accounts for 2.1 per cent of the variance in time spent with familiar object. There was a very small positive correlation between ambulation and time spent investigating the novel object, $r = .047$, $n = 27$, $p = .82$. Ambulation therefore accounts for 0.2 per cent of the variance in time spent with novel object. These findings indicate no significant confounding effect of ambulation in the NOP test.

There was no statistically significant difference between treatments on the combined dependent variables, $F(6, 46) = 1.603$, $p = .168$; Pillai’s Trace = .346; partial eta squared = .173. As no significance was found, further post hoc tests are not presented. However, independent-samples t-tests were conducted to assess for significant differences within treatment groups for time spent with familiar versus novel objects. These tests revealed no significant differences (see figure 4.7: A, C, E, G). The largest difference was seen for the DBS treated animals for time spent with familiar ($M = 25.8$, $SD = 17.07$) versus novel ($M = 61.88$, $SD = 50.95$) objects; $t(10) = 1.65$, $p = .13$ (two-tailed). The magnitude of the differences in the means was very large (mean difference = 36.08, 95% CI -84.95 to 12.79), $R$ square of 0.21. This indicates that DBS treated animals may have an increased memory capacity; however, given the high variation in the sample, this cannot be confirmed with the current data.

To combat any potential effect of testing fatigue, the first half (150 seconds) of the novel object test was also analysed (Mumby, 2001). Independent-samples t-tests were conducted to assess for significant differences within treatment groups for time spent with familiar versus novel objects (see figure 4.7: B, D, F, H). These tests revealed that animals in the DBS treatment group spent significantly more time in the first half of the test interacting with novel ($M = 3.48$, $SD = 3.66$) versus familiar ($M = 10.52$, $SD = 6.24$); $t(10) = 2.38$, $p =$
.04 (two-tailed) objects. The magnitude of the differences in the means was very large (mean difference = 7.04, 95% CI -13.62 to -0.46), R square of 0.36 indicating this is a robust effect. Furthermore, it is notable that given the choice, all animals displayed a consistent preference between groups and across time for the novel object. This supports the underlying theory of this behavioural test that rats will innately tend toward exploring new items in the environment given the chance.

To assess the impact of treatment on willingness to engage with objects in a familiar environment, total time interacting with both objects was also analysed using a one-way between-groups analysis of variance. There was a trend toward significance for the effect of treatment: F(3, 23) = 2.84, \( p = .06 \). This trend is further supported by the large effect size, calculated using eta squared, of .27. In light of these findings, independent t-tests were conducted to assess individual between group effects (see figure 4.8).
Figure 4.7. Time spent with familiar and novel objects by treatment group. Control 300s(A), 150s(B); ACTH 300s(C), 150s(D); SDBS 300s(E), 150s(F); DBS 300s(G), 150s(H). All values are group average +/- SEM. (control n = 7, ACTH n = 8, SDBS n = 6 and DBS n = 6) *P ≤ .05
Significant scores were found for ACTH treated animals ($M = 35.76, SD = 19.02$) and DBS treated animals ($M = 88.15, SD = 56.65$); $t(12) = -2.47, p = .03$ (two-tailed). The magnitude in the differences in means (mean difference = -52.85, 95% CI: -98.69 to -6.08) was large (eta squared = .337). Significant scores were also found for ACTH treated animals ($M = 35.76, SD = 19.02$) and SDBS treated animals ($M = 66.82, SD = 17.29$); $t(12) = -3.14, p = .009$ (two-tailed). The magnitude in the differences in means (mean difference = -31.06, 95% CI: -52.62 to -9.50) was large (eta squared = .451). These results suggest that chronic ACTH treatment, without clinically effective antidepressant intervention, decreases time spent exploring objects in a familiar environment in the NOP testing paradigm.

**Figure 4.8.** Total time spent investigating familiar and novel objects in the novel object preference test. Total test time was 5:00 minutes (equivalent to 300 seconds). All values are expressed as the group average +/- SEM. (control n = 7, ACTH n = 8, SDBS n = 6 and DBS n = 6)

**Discussion**

Consistent with the literature, the results of the FST confirm the effect of imipramine in saline versus ACTH treated animals, supporting the efficacy of the treatment protocol in producing an antidepressant resistant state in these animals (Kitamura, et al., 2002; Walker et al., 2013). The antidepressant effect of DBS in chronically ACTH treated animals is also
confirmed, with these animals displaying levels of immobility below that of control animals, indicating a pharmacologically effective antidepressant treatment. Animals in the SDBS group also showed reduced immobility time, although these levels did not reach significance. This could be indicative of a therapeutic response to the electrode insertion and its associated local lesioning effect. Such an effect may be conceptualised as sharing properties similar to the surgical procedure of cingulotomy. This so-called *insertional effect* causes an inflammatory response at the site of implantation which has been linked to increased serotonin function and reactive gliosis, both of which are believed to impart antidepressant effect (Perez-Caballero *et al.*, 2014).

An interesting observation of this particular data is the preferred active coping mechanism employed by DBS versus control animals, with the former group favouring swimming and the latter climbing. This finding informs on an underlying mechanism of DBS as within the FST, serotonin is purported to mediate swimming, while noradrenaline mediates climbing behaviours (Detke *et al.*, 1995). These results support that DBS may act, in part, via serotonergic mechanisms (Hamani & Nobrega, 2012). While speculative, DBS may modulate serotonin in a way that is more effective or direct than other conventional antidepressant treatments, thereby imparting antidepressant effect in this antidepressant resistant model. It is also important to note that DBS enables modulation of the neural network directly which is not achievable with pharmacotherapeutics. This combination of direct, targeted network modulation together with alterations in neurotransmitter signaling may be important for overcoming the antidepressant resistant state. The fact that SDBS animals were shown to have the second highest swimming rate, perhaps due to a serotonin mediated insertional effect, lends support to this hypothesis. A recent study examining the therapeutic mechanisms mediating this acute antidepressant-like response to DBS electrode insertion in the infralimbic cortex supports this. Specifically, it was demonstrated that changes in
serotonergic synaptic efficiency were modulated in response to inflammation occurring in response to electrod placement (Perez-Caballero et al., 2014). The response lasted for at least 2 weeks and had abated by 6 weeks. Future work is needed to assess whether this acute antidepressant response can be facilitated to enhance long-term therapeutic outcomes to DBS.

The similar performance by all treatment groups in the T-maze test indicates no apparent deficit or enhancement of hippocampal mediated spatial working memory and long-term reference memory from either ACTH treatment, electrode implantation or DBS. These results are consistent with other findings of no cognitive benefit or decline upon implantation of an electrode, with or without high frequency stimulation. This addresses potentially serious ethical issue facing the field of neurosurgery for refractory illness, namely the concern that implantation of the stimulating leads or stimulation itself may cause unwanted changes to the abilities of the patient reminiscent of early psychosurgeries (Jotterand & Giordano, 2011; Hariz, 2012). Further concerns on the other end of the spectrum regard imparting hyperfunctioning cognitive ability, so called brain doping, (Fuchs, 2006; Ford, 2007 as cited in Schlaepfer et al., 2011) as a result of DBS treatment. At present, these concerns have been largely refuted, as no impacts, positive or negative, have been found to occur with acute or chronic DBS of BA25 for treatment resistant depression in humans (Dunn et al., 2011; Kennedy et al., 2011; Lozano et al., 2011; McNeely, Mayberg, Lozano, & Kennedy, 2008).

The results of the NOP test lend further support to the safety of DBS use as it neither improved nor debilitated reference memory based object recognition skills in DBS or SDBS treatment groups. Furthermore, although not significant, all groups did exhibit increased time with the novel object, lending support to the validity of the basis of this test, namely the inherent novelty seeking behaviour of rattus norvegicus.
Interestingly, the results of the NOP test may indicate an anxiogenic effect of ACTH, as the ACTH treatment group spent the least amount of time interacting with objects overall, although this was not confirmed by the OFT. Furthermore, animals receiving SDBS and DBS exhibited the highest time for overall interaction with objects and, although not significant, DBS animals showed the highest level of interaction, suggesting that DBS may be subtly affecting this behaviour, and that implantation of the electrode without stimulation has a similar, although less intense effect, indicating a short-lived insertional effect. Given these observations, the anxiety profile of this animal should be further assessed as should the effects of DBS in potentially attenuating such states.

It must be noted that the novel object preference test has been largely discredited as a true test of hippocampal memory ability, as have most the visual object recognition tests (Mumby, 2001). It is now widely accepted that reference memory is not a localised function but relies on several structures. Bearing this in mind, future work aimed at the assessment of reference memory in this animal model should ideally be continued using various sensory recognition stimuli over several paradigms.

Finally the OFT results indicate that findings of these behavioural tests are likely not attributable to differences in locomotor activity, as ambulation was not found to differ significantly between groups. Situational anxiety was also found to be similar across groups, as no significant difference was found between time spent in the centre or periphery between groups. However, given the variation between the control and DBS groups on this measure, further investigation in a larger cohort is a necessary next step. Incidental observation of other behaviours in the OFT, such as ACTH and SDBS groups spending the most time in the centre with the latter also exhibiting the highest number of rears could indicate increased exploratory behaviour as a result of chronic ACTH treatment (Walker et al., 2013). These findings should be addressed in future work as they may indicate an underlying mechanism
mediated by ACTH administration that could inform on symptomatology and etiopathology of human TRD.

The safety of the infralimbic stimulation protocol with regards to unwanted alterations in memory and cognitive ability are supported by this work. The inherent low risk of DBS treatment stems from the fact that it is reversible, as the device can be turned off at any time, and removal of the electrical probes causes no deficits in patient abilities (Mayberg, personal communication). Furthermore, compared to pharmacotherapy which increases neurotransmitters throughout the body, DBS is an extremely focused and local treatment. Obviously the complications inherent to neurosurgery must not be underscored, and the ethical ramifications of any medical treatment should always be taken into consideration, hence research into these issues will continue to be collected with the aim of providing a complete understanding of the influence of BA25 DBS for TRD.
Works Cited


Wenk, G. L. (2001). Assessment of spatial memory using the T maze. *Current Protocols In Neuroscience / Editorial Board, Jacqueline N. Crawley ... [Et Al.], Chapter 8, 8.5B.*
Chapter V: Effect of Adrenocorticotropic Hormone and Deep Brain Stimulation on Glial Cell Line Derived Neurotrophic Factor Levels in the Dorsal and Ventral Hippocampus
Abstract

The neurotrophic hypothesis of depression suggests a role for neurotrophic factors in buffering against stress-induced onset and maintenance of depressive disorders, as well as in mediating antidepressant effects. Here we investigate levels of hippocampal glial cell line derived neurotrophic factor (GDNF) using a chronic adrenocorticotropic hormone (ACTH)-induced animal model of antidepressant resistance. Male Sprague-Dawley rats (n = 28) were treated with daily injections of either saline (0.9%) or ACTH (100μg/day) for 18 days. One experimental group also received infralimbic deep brain stimulation (DBS) for 11 days at 130 Hz, 100μA, 90μSec for 6 hours per day to assess effects of this antidepressant treatment on hippocampal GDNF levels. A sham DBS group was also included as surgical control. On the eighteenth day, animals were euthanized and brains extracted and frozen in liquid nitrogen. The dorsal and ventral hippocampus were later dissected over ice and GDNF protein levels quantified by enzyme linked immunosorbent assay (ELISA). Significant differences in GDNF were not observed between chronically ACTH-treated or DBS-treated relative to saline treated animals. Limitations and implications of this work are discussed.
The same chemical cascade mediating the stress response can simultaneously reduce levels of neurotrophic factors (Duman & Monteggia, 2006), and while this relationship is not completely understood, a role for neurotrophic factors in stress, depression and the antidepressant response has been demonstrated (Duman & Monteggia, 2006; Kozisek, Middlemas, & Bylund, 2008; Saarelainen et al., 2003). The role of brain derived neurotrophic in MDD is well established; however the neurotrophic hypothesis of depression would suggest other neurotrophic factors may also contribute to the pathophysiology of this psychiatric illness. Glial cell line-derived neurotrophic factor (GDNF) is a promising candidate. Its considerable therapeutic benefit in attenuating the degeneration associated with Parkinson’s disorder (Hamani et al., 2007 for review) is due to the extensive trophic support GDNF exerts upon the maintenance of glia, catecholaminergic and serotonergic neurons (Michel et al., 2008), substrates also critical in MDD. Infusion of GDNF into the striatum of rats significantly increased cell proliferation within the hippocampus and upregulated dopamine in existing cells (Chen et al., 2005). Intrahippocampal administration of human GDNF to aged Fisher 344 rats resulted in increased synthesis of serotonin, dopamine and acetylcholine with corresponding cognitive improvement in the Morris water maze compared to control (Pertusa et al., 2008). GDNF may also protect against the neurodegenerative effects of HPA axis activation via contributions to the regulation of noradrenergic and GABA-ergic pathways and buffering against oxidative stress (Michel et al., 2008). Furthermore, GDNF exhibits unique signalling properties that may facilitate neuron-glia cross-talk, increasing and enhancing survival and integration in key affective networks (Ledda et al., 2007; Paratcha et al., 2003).

Reduced GDNF levels have been found in some models of stress which may contribute to maladaptive stress reactivity in later life (Kawano et al., 2008), while GDNF heterozygous models have presented with a deficit in maze negotiation (Gerlai et al., 2001).
Significantly decreased levels of serum GDNF have been found in treatment resistant patients as compared to other depressed subjects (Tseng et al., 2013) and in complement, GDNF has been shown to increase to a greater degree in those who respond to antidepressant treatment compared to those who do not (Zhang et al., 2009).

Together, these data suggest that GDNF activity may be important in mediating antidepressant response and consequently influencing individual susceptibility to relapse and recovery. In support of this, low GDNF levels have been found in remitted MDD patients (Takebayashi et al., 2006), while lithium has been shown to differentially affect GDNF in the hippocampus, frontal cortex and occipital cortex of Flinders Resistant Line as compared to Flinders Sensitive Line rats (Angelucci et al., 2003) suggesting an innate aberrancy in the GDNF response to antidepressants in susceptible individuals. While the mechanism underlying the role of GDNF in MDD and antidepressant activity is as yet unclear, evidence suggests it may rely on glia and trophic intracellular pathway activation.

The tricyclic imipramine has been shown to induce GDNF upregulation in astrocytes via early growth response factor 1 and the MAPK (mitogen-activated protein kinase) pathway (Kim et al., 2011). Amitriptyline, imipramine, desipramine and citalopram have also been shown to increase GDNF in rat C6 glioblastoma cells via MAPK-CREB pathway (Hisaoka et al., 2001, 2008) as well as the β-arrestin1-CREB pathway (Golan et al., 2011). Expanding on these findings, chronic clomipramine restored hippocampal GDNF protein levels in a model of chronic stress (Liu et al., 2012). ECT was shown to increase mRNA levels of the preferential GDNF receptors, GFRα- 1/2, in hippocampal subregions of adult male Sprague Dawley rats and this effect was enhanced with chronic ECT treatment (Chen et al., 2001), while humans receiving ECT showed an increase in serum GDNF, which was 58% greater for responders compared to non-responders (Zhang et al., 2009). These data suggest that antidepressant induced increases in neurotransmitters may mediate downstream
transcriptional regulation of GDNF expression. Increased transcription of GDNF could then act in a retrograde fashion to increase expression of migratory and differentiation genes (Pahnke et al., 2004) culminating in protective and trophic support throughout dysregulated networks aiding the return of a normal behavioural state.

While the insight into the role GDNF in MDD onset and recovery is promising, it must be noted that the variability of findings and methodology used is quite large. For instance, other models of stress have presented with increased GDNF levels in the hippocampus and medial prefrontal cortex (Bian et al., 2012). To contribute to the understanding of hippocampal GDNF in stress mediated depressive illness, acute ACTH exposure was chronically administered to adult male Sprague-Dawley rats for 18 days during circadian nadir. Administered in this manner, ACTH acts to stimulate HPA axis activation, hereby mimicking a state of repeated exposure to an acute stressor. This model has shown to result in an antidepressant resistant phenotype (Kitamura et al., 2002; Walker et al., 2013). To address the influence of antidepressant therapy on hippocampal GDNF levels in this model, infralimbic deep brain stimulation (DBS) was applied for 6 hours per day, beginning on day 7, alongside a sham control stimulation protocol. Stimulation of the infralimbic cortex in the murine model is homologous to stimulation of the anterior cingulate cortex currently in use for DBS of patients with treatment resistant depression. A plasma sample was collected prior to ACTH administration every 72 hours and used to assess the endogenous corticosterone levels of all animals throughout the study protocol.

To the best of our knowledge, the work presented here is the first to assess the effects of infralimbic DBS on hippocampal GDNF levels in an animal model of antidepressant resistance. The data presented here was able to be interpreted in light of previously collected behavioural measures of the forced swim test (see Chapter IV) to assess any relationship of
GDNF with ACTH-induced antidepressant resistance and DBS-induced antidepressant efficacy. Limitations and future directions are also discussed.

Methods

5.2.1 Animals

Male Sprague Dawley rats, aged 9-12 weeks through the duration of experimental protocol (337-478g), were communally housed 2 per cage, under a 12 hour light/dark cycle (lights on at 7:00am), with food and water available *ad libitum*. Animals were permitted to acclimatise to the facility for seven days prior to initiation of experimental protocol, during which time all animals were allocated by caged pairs to one of four experimental groups: control, ACTH, ACTH plus sham infralimbic deep brain stimulation (SDBS) or ACTH plus high frequency infralimbic deep brain stimulation (DBS). All procedures were approved by Deakin University Animal Ethics Committee.

All animals received 18-days of ACTH-(1-24) (AnaSpec, California, USA) 100μg/day dissolved in biological saline or 0.09% saline vehicle for control (Walker *et al*, 2013; Kitamura *et al.*, 2002). All injections were administered intraperitoneally (i.p.) at approximately 10:00am (+/- 2 hours) over the 18 days. Also beginning on day 1 of the experimental protocol, and once every 72 hours thereafter for a total of 6, a sample of 500μL of tail blood was collected into a heparinised tube for corticosterone analysis; these samples were stored on ice during collection, centrifuged at 3000g for 15 minutes, and supernatant collected, stored at -80°C. An additional blood sample was collected post-mortem via cardiac puncture, stored on ice during collection, centrifuged at 3000g for 15 minutes at 4°C, and supernatant collected and stored at -80°C. Corticosterone analysis was conducted using the commercially available corticosterone ELISA kit (Assaypro St. Charles, MO USA). All tail blood was collected prior to ACTH administration, while post-mortem blood was
collected a minimum of 8 hours after injection. Over the 18-day ACTH administration, animals also underwent a number of behavioural assessments to gauge aspects of memory, locomotor function and antidepressant resistance (see Chapter IV).

On day 18 of the protocol, animals were humanely killed via pentobarbitone (Virbac, NSW, Australia) overdose and brain tissue extracted. The brain was immediately rinsed with ice cold distilled water, wrapped in foil and placed in liquid nitrogen. All tissue was stored at -80°C until prepared for analysis. Study protocol is outlined in Appendix B.

5.2.2 Stereotaxic Surgery

After 1 week of acclimatization, rats were anaesthetised using 3% isoflurane and maintained with 1-3% isoflurane throughout surgery. Polymide-insulated stainless steel monopolar electrodes (250 μm in diameter with 0.6 mm of surface exposed, Plastics One Roanoke, VA, USA) were implanted unilaterally into the left infralimbic area (AP: +/- 3.0, ML: +/- 0.4, DV:-5.6 (Paxinos & Watson, 2007). Small jewellers’ screws of approximately 2mm in length were fixed into the skull to act as scaffolding for dental cement that was placed around the stimulating electrode to prevent displacement or damage. All animals were designated SDBS or DBS post-operatively ensuring surgical procedures remained the same across experimental groups. Stimulation was conducted using the AMPI / Master-9 system, which was attached via the ISO-Flex Stimulus Isolator (A.M.P.I., Jeruselum, Israel) to the implanted electrode of the singly housed animal at 130 Hz, 100μA, 90μSec for 6 hours per day, beginning on day 7 of study protocol, as outlined in the appendix.

5.2.3 Plasma Corticosterone

To assess the effects of exogenous ACTH administration on corticosterone levels, the blood collected throughout the study was analysed according to manufacturer’s specifications
using commercially available ELISA kits (Assay Max Corticosterone ELISA Kit AssayPro, Saint Charles, MI USA). All reagents were provided by the manufacturer and prepared prior to use according to instructions.

Briefly, standards were prepared with EIA diulent via serial dilution (100-0 ng/ml) for a total of 6 corticosterone standards. Plasma samples were prepared at a 1:4 concentration with EIA diluents. 25μl of samples and standards were loaded in triplicate into the pre-coated 96-well plate (polyclonal antibody against corticosterone), on top of which, 25μl of biotinylated corticosterone was also loaded. The plate incubated for 2 hours at room temperature.

Near the end of the 2 hour incubation period, streptavidin-peroxidase (SP) conjugate was prepared, 1:100 with EIA diluent. At the end of 2 hours, the 96-well plate was washed 5 times with wash buffer, following which excess liquid was carefully removed from the wells. Once clean, 50μl of SP conjugate was added to each well and incubated at room temperature for 30 minutes and then washed. 50μl chromogen substrate was then added to each and incubated at room temperature for 20 minutes. Finally, 50μl of stop solution was added to each well, and the plate was immediately read for optical density (OD) at 450nm and again at 570nm with a pathlength correction of 100μl (Bio-Rad xMark Microplate Spectrophotometer, Hercules, CA USA). Final readings were obtained by OD450-OD570. Samples were averaged and compared against the standard curve and multiplied by the dilution factor for final concentration of plasma corticosterone.

5.2.4 Brain Dissection and Preparation

Brain tissues were removed from -80°C and kept on dry ice until dissected at which time the brain was allowed to thaw on ice for approximately 60-90 seconds. This slight reduction in temperature allowed more precise cutting and reduced the risk of fracturing to
the whole brain. Dissections were completed on a sterilised metal plate over ice to ensure a sufficiently cold work surface.

The first cut removed the frontal lobe, approximately the first 2 mm of tissue, demarcated on the ventral side by the optic chiasm. The pituitary was then removed, following which the remaining cortex was disarticulated from the internal structures and the rhombencephalon was removed (see section 2.4.1). In this state the hippocampus is clearly delineated by the corpus callosum and can be gently rolled away from underlying white matter as a complete structure. Once fully separated, the hippocampus was laid out in a horizontal line, divided evenly into left and right hemisphere sections, which were again evenly divided into dorsal (medial) and ventral (lateral) sections (see figure 2.8 E). All tissue was stored at -80°C unless analysed within 24 hours, in which case samples were kept at -20°C.

All tissue samples were kept ice cold during preparation for GDNF ELISA. Samples were homogenized in 500μL ice cold lysis buffer with added protease inhibitors (150mM NaCl, 50mM Tris 1% Triton x 100, 40mM PMSF, 1.7mg/mL aprotinin, 1mg/mL leupeptin and 1mg/mL pepstatin) using a hand held homogenizer (Pro Scientific, Inc. Oxford, CT). Samples were allowed to sit on ice for 10 minutes to ensure that all tissue had been completely blended. Samples were centrifuged at 4°C for 20 minutes at 9000g. Supernatant was then collected and stored at -20°C until used in ELISA analysis.

5.2.5 GDNF ELISA

GDNF was measured using the commercially available Rat GDNF ELISA Kit (EK0363, Insight Genomics, Falls Church, VA, USA). The kit included a 96-well plate, pre-coated with anti-rat GDNF antibody, and requisite reagents and buffers, save the washing buffer which was prepared according to manufacturer’s instructions prior to conducting the
Rat GDNF standard was prepared no more than 2 hours prior to beginning the ELISA at concentrations of 1000 pg/ml - 31.2 pg/ml. Hippocampus samples were diluted at a concentration of 1:2 using kit provided sample diluent buffer. Samples and standards, including one blank of only diluent, were loaded in duplicate at a volume of 0.1 ml per well to the pre-coated 96-well plate, sealed with aluminium foil and allowed to incubate at 37°C for 90 minutes. After this incubation period, the well plate was emptied, then loaded with 0.1 ml of the kit provided biotinylated anti-rat GDNF antibody working solution and incubated at 37°C for 60 minutes. When incubation was complete, the plate was washed 3 times with the prepared buffer which sat in the well for 1 minute. After the third wash, 0.1 ml of ABC working solution (Avidin-Biotin-Peroxidase Complex) was added to each well, and incubated at 37°C for 30 minutes. After 30 minutes, the wells were washed 5 times as before. After the fifth wash, 90 μl of TMB colour developing solution was added to each well. The plate was then incubated for 15 minutes at 37°C, after which 0.1 ml of TMB stop solution was added to each well. The plate was immediately read for OD absorbance at 450 nm (Bio-Rad xMark Microplate Spectrophotometer, Hercules, CA, USA). GDNF levels were calculated by zeroing each well value; i.e. O.D. reading - OD of blank well and calculated against the standard curve.

GDNF levels were evaluated as ng/mg of protein. Total protein was assessed using commercially available bicinchoninic acid (BCA) ELISA kits (Pierce® BCA protein assay kit, Thermo Scientific, Rockford, IL USA). According to previously published work, total GDNF, as opposed to free, content was measured.
Results

5.3.1 Plasma Corticosterone Levels

A mixed between-within subjects analysis of variance was conducted to assess the impact of 3 treatments and a control on plasma corticosterone levels across 7 time periods during daily ACTH administration. Levene’s tests for equality of variance were not violated. Although Box’s test could not be computed, the sample size in each cell was larger than 20, indicating that the data are robust to violations of normality and equality of variance allowing for the use of the this statistical technique (Tabachnick & Fidell, 2007). However, owing to this violated assumption, the more conservative Pillai’s Trace statistic was interpreted.

There was no significant interaction between treatment group and time, Pillai’s Trace = 1.031, F(18, 45) = 1.308, p = .228, partial eta squared = 0.344. There was a significant effect of time, Pillai’s Trace, F(6, 13) = 7.898, p = .001 (Greenhouse-Geisser = 3.043, p = 0.026, partial eta squared = 0.145,) with all groups showing a general decrease in corticosterone levels across the seven time periods (see Table 5.1). This indicates a tolerance effect by all groups to the administration of the testing protocol. The main effect comparing treatment group was not significant, F(1,3) = .489, p = .694, partial eta squared = .075 suggesting no difference in the effect of treatment group on corticosterone levels, however the large effect size of treatment group indicates that significance may be attained with increased power.
### Table 5.1.

**Plasma corticosterone levels for all treatment groups through study protocol**

<table>
<thead>
<tr>
<th>Time period</th>
<th>Control</th>
<th>ACTH</th>
<th>SDBS</th>
<th>DBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>M</td>
<td>SD</td>
<td>n</td>
</tr>
<tr>
<td>Day 1</td>
<td>6</td>
<td><em>266.178</em></td>
<td>137.28</td>
<td>7</td>
</tr>
<tr>
<td>Day 4</td>
<td>6</td>
<td>145.85</td>
<td>82.475</td>
<td>7</td>
</tr>
<tr>
<td>Day 7</td>
<td>6</td>
<td>207.829</td>
<td>110.004</td>
<td>7</td>
</tr>
<tr>
<td>Day 10</td>
<td>6</td>
<td>123.887</td>
<td>84.783</td>
<td>7</td>
</tr>
<tr>
<td>Day 13</td>
<td>6</td>
<td>112.229</td>
<td>121.849</td>
<td>7</td>
</tr>
<tr>
<td>Day 16</td>
<td>6</td>
<td>171.916</td>
<td>100.976</td>
<td>7</td>
</tr>
<tr>
<td>Postmortem (Day 18)</td>
<td>6</td>
<td>130.171</td>
<td>69.63</td>
<td>7</td>
</tr>
</tbody>
</table>

* *P* ≤ .05
In light of the effect of time, further one-way ANOVAs were conducted to determine at which time points statistically significant differences occurred (see figure 5.1). Results indicate a significant effect of group for day 1 ($F(3, 23) = 3.227, p = .041$). Further independent $T$-tests revealed that on day 1, the control group displayed significantly higher levels of plasma corticosterone ($M = 285.78$, $SD = 135.62$) than the DBS group ($M = 110.17$, $SD = 63.57$), $t(27) = 3.06$, $p = .014$. These results indicate that the group designated as control had significantly higher plasma corticosterone levels prior to treatment protocol which must be taken into consideration when interpreting all results (see table 1).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control; 18 days saline IP</td>
<td>180</td>
</tr>
<tr>
<td>ACTH; 18 days ACTH IP</td>
<td>100</td>
</tr>
<tr>
<td>SDBS; 18 days ACTH IP plus sham deep brain stimulation</td>
<td>200</td>
</tr>
<tr>
<td>DBS; 18 days ACTH IP plus 6 hours high frequency deep</td>
<td>300</td>
</tr>
</tbody>
</table>

**Figure 5.1.** Day 1 plasma corticosterone levels for all treatment groups. All values are expressed as group average +/- SEM. (control $n = 6$, ACTH $n = 7$, SDBS $n = 4$ and DBS $n = 5$) **$P \leq .01$**

### 5.3.2 Hippocampal GDNF Levels

A one way ANOVA was carried out to assess the effect of treatment on GDNF levels in the dorsal and ventral hippocampus. Protein concentration was assessed as nanograms of GDNF per milligram of protein in total sample loaded for GDNF ELISA. In the dorsal hippocampus (see Figure 5.2 A), data were not normally distributed; therefore the Kruskal-Wallis test was used to assess for an effect of treatment on GDNF levels in this structure. Kruskal-Wallis test revealed no significant difference in dorsal GDNF levels across the four
treatment groups (Gp1. n = 7: control, Gp2 n = 8: ACTH, Gp3 n = 6: SDBS, Gp4 n = 6: DBS), \( \chi^2 (3, n = 27) = 1.30, p = .728 \). The DBS treatment group recorded the highest median score (Md = 5.497) followed by the ACTH treatment group (Md = 5.432), the Control treatment group (Md = 5.271) and finally the SDBS treatment group (Md = 4.757). Further Mann-Whitney U tests revealed no significant differences in GDNF levels between any treatment groups for the dorsal hippocampus.

In the ventral hippocampus, (see figure 5.2 B), data were normally distributed allowing for the use of the ANOVA technique. Levene’s test for equality of variance was not violated, \( W(3, 23) = .813, p = .500 \), which allowed for the use of the more robust Bonferroni post test. A one-way between groups ANOVA was conducted to explore the impact of treatments on GDNF levels as measured by ELISA. There was no statistically significant effect of treatment on GDNF levels \( F(3, 23) = 1.59, p = .219 \). However, the actual difference in mean scores between the groups was quite large. The effect size, calculated using eta squared, was .17. Therefore, with increased power, a significant effect of treatment may be found for GDNF levels in the ventral hippocampus. Further Independent-samples t-tests were conducted to assess for significant difference in GDNF levels between treatment groups. There was no significant difference found in GDNF levels between any treatment groups for the ventral hippocampus.
Figure 5.2. GDNF levels (ng/mg protein) for dorsal (A) and ventral (B) hippocampus. Data are expressed as the group percent average +/- SEM of the mean. (control n = 6, ACTH n = 7, SDBS n = 4 and DBS n = 5)

5.3.3 Relationships Between Behavioural and Biochemical Analyses

5.3.3.1 Corticosterone and GDNF

The relationship between plasma corticosterone levels and GDNF levels of the hippocampus at time of death were calculated using Pearson product-moment correlation coefficient. Preliminary analysis showed no serious violation of the assumptions of normality, linearity and homoscedasticity. There was a positive medium strength relationship between corticosterone levels and total hippocampal GDNF levels, $r = .39, n = 27, p = .042$ with higher levels of corticosterone associated with higher levels of combined (total ventral and dorsal) hippocampal levels of GDNF. The coefficient of determination indicated these variables share 15.25% of the variance. There was also a positive medium strength relationship between corticosterone levels and total ventral GDNF levels, $r = .43, n = 27, p =$
.026 with higher levels of corticosterone associated with higher levels of ventral hippocampal levels of GDNF. The coefficient of determination indicated these variables share 18.32% of the variance. These results indicate that as the endogenous levels of corticosterone increase, so do the levels of GDNF in the hippocampus. Furthermore this overall increase in hippocampal GDNF is likely driven by an increase in ventral hippocampal GDNF levels.

5.3.3.2 Corticosterone and Forced Swim Test

The potential relationship between corticosterone levels and immobility in the forced swim test was also assessed using Pearson product-moment correlation coefficient. As the FST was conducted on the final day of the experimental protocol, immobility time was correlated with blood collected at time of death, which was a minimum of one hour after completion of the FST. Preliminary analysis showed no serious violation of the assumptions of normality, linearity and homoscedasticity. There was an insignificant negative medium strength relationship between corticosterone levels and immobility time in the FST, \( r = -0.30, n = 27, p = 0.127 \). These results indicate there is no significant relationship between plasma corticosterone levels one hour post FST and immobility time in the FST.

5.3.3.3 GDNF and Forced Swim Test

Pearson product-moment correlation coefficient was also calculated to assess if any relationship existed between hippocampal GDNF levels at time of death and immobility time in the FST. There was an insignificant positive medium strength relationship between combined total bilateral hippocampal GDNF levels and immobility time in the FST, \( r = 0.244, n = 27, p = 0.220 \). These results indicate there is no significant relationship between hippocampal GDNF levels one hour post FST and immobility time in the FST.
Discussion

Consistent with the literature, corticosterone levels displayed a decrease across groups over time, likely due to both procedural habituation and system feedback (Walker et al., 2013). These data do not support an effect of acute ACTH exposure, chronically administered during circadian nadir over 18 days in moderating hippocampal GDNF levels of adult male Sprague Dawley rats despite the positive correlation between plasma corticosterone levels and GDNF levels within this structure. Furthermore, neither corticosterone nor GDNF levels were found to correlate with immobility time in these animals one hour after the forced swim test.

Differences observed in day one plasma corticosterone levels, however, present a particularly unexpected and potentially confounding finding; given control animals displayed significantly higher levels of corticosterone relative to DBS-treated animals. Control levels were also elevated as compared to ACTH and SDBS animals, and remained the highest of all experimental groups until day 10 (see table 5.1) albeit not significantly. This indicates that animals designated control had, by chance, a stronger reactivity to stress than other animals. As day one levels were taken prior to the administration of any drug, and all animals were handled prior to initiation of research protocol as part of acclimatisation, this appears to be an unanticipated artefact, perhaps resulting from procedural restraint. All other results must be interpreted in light of this elevated baseline finding. This initial difference suggests that an effect of ACTH may have gone undetected due to the hyper-reactive stress response of control animals. These animals could therefore be exhibiting behaviour similar to those of ACTH animals resulting in a type II error.

When considered in light of previous work by our group (Walker et al., 2013) in which plasma corticosterone samples were collected after ACTH administration, details of the HPA axis reactivity begin to emerge. The combined data suggest that while ACTH
induced changes can be observed immediately post administration, given 24 hours to adjust, corticosterone levels recover, even with chronic treatment (see figure 5.3). However, given that these animals display tricyclic antidepressant resistance and antidepressant response with infralimbic DBS, as per FST, basal cortisol levels may not be a reliable predictor of depression or antidepressant reaction, but instead suggest that the reactivity of an organism to a stressor challenge is a more accurate measure. This is fitting with results from HPA manipulation in clinical populations (Carroll et al., 2007; 2012) and further supported by the chance rate of hypercortisolism and depression comorbidity suggesting cortisol is not a trait of the illness (Lee, Ogle, & Sapolsky, 2002). Taken together, corticosterone per se may not be responsible for the antidepressant resistant phenotype, but instead dysregulation of feedback within the HPA system could be especially important, particularly when paired with a stressful environmental challenge as occurs in the forced swim test.
Figure 5.3. Mean plasma corticosterone (ng/ml, from Table 5.1) as measured prior to or after ACTH administration (inset, Walker et al., 2013).

Together these data suggest that ACTH-mediated changes in HPA-axis reactivity were induced, as seen when measured post-ACTH (inset), but system normalisation occurred within 24 hours. This indicates that plasma cortisol levels may not be a reliable predictor or marker of MDD or antidepressant response. Data are presented as mean ± SEM. *$P \leq .05$, (control n = 6, ACTH n = 7, SDBS n = 4 and DBS n = 5) **$P \leq .01$
GDNF analysis revealed no significant difference between groups for levels in the dorsal hippocampus. The same analysis for the ventral hippocampus also revealed no significance, however, a large effect size was found for this structure indicating that with increased power a significance level may be attained. This trend is in keeping with the theory that the ventral hippocampus is more closely associated with emotional memory, whereas the dorsal hippocampus is more involved with mnemonic process, especially spatial working memory. While speculative, these findings are in line with the normal function exhibited by all animals in the spatial memory tasks (Chapter IV), and support an effect of behavioural test stress on the ventral emotive system. Alternatively, as studies have found changes in mRNA, not protein (Hisaoa et al., 2001, 2008; Golan et al., 2011) this trend may indicate that with longer duration, a change in GDNF may be measureable or that a change could have previously occurred and returned to baseline levels.

There is evidence to suggest that these findings may be accurate in a model of depression. Work by Uchida et al. (2011) found no change in GDNF levels in the hippocampus under the chronic unpredictable mild stress paradigm; they did however find changes of the neurotrophic factor in the striatum. This group also induced modified behaviour in the BALB/c strain by over-expressing GDNF in the nucleus accumbens and confirmed epigenetic changes in the promoter region for GDNF in this structure. While these findings are complementary, several key differences exist in that Uchida’s work used the genetically distinct mouse strain, BALB/c, which are known to be stress-vulnerable, and a behavioural rather than biochemical stress protocol. Despite these differences, this murine model supports the need for further analysis of the role of GDNF in the ACTH model. It is also important to note that we are focusing here solely on memory, not behavioural response, and with this new information, new protocols can be developed bearing these findings in mind.
Limitations to this study include small numbers and a potential confound of housing. Animals in this study were cage paired by treatment group to avoid accidental administration of the treatment drug to a non-treatment animal. It is possible that ACTH influenced the behaviour of the animals toward the cage mate and somehow altered the stress reactivity of those animals receiving ACTH. This point was made particularly relevant by a small group of ACTH animals who began displaying overtly aggressive and dominant behaviour towards cage mates. This behaviour was displayed between all of the animals of this particular group at all times throughout the study, making this unlikely to be an attempt at hierarchy establishment. Due to this drastically anomalous behaviour, these animals were not included in any data analysis, but have highlighted a potentially unexpected avenue of further research regarding this antidepressant resistant model. In the future, this can be addressed by cross-caging animals or monitoring their behaviour during the wake cycle.

In light of these findings, future work using this model should involve pre-screening animals for stress reactivity by collecting a blood sample for corticosterone analysis prior to assigning groups. After analysis of stress reactivity, animals could then be balanced across groups. To further address the role of HPA axis reactivity in this model, corticosterone samples should also be collected after ACTH administration and differential fluctuations in corticosterone across the circadian rhythm should be quantified. It may also be a useful endeavor to explore future stress reactivity results as normalized values, for example the percentage of original value at time 1 for each animal. This may control for between subjects variation which could confound the group to group comparisons, however as analyzed here, between group comparisons are still relevant. A time course method should also be employed to assess for any changes in GDNF levels that may have normalised by day 18, while alternate methods of assessing mRNA levels may also be of value. Additionally it will be important to understand the role of GDNF in interaction with dopaminergic signaling. Given
that GDNF has been shown to promote survival and differentiation of dopamine neurons, combined with the established role of dopamine in mediating pleasure, a comprehensive understanding of this relationship in vivo could offer a potential means of therapy to address the core symptom of MDD, anhedonia (Nestler & Carlezon, 2005). Neurotrophic mediated mechanisms of treatment response may then be the result of the neuron or network response to GDNF, rather than a change in the protein per se. Measuring such changes involves the assessment intracellular signaling pathways and post-synaptic changes in response to pro-depressive and antidepressant protocols, which is the focus of the subsequent research presented in this thesis.
Works Cited


Chapter VI: Effects of Adrenocorticotropic Hormone, Forced Swim Test and Deep Brain Stimulation on Neurotrophin Intracellular Signalling Pathway Gene Expression Levels in the Prefrontal Cortex
Abstract

Atrophy of the frontal lobes is one of the most consistent and robust neurophysiological abnormalities associated with major depressive disorder (MDD) and prefrontal activity displays an inverse relationship with depression severity. Through reciprocal connections to limbic and circadian networks, this hypometabolism functionally manifests as the mood and cognitive symptoms of MDD, described in the DSM-5. Furthermore, vulnerability of these innervated limbic structures to glucocorticoids contributes to the continuation of depressive states through stress-induced neuronal remodelling and reduction in plasticity. These alterations in activity, metabolism and symptomatology are attenuated with effective antidepressant treatment. The mechanisms mediating these antidepressant-induced changes remain unknown and represent one of the foremost challenges of current MDD research. To contribute to this understanding, global gene expression profiling using the microarray platform was conducted to assess alterations in gene expression of the neurotrophic factor intracellular signalling pathways. Sprague Dawley rats were subjected to behavioural (forced swim test, FST) and/or hormonal (adrenocorticotropic hormone, ACTH) stressors and compared with saline treated control animals. An additional group of ACTH-treated animals were also treated with infralimbic (IL) deep brain stimulation (DBS) as antidepressant therapy and underwent FST stress. Alterations in the expression of genes within intracellular neurotrophic factor pathways of the infralimbic region of the brain were compared across groups. The data demonstrate differential expression of genes within neurotrophin signaling pathways in behaviorally stressed animals relative to stress naïve controls, which are distinct between ACTH- and saline-pretreated animals. That is, the cellular responses following FST stress are distinct in antidepressant resistant and control animals. Moreover, DBS modulated these responses, highlighting an important cellular action of DBS in the brains of animals non-responsive to antidepressants that may facilitate therapeutic neuroadaptations.
A substantial sub-group of individuals suffering with MDD receive little or no therapeutic benefit from first line antidepressant treatments (Fava & Davidson, 1996; Hindmarch, 2001; Ressler & Mayberg, 2007). The reasons for this lack of response remain poorly understood and exploratory approaches are required to assist in the identification of novel therapeutic mechanisms. Antidepressant resistance, when taken into consideration with the minimal 3 weeks therapeutic lag time of most antidepressant medications, indicates that therapeutic mechanisms of antidepressant efficacy likely lie beyond the known modulation of neurotransmitter dynamics at the synapse. Neural adaptations occurring in response to antidepressant exposure and alterations in neurotransmitter levels may be impaired. Such responses are mediated by intracellular signaling cascades, including neurotophin signaling. Determining how these cellular mechanisms mediating adaptive responses to antidepressant treatments are impaired in treatment resistance will both broaden our understanding of therapeutic pathways for MDD and improve available treatment options and efficacy. DBS provides a novel approach for achieving this as it has been effective in cases where all other treatments have failed (Mayberg et al., 2005). Unfortunately, recent multisite clinical trials have failed to replicate the efficacy observed by experienced groups, suggesting there is still much we need to learn about the mechanisms mediating both antidepressant resistance and DBS-mediated antidepressant response therein to ensure these interventions can be effectively translated to the broader community of practicing clinicians.

The neurotrophic hypothesis of depression and antidepressant response purports that efficacious treatments engage cell signaling cascades that mediate cell growth, repair and plasticity (i.e neurotrophic signaling), downstream of elevated synaptic monoamine (Duman & Monteggia, 2006). This plasticity is achieved through activation of several intracellular pathways that are stimulated by neurotrophic factors (refer to figure 1.7), most notably p42/44-MAPK (mitogen-activated protein kinase, hereafter, MAPK), PLC-γ (phospholipase
Cγ), PI3K (phosphatidylinositol 3-kinase) and p38-JNK (Jun N-terminal kinase) pathways, the latter being activated through neurotrophin binding to the p75 nerve growth factor receptor (p75NTR). These cell signalling pathways in turn modify gene expression through CREB (cyclic AMP response element binding protein), BCL2 (B-cell lymphoma 2) and TNFSF6 (tumour necrosis factor ligand superfamily member 6) to ultimately mediate cell survival and proliferation (Chao, 2003; Duman et al., 2007). Such changes at the cellular and network level manifest as clinical improvement in depressive symptoms (Charney & Manji, 2004).

In line with this, functional imaging supports a role for plasticity in mediating the onset of depression via hyperfunctioning limbic regions and hypofunctioning dorsal regions. Specifically, functional hypometabolism of the rostral cingulate acts as a predictor of poor antidepressant efficacy based on a reduced adaptive potential (Helen S. Mayberg, 1997). Furthermore, as a stress related psychiatric illness, glucocorticoids have been linked to reduced neural survival and function, especially within the neurogenic hippocampal region in depression (Sapolsky et al., 1986; Sapolsky, 2000). Together, these data highlight a role for the interplay of glucocorticoids, neurotrophic factors and neurotransmitters in mediating intracellular pathways governing synaptic plasticity as mechanisms of depression and antidepressant response (Pittenger & Duman, 2008).

Microarray studies have shown that several components of the intracellular neurotrophic factor signalling pathway are dysregulated in both animal models of stress and depressive behaviour, as well as in post-mortem brain tissue from individuals who have committed suicide during a depressive episode. Specifically, postmortem data have measured reduced levels of CAMK2A (calcium/calmodulin-dependent protein kinase II alpha), AKT (serine/threonine kinase Akt/PKB), BDNF (brain derived neurotrophic factor), TrkB (neurotrophic tyrosine kinase, receptor, type 2), RAF (V-raf murine sarcoma viral oncogene
homolog B), MEK (aka Map2k, mitogen-activated protein kinase kinase), CREB and ERK1/2 (aka Mapk, mitogen-activated protein kinase 1 / 2) in the hippocampus and prefrontal cortex of suicide victims (Tochigi et al., 2008, Hsiung et al., 2003; Karege et al., 2007; Dwivedi et al., 2001, 2003, 2006; Grønli et al., 2006; Lanni, Govoni, Lucchelli, & Boselli, 2009; Shirayama et al., 2002). These findings have been confirmed using various animal models of stress, that present with reduced phospo-ERK, BDNF, TrkB, FZD6 (frizzled family receptor 6) and MAPK activity (First et al., 2010; Dwivedi et al., 2003; Duman et al., 2007); in turn, these trends are reversed with antidepressant treatment, especially within the hippocampus (Okamoto et al., 2010; Newton et al., 2003; Duman, Henniger & Nestler, 1997; Duman et al., 2000; Nibuya, Nestler & Duman, 1996; First et al., 2010; Beurel, Song & Jope, 2011; Saarelainen et al., 2003; Chen & Chuang, 1999; Cao et al., 2004; Kodama, Russell & Duman, 2005; Mercier et al., 2004; Larsen et al., 2008). These findings have been confirmed by in situ hybridization, real-time polymerase chain reaction (RT-PCR) as well as western blotting, indicating that both mRNA and protein levels are affected in active depression and antidepressant response. Interestingly MKP-1 (aka DUSP1, dual-specificity phosphatase-1) a negative regulator of the MAPK pathway, was significantly dysregulated according to whole-genome expression microarray analysis (Duric et al., 2010). This was further assessed through quantitative real-time polymerase chain reaction (qPCR) and in situ confirming that mRNA expression and levels, respectively, of MKP1 were increased in the hippocampus of both human suicide and animal models of depression, while an MKP1 null transgenic mouse model, having no MKP1 expression, shows resilience to stress (Duric et al., 2010). This is of particular interest regarding recent insight into the rapid antidepressant effects of ketamine, which also utilises PI3K and MAPK pathways (Beurel et al., 2011; Duric and Duman, 2013; Duman et al., 2012). The influence of these intracellular pathways in rapid DBS-mediated antidepressant efficacy, however, remains to be determined,
though evidence indicates its ability to modulate shared signaling mechanisms (Jimenez-Sanchez, Castane, et al., 2016).

To investigate the role of the neurotrophin signalling system in antidepressant-resistance and DBS-mediated antidepressant efficacy, we have quantified mRNA expression for this cell signalling pathway in the infralimbic (IL) region of the prefrontal cortex of adult male Sprague-Dawley rats treated with ACTH over 14 days (100μg/day), subjected to the FST or both treatments combined, half of which also received IL-DBS. The ACTH treatment results in a tricyclic antidepressant resistant phenotype (Kitamura et al., 2002; Walker et al., 2013) in which IL-DBS has demonstrated efficacy as measured with the forced swim test (FST). The results of this work will guide future studies aimed at elucidating mechanisms of both antidepressant resistance and DBS-mediated antidepressant response.

Methods

6.2.1 Animals

Male Sprague Dawley rats, 8-10 weeks through the duration of experimental protocol, weighing 200-350g during testing, were communally housed 2 per cage under a 12 hour light/dark cycle (lights on at 7:00am) with food and water available ad libitum. Animals were permitted to acclimatise to the facility for seven days prior to initiation of experimental protocol, during which time all animals were randomly allocated to one of five experimental groups: saline/naïve, ACTH/naïve, saline/FST, ACTH/FST and ACTH/FST/DBS (see table 6.1). All procedures were approved by Deakin University Animal Ethics Committee.

Animals designated naïve experienced no behavioural testing, thus saline naïve received 1 millilitre of 0.09% saline (Baxter, NSW, Australia) vehicle and animals designated ACTH naïve received 14-days of ACTH-(1-24) (Sigma, AnaSpec, California, USA) 100μg/day dissolved in 1 millilitre distilled water (Walker et al, 2013; Kitamura et al., 2002).
Animals designated FST were subjected to the Porsolt forced swim test in addition to a saline injection protocol.

**Table 6.1.**

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Abbreviation</th>
<th>14-Day Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Naïve</td>
<td>Ctl (n = 8)</td>
<td>1 ml 0.9% saline per day</td>
</tr>
<tr>
<td>ACTH Naïve</td>
<td>ACTH (n = 8)</td>
<td>100μg ACTH per day</td>
</tr>
<tr>
<td>Saline/FST</td>
<td>FST (n = 8)</td>
<td>1 ml 0.9% saline per day + FST</td>
</tr>
<tr>
<td>ACTH/FST</td>
<td>AFST (n = 8)</td>
<td>100μg ACTH per day + FST</td>
</tr>
<tr>
<td>ACTH/FST/DBS</td>
<td>DBS (n = 7)</td>
<td>100μg ACTH per day + FST + IL DBS</td>
</tr>
</tbody>
</table>

Animals designated AFST received 14-days of ACTH, as above, and were subjected to the FST. Animals designated DBS received 14-days of ACTH, as above, were subjected to the FST and underwent 4 hours of unilateral (left) IL DBS on day 14 and 2 hours of IL DBS on day 15 (see table 6.1). All injections were administered intraperitoneally (i.p.) during circadian nadir, at approximately 9:00 am over the 14 days. Study protocol is outlined in Appendix C.

Exactly 1 hour after exposure to the FST, or at the equivalent time of day for naïve groups, animals were humanely killed via pentobarbitone overdose (Virbac, NSW, Australia) beginning with 4ml i.p. and supplied as needed. Brain tissue was extracted, immediately rinsed with ice-cold distilled water, wrapped in foil and placed in liquid nitrogen. All tissues were stored at -80°C until prepared for analysis. A blood sample was also collected at this time through cardiac puncture, centrifuged at 3000g at 4°C for 15 minutes, supernatant collected and stored in -80°C for plasma corticosterone analysis using the commercially available corticosterone ELISA kit (Assaypro St. Charles, MO USA).
6.2.2 Stereotaxic Surgery

After 1 week of acclimatisation, rats were anaesthetised with ketamine (25mg/kg) and xylazine (5mg/kg) mixture. Anaesthesia was maintained throughout surgery as needed. Polymide-insulated stainless steel monopolar electrodes (250 μm in diameter with 0.6 mm of surface exposed, Plastics One, Roanoke, Va USA) were implanted unilaterally into the left IL (AP: +/- 3.0, ML: +/- 0.4, DV:-5.6; Watson and Paxinos, 2007). Small jewellers screws of approximately 2mm in length were fixed into the skull to act as scaffolding for dental cement that was placed around the stimulating electrode to prevent displacement or damage. Stimulation was conducted using the AMPI / Master-9 system, which was attached via the ISO-Flex Stimulus Isolator (A.M.P.I., Jeruselum, Israel) to the implanted electrode of the singly housed animal at 130 Hz, 100μA, 90μSec from approximately 9:00am for a total of 4 hours on day 14 and 2 hours on day 15. Study protocol is outlined in Appendix C.

6.2.3 Microarray

Total RNA was extracted from relevant tissue sample using TRIzol reagent, and purified using RNeasy-Mini Kit columns (Qiagen, Mannheim, Germany). Quality and quantity of purified RNA was determined using the Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA) and RNA6000 NanoAssay Kit (Agilent, Melbourne, Australia). Fluorescently-labelled cDNA was prepared from 800ng total RNA using Agilent’s Quick-Amp Labelling and One-Color RNA Spike-In kits. The cyanine 3-CTP-labelled cDNA was hybridised for 17h to Agilent Whole Rat Genome (4x44k) Oligo Microarray Slides (44,000 genes and transcripts) using Agilent Gene Expression Hybridisation kit. Fluorescent images of microarrays were acquired using GenePix 4000B scanner, with data extraction performed via GenePix 5.1 software (Molecular Devices, Melbourne, Australia).
6.2.3.1 Analysis of Microarray Data

Normalisation and primary analysis of microarray data was performed using Aequity 4 software (Molecular Devices). Briefly, the fluorescent reading for all duplicate genes were averaged, leaving 37,047 unique array gene identification numbers. Data from each array was scaled by normalising the expression values against the mean of all features that pass the quality control criteria, which define an acceptable expression value as one that is 2 standard deviations above the background noise and is not derived from a saturated signal. Each array dataset sample was normalised so that the median expression value in each array was 1.0. The relative mRNA expression levels were expressed as high or low depending on the amount of fluorescence detected. Data was analysed using SPSS version 20.0 (IBM Corp., Armonk, NY USA). Distribution of the data was assessed using the Kolmogorov-Smirnov test. Normally distributed data was analysed using one-way ANOVA analysis to test for mean differences between treatment groups, and $p$ values were adjusted for multiple comparison testing using the Bonferroni correction to identify differentially expressed genes. Genes were determined to display differential gene expression if $p \leq 0.05$ following Bonferroni correction. Data that were not normally distributed were assessed using Kruskal-Wallis H test to determine differences in group means.

Due to the multiple comparisons made when total data from the microarray chip was analysed, it is plausible that type II errors are present when looking at a small subset of data as was done here, making it necessary to differentiate between statistical significance and biological significance. Therefore, pathway analysis began at points where data showed consistent and replicable significance, the latter dependent on the number of repeated hybridized sequences for any given gene on the microarray chip. From there, points up and downstream of those statistically robust gene expressions were assessed by fold change, in an attempt to identify potential pathways of interest, rather than points throughout the entire
signalling profile. In doing this, a more complete picture may be more accurately ascertained for future protein confirmation analysis (Surget et al., 2009).

Results

Overall, 30 genes displayed statistically significant changes in mRNA expression levels, of these, 17 represented changes incurred by ACTH, FST or AFST as compared to Ctl (see table 6.2). These changes were predominant to the MAPK and p75NTR pathways, the latter exhibiting increased p75 receptor mRNA level for the AFST group indicating a potential “2-hit” effect of doubled stress in upregulating the apoptotic component of the neurotrophin intracellular pathway. The overall significant differences also show that Psen1 (presenilin 1) and Akt are down regulated while CREB2 is upregulated with stress, and these are not attenuated with the application of DBS, suggesting that these points may be important in mediating antidepressant resistance.

Thirteen significant mRNA expressions represented changes that appeared to be regulated by the application of DBS as compared to each other treatment group (see table 6.2). DBS resulted in changes of Matk/CHK (megakaryocyte-associated tyrosine kinase) and Ripk2 (receptor-interacting serine-threonine kinase 2) expression levels, indicating DBS may preferentially down regulate Matk and up-regulate Ripk2 as a mechanism of efficacy. Also, an up-regulation of RhoA (ras homolog family member A) by DBS in only stress groups is seen, suggesting a preferential modulation of this gene and/or pathway by DBS in symptomatic neurophysiology only- a modulation which results in normalization to control levels.

Pathway analysis was also conducted for MAPK and p75NTR intracellular signalling pathways to ascertain more specific points of interest. Due to the robust decreased expression of AKT, the PI3K and PLCγ pathways were also more closely examined to assess potential
effects of treatment on plasticity and cell survival. All abbreviation definitions for genes can be found in appendix 9.5.
### A. Fold change in gene expression of stressor versus control

<table>
<thead>
<tr>
<th>Gene</th>
<th>ACTH</th>
<th>FST</th>
<th>AFST</th>
<th>DBS</th>
<th>p&lt;.05/repeat</th>
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<td>.8</td>
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<td></td>
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<td>1/1</td>
</tr>
<tr>
<td>Bax</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td></td>
<td></td>
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### B. Fold change in gene expression of DBS versus each group

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
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<th>FST</th>
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### C

<table>
<thead>
<tr>
<th>MAPK</th>
<th>p75NTR</th>
<th>PI3k</th>
<th>PLCγ</th>
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#### Table 6.2.
Differential mRNA expression levels of neurotrophin signalling pathway genes as affected by **A**: stress, values are fold change as compared to control. **B**: DBS, values are fold change in DBS as compared to each treatment. Right hand column expresses number of t-tests that were statistically significant for all replicates of a given gene. **C**: Colour coded boxes indicate intracellular pathway of each gene in **A** and **B**. See appendix 9.5 for abbreviation definitions. (control n = 8, ACTH n = 8, FST n = 8, AFST n = 8 and DBS n = 7)
6.3.1 Differential Regulation of Neurotrophins and Receptors

No robust changes in mRNA expression levels were found for BDNF in this microarray data set. Nerve growth factor (NGF) showed reduced expression in all treatment groups exposed to stress, including the DBS group, indicating this neurotrophin may be critical in antidepressant resistance. The FST group showed the largest increases in NT3 and NT4/5 expression levels and DBS exerts the opposite effect (see table). Overall, stress increases expression levels of all receptors, which is reversed by DBS; however, missing data prohibits the accurate conclusion of the overall trend for TrkA.

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Figure 6.1. Schematic of neurotrophins (green circles) and their preferential receptors (grey boxes) as regulated by stress (red arrows) and DBS (blue arrows). Direction of arrows indicates direction of change in gene expression levels for the adjacent gene and, where noted, group. Table displays changes in mRNA expression (*P ≤ .05). Each column represents the fold change seen with treatment; e.g., TrkB expression showed a 1.1 fold change with AFST as compared to control and this was attenuated with DBS, which expressed a .9 fold change compared to AFST and expression equivalent to control. See appendix 9.5 for abbreviation definitions. (control n = 8, ACTH n = 8, FST n = 8, AFST n = 8 and DBS n = 7)
6.3.2 Differential Regulation of MAPK Pathway

Due to the high number of significant genes found within the MAPK pathway, further data mining was conducted. The results indicate an overall opposite regulation of gene expression by stressors and DBS (see figure 6.2). This suggests that the MAPK pathway may be mediating the effects of stress and antidepressant effect in this model.
Figure 6.2. Schematic of the genes (grey boxes) in the MAPK intracellular pathway as regulated by stress (red arrows) and DBS (blue arrows). Direction of arrows indicates direction of change in mRNA expression levels for the adjacent gene and, where noted, group. See appendix 9.5 for abbreviation definitions.

There are notable exceptions to the overall opposition trend of gene expression levels by stress and DBS, as the fold change of genes indicates points in the pathway that may be modulated by a specific treatment type (see table 6.3). Specifically, these data suggest that Rap1b (a member of RAS oncogene Family) and Mapkap2k (mitogen-activated protein kinase-activated protein kinase 2) may be targets of DBS as they show a minimum of 1.3 fold
increased expression with DBS against all other treatment groups. The CREB-2 gene showed a statistically significant and robust increase across treatments, indicating that CREB-2 may be regulated by stress and this is not attenuated by DBS. Alternatively, this may be due to stimulation resulting from handling and behavioural testing. Finally AFST predominately shows the least number of significant changes when compared with Ctl, suggesting there may not be a cumulative effect of combined ACTH and FST stressors on the system.

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Table 6.3. Significant changes (*P ≤ .05) in mRNA expression and relevant biological changes of interest in the MAPK pathway. Each column represents the fold change seen with treatment; e.g., Mapkap2k expression showed a 0.9 fold change with AFST as compared to control and this was reversed with DBS, which expressed a 1.3 fold increase in Mapkap2k compared to AFST and expression relatively equivalent to control. See appendix 9.5 for abbreviation definitions. (control n = 8, ACTH n = 8, FST n = 8, AFST n = 8 and DBS n = 7)
6.3.3 Differential Regulation of p75NTR Pathway

The p75NTR pathway exhibits a similar pattern to that of the MAPK pathway, in that ACTH, FST and AFST result in a global down regulation of gene expression levels while DBS exhibits the opposite effect on these same genes. DBS also exerts numerous changes within the pathway (see figure 6.3) including modulating JUND/AP-1 (Jun D proto-oncogene) expression via RIPK2 and IKBKB (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta), a pathway known to result in cell survival. Further to this, stress and DBS regulate RhoA in opposition, indicating a role for cytoskeletal reorganisation in these processes.

The p75 pathway also displays treatment specific modulation (see table 6.4). SC-1 mRNA levels are significantly reduced in the FST and AFST group, but not the ACTH group, suggesting this gene may be susceptible to the stress of the FST, an effect that is normalised with DBS, which displays mRNA levels similar to control. The gene p53 shows a reduction in expression levels in the ACTH and FST groups, but not the AFST group, indicating there is no compounded effect of both stressors on gene expression levels. DBS resulted in increased p53 mRNA levels when compared to any other treatment group, suggesting this gene may be a therapeutic target of DBS treatment. The p75NTR gene exhibits the opposite profile to the p53 gene, displaying a significant increased mRNA level in the AFST group only, suggesting a compounded effect of ACTH and FST, potentially a “2-hit” stress effect. Finally, RhoA gene expression levels were increased with DBS when compared to any other treatment group, suggesting regulation of this gene is a mechanism of action of DBS. The gene PSEN1 shows a significant reduction in gene expression levels in all stress groups and this is not attenuated with DBS, suggesting that this gene may mediate effects of stress.
Figure 6.3. Schematic of p75 intracellular pathway genes (grey boxes) as regulated by stress (red arrows) and DBS (blue arrows).

Direction of arrows indicates direction of change in mRNA expression for the adjacent gene, blue arc indicates inhibition of RhoA. See appendix 9.5 for abbreviation definitions.
### Table 6.4.

Significant changes (*P ≤ .05) in mRNA expression levels and relevant biological changes in the p75NTR pathway. Each column represents the fold change measured with treatment; *e.g.* p75NTR expression showed a 0.8 fold change with ACTH as compared to control. DBS treatment resulted in a 1.7 fold change in p75 expression when compared with ACTH and expression maintained elevation compared to control. See appendix 9.5 for abbreviation definitions. (control n = 8, ACTH n = 8, FST n = 8, AFST n = 8 and DBS n = 7)

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6.3.4 Differential Regulation of PI3K Pathway

The PI3K pathway revealed a global effect of stress. AFST resulted in a robust up-regulation of the mRNA levels of IRS1 (insulin receptor substrate 1) and FASL (Fas ligand) (see table 6.5). The decrease in AKT1 expression levels with stress were not attenuated with DBS, despite a reduction in PDK1 (3-phosphoinositide dependent protein kinase-1) expression levels, the upstream kinase to AKT1. This suggests that regulation AKT1 may be implicated in stress and depression. Furthermore, the lack of increased gene expression levels upstream of IKB (aka Nfkbia, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) and NFKB (v-rel avian reticuloendotheliosis viral oncogene homolog A) here supports that these genes may be modulated by DBS via the p75 pathway.
Figure 6.4. Schematic of PI3K intracellular pathway genes (grey boxes) as regulated by stress (red arrows) and DBS (blue arrows). Direction of arrows indicates direction of change in mRNA expression for the adjacent gene, and, where noted, group. Blue arc indicates inhibition of BAD (BCL2-associated agonist of cell death) and GSK3β (glycogen synthase kinase 3 beta) by phosphorylated AKT. See appendix 9.5 for abbreviation definitions.
Table 6.5. Significant changes (*P ≤ .05) in mRNA expression and relevant biological changes in the PI3K pathway. Each column represents the fold change seen with treatment; e.g., AKT expression showed a 0.7 fold change with AFST as compared to control. DBS treatment resulted in no change in AKT expression as compared to any group. See appendix 9.5 for abbreviation definitions. (control n = 8, ACTH n = 8, FST n = 8, AFST n = 8 and DBS n = 7)
6.3.5 Differential Regulation of PLCγ Pathway

The PLCγ intracellular pathway (see figure 6.4) continued the trend of opposing regulation of mRNA expression by stress and DBS. These patterns could be clearly seen in the fold change of gene expression (see table 6.5), where DBS-affected changes in gene expression were similar to that of control, save p90RSK (aka Rps6ka2, ribosomal protein S6 kinase polypeptide 2) and CREB2, which are also affected through the MAPK pathway. Notably, p90RSK and CREB2 exhibited the same pattern of gene expression for all treatment groups when compared to control. However, while the p90RSK gene expression level was down regulated by DBS when compared to any stress treatment group, when compared to control, the expression level of the p90RSK gene in the DBS group is elevated when compared to control, suggesting that DBS does not return p90RSK mRNA levels to control and may be a target of stress (see table 6.5).
Figure 6.5. Schematic of PLCγ intracellular pathway gene (grey boxes) expression levels as regulated by stress (red arrows) and DBS (blue arrows). Direction of arrows indicates direction of change in mRNA expression for the adjacent gene and, where noted, group. See appendix 9.5 for abbreviation definitions.
Table 6.6. Significant changes (*$P \leq .05$) in mRNA gene expression and relevant biological changes of interest in the PLC$\gamma$ pathway. Each column represents the fold change seen with treatment; eg CALM expression showed a 1.4 fold change with FST as compared to control, while DBS treatment resulted in a .8 change in CALM expression as compared to FST and was only .1 increased compared to control, suggesting DBS normalised CALM expression. See appendix 9.5 for abbreviation definitions. (control n = 8, ACTH n = 8, FST n = 8, AFST n = 8 and DBS n = 7)

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Discussion

This work provides insight into the mechanisms mediating antidepressant resistance resulting from behavioural stress of the FST, 14-day ACTH administration and both ACTH and FST stress combined. This work also begins to elucidate how DBS is affecting the prefrontal cortex in a model of antidepressant resistance. Genes of the MAPK and p75 pathways showed the largest number of significant changes for all treatment groups while the AKT gene also appears to play a significant role. Overall, mRNA gene expression appeared to be regulated in opposition by stress, whether ACTH, behavioural, or combined when compared with DBS. CREB2, AKT1, and PSEN1 are notable exceptions, with the former
showing increased expression in all stress groups through the MAPK pathway, and the latter two genes showing a decreased expression in all stress groups that was not normalized by DBS. As a result, these genes, or their target proteins may not be implicated in DBS antidepressant response, though this speculation warrants further research.

The findings within the MAPK pathway are in keeping with current published knowledge regarding the role of MAPK and, ultimately CREB2, in cellular proliferation, survival and antidepressant response; however, the numerous changes exerted by DBS in the p75NTR pathway may be initially unexpected. Though largely considered a trigger for apoptosis, the p75NTR pathway also can function to promote survival. The data presented here support this pathway utilising both roles, as there is an indication that DBS up-regulates both NFKB and RHOA while down regulating SORT1 and PSEN1. NFKB can also be activated through the PI3K pathway; however, data from the DBS group did not have robust affects upstream of NFKB through the PI3K pathway, and critically did not show an attenuation of decreased AKT expression that was seen with stress. It is therefore likely that NFKB mRNA expression is being modulated by DBS via the p75NTR pathway, as genes upstream of this gene also show increased expression with DBS. Furthermore, RIPK has been shown to act as a switch that governs death or survival in Schwann cells by binding to the death domain of p75 using the caspase recruitment domain (CARD), and in turn RIPK can enhance NFKB activity in an NGF-dependent manner (Khursigara et al., 2001) and this could be the case here. Other studies suggest that p75 can enhance Trk signalling and thus facilitate trophic properties of mature neurotrophin ligand binding (Roux & Barker, 2002; Skeldal et al., 2012). Epa et al. (2004) showed that p75 and Shc can be co-immunoprecipitated, resulting in enhanced signaling of AKT through Shc.

While it is known that NFKB increases cell survival, the role of RHOA is more complex. When in the active GDP-bound form, RHOA inhibits axonal growth and filopodial
dynamics, which is thought to occur when p75 is unoccupied or activated by a pro-
neurotrophin, such as proNGF (Sun et al., 2012; Gehler et al., 2004). However, in the
absence of preferential receptors, mature neurotrophins are able in increase filopodial
dynamics and relax cytoskeletal rigidity by binding p75 and inactivating RHOA (Gehler
et al., 2004). One can only speculate at this point the effects of the increased RHOA gene
expression levels, and certainly cannot rule out the possibility that rather than having any
therapeutic benefit, this is a side-effect of stimulation-induced effects on myelin of the tissue
surrounding the electrode, as myelin derived proteins activate RHOA as well (Yamashita &
Tohyama, 2003).

Further complicating the role of p75 in this model is the decreased expression of
SORT1 and PSEN1 with stress, which is not normalized by DBS. It has been shown that
SORT increases the affinity of p75 for pro-neurotrophins, thus contributing to cell death and
this is further exacerbated if the ratio of p75 to Trk receptors is high (Skeldal et al., 2012).
However, sortilin can also bind with Trk receptors and transport them anterogradely to
synaptic membranes thereby potentiating survival signalling (Skeldal et al., 2012). While it
cannot be confidently stated what the decreased expression of these mRNA levels means
functionally, one possibility is a compensatory action by the system to protect against cell
death in the face of stress. However, the ability to chauffer Trk receptors would also be
negatively affected by this action, and thus it would seem there is a complex and
dichotomous function of the p75 pathway in mediating prefrontal cortex response to stress
and the antidepressant treatment of DBS, and that this response is not necessarily aligned
with anticipated therapeutic mechanisms.

The data here would suggest that cell survival and proliferation may be important,
largely through the MAPK pathway which is regulated in opposition by stress and DBS.
Furthermore, additional complex neurophysiology involving the dynamic capabilities of p75
may prove more critical than previously thought. One possibility, though speculative at this stage, is that stimulation of the electrode may act as a sudden insult, which is also known to incite p75 and neurotrophin expression (Roux & Barker, 2002) and the equal affinity possessed by the p75 receptor for all neurotrophins may impart benefit more rapidly than relying on one pathway alone. Based on the data presented in this chapter, future work should confirm the protein expression of all neurotrophic factors, including both mature and pro forms, as well as receptors in each treatment group. In doing so, the specific involvement of MAPK and p75 pathways can be further delineated, especially as MAPK results in cell survival when bound to mature neurotrophins and cell death when bound to pro-forms. Protein confirmation levels will also provide the post-translational effects of the gene expressions found here and thereby give an indication of the functional effects of gene expression levels. Furthermore, the role of actin regulation through p75/Rhoa in this model should be investigated. This could be achieved by using the peptide ligand, GST-PEP5 (Glutathione S-transferase fusion protein containing Pep5) which inhibits the association of p75 with RHO-GDI through binding the fifth helix of the p75 intracellular domain (Yamashita & Tohyama, 2003), thereby, prohibiting the activation of RhoA. Finally, increasing the time course of DBS treatment may offer additional insights into how this treatment modulates prefrontal cortex dynamics, as patients receiving DBS exhibit a more extensive network change with longer treatment (Giacobbe et al., 2009).
Works Cited


of p75(NTR). Bioessays: News And Reviews In Molecular, Cellular And Developmental Biology, 33(8), 614-625. doi: 10.1002/bies.201100036


Chapter VII: Antidepressant Actions of Chronic High Frequency Infralimbic Deep Brain Stimulation are Associated with Increased AKT, mTOR and NF-κB Activation in an Animal Model of Chronic Adrenocorticotropic Hormone Administration
Abstract

Deep brain stimulation (DBS) is currently being explored as an antidepressant therapy for treatment resistant depression (TRD). Intracellular signalling pathways utilizing AKT, mTOR and NF-κB have been implicated in these processes. To assess this theory, chronic bilateral infralimbic DBS was applied to adult male Wistar rats pre-treated with adrenocorticotropic hormone (ACTH) for 14 days. AKT, mTOR, and NF-κB levels of the target stimulation site were quantitated by western blot for control (n= 9), ACTH only (n = 9), sham DBS (n = 10), and DBS (n = 6) groups. Forced swim (FST) and open field (OFT) tests were conducted to evaluate antidepressant response and locomotor differences respectively. FST data support the antidepressant effect of DBS (F(3, 30) = 6.54, p = 0.002), and OFT indicated this was not due to difference in locomotor ability between groups (F(3, 30) = 0.705, p = 0.6). Western blot data indicate that DBS selectively increases activated levels of AKT (H(3) = 13.26, p = 0.004), mTOR (F(3, 27) = 3.77, p = 0.02) and total NF-κB levels (F(3, 27) = 3.97, p = 0.02). These results suggest a role for chronic DBS in modulating downstream mediators of antidepressant response and have significant implications for the ongoing investigation into novel and improved therapies for depressive disorders.
Approximately 30% of individuals suffering with major depressive disorder (MDD) receive little or no therapeutic benefit from first line antidepressant treatments (Fava & Davidson, 1996; Furukawa et al., 2009; Hindmarch, 2001; Ressler & Mayberg, 2007). The reasons for this lack of response remain poorly understood; however, antidepressant resistance, in consideration with 2-6 week therapeutic lag time of most antidepressant medications, indicates that therapeutic mechanisms of antidepressant efficacy likely lie beyond the known modulation of neurotransmitter dynamics at the synapse. Recent evidence suggests a role for neuroplasticity, neuroenergetics, and neuroimmunology in mediating depressive symptomatology and antidepressant response (Bliss et al., 2004; Brunoni, Lopes, & Fregni, 2008; Capuron & Miller, 2011; Carlson, Singh, Zarate, Drevets, & Manji, 2006; Catena-Dell'Osso et al., 2011; D'Sa & Duman, 2002; Eyre & Baune, 2012; Hyman & Nestler, 1996; Janssen, Caniato, Verster, & Baune, 2010; Leonard, 2010; Pittenger & Duman, 2008; Raaijmakers & Shiffrin, 2002; Roy & Sapolsky, 2003). Furthermore, the impact of stress, an established risk factor for the development and maintenance of MDD, is known to directly influence aberrations in these neural processes and contribute to disease state (Charney & Manji, 2004; Chih-Hao, Chiung-Chun, & Kuei-Sen, 2004; Ehlert, Gaab, & Heinrichs, 2001; Krishnan & Nestler, 2008; Munhoz et al., 2008; Pittenger & Duman, 2008; Willner, 1997).

The cytokine, or inflammatory, depression hypothesis is based on empirical evidence of increased inflammatory factors in patients with active MDD, and is positively correlated with neurovegetative symptoms of depression, often referred to as sickness behaviour (Capuron & Miller, 2011; Leonard, 2010; Miller, Maletic, & Raison, 2009; O'Brien, Scott, & Dinan, 2004; Sapolsky, Krey, & McEwen, 1986). Additionally, increased cytokine levels are correlated with the somatic comorbidities often associated with depression (Himmerich et al., 2008; Numakawa et al., 2014). Interestingly, the relationship may be bidirectional as cytokine levels have also been reported to be down regulated by effective antidepressant
medications (Janssen et al., 2010; Munhoz et al., 2008; O'Brien et al., 2004). In particular, the tumor necrosis factor receptor (TNFR) and Toll-like receptor/interleukin-1 receptor superfamilies (Napetschnig & Wu, 2013) have emerged as primary mediating factors of the pro-inflammatory effects of stress via the hypothalamic pituitary adrenal axis (Leonard, 2010; O'Brien et al., 2004).

Recently, functional neuroimaging has captured the neural maladaptations of MDD at a systems level, pinpointing the subcallosal cingulate (SCG) as a key node of hypermetabolic activity in depression neural networks (H. Mayberg, 2003; H. S. Mayberg, 1997, 2006; Stone, Lin, & Quartermain, 2008). Importantly, antidepressant responses were observed to occur concurrent with reductions in SCG regional metabolism. These investigations ushered in a new era of psychiatric neurosurgery wherein chronic high frequency deep brain stimulation (DBS) was applied to the SCG to reduce local activity in this region. To date, this treatment has been reported to provide effective therapeutic benefit for chronic and unremitting unipolar and bipolar depression (treatment resistant depression: TRD) and has shown predictive validity in animal models of antidepressant resistance (Grill, Snyder, & Miocinovic, 2004; C. Hamani & Nóbrega, 2010; Hariz, 2012; Holtzheimer & Mayberg, 2010; Kennedy et al., 2011; Lim et al., 2015; Lozano et al., 2011; Lozano et al., 2008; H. S. Mayberg et al., 2005; Puigdemont et al., 2012; Schlaepfer & Lieb, 2005). Although the mechanisms are not well understood and multisite clinical trials have thus far failed to translate this promising treatment modality more broadly, the use of DBS for severe TRD still holds substantial promise for this patient group. Studies aimed at elucidating the antidepressant mechanisms of DBS action may help to identify critical mechanisms and novel therapeutic targets outside of current monoaminergic paradigms which in turn may be used to facilitate future attempts for clinical translation (Clement et al., 2010; Clement Hamani et al., 2012; Slattery, Neumann, & Cryan, 2011). Potential targets of interest include known
mediators of plasticity and immune/inflammatory regulation, such as RAC serine/threonine-protein kinase (AKT), nuclear factor kappa B (NF-κB), and mammalian target of rapamycin (mTOR). AKT is a common kinase protein that lies upstream of both NF-κB, a ubiquitous transcription factor target primarily associated with inflammatory and immunological responses as well as cell survival and plasticity, and mTOR, which is associated with lymphatic immune-reaction and mediates the rapid antidepressant effects of ketamine (Delaloye & Holtzheimer, 2014; R. S. Duman, Li, Liu, Duric, & Aghajanian, 2012; Mitchelmore & Gede, 2014). Furthermore, these proteins are downstream of tumor necrosis and tyrosine kinase receptor signalling, and as such, may interact to mediate depressive pathology and/or antidepressant response. Indeed, evidence from oncogenic research has found support for regulation of mTOR/NK-κB interaction by AKT, which ultimately promotes cell survival independent of translational mechanisms (Lopez & Murray, 1998).

Taken together, these proteins and their target genes may moderate synaptic plasticity, now believed to be a critical and necessary factor for antidepressant response.

The five NF-κB proteins are divided into two subfamilies: NF-κB (p65, RelB, c-Rel) and Rel (p50 and p52), which contain the highly conserved Rel homology domain, enabling the proteins to homo- or heterodimerize once released from the sequestering protein, inhibitor of NF-κB (IκB) (Gilmore, 2006; Napetschnig & Wu, 2013). The release of NF-κB and subsequent nuclear translocation occurs via the canonical (classical) or non-canonical (alternative) pathways; the latter is utilized during B- and T-cell organ development (Gilmore, 2006; Napetschnig & Wu, 2013) and thus much research regarding the role of NF-κB in MDD focuses on the canonical signalling pathway. In addition to modulating effects of stress and subsequent inflammatory reactions, NF-κB is also a target transcription factor for neuroplasticity and cell survival (Eyre & Baune, 2012; Gutierrez & Davies, 2011; Koo, Russo, Ferguson, Nestler, & Duman, 2010; Mattson, 2005). Indeed, the p65 subunit has
recently been discovered to mediate plasticity in the developing and adult brain (Gutierrez & Davies, 2011). This plasticity is, in part, mediated via the neurotrophic signalling pathway, which has recently been heavily implicated in antidepressant response, through which NF-κB can be activated via the phosphatidylinositol 3-kinase (PI-3K) and p75 nerve growth factor receptor (p75NTR) signalling pathways (Carter et al., 1996; Khursigara et al., 2001; Marini et al., 2004). The evolutionarily conserved mTOR is a serine-threonine kinase that nucleates as two separate complexes, mTORC1 and mTORC2, which are composed of 5 and 6 subunits, respectively. The majority of research, and thus our understanding of these proteins, has focused on mTORC1 which is associated with protein and lipid synthesis via translation, the regulation of mitochondria and cell survival and plasticity (for comprehensive review see (Laplante & Sabatini, 2009)). Critically, mTOR has been established as a mechanism of action of low-dose antidepressant ketamine administration in critically depressed patients, which is in part mediated via rapid synaptogenic effects that likely utilize neurotrophic factors and NF-κB (Delaloye & Holtzheimer, 2014; Dong, Ruzicka, Grayson, & Guidotti, 2014; Li et al., 2010; Mitchelmore & Gede, 2014; Moreines, McClintock, Kelley, Holtzheimer, & Mayberg, 2014; Niciu et al., 2014; Rybakowski, Permoda-Osip, Skibinska, Adamski, & Bartkowska-Sniatkowska, 2013).

The role of these proteins in mediating immunological responses, synaptic plasticity, and ultimately influencing the dispensation of energy demands within the cell to enable synaptic growth may be important for facilitation of antidepressant response in the otherwise treatment resistant state. To investigate this, adult male rats received chronic adrenocorticotropic hormone (ACTH) administration, which induces a behavioural phenotype of tricyclic antidepressant resistance (Kitamura, Araki, & Gomita, 2002; Walker et al., 2013). These animals were then treated with DBS of the infralimbic cortex, rodent homologue of the SCG, a well-established DBS target for pre-clinical research in this field.
(Clement Hamani et al., 2014; Clement Hamani et al., 2012; C. Hamani et al., 2010; Clement Hamani, Diwan, Silvia, Lozano, & Nobrega, 2010; C. Hamani & Nóbrega, 2010; Lim et al., 2015). Drug-free and sham DBS (electrode placement, no stimulation) groups were also used for baseline comparison. Western blot was used to quantitate AKT, NF-κB and mTOR levels within the infralimbic cortex of all experimental groups. Results indicate that these signalling mechanisms are modulated locally by DBS.

Methods

7.2.1 Animals

Male albino Wistar rats, aged 5-7 weeks through the duration of experimental protocol, were pair-housed under a 12-hour light/dark cycle (lights on at 6:00am) in a temperature controlled facility with food and water available ad libitum. Animals were permitted to acclimatise to the facility for three days prior to initiation of the experimental protocol, during which time all animals were randomly allocated to one of four experimental groups as shown in Table 1. Study protocol was carried out over 15 days, key test points are described below. Animals were humanely euthanized approximately one hour after completing the final test on day 15. Procedures were conducted in accordance with Mayo Clinic Institutional Animal Care and Use Committee guidelines and animal use was minimized.

7.2.2 Drugs

ACTH (1-24) (AnaSpec, San Jose, California, USA) was dissolved in distilled water and administered i.p. at 100μg/day to AS, sham, and DBS animals. Pentobarbitone (.70 -1.10 cc. 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) overdose was administered i.p. as euthanization at the end of study protocol.
Animals designated sham and DBS were anaesthetised via isoflurane inhalation (1-3%) in an induction chamber (World Precision Instruments), then placed in a stereotaxic frame (Model 1430, David Kopf Instruments, CA, USA) with maintenance isoflurane. The skull was secured with a nose clamp, incisor bar, and atraumatic ear bars. Constant body temperature (36.5°C) was maintained using a heat pad and the animal was monitored by respiratory rate and hind-paw pinch to ensure maintenance of surgical level of anaesthetization.

A midline 1.5-2cm incision was made starting just caudal to the eyes and ending rostral to the ears to expose the two skull landmarks: bregma and lambda. A 2-mm diameter trephine hole was broached in the skull at the site corresponding to the targets. The dura mater was opened using a fine needle. Polymide-insulated stainless steel monopolar electrodes (250 μm in diameter, 10mm for DBS) or 7 or 5.6mm (for sham) in length, Plastics One, Roanoke, VA, USA) were implanted into the infralimbic regions (AP: +/- 3.0, ML: +/-...
DBS MEDIATES NEUROTROPHINS IN ANTIDEPRESSANT RESISTANCE

0.4, DV:-5.6, Watson and Paxinos, 2007). DBS animals received bilateral electrode placement, while sham animals received only left electrode placement. The use of longer electrodes for DBS animals allowed for the wires to be bent, and electrode mounts to be placed further apart allowing both leads to be attached and turned on simultaneously to achieve constant bilateral stimulation. Tests conducted within our laboratory do not show any loss of conductance due to this method. Small mounting screws (3-4) (Eicom, San Diego, CA, USA) of approximately 2mm in length were fixed into the skull to act as scaffolding for dental cement, which was placed around the stimulating electrode to prevent displacement or damage. Stimulation was conducted using the AMPI / Master-9 system, which was attached via the ISO-Flex Stimulus Isolator (A.M.P.I., Jeruselum, Israel) to the implanted electrodes of the singly housed animal constantly from day 10 at 130 Hz, 100µA, 90µSec.

7.2.4 Behavioural Tests

7.2.4.1 Forced Swim Test

The Porsolt Forced Swim Test (1977) was used to evaluate the effects of the antidepressant interventions executed in this study. The test has been extensively validated and previously used in this research paradigm (Castagné, Moser, Roux, & Porsolt, 2011; Kitamura et al., 2002; Porsolt, Le Pichon, & Jalfre, 1977; Walker et al., 2013). Briefly, a tall plexiglass cylinder (60.5cm in height, 20cm diameter on a base measuring 30 x 30cm) was filled to a depth of 35cm with 25°C +/- 1°C clean tap water. All animals underwent a 10-minute habituation session on day 14 of experimental protocol. The following day, a 6-minute test session was conducted under the same conditions, save antidepressant intervention being administered no more than 30 minutes prior to testing. All sessions were recorded by video camera. Test sessions for each animal were analysed in 1-2 second intervals, blind to experimental condition. Behaviours of interest included ‘immobility’ and ‘passive diving’
(passive behaviours), as well as ‘swimming’, ‘climbing’ and ‘diving’ (active behaviours). The final four minutes of the FST were used for data analysis.

7.2.4.2 Open Field Test

The open field test (OFT) was used to assess locomotor, anxiety, and exploratory behaviours of all animals. The apparatus was a large blue box (50cm x 50cm x 40cm) with affixed TopScan (CleverSys, Reston, VA, USA) tracking software. The OFT was completed in one 10-minute free exploration session.

7.2.5 Western Blotting

The infralimbic region was dissected out over dry ice by hand using a BioCision ThermalTray LP then lysed in radioimmunoprecipitation assay (RiPA) lysis buffer for Western blotting. For animals that received only unilater electrode placement, tissue from the implanted side was used in analysis. Protein concentration of each sample was measured using the Bradford spectroscopic method. Lysate was then aliquoted according to antibody manufacturer specifications and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P). Membranes were then probed with antibodies for AKT, phosphorylated AKT, mTOR, phosphorylated mTOR, and NF-κB (p65).

7.2.6 Statistical Analysis

Data were analyzed using SPSS (Statistical Package for the Social Sciences, version 20) and GraphPad (version 5.04). Unless otherwise stated, data were analyzed using one-way between-groups analysis of variance (ANOVA) with Bonferroni post hoc test. Significance was set at $p \leq .05$. 
Results

7.3.1 Forced Swim Test

A one-way between-groups analysis of variance (ANOVA) was carried out to assess the effect of treatment on average immobility time in the forced swim test (Figure 1a). Levene’s test for equality of variance was not violated ($W(3, 30) = 0.846, p = 0.48$), which allowed for the use of the more robust Bonferroni post-test. Results showed there was a significant effect of treatment ($F(3, 30) = 6.54, p = 0.002$) on immobility in adult male Wistar rats. Bonferroni post hoc tests revealed that immobility time was lower for the DBS treated animals ($M = 45.67, SD = 32.72$) compared to the NS group ($M = 129.11, SD = 42.47$) $p = 0.006$, and the AS group ($M = 126.89, SD = 54.94$) $p = 0.007$. This indicates that bilateral infralimbic DBS had antidepressant effects in comparison to drug naive animals and ACTH treated animals. The average total immobility time was mirrored by latency to immobility ($F(3, 30) = 9.37, p < 0.001$). Bonferroni post tests indicated that NS ($M = 85.11, SD = 40.82$) and AS ($M = 100.78, SD = 66.31$) animals adopted immobile behaviour significantly earlier than DBS ($M = 243.33, SD = 92.36$) animals when performing the FST ($p < 0.001$ and $p = 0.002$, respectively (Figure 1b, Levene’s $W(3, 30) = 1.77, p = .17$)). ANOVA of the open field test supports that these differences were not due to changes in locomotion, as there was no effect of treatment on total distance travelled ($F(3, 30) = 0.705, p = .6$ (Figures 1c, d)), nor on time spent in center ($F(3, 30) = .813, p = .5$ (Fig 1D)).
Figure 7.1. Forced swim test analysis displayed as average for treatment group. (a) Total immobility in seconds. DBS significantly decreased total immobility time compared to NS and AS groups. Sham animals display a mild, though insignificant decrease in immobility. (b) Latency to immobility in seconds. NS and AS groups displayed immobility significantly earlier than the DBS group. (c & d) OFT analyses indicate differences in the FST were not due to difference in locomotor ability. Data are presented as mean ± SEM. (NS n = 9, AS n = 9, Sham n = 10 and DBS n = 6) **P ≤ .01

7.3.2 AKT

ANOVA was carried out to assess the effect of treatment on total AKT levels as measured by Western Blot (Figure 2a). Levene’s test for equality of variance was not violated (W(3, 27) = 1.50, p = 0.24). Results showed there was no significant effect of treatment (F(3, 27) = 0.51, p = 0.68) on AKT levels in adult male Wistar rats. As no
significance was found, *post hoc* tests were not conducted. Relative expression data for phosphorylated AKT was neither normally distributed nor homogenous (Levene’s test: $W(3, 27) = 3.18, p = .04$), as such the non-parametric Kruskal-Wallis statistical analysis was performed (Figure 2b). These analyses indicated a significant difference between treatment groups for relative expression level of phosphorylated AKT within the infralimbic cortex ($H(3) = 13.26, p = 0.004$) with a mean rank of 27.67 for the DBS group, 14.90 for the Sham group, 13.71 for NS group, and 10.63 for the AS group. Overall, DBS animals exhibited the highest relative expression of phosphorylated AKT compared to all other groups and Dunn’s multiple comparison indicated this was statistically significant ($p < .05$). Kruskal-Wallis statistical analysis was also used to analyse relative expression data for the ratio of phosphorylated AKT to total AKT (Levene’s test: $W(3, 27) = 3.52, p = 0.03$, Figure 2c). There was a significant difference between treatment groups for relative expression level of the ratio of phosphorylated AKT to total AKT within the infralimbic cortex ($H(3) = 9.63, p = 0.02$) with a mean rank of 25.67 for the DBS group, 15.14 for NS group, 15.0 for the Sham group, and 10.75 for the AS group. Overall, DBS animals exhibited the highest ratio of phosphorylated to total AKT compared to all other groups. Dunn’s multiple comparison indicated this was only statistically significant for the DBS as compared to AS group ($p < 0.05$).
Figure 7.2. Western blot analysis of AKT displayed as relative expression for treatment group. (a) Total AKT was not different between treatment groups. (b) Phosphorylated AKT was significantly increased in DBS treated animals, as was the ratio of phosphorylated to total AKT (c). Data are presented as mean ± SEM. (NS n = 9, AS n = 9, Sham n = 10 and DBS n = 6) *$P \leq .05$

7.3.3 mTOR

ANOVA was carried out to assess the effect of treatment on total infralimbic mTOR levels as measured by Western Blot (Figure 3a). Levene’s test was not violated ($W(3, 27) = 2.636, p = 0.07$). Results showed there was no significant effect of treatment ($F(3, 27) = 0.37, p = 0.773$), on total mTOR levels in adult male Wistar rats. As no significance was found, post hoc tests were not conducted. ANOVA was carried out to assess the effect of treatment on infralimbic phosphorylated mTOR levels as measured by Western Blot (Figure 3b). Levene’s test was not violated ($W(3, 27) = 1.55, p = 0.23$). Results showed a significant effect of treatment ($F(3, 27) = 3.77, p = 0.02$) on phosphorylated mTOR levels in adult male Wistar rats. Bonferroni post hoc tests revealed that the level of mTOR was significantly higher in DBS treated animals ($M = 0.71, SD = 0.14$) compared to NS animals ($M = .48, SD = .15, p = .05$) and AS animals ($M = 0.48, SD = 0.09, p = 0.02$). This indicates that bilateral infralimbic
DBS had significant effects on phosphorylated mTOR expression compared to these treatment groups. ANOVA was conducted to test for a significant difference between groups in the ratio of phosphorylated to total mTOR (Figure 3c). Levene’s test was not violated ($W(3, 27) = 1.55, p = 0.23$). Results indicated no significant effect of treatment ($F(3, 27) = 0.30, p = 0.82$), on the ratio of phosphorylated to total mTOR levels in adult male Wistar rats. As no significance was found, *post hoc* tests were not conducted.

![Figure 7.3](image)

**Figure 7.3.** Western blot analysis of mTOR displayed as relative expression for treatment groups. (a) Total mTOR was not different between treatment groups. (b) Phosphorylated mTOR was significantly increased in DBS treated animals, compared to NS and AS animals. (c) The ratio of phosphorylated to total mTOR was not different between treatment groups. Data are presented as mean ± SEM. (NS n = 9, AS n = 9, Sham n = 10 and DBS n = 6) *P ≤ .05

### 7.3.4 NF-κB

ANOVA was carried out to assess the effect of treatment on NF-κB levels as measured by Western Blot (Figure 4). Levene’s test was not violated ($W(3, 27 ) = 0.79, p = 0.51$). Results showed there was a significant effect of treatment ($F(3, 27) = 3.97, p = 0.02$) on NFKB levels in adult male Wistar rats. Bonferroni *post hoc* tests revealed that the level of NFKB was significantly lower in DBS treated animals ($M = 0.41, SD = 0.24$), compared to
NS animals ($M = 0.97, SD = 0.30 \ p = 0.05$) and sham animals ($M = 0.98, SD = 0.38 \ p = 0.02$). This indicates that bilateral infralimbic DBS had significant effects on NFκB expression compared to these behaviourally naive animals and animals that received electrode implants without stimulation. Although not significant, there was a trend toward significance for reduced NF-κB levels in the AS group ($M = 0.92, SD = 0.43, p = 0.07$) as compared to the DBS group. This analysis may have been significant with increased power.

![Infralimbic NF-κB](image)

**Figure 7.4.** The relative expression of NF-κB in the infralimbic cortex of all treatment groups as measured by western blot. DBS resulted in significantly reduces NF-κB as compared behavioural NS and Sham animals. Data are presented as mean ± SEM. (NS n = 9, AS n = 9, Sham n = 10 and DBS n = 6) *$P \leq .05$

**Discussion**

Chronic DBS of the IL in ACTH pre-treated animals is able to significantly reduce immobility in the FST compared to both control and ACTH pre-treated animals, and these results do not appear to be associated with variations in exploratory behaviours or locomotor
activity. Given the pharmacological predictive validity of the FST (Castagné et al., 2011),
this work lends support to the growing body of evidence that DBS has therapeutic
antidepressant actions. These data indicate that regulation of AKT, NF-κB, and mTOR within
the target stimulation area may be implicated in the antidepressant response of DBS. This
work precedes, and is in agreement with, other work that found increases mTOR and Akt in
the IL region after DBS using an olfactory bulbectomized model of depression (Jimenez-
Sanchez et al., 2016). Understanding the functional implications of these molecular actions is
an important area for future research investigating the antidepressant mechanisms of DBS.
This is of particular relevance given the role of these signalling pathways in the regulation of
neurotrophic factor mediated neural activity, as well as inflammatory processes, which have
recently been found to be correlated with antidepressant response and symptom
improvement.

Neurotrophic factors are potent mediators of cell survival and plasticity, and are
believed to greatly impact the alterations in neural substrates necessary for effective
antidepressant response (Berlim, McGirr, Van den Eynde, Fleck, & Giacobbe, 2014; D'Sa &
Duman, 2002; Dias, Banerjee, Duman, & Vaidya, 2003; C. H. Duman, Schlesinger, Kodama,
Russell, & Duman, 2007; Duric et al., 2010; Duric & Duman, 2013; Pittenger & Duman,
2008; Schmidt, Banasr, & Duman, 2008). NF-κB can be activated through the PI-3K and
p75NTR neurotrophic factor signalling cascades, the latter also being member 16 of the
tumour necrosis factor family. Although speculative, it is possible that modulation of NF-κB
expression by DBS in the present work is affecting neuronal plasticity through any number of
mechanisms of the neurotrophic signalling pathway. For example, receptor-interacting
serine/threonine-protein kinase 2 (RIPK2) lies upstream of NF-κB in the p75NTR pathway.
This protein has been shown to act as a switch that governs death or survival in Schwann
cells by binding to the death domain of p75 using the caspase recruitment domain (CARD),
in turn allowing RIPK to enhance NF-κB activity in a neurotrophic growth factor-dependent manner (Khursigara et al., 2001). Other studies suggest that p75 can enhance neurotrophic factor receptor signalling, thus facilitating trophic properties of mature neurotrophin ligand binding (Roux & Barker, 2002; Skeldal, Matusica, Nykjaer, & Coulson, 2011); for example, p75NTR and Shc can be co-immunoprecipitated, resulting in enhanced signaling of AKT through Shc, which also activates NF-κB via the PI-3K pathway (Epa, Markovska, & Barrett, 2004). This latter route is additionally supported by the finding that phosphorylated AKT is increased in these samples, however, the involvement of Shc would need to be confirmed, as disregarding a role for p75NTR/Shc co-immunoprecipitation and increased phosphorylated AKT alongside decreased total Nf-κB (p65) may indicate activation of the AKT-NF-κB pathway via PI-3K alone, and increased nuclear translocation of the transcription factor. The increased active form of AKT is also relevant to the increased active mTOR, indicating a potential overall increase in this signalling pathway and modulation of cell growth and metabolism. Finally, reduced total NF-κB could indicate an influence of the IκB-NF-κB complex on tuberous sclerosis complex (TSC) activity within the mTOR cascade. Activation of AKT together with mTOR, further indicates a potential overall increase in the concurrent modulation of cell growth and metabolism. We have previously shown that DBS of the nucleus accumbens has similar antidepressant effects and upregulates mitochondrial efficiency in the prefrontal cortex (Yen, KS et al., In press) supporting its facilitatory effect on metabolic processes.

Additionally relevant is the display of a behavioral and molecular response to electrode insertion, referred to as the insertional effect (Clement Hamani et al., 2006). It is becoming increasingly apparent in rodent studies that a therapeutic response can be achieved as a result of electrode placement in rats pre-treated with stress hormones or a stress paradigm (Perez-Caballero et al., 2014). Although not significant, sham animals exhibited a
reduction in immobility in the FST without comorbid locomotor abnormalities. The insertion of a DBS electrode into brain tissue of pre-stressed rats may induce an inflammatory response at the site of electrode implantation that in and of itself has therapeutic antidepressant actions. In line with this, inflammatory challenge has been shown to reactivate depleted microglia to upregulate synaptic plasticity and have antidepressant-like effects in animals pre-treated with chronic mild stress (Kreisel et al., 2014). Within the context of DBS, an acute inflammatory response following surgery has been shown to increased serotonin synaptic efficiency and reactive gliosis, both of which are believed to impart a transient antidepressant effect, and can be attenuated with postoperative anti-inflammatory medication therapy (Perez-Caballero et al., 2014). While our data support this behavioural effect, they further suggest the observed actions on AKT, NF-κB and mTOR protein expression occurring in response to active DBS only, may be important for more robust and enduring antidepressant responses. Further unbiased proteomic and microarray analyses could help to delineate the molecular actions of active DBS, and any interplay between trophic, metabolic and inflammatory mechanisms under control of these specific intracellular proteins.

Future work using this model should take into consideration a broader scope of behavioural assessments to include more direct measures of both positive and negative valence systems, for example, anhedonia and threat vigilance. Such research may provide additional information on mechanisms involved in DBS modulation of behavioural phenotypes more transnationally relevant to human depression, and how they relate to predictive antidepressant screens such as the forced swim test. The present study’s identification of a role for local AKT, NF-κB and mTOR modulation by DBS suggest that active electrical stimulation directly modulates the key antidepressant target proteins to reduce immobility in the FST.


Dong, E., Ruzicka, W. B., Grayson, D. R., & Guidotti, A. (2014). Dna-methyltransferase1 (dnmt1) binding to cpg rich gabaergic and bdnf promoters is increased in the brain of schizophrenia and bipolar disorder patients. *Schizophrenia Research.*


DBS MEDIATES NEUROTROPHINS IN ANTIDEPRESSANT RESISTANCE


Mitchelmore, C., & Gede, L. (2014). Brain derived neurotrophic factor: Epigenetic regulation in psychiatric disorders. *Brain Research, 1586*(0), 162-172. doi: [http://dx.doi.org/10.1016/j.brainres.2014.06.037](http://dx.doi.org/10.1016/j.brainres.2014.06.037)


Chapter VIII: General Discussion

8.1 Antidepressant Resistance in ACTH-treated Animals

The works presented in this thesis confirm the utility of 100μg/day of adrenocorticotropic hormone (ACTH, 1-24) administered during the circadian nadir in producing imipramine antidepressant resistance in the forced swim test (FST), a well established, pharmacologically predictive, behavioural screen for antidepressant efficacy. Other work using this model (Walker et al., 2013) has demonstrated that this ACTH administration schedule elicits acute release of corticosterone. The work presented herein builds on this, to show that this protocol does not result in a consistently hyperactive hypothalamic-pituitary-adrenal (HPA) axis, as elevated corticosterone levels return to normal baseline levels within 24 hours of acute ACTH administration. In fact, our findings indicate that the system is very capable of enacting negative feedback mechanisms, as corticosterone levels were attenuated over time whether samples were collected prior to, or after, ACTH injection. This suggests that changes in responsivity or flux within the HPA-axis upon stimulation may be important in this model and implicated in antidepressant resistance, rather than changes in overall basal tone such as hypercorticoleemia.

Disruption of antidepressant treatment response appears to be the hallmark phenotypic feature of this model. In line with this, impaired memory function, a core symptom of major depressive disorder (MDD), did not appear to be disrupted by the stress hormone treatment. Memory deficits induced via chronic corticosterone treatment, usually administered over the full diurnal rhythm via drinking water, are typically associated with neuronal atrophy and apoptosis, particularly in the hippocampus, which is thought to contribute to the associated functional memory impairments of MDD (Conrad, 2008; McEwen & Magarinos, 2001). This suggests that the physiological adaptations contributing to ACTH-induced antidepressant resistance do not result from potentially neurotoxic, chronically elevated, glucocorticoid
signaling sufficient to damage the neural systems underlying spatial, working and reference memories. Instead the work presented here implies that such treatment resistance may be mediated via neural adaptations in stress-sensitive systems that impair the ability of the brain to respond to first line antidepressant pharmacotherapies. That is, a differential response of the primed HPA-axis to a stressor, such as the FST, may underlie the adoption of passive depression-like coping behaviors and an overall reduction of active coping response, requiring the exertion of physical effort under duress.

The adoption of passive coping behaviors in response to stress is associated with stress resiliency, such that less resilient individuals are unable to effectively engage active coping strategies in response to stress. The neurobiology of resiliency is a highly complex and interdependent process of neurotransmitter activity, neuroimmune and stress hormone responsivity, together with genetic and epigenetic mechanisms across local synaptic microcircuits and wider neural network interactions that appear to be age, sex and stress exposure dependent (Bale & Epperson, 2015; Elliott, Ezra-Nevo, Regev, Neufeld-Cohen, & Chen, 2010; Isingrini et al., 2016; Russo, Murrough, Han, Charney, & Nestler, 2012; Shin et al., 2015; Vialou et al., 2010). These processes physiologically manifest as spatiotemporal patterns of plasticity that have been outlined in detail in this thesis (Chattarji, Tomar, Suvrathan, Ghosh, & Rahman, 2015). Briefly, regions offering inhibitory input, such as the hippocampus and medial prefrontal cortex (mPFC), show reduced long term potentiation (LTP), and increased long term depression (LTD), while regions promoting the stress response, such as the amygdala and nucleus accumbens, present the opposite (Arnsten, 2015; McEwen, Eiland, Hunter, & Miller, 2011). These region specific changes are associated with behavioural outcomes consistent with symptoms of MDD, such as reduced motivation, poorer cognitive function, and habitual negativity in action selection and rumination (Floresco, Tse, & Ghods-Sharifi, 2007; Gunaydin et al., 2014; Hollon, Burgeno, & Phillips,
2015; Nicola, 2007), which act to exacerbate and maintain the initial depressive episode. Importantly, currently available antidepressants appear to facilitate the re-establishment of favorable synaptic plasticity and the time course of clinical response is concurrent with antidepressant induced neural adaptations involved in this process, such as dendritic branching (Andrade & Rao, 2010; Bessa et al., 2008; D'Sa & Duman, 2002).

Taken together, the behavioural and corticosterone profile of this model do not support it as one of depression *per se*, but instead suggest this model as offering a window into the failure of the brain to adapt to typical antidepressants under continued stress, here, the FST. This is critically important and highlights a unique function of this ACTH model to be utilized in elucidating a mechanism of failed antidepressant response initiation which must precede the neural adaptation to chronic antidepressant treatment in order to impart behavioural improvement (Hyman & Nestler, 1996). These cellular adaptations are the consequence of homeostasic mechanisms at the synapse, which function to maintain stability within the network following significant perturbations. This model predicts that when synaptic neurotransmitter levels are significantly elevated (as occurs with inhibition of neurotransmitter transporter function), cellular adaptations ensue, including changes in membrane receptor expression. Additionally, intracellular pathways are modulated in an attempt to regulate cellular processes necessary for these adaptations. The work presented herein suggests that repeated ACTH challenge may impair these adaptive mechanisms. This is in accordance with other studies demonstrating that altered glucocorticoid receptor sensitivity via neuroendocrine (Anacker, Zunszain, Carvalho, & Pariente, 2011; Ehlert, Gaab, & Heinrichs, 2001) and neuroimmune (Hodes, Kana, Menard, Merad, & Russo, 2015; Martino, Rocchi, Escelsior, & Fornaro, 2012) maladaptations, can aberrantly impact epigenetic regulation of stress response (Vialou, Feng, Robison, & Nestler, 2013; Witzmann, Turner, Mériaux, Meijer, & Muller, 2012). The contribution of these neuroendocrine and
neuroimmune influences on regional brain cell responses to stress and the contribution of this to antidepressant resistance and neuroprogression warrants further research. The FST paradigm, although limited in its face validity for depression-like behavior on the whole, may be a useful tool for investigating these stress-sensitive mechanisms.

8.2 Behavioral Effects of DBS in ACTH-treated Animals

The efficacy of unilateral and bilateral infralimbic deep brain stimulation (IL-DBS) (130 Hz, 100μA, 90 μSec) in attenuating depression-like behaviours in this antidepressant resistant phenotype was confirmed using the FST, well-validated for its predictive validity as an antidepressant screen (Castagné, Moser, Roux, & Porsolt, 2011; Nestler & Hyman, 2010). This antidepressant response was seen using both acute and chronic IL-DBS stimulation. The display of swimming as the dominant active coping mechanisms by DBS animals contributes additional support to the hypothesis that DBS may upregulate serotonin neurotransmission (Cryan, Valentino, & Lucki, 2005; Detke, Rickels, & Lucki, 1995; Clement Hamani et al., 2012; C. Hamani et al., 2010; Clement Hamani & Nobrega, 2012). Furthermore, the safety of DBS with regards to diminishing cognitive or recall performance has also been supported by this work as there were no serious detriments seen with either DBS or sham DBS (SDBS) nor were further behavioural anomalies noted in these groups, such as locomotor complications or difficulty performing daily activities.

Complementary to this, there was no robust indication of improved ability with SDBS or DBS. It must, however, be noted that DBS animals did display a preference for novelty at the midpoint of the novel object preference test (NOP), although this finding did not hold for the remainder of the test. The NOP also revealed a pattern for overall interaction time with objects. ACTH-treated animals displayed a significantly reduced level of interaction time with objects compared to both SDBS and DBS groups, despite the increased exploratory
rearing behaviours displayed in the open field test (OFT). Together, this suggests a pattern of cognitive and/or anhedonic improvement garnered by DBS, that may be difficult to measure in pre-clinical models, but may be an essential component of human treatment response. Such changes to novelty seeking and social or object engagement are more often associated with the dopaminergic system, which is known to be modulated by other DBS targets, such as the nucleus accumbens, to successfully reduce anhedonia (Schlaepfer et al., 2008), but the modulation of this system through IL-DBS remains to be more thoroughly clarified in both pre-clinical and clinical research. Furthermore, not only does this indicate that reduced interaction was not due to decreased engagement or physicality, but suggests a potential mediating role for anxiety in this model and treatment modality. This is relevant given the high comorbidity of this disease with MDD (Brown, Campbell, Lehman, Grisham, & Mancill, 2001). Further tests should be carried out to assess anxiety behaviours present in the ACTH model, such as the novelty suppressed feeding test and the elevated plus maze test. If these tests confirm the existence of anxiety behaviours, the effects of DBS in attenuating anxiety when applied to the IL cortex should also be investigated as neither sham nor DBS animals displayed high levels of anxiety-like behaviour.

8.3 Neurotrophic Factors and Antidepressant Response to DBS in ACTH-treated Animals

These data do not support a role for hippocampal glial cell-line derived neurotrophic factor (GDNF) in mediating ACTH-induced antidepressant resistance, nor DBS-induced antidepressant effects as no changes in GDNF protein were observed between groups. Furthermore, hippocampal GDNF does not appear to be correlated with the memory tasks carried out here or behaviours of the FST and is therefore likely not significantly impacted by the ACTH paradigm executed in this study nor a mechanism of action of IL-DBS. Similarly,
these data do not indicate that alterations in hippocampal brain derived neurotrophic factor (BDNF) is a mechanism of antidepressant resistance in this model.

These findings contribute to current literature indicating opposing profiles for neurotrophic factors in MDD and antidepressant response as discussed in detail earlier in this thesis. These variations are likely due to differences in treatment modality, depression subtype, and general methodology and indicate an important area for clarification through future research. For instance, although not significant, the elevated level of ventral (as compared to dorsal) hippocampal BDNF and increased proportion of phosphorylated tropomyosin receptor kinase B (pTrkB) to full length (FLTrkB) in this same structure support a potential role for the ventral hippocampus in mediating environments of chronic stress. In light of the network model of depression, such differentiation in neurotrophic activity could underscore the negative ventral shift exhibited in the depression. Whether pre-existing organically or induced by stress, elevated BDNF activity could prime the neural network towards a “stress ventral” profile leading to depressive symptomatology. Such a theory gains support when considered with the observed increase of ventral (as compared to dorsal) GDNF levels seen with ACTH; while again not significant, this difference displayed a large effect size. Furthermore, these changes in ventral neurotrophic levels could potentially be involved in the increased rearing in the OFT and lower object interaction times in the NOP that were observed in this group. While BDNF levels may not be directly affecting these behaviours, the intimate relationship between BDNF and stress hormones, based on the increased adaptive potential associated with these molecules and their signaling pathways, highlight an important avenue of exploration, especially in regards to resilience.

The implications of region specific neurotrophic changes are supported by the microarray findings presented here, as despite no significant changes being observed in the hippocampus, microarray analyses revealed an effect of stress and DBS on mRNA gene
expression levels of the neurotrophic signaling pathway in the prefrontal cortex. These data indicated that ACTH and FST, both individually and combined, exert influences on gene expression at the mRNA level within this pathway. This may be one of the mechanisms through which corticosterone appears to prime the system to adapt poorly to future stressors despite not elevating corticosterone levels *per se*. This is also in line with previous work by our group (Walker *et al.*, 2013) which established that acute ACTH administered over 14 days alters prefrontal monoamine levels, which likely influence and are influenced by the neurotrophic signaling changes seen here. This microarray data furthermore revealed potential mechanisms of action of DBS in this model, most notably through modulating gene expression in opposition to that observed with stress. Key genes that seem to be strongly influenced by DBS were also noted, such as RhoA which indicates a role for the dynamic effects of the p75 receptor in this treatment modality or remodeling of structural matrices to support plasticity. These findings are in line with multiple studies, including those presented here, that have shown the infralimbic region to be important in mediating active coping response in the FST (Chang, Chen, Qiu, & Lu, 2014; Slattery, Neumann, & Cryan, 2011). Future work should confirm these findings through protein analysis as well as aim to assess the relationship between prefrontal monoamine levels and neurotrophins using both ACTH and ACTH pre-treated DBS cohorts.

It is important to note, that much of this work highlighting a critical role for neurotrophic factors in depression, and antidepressant recovery of the depressed phenotype, has utilized stress-induced models of depression that are responsive to first line antidepressants, for example the chronic mild unpredictable stress paradigm. The phenotypes of such an animal model often exhibits elevated levels of anhedonia and impaired cognitive function and are fundamentally different from the model utilized in the series of experiments presented herein. Thus, the distinctions may pertain more to the FST behavioral stress
mediated induction of depression-like behaviors, than to the ACTH-induced disruption of behavioral response to imipramine, or perhaps the combination of both.

8.4 Metabolic Factors and Antidepressant Response to DBS in ACTH-treated Animals

Preliminary assessment of key intracellular signaling components, AKT, Nf-κB and mTOR does indeed indicate that they are effectively modulated by DBS at the level of protein expression within the target stimulation site. Given the inherent complexity of intracellular signaling cascades, additional work is necessary to confirm both the original target systems of these mid-stream changes as well as the final result; however, changes in Nf-κB point to alterations in gene translation. The role of neurotrophic factors in these changes appears to be critical, though it is not yet clear how (Dell’Osso et al., 2014; Dias, Banerjee, Duman, & Vaidya, 2003; Clement Hamani et al., 2012; Clement Hamani & Nobrega, 2012; Mitchelmore & Gede, 2014; Ramasubbu, Vecchiarelli, Hill, & Kiss, 2015). These data concur with other work from our lab that indicate intracellular changes activated by DBS facilitate cellular metabolic mechanisms through restoration of mitochondrial function and lipid regulation, furthermore these changes are seen in the IL with local and regional stimulation, such as lateral habenula and nucleus accumbens (Kim et al., 2016). Together these outline a critical role of bioenergetics in mediating necessary adaptations to DBS antidepressant response.

8.5 Future Directions and Limitations

A clearer understanding of the mechanisms through which ACTH treatment disrupts HPA-axis circadian rhythms and imparts antidepressant resistance in this model could potentially be gained if plasma samples were collected at several time points over a single 24 hour period, ideally at the beginning, middle and end of the 14 day protocol. In addition to
this amended repeated-measures design, it may also be informative to extend the protocol beyond 18 days. Prolonged ACTH administration may inform on whether behavioural symptoms precede, or perhaps are not linked to, quantifiable physiological changes in circulating corticosterone levels and inflammatory processes. Such findings could offer one possible explanation as to why elevated cortisol is not consistently present in individuals suffering from MDD, whether antidepressant responsive or not, and contribute to the wider body of ongoing research into this paradoxical physiological contribution to stress-related mental illness (Stetler & Miller, 2011).

A semi-longitudinal study design would also prove beneficial regarding the insertional effect - a short-lived, but measurable antidepressant response attributed to simply surgically implanting the stimulating electrodes into the brain of the patient. This effect is believed to subside at approximately 6 weeks and has been found to be a measurable confound for subgenual cingulate DBS (Perez-Caballero et al., 2014). Indeed the work presented here consistently shows an antidepressant trend of sham DBS surgery, while work using DBS to treat chronic pain has found a significant effect of electrode insertion that was predictive of successful treatment and in one case sufficient to treat the symptoms of pain (Hamani et al., 2006). This phenomenon is somewhat paradoxical, especially regarding MDD, as elevated inflammatory factors are the primary mediator of HPA activity and are more often than not associated with MDD (Hodes et al., 2015). As is the case with the neurotrophic expression profile of MDD, it may be that inflammation, at least as it pertains to the effective application of DBS, exhibits region and time specific modulations in relation to symptomatology. Furthermore, as previously mentioned, *apriori* knowledge suggests these effects are similar to lesion surgeries, such as the cingulotomy, historically used with rare success to treat psychiatric disorders. Given the steady presence of this phenomenon, work is currently underway to better characterize the insertional effect. In agreement with the
mechanisms of action proposed in this work, a recent mouse model of IL-DBS has confirmed 
neuroanatomical remodeling at the cellular level, both locally and distally, with electrode 
insertion (Chakravarty et al., 2016). This work further confirmed remodeling of the vascular 
architecture with long-term stimulation, and in agreement with propositions here, cite 
neuroplasticity and the implantation of neurotrophic factors as key mediators of these 
responses (Chakravarty et al., 2016). While there are serious methodological differences 
between the cited studies and those presented here, research in this comparatively novel field 
appears to be in agreement as to the mechanism underlying TRD and antidepressant response 
mediated by DBS, which sheds light on efforts to treat MDD in general. An extended 
timeline executed in the pre-clinical model utilized here would allow for assessment of the 
neural properties that may underlie the insertional effect, while also allowing for the 
evaluation of mechanisms that change over time to abrogate this effect. These findings could 
yield critical information on mechanisms of antidepressant action, for example initiation, that 
may be useful in drug development and optimal selection of patient treatment toward 
individualized medicine.

In addition, a longer stimulation protocol could yield a more detailed view of the 
effects of DBS which presents with progressive behavioural improvements, suggesting 
modulation of plastic mechanisms (Giacobbe, Mayberg, & Lozano, 2009; Merkl et al., 2013). 
For example, some patients achieve improvement in 3 months, while others require 6 or even 
12 months for a measureable behavioural response, during which time those who exhibited 
symptom improvement at 3 months commonly display even greater improvement (Lozano et 
al., 2008; Mayberg et al., 2005). Furthermore, there are indications from ratings on the 
Hamilton Rating Scale of Depression (Hamilton, 1980) that mood symptoms may be the first 
to improve at 3 months, while anxiety, sleep and somatic symptoms may require 6 months for 
measureable improvement (Giacobbe, Mayberg, & Lozano, 2009). Research suggests that the
amelioration of mood may be mediated by proximity to the stimulator as evidenced by acute deactivation of BA25 during intra-operative electrode placement and consequent immediate symptomatic relief (Mayberg et al., 2005). The later improvements are believed to lie in both proximity to the stimulator and the strength of connections of BA25 to other limbic-cortical regions. Medial and orbital frontal decreases were noted at 3 months and sustained at 6 months whereupon more distal regions, such as the ventrolateral and dorsolateral prefrontal, parietal mid- and posterior cingulate were also noted to have changed (Lozano et al., 2008). The pathways leading from the site of stimulation to the hypothalamus, nucleus accumbens, hippocampus/amygdala, orbitofrontal cortex and anterior midcingulate cortex have been traced using diffusion weighted imaging tractography and are in line with symptom improvement (Johansen-Berg et al., 2008; Merkl et al., 2013) and we too have shown that DBS executed in this animal model elicits effects outside of the region of stimulation. By expanding the course of IL DBS in ACTH pre-treated animals and attempting to match methodological imaging measures used in human research, it may be possible to detect mechanisms of specific symptom attenuation and construct a time line for such changes. PET has shown that DBS modulates the same neural substrates as cognitive behavioural therapy and SSRIs simultaneously (Helen S. Mayberg, 2006; Mayberg et al., 2005). It may therefore be possible to use DBS in a way that addresses a patients’ specific symptomatology more quickly, rather than waiting months for the progressive response of symptoms that is associated with current DBS protocols used for depression.

Additional limitations of the studies conducted for this thesis include the small sample size of the animal groups, the use of only male animals, the age variability in animals used and the use of two different strains of Rattus norvegicus. The latter serves to both confirm the original ACTH studies conducted by Kitamura et al. (2002) whilst increasing the reproducability and validity of the ACTH model in consistently producing antidepressant
resistance in other strains. The variability in age of animals at onset and duration of experimental protocol does indicate the need for a measure of caution when comparing results across studies presented herein. The translational quality of the data must also be regarded as best applied to young adolescent human patients, as the average age of animals used, 8.4 weeks, corresponds roughly to peri-pubescent human development (Sengupta, 2013). It is, however, important to note that the average age of onset for diagnosis in humans precedes complete social development, and is currently rapidly decreasing, thus these results maintain a credible translational validity. It must also be noted that, unlike human DBS, active DBS was halted in these research projects during behavioural tests, which has presented with immediate metabolism changes in the target and adjacent network areas, but importantly did not result in a return of depressive symptomatology in a time frame that indicates cessation of stimulation would effect the measures conducted here (Martin-Blanco et al., 2015). Future work should strive to include higher numbers of animals, broken into relevant age groups, of both sexes and various strains to increase the overall translational validity of the work.

There is also a need for the method of hippocampal dissection to be addressed in future work. A more precise dissection of this structure could prove beneficial as the established complexity of this widely innervated structure is likely to bear intra-region specific changes that are correlated with various symptom and response patterns (Fanselow & Dong, 2010). Finally, in light of current endeavours toward individualized medicine, together with recent trends to suspend the application of DBS to treat mental health conditions, the pattern of responders and non-responders, even in pre-clinical research, should be acknowledged. In doing so, it is likely that greater insight will be gained into the underlying pathophysiology of MDD and the mechanism of action of effective DBS.
8.6 Summary and Conclusion

Chronic ACTH administered *i.p.* at circadian nadir to adult male Sprague-Dawley rats does not result in altered plasma corticosterone profile at the end of a 14 or 18 day administration protocol. This treatment regimen did not change the protein expression of mature BDNF, full length TrkB or phosphorylated TrkB within the ventral or dorsal hippocampus compared to untreated animals. However, when ACTH-treated and untreated samples were combined, data indicate that ventral hippocampal phosphorylated TrkB levels are increased. When ACTH pre-treated adult male Sprague-Dawley and Wister rats were simultaneously administered infralimbic DBS, a behavioral antidepressant response was evidenced using the pharmacologically predictive, preclinical FST behavioral paradigm. This response was achieved with both acute and chronic DBS and did not present with obvious cognitive changes. This antidepressant response did not appear to be mediated by changes in hippocampal GDNF levels, however further investigation revealed that ACTH and infralimbic DBS do modulate gene and protein expression of neurotrophic factor intracellular signaling pathways in the IL. Specifically, according to microarray analysis, hormonal and environmental stress modulate mRNA in opposition to DBS. These changes, found primarily in the MAPK and p75 pathways, suggest a role for synaptic plasticity as an etiopathological mechanism of stress-mediated disorders and antidepressant response. Western blot probing of the stimulation site confirmed modulation of key components at the protein level. Increased activation of the neurotrophic factor PI3K pathway component, AKT, was found, as was increased activation of mTOR, known to be critical for rapid antidepressant effects. Decreased overall levels of NF-κB were also associated with infralimbic DBS. These protein findings confirm the indications from microarray that plastic mechanisms and metabolic regulation are, at least in part, a mechanism of infralimbic DBS antidepressant action.
DBS is a relatively novel antidepressant technique reserved for only the most extreme cases of refractory depression. Clinical data aimed at elucidating mechanisms of efficacy are thus difficult to obtain and is further complicated by the FDA suspension of DBS research trials. It is therefore critical that the findings reported in this thesis be utilized in further research using animal models so that investigations into the mechanisms mediating both DBS efficacy and the underlying neuropathology of antidepressant resistance are continued. The model used here has the potential to reveal some of these mechanisms; however, this work also highlights key points that must be addressed when using animal models to research mental health disorders. Overall, these findings impact future work on the ACTH model and contribute to the current understanding of the mechanisms of action of DBS in antidepressant resistance.

Together, these findings stand to make important positive impacts on treatment strategies for one of the most debilitating and prevalent disorders of the modern era and suggest that antidepressant treatment response may involve mechanisms distinct from chronic stress-mediated induction of depression-like behavioral phenotypes. The demonstration that ACTH-disrupts cyclic antidepressant treatment responsivity, which can seemingly only be ‘reacquired’ through IL-DBS via the engagement of plastic and metabolic mediators to facilitate local neurotrophic signaling pathways makes an important contribution to the field. Specifically, confirming the downstream adaptations necessary for antidepressant response. Importantly, the work presented herein underscores the need to approach the study of ‘depression’ and ‘antidepressant response’ as interdependent and parallel processes, which clearly have relevance to each other, but are not necessarily opposite faces of the same coin.


Mitchelmore, C., & Gede, L. (2014). Brain derived neurotrophic factor: Epigenetic regulation in psychiatric disorders. *Brain Research, 1586*(0), 162-172. doi: http://dx.doi.org/10.1016/j.brainres.2014.06.037


Appendices

9.1 Appendix A

Study Protocol, Chapter III

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<td>Day 10 – i.p. 3</td>
<td>Day 11 – i.p. 4</td>
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*i.p.* intraperitoneal; *ACTH* adrenocorticotropic hormone
### Study Protocol Chapters IV and V

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<td>FST, Humane Euthanisation, Cardiac Blood &amp; Brain Collection</td>
<td>Pre-FST treatment: DBS up to 5 minutes prior to FST; *imipramine10mg/kg</td>
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*i.p.* intraperitoneal; *ACTH* adrenocorticotropic hormone; *T.B.* Tail Blood; *OFT* Open Field Test; *NOP* Novel Object Preference test; *TMT* T-Maze Test; *FST* Forced Swim Test; *Control and ACTH only*
9.3 Appendix C

Study Protocol, Chapter VI

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
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<td>Day 8</td>
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<td>Day 10</td>
<td>Day 11</td>
<td>Day 12</td>
<td>Day 13</td>
<td>Day 14</td>
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<tr>
<td>– Begin i.p. 0.9% saline or 100μg ACTH</td>
<td>– i.p. 2</td>
<td>– i.p. 3</td>
<td>– i.p. 4</td>
<td>– i.p. 5</td>
<td>– i.p. 6</td>
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<td>Day 18</td>
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<td>– i.p. 14; OFT</td>
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<td>Pre-OFT treatment: Ctl, ACTH, FST &amp; AFST - imipramine 10mg/kg i.p.; DBS – 4 hours unilateral DBS up to 5 minutes prior to FST</td>
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**Day 22** – FST, Humane Euthanisation & Brain Collection

Pre-FST treatment – Ctl, ACTH, FST & AFST - imipramine 10mg/kg i.p.; DBS – 2 hours unilateral DBS up to 5 minutes prior to FST

*i.p. intraperitoneal; ACTH adrenocorticotropic hormone; OFT Open Field Test; FST Forced Swim Test; Ctl Saline Naive; ACTH ACTH Naive; FST Saline +FST; AFST ACTH + FST; DBS ACTH + FST + DBS*
### Study Protocol, Chapter VII

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4 - Begin i.p.</th>
<th>Day 5 – i.p. 2</th>
<th>Day 6 - i.p. 3</th>
<th>Day 7 - i.p. 4</th>
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<td>0.9% saline or 100μg ACTH</td>
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<td>Sham and DBS surgeries</td>
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<td>Begin DBS stimulation</td>
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<td>Pre-FST treatment: NS- saline; AS - saline; Sham – N/A; DBS – bilateral DBS up to 5 minutes prior to FST</td>
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</table>

*i.p.* intraperitoneal; *ACTH* adrenocorticotrophic hormone; *FST* Forced Swim Test; *NS* Naïve saline; *AS* – ACTH pre-treated; *Sham* Sham deep brain stimulation; *DBS* High Frequency Deep Brain Stimulation
Abbreviation Key for Gene/Protein Names, Chapter VI

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
<th>Relevant Pathway</th>
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<tr>
<td>Akt1</td>
<td>RAC serine/threonine-protein kinase</td>
<td>PI3K</td>
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<tr>
<td>BAD</td>
<td>Bcl-2-antagonist of cell death</td>
<td>PI3K</td>
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<tr>
<td>Bax</td>
<td>apoptosis regulator BAX</td>
<td>p75NTR</td>
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<tr>
<td>Bcl2</td>
<td>apoptosis regulator Bcl-2</td>
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<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
<td>TrkB</td>
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<tr>
<td>Braf</td>
<td>B-Raf proto-oncogene serine/threonine-protein kinase</td>
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<td>NF-kappa-B inhibitor beta</td>
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