A Study of Brain-Derived Neurotrophic Factor in Sprague-Dawley Rats Trained on a Learned Helplessness Procedure

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ABSTRACT

A STUDY OF BRAIN-DERIVED NEUROTROPHIC FACTOR IN SPRAGUE-DAWLEY RATS TRAINED ON A LEARNED HELPLESSNESS PROCEDURE

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Inescapable stress contributes to learned helplessness (LH), a behavioural component of depression. Also implicated is altered plasma and limbic brain-derived neurotrophic factor (BDNF). The general objective of this study was to explore whether LH is associated with changes in plasma and brain BDNF. Therefore, parametric experiments were conducted to identify testing conditions that generate LH. Then, plasma BDNF and BDNF mRNA in limbic regions were investigated due to their implication in mood disorders. It was found that: 1) inescapable shock lead to transient LH effects; 2) stress intensity interacted with stress inescapability to influence LH; and 3) limbic BDNF mRNA, but not plasma BDNF, was sensitive to different aspects of stress induced LH. Lastly, LH was not linked to anxiety-like behaviour and impaired locomotion. These experiments present effective parameters for a LH procedure, transient LH influenced by shock inescapability and amount, and differential BDNF mRNA alterations following stress induced LH.
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1. General Introduction

Depression is a highly prevalent and detrimental mood disorder, that can largely impact an individual’s quality of life (Beddington et al., 2008; Kessler et al., 2005). Though the cause of depression is multifaceted, the monoamine hypothesis of depression postulates low levels of serotonin (and other monoamines) are associated with depression (Martinowich & Lu, 2008). Another recognized, and possibly related model is the neurotrophic model of depression. This model posits low levels of neurotrophins are linked to mood disorders and antidepressants exert therapeutic effects by modulating this decline (Duman, Heninger & Nestler, 1997; Duman & Monteggia, 2006). Broadly, it is thought that neurotrophins may lead to restoration by regulating many aspects of neuronal development, function, plasticity, repair, dendrite growth and synaptogenesis (Altar, 1999; Ninan, 2014; Zagrebelsky & Korte, 2014). The family of neurotrophins include: brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (Castrén, Võikar, & Rantamäki, 2007). Of these, BDNF is the most widely studied and has received the most attention in relation to depression (Park & Poo, 2013; Martinowich & Lu, 2008).

BDNF is an endogenous protein that has a high affinity for the tropomyosin-related kinase B (TrkB) receptor (Altar, 1999; Ebendal, 1992; Park & Poo, 2013). BDNF can also bind to the p75 receptor but with a lower affinity than TrkB (Meeker & Williams, 2015). BDNF-TrkB modulates neurotransmission and synaptic transmission by interacting with both pre-synaptic and postsynaptic mechanisms (Bjorkholm & Monteggia, 2016). BDNF binding to TrkB regulates multiple intracellular pathways: the phospholipase C-gamma (PLC) pathway leading to protein kinase C (PKC) activation,
the Ras-mitogen-activated protein (MAP) kinase pathway, and the signaling pathway involving phosphoinositide 3-kinase (PI3K) that can activate the AKT-mTOR pathway (Björkholm & Monteggia, 2016; Park & Poo, 2013). As a result, BDNF is a major regulator of neural circuitry, gene expression and function.

BDNF is found widely throughout the mammalian body and brain (Duman, Heninger & Nestler, 1997). In the periphery, serum levels are reported to be higher than in plasma (Radka, Holst, Fritsche, & Altar, 1996), coinciding with the finding that BDNF is released from platelets during clotting processes (Fujimura et al., 2002). Notably, BDNF is a moderately sized, charged protein and therefore it is challenging for BDNF to cross the blood-brain barrier following peripheral administration (Nagahara & Tuszynski, 2011). Nonetheless, it has been shown that peripheral BDNF can cross the blood-brain barrier and vice versa (Pan et al., 1998), BDNF can cross in-vitro when aided by a magnetic nanoparticle-based carrier (Nair et al., 2013) and BDNF in rodents can cross using vector-mediated peptide drug delivery in-vivo (Pardridge, Kang & Buciak, 1994). In humans, permeability is more difficult to ascertain. Hence, a novel method aimed to quantify brain BDNF in humans has been established by Dawood and colleagues (2007). That is, blood is collected from the brachial artery and the internal jugular vein simultaneously and it is thought that the veno-arterial plasma BDNF concentration gradient can be used as an index of brain BDNF production (Dawood et al., 2007). Though this methodology is assumptive and indirect, low BDNF overflow from the brain was associated with depressive symptoms (Dawood et al., 2007). However, due to the difficulty in assessing human brain BDNF levels and the inconsistent preclinical research findings (Karege, Schwald, & Cisse, 2002; Luo et al.,
2010; Klein et al., 2011), a debate remains as to whether measuring peripheral BDNF levels is a reliable indicator of brain levels.

1.1. Biomarker Potential of BDNF

Brain-derived neurotrophic factor (BDNF) is one of the potential biomarkers of depression. A “biomarker” of depression is a biological substrate that can be measured quantitatively and reliably (Strimbu & Tavel, 2011), that has been proven to be predictive of presence/severity of symptoms, treatment response and/or treatment non-response. Since there is high heterogeneity in treatment response to commonly prescribed antidepressants (Souery et al., 2006), with remission in less than half of treated patients (Berton & Nestler, 2006), biomarkers are essential to investigate. Through identifying biomarkers, diagnoses can be readily made, and individualized treatment can be prescribed (Kennedy et al., 2012).

There are several lines of evidence suggesting BDNF is a viable biomarker of depression. The first line of evidence is apparent in clinical studies that report individuals suffering from depression have significantly lower levels of serum BDNF when compared to healthy counterparts (See Matrisciano et al., 2009; Molendijk et al., 2014 and Sen, Duman & Sanacora, 2008 for reviews). These findings corroborate low BDNF mRNA levels observed post-mortem in the hippocampus and prefrontal cortex regions of depressed, un-medicated individuals (Dwivedi et al., 2003). Notably however, while serum BDNF levels may serve as a potential biomarker for depression, levels do not correlate with depression severity (Molendijk et al., 2014). This highlights the complex relationship between BDNF and depression.

The second line of evidence is from research that reports increased BDNF levels
following a course of treatment of depression (See Duman & Monteggia 2006, Bjorkholm & Monteggia, 2016, Brunoni et al., 2008 and Polyakova et al., 2015 for reviews). In human and preclinical models, treatments such as electroconvulsive seizure therapy, monoamine oxidase inhibitors (MAOI’s), selective serotonin reuptake inhibitors (SSRI) and norepinephrine selective reuptake inhibitors (NESRI) have all promoted the up-regulation of serum BDNF levels (Duman & Monteggia, 2006). However, the relationship may be more complex than initially proposed: BDNF levels after treatment have been shown to be sensitive to dose, time of measurement and type of antidepressant medication. For example, sertraline (a SSRI) increased serum BDNF levels in depressed individuals after 5 weeks and 6 months of treatment, whereas venlafaxine (serotonin-norepinephrine reuptake inhibitor; SNRI), increased levels only after 6 months (Matrisciano et al., 2009). Additionally, escitalopram (another SSRI), has shown differential effects on BDNF, both not affecting and increasing serum levels (Matrisciano et al., 2009; Park, Lee, Um, & Kim, 2014; Wolkowit et al., 2011). Further, Deuschle and colleagues (2013), examined serum BDNF levels in patients treated with venlafaxine and mirtazapine (a noradrenergic and specific serotonergic antidepressant; NaSSA). They reported a decline of BDNF in venlafaxine-treated patients and an increase in mirtazapine-treated patients. Despite these differences, the predominant argument remains: an increase in serum BDNF levels can be seen after a course of treatment (Björkholm & Monteggia, 2016; Brunoni et al., 2008).

The third and final line of evidence is that changes in depressive symptoms following a course of treatment have been linked to BDNF. For example, a clinical study by Wolkowitz and colleagues (2011) confirmed that un-medicated depressed individuals
had low serum BDNF levels, which increased following escitalopram treatment. Higher pre-treatment serum levels of BDNF were also correlated with improved antidepressant responses following treatment (Wolkowitz et al., 2011). In another investigation, patients treated with duloxetine (another SNRI) had increases in serum BDNF in the first and second week of treatment (Delini-Stula et al., 2012). Notably, individuals with an early increase in serum BDNF were more likely to have decreases in depressive symptomology and were more likely to later achieve remission when compared to individuals with no BDNF increase (Delini-Stula et al., 2012). Further, serum BDNF levels were lower in patients who did not show an alleviation of symptoms when compared to controls and remitted subjects (Park et al., 2014). Thus, the absence of an increase in serum BDNF levels following a course of treatment is associated with a failure to achieve remission in depressed patients. Finally, a 2015 meta-analysis once again confirmed that serum BDNF levels are low in patients with major depression and that levels are increased, specifically in those who respond to treatment (Polyakova et al., 2015). Taken together, these studies demonstrate the biomarker potential of BDNF in treatment response.

1.2. Learned Helplessness (LH)

1.2.1. Humans. A key behavioural component of depression is helplessness (Beck, Riskind, Brown & Steer, 1988). Subjectively, people can report a feeling that external circumstances and negative stressors are beyond control and cannot be stopped through any effort (Alloy & Seligman, 1979). Indeed, high levels of helplessness have been reported in depressed patients (Beck, Riskind, Brown & Steer, 1988; Grote, Bledsoe, Larkin, Lemay & Brown, 2007), in individuals displaying
depressive symptoms (Maier & Seligman, 2016), and more severe feelings of helplessness have been associated with more severe feelings of depression (Ozment & Lester, 1998). Although helplessness can have a trait-like quality in depressed individuals (Klein, Fencil, Morse & Seligman, 1975; Klein & Seligman, 1976), there is evidence that it can be learned following exposure to inescapable stressors and life traumas (termed learned helplessness; LH) (For review see Pryce et al., 2011). That is, inescapable stressors have been found to predict depression (Brown, Harris & Heppworth, 1995; Keller, Neale & Kendler, 2007; Kendler & Gardner, 2010; Kendler, Gardner & Prescott, 2002), and it is proposed that these inescapable stressors/life events can lead to generalized LH if they are likely to lead to highly aversive outcomes (Abramson, Seligman & Teasdale, 1978).

1.2.2. Animals. LH was first documented in animals by researchers Seligman and Overmier (1967). In their original experiment, four experimental groups were used. The first group was a no shock control group (NS). The second group received 64 trials of inescapable foot-shock (5-second duration, intertrial interval [ITI] of 60-120 seconds). The third group received 640 trials of inescapable foot-shock (0.5 second duration, ITI of 4.5-18 seconds). The fourth group received 64 trials of inescapable foot-shock (0.5 second duration, ITI of 60-120 seconds). The next day, all groups underwent avoidance-escape training. After being exposed to the inescapable shocks, subjects displayed lowered and delayed avoidance-escape responding. Interestingly, the higher shock density groups showed more impairments than the low-shock density groups. Researchers concluded that impairments were a general phenomenon resulting from exposure to an inescapable shock. Therefore, LH was defined as a behavioural
passivity to escapable and aversive stressors, resulting from previous experience with inescapable stressors (Overmier & Seligman, 1967).

Since the discovery of LH, two different LH procedures have been created. The first procedure uses three experimental groups (See Pryce et al., 2011). The first group is a no shock (NS) group, where subjects experience all procedures, but are not exposed to any shock. This group is mainly used for neurological and biological controls. The second group is the escapable shock group (ES). This group is exposed to unpredictable, escapable stressors (i.e., foot shocks). The third group is the “yoked” or inescapable shock group (IES). Initially, durations of the foot-shock experienced by the IES group are determined by and equal to the ES group. That is, each IES subject is paired with an ES subject, and simultaneously, each subject is placed into its own chamber. The two chambers are electrically linked, and the ES subject terminates the shock for both itself and the paired IES subject. Therefore, the amount of shock exposure is the same in both groups, making the escapability of the stressor the only difference between groups.

After exposure to inescapable (IES) and escapable shock (ES), another behavioural test is conducted (i.e., an avoidance or escape task), providing groups an opportunity to learn to avoid and escape a stressor. This test is used to examine effects of shock escapability on subsequent learning and behaviour. Further, an additional behavioural task (i.e., avoidance or escape task) can be executed, typically after a delay period of 7-14 days. This test is used to determine if the LH effects are chronic in nature (sustained deficits), and if they can be alleviated (i.e., with drug treatments) or exacerbated with pharmacological manipulations.
The second procedure consists of a NS group and a group that is subjected to IES. Again, in this procedure the NS group is exposed to the same experimental procedures as the IES group, but they are not exposed to any shock. Next, all subjects undergo a behavioural test (i.e., avoidance test) to examine the effects of the IES and NS on behaviour and learning. The IES subjects that seem to display behavioural impairments and learning deficits in the task are believed to have developed LH and are used for further experimental procedures (i.e., drug treatments, further behavioral tests). Typically, initial exposure to IES or NS is termed the pre-exposure phase and the second behavioural task is termed the post-shock screening test, as it screens for the subjects who have adopted LH (Pryce et al., 2011).

Typically, if the IES group has developed LH, it is expected they will show low escape effort (i.e., high escape latencies or no effort to terminate the shock) (Overmier & Seligman, 1967). In addition to observing a decreased escape effort, other evident depressive-like behaviours include weight loss, sleep disturbances (See Vollmayr & Henn, 2003 for review) and decreased sucrose and saccharine intake (Vollmayr et al., 2003; Minor et al., 1994).

1.3. Individual Differences in LH

Individual differences in vulnerability and resiliency to both inescapable and escapable stress have been extensively studied (Minor, Dess, Ben-David & Chang, 1994; Mansuy, 2012). Indeed, in LH experiments sub-groups of vulnerable and resilient subjects can be identified (Yang et al., 2015; Muneoka et al., 2013; Ronan, 2000). When compared to resilient (or “non-LH”) subjects, vulnerable (or “LH” subjects) subjects presented with differential neurotransmitter and amino-acid expression in
regions associated with depression (Muneoka et al., 2013; Ronan, 2000). As well, regional differences in BDNF in the dentate gyrus (DG), CA3, medial prefrontal cortex (mPFC) and nucleus accumbens (NAcc) could play a role in the manifestation of individual differences in LH (Yang et al., 2015). Other factors as well, such as sex (Shors et al., 2008), strain (Lima et al., 2009) and reactivity to novel environments (i.e., open field test) have been predictive of individual differences in LH, but locomotion, habituation styles (Lima et al., 2010) and hormonal differences (Shors et al., 2008) have not.

1.4. Manipulation of BDNF and TrkB

In rodent studies, researchers can manipulate and alter BDNF levels either in the periphery, entire brain, or in specific brain regions. To decrease BDNF levels, deletion of BDNF can be region specific (Adachi, Barrot, Autry, Theobald, & Monteggia, 2008) or BDNF knockout rodents (typically mice with a 50% deletion of the BDNF gene) can be utilized (Benmansour et al., 2008; Chen et al., 2007). To increase BDNF levels, researchers can either directly inject BDNF into various brain regions (Shirayama, Chen, Nakagawa, Russell, & Duman, 2002), over express BDNF gene using specific lentiviral vectors (Taliaz, Nagaraj, Haramati, Chen, & Zangen, 2013) or implant peripheral subcutaneous mini-pumps containing BDNF (Schmidt & Duman, 2010). As well, enhancing BDNF-TrkB is a commonly used methodology. Increased activation of TrkB receptors can be induced via intraperitoneal injections or intracerebral microinjections of TrkB agonists (Yang, Shirayama, Zhang, Ren, & Hashimoto, 2015). Overexpression of TrkB gene has been established by electroporating a non-viral plasmid into specific brain regions (De Vry et al., 2016). Similar methods can be used to
decrease BDNF-TrkB signalling, using a TrkB antagonist (Cazorla et al., 2014; Shirayama et al., 2015).

1.5. Effects of Manipulations on Depressive-Like Symptoms & Treatment Response

By implementing the aforementioned manipulations, BDNF-dependent regulation of treatment response has been linked to an interaction between BDNF and serotonin (See Homberg et al., 2014 For Review). That is, not only does the upregulation of serotonin increase levels of BDNF, but BDNF has also demonstrated a role in modulating serotonergic transmission and function. For example, when BDNF is injected intracerebrally, an enhancement of serotonin transporter (SERT) function can be observed. Also, modifications in BDNF transmission have been shown to decrease the effectiveness of SSRI’s (i.e., delay their effect), by altering serotonergic signaling cascades (Benmansour et al., 2008). Additionally, a decrease or increase in hippocampal BDNF has resulted in differential alterations of serotonergic neurotransmission (Delttheil et al., 2008). Specifically, when hippocampal BDNF was decreased by 50%, the capacity of serotonin reuptake was reduced, and an SSRI failed to increase hippocampal extracellular serotonin. In contrast, when BDNF was increased this resulted in an increase in SSRI response on extracellular serotonin in the hippocampus, which was then correlated with depressive-resistant behavioural responses (Delttheil et al., 2008). Therefore, it is recognized that BDNF and serotonergic systems interact to regulate neurobiological mechanisms involved in treatment effects; the monoamine hypothesis of depression and neurotrophic model of depression interact to modulate not only depressive symptomatology, but also treatment efficacy.
Manipulation of BDNF and/or BDNF-TrkB alters depressive-like behaviours and responses to antidepressant drugs. For example, a single bilateral infusion of BDNF into the rat DG reduced both LH and immobility in the forced swim tests (FST) (Shirayama et al., 2002). In line with this, lowered BDNF was found in the mPFC, CA3 and DG in LH rats displaying depressive-like phenotypes (Yang et al., 2015). However, decreasing BDNF in this area does not induce behavioural despair or depressive-like symptoms. Rather, the selective loss of BDNF in the DG decreases response to desipramine (a tricyclic) and citalopram (a SSRI) in the FST (Adachi et al., 2008). This was the first record of the role of BDNF in the DG response to antidepressant drugs. Taken together, these studies suggest increases of BDNF in the DG create a depression-resilient phenotype, whereas the selective loss of BDNF in the DG results in decreased effectiveness of antidepressant treatments.

Interestingly, different effects are seen when examining BDNF in the NAcc, ventral tegmental area (VTA) and amygdala. Specifically, increasing BDNF levels in the VTA results in depressive-like behaviour, and blocking TrkB in the NAcc results in a depression-resilient phenotype in the FST (Eisch et al., 2003). Recently, Shirayama and colleagues (2015) explored the role of BDNF-TrkB in depressive-like behaviours using a LH procedure. Through administering a TrkB antagonist or agonist into multiple rat brain regions, it was concluded that TrkB agonists exert effects by stimulating TrkB in the infralimbic mPFC, the CA3 and DG of the hippocampus. Conversely, they found TrkB antagonists exert effects by blocking TrkB in the NAcc. Lastly, BDNF in the basolateral amygdala (BLA) is increased following stress exposure when compared to controls (Fanous et al., 2010; Lakshminarasimhan & Chattarji, 2012). Together these
1.6. Objectives

The working hypothesis of the current thesis is as follows: Expression of limbic BDNF is differentially affected by uncontrollable stress. Therefore, the thesis aimed to first, establish effective parameters for a LH procedure (See Parametric Experiments 1, 2 and 3). The second objective was to investigate whether the LH effect is stable and associated with altered plasma BDNF protein and limbic BDNF mRNA. Specifically, emphasis was placed on BDNF mRNA in the DG, NAcc and BLA due to their implication in mood disorders. The third objective of this thesis was to investigate whether LH is linked to anxiety-like behaviour or impaired motor functions. Finally, individual differences in LH were also assessed, which allowed a relative weighing of stress escapability and stress amount on subsequent behaviour.

2. Parametric Experiments

2.1. Materials and Methods

2.1.1. Animals

Adult male Sprague-Dawley rats weighing 175-200 grams upon arrival (Charles River, Quebec, Canada) were individually housed (cages made of polycarbonate with standard bedding and environmental enrichment) and maintained on a 12-hour reverse light-dark schedule (lights off at 7:00 AM, on at 7:00 PM). Water and rat chow (Purina rat chow 18% protein) was available ad-libitum. Before any experimental procedures began animals were given one week of acclimatization to the facility, adequate handling and habituation to experimental apparatuses. All experiments were approved by the
Animal Care Committee of the University of Guelph and were carried out with the recommendations of the Canadian Council on Animal Care.

2.1.2. Escape and Avoidance Training Apparatus

Escape and avoidance training was carried out using the commercially available Gemini Avoidance Chambers. These chambers (composed of acrylic and aluminum) consist of two compartments divided by a retractable stainless-steel gate (7 cm wide x 10 cm high), with 28 stainless-steel floor grids. The dimensions for the chambers on the outside were: 66 cm wide x 33 cm long x 44 cm high, and inside: 24 cm wide x 20 cm long x 20 cm high. The shock is delivered to the grid flood with a solid-state feedback controlled, constant current shocker. A screen on each box allowed a real-time visualization of the current being administered to the subject. Eight infrared photobeams in each compartment allowed detection of animal location. The Gemini software automatically recorded response latency and categorized each response as an avoidance, escape or non-response (Gemini Avoidance System: Microsoft Access, San Diego Instruments, SD USA).

3. Procedures and Results

3.1. Overview

Parametric experiments were conducted on tasks commonly used in LH procedures to identify testing conditions and parameters that would generate significant LH. Specifically, three parametric experiments were conducted to investigate how escape (Parametric Experiment 1) and avoidance (Parametric Experiments 1 and 2) responding would develop over multiple and consecutive training days.
3.2. Parametric Experiment 1

Subjects (n=12) underwent 7 days of training that consisted of 100 trials of a 30-second foot-shock (0.8mA, inter-trial interval of 22-38 seconds). The first five trials were carried out using a fixed ratio 1 schedule (FR1; where one cross to the adjacent compartment of the chamber terminated the shock), while the remaining trials employed a fixed ratio 2 schedule (FR2; where two crosses were required to terminate the shock). An FR1 schedule was implemented before a FR2 schedule to help ensure subjects would learn that shock termination was contingent on their behavioural response. After completing the 7 days of training, it was clear that the highest escape responding (i.e., the most escapes and least amount of total shock exposure) was achieved by day 4 of training (data not shown).

3.3. Parametric Experiment 2

Subjects (n=12) underwent 30 trials where a 3-second light presentation preceded a foot-shock (0.8 mA, 30 second duration, with an ITI between 22-38 seconds). All subjects were given the opportunity to avoid the stressor by crossing to the adjacent compartment during the 3-second light presentation or escape the stressor during the 30-second shock administration. Avoidance responding on this task did not reach higher than 32% by day 6 of training (data not shown).

3.4. Parametric Experiment 3

Aiming to increase the percent of avoidances, subjects (n=12) underwent 30 trials where a 6-second tone preceded and terminated with a foot-shock (0.8 mA, 30 second duration, with an ITI between 22-38 seconds). All subjects were given the opportunity to avoid the stressor by crossing to the adjacent compartment during the 6-
second tone presentation or escape the stressor during the 30-second shock administration. As expected, using tone for a longer duration (rather than a light) resulted in more avoidance responding, reaching 46% by day 6 of training (data not shown).

3.5. Final LH Procedure

On the basis of the results of these parametric experiments, the final LH procedure consisted of: four days of the escape task (as described in Parametric Experiment 1), followed by a seven day wait period, followed by six days of an avoidance task (as described in Parametric Experiment 3; tone as a cue).
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Abstract

Inescapable stress plays a role in the development of learned helplessness (LH), a feature of major depression. Considering possible biomarkers, depression has been linked to altered brain and plasma brain-derived neurotrophic factor (BDNF). Therefore, this study measured BDNF levels in the plasma, dentate gyrus (DG), nucleus accumbens core (NAcc) and basolateral amygdala (BLA) of male Sprague-Dawley rats trained in LH.

In Experiment 1, groups (n=12-18 each) were trained for 4 days in an escape task. The “escapable” group (ES) could escape foot-shocks by performing a shuttle response (100 trials, 0.8 mA, 30 sec each). The “inescapable” group (IES) was yoked to the ES group, and hence escape behaviour was without consequences, but the subjects received the same number and duration of shocks as the ES group. When tested on an avoidance task (30 trials, 0.8 mA, 30 sec each), the IES group displayed a transient escape deficit.

Experiment 2 demonstrated that LH differences between ES and IES groups could not be explained by anxiety-like behaviours or locomotion impairments. BDNF mRNA levels in DG and NAcc was lower and higher, respectively, in shock groups (ES and IES) regardless of escapability. However, in the BLA, BDNF mRNA expression was higher only in the IES group. Plasma BDNF was not affected by any manipulation. These data in rats suggest that stress inescapability influences LH, and that BDNF levels in the BLA, but not plasma, hippocampus or NAcc, are correlated with this behavioural response.

Keywords: Depression, Biomarker, Brain-derived Neurotrophic Factor (BDNF), Stress, Learned Helplessness, Rat
1. Introduction

Depression is a highly prevalent and detrimental mood disorder that can largely impact an individual’s quality of life (Beddington et al., 2008; Kessler et al., 2005). However, there is a high degree of heterogeneity in treatment response to commonly prescribed antidepressants (Souery et al., 2006), with remission in less than half of treated patients (Berton & Nestler, 2006). Hence, it is essential to identify biomarkers of depression (Kennedy et al., 2012). A “biomarker” of depression is a biological substrate that can be measured quantitatively and reliably (Strimbu & Tavel, 2011), that has been proven to be predictive of presence/severity of symptoms, treatment response and/or treatment non-response. By identifying biomarkers, diagnoses can be made, and individualized treatment can be prescribed (Kennedy et al., 2012).

Brain-derived neurotrophic factor (BDNF) is one of the potential biomarkers of depression. BDNF is a neurotrophic protein found throughout the mammalian body and brain, that is involved in synaptic plasticity and neurogenesis (Duman et al., 1997; Altar, 1999). In support of BDNF as a biomarker, it is known that individuals suffering from depression have lower levels of serum BDNF when compared to non-depressed counterparts (See Matrisciano et al., 2009; See Molendijk et al., 2014 and Sen et al., 2008 for reviews). Further, mood disorder treatments promote the up-regulation of serum BDNF levels (See Duman & Monteggia 2006, Bjorkholm & Monteggia, 2016, Brunoni et al., 2008 and Polyakova et al., 2015 for reviews), and both pre-treatment (Park et al., 2014; Wolkowitz et al., 2011), and post-treatment serum BDNF levels have been found to be predictive of treatment response (Delini-Stula et al., 2012).

A key behavioural component of depression is helplessness (Beck et al., 1988).
Subjectively, people can report a feeling that external circumstances and negative stressors are beyond control and cannot be stopped through any effort (Alloy & Seligman, 1979). Indeed, high levels of helplessness have been reported in depressed patients (Beck et al., 1988; Grote et al., 2007), and in individuals displaying depressive symptoms (Maier & Seligman, 2016), and more severe feelings of helplessness have been associated with more severe feelings of depression (Ozment & Lester, 1998).

Although helplessness can have a trait-like quality in depressed individuals (Klein et al., 1975; Klein & Seligman, 1976), there is evidence that it can be learned following exposure to inescapable stressors and life traumas (termed learned helplessness; LH) (For review see Pryce et al., 2011). That is, inescapable stressors have been found to predict depression (Brown et al., 1995; Keller, Neale & Kendler, 2007; Kendler & Gardner, 2010; Kendler et al., 2002), and it is proposed that these inescapable stressors/life events can lead to generalized LH if they are likely to lead to highly aversive outcomes (Abramson et al., 1978).

In rodents, LH can be studied using a procedure pioneered by Overmier and Seligman (1967). LH is defined as a behavioural passivity to escapable and aversive stressors, resulting from previous experience of inescapable stressors. Typically, in LH experiments three experimental groups are used: 1) a no-shock (NS) control group; 2) an escapable shock group (ES) and 3) an inescapable shock group (IES). Shock chambers are electrically linked, and the ES subject terminates the shock for itself and its paired IES subject (yoked design). Therefore, the escapability of the stressor is the only difference between the two groups. In addition to observing a decreased escape effort in the IES group (Overmier & Seligman, 1967), rodents display other depressive-
like behaviours including weight loss, sleep disturbances (See Vollmayr & Henn, 2003 for review) and decreased sucrose and saccharine intake (Vollmayr et al., 2004; Minor et al., 1994). Interestingly, antidepressant medications can reverse LH in rodents (Benatti et al., 2014; Sherman et al., 1982).

It is hypothesized that expression of limbic BDNF is differentially affected by uncontrollable stress. Therefore, to explore this hypothesis, Experiment 1 quantified BDNF mRNA expression in the brain and plasma BDNF of rats that underwent a LH procedure. BDNF mRNA was quantified in the dentate gyrus (DG), because lower BDNF in this region is associated with depressive-like symptoms (Yang et al., 2015), and intra-DG BDNF infusions reduce LH (Shirayama, 2002). BDNF mRNA was also measured in the nucleus accumbens (NAcc), because LH subjects have high BDNF expression in this region (Yang et al., 2015), and blocking BDNF here results in a depression-resilient phenotype in rodents (Eisch, 2003). Experiment 2 investigated whether LH is linked to anxiety-like behaviour and impaired motor functions. As well, in addition to the DG and NAcc, BDNF mRNA expression was assessed in the basolateral amygdala (BLA) because stress elevates BDNF in this region (Fanous et al., 2010; Lakshminarasimhan & Chattarji, 2012).

Finally, typical LH experiments identify sub-groups of resilient and vulnerable subjects (Yang et al., 2015). Individual differences in vulnerability to IES has been studied for decades (Minor et al., 1994) and interestingly, regional differences in BDNF protein in the DG, CA3, mPFC and NAcc could play a role (Yang et al., 2015). Therefore, in both experiments individual differences in ES and IES groups were assessed.
2. Materials and Methods

2.1 Animals

Male Sprague-Dawley rats (Charles River, Quebec, Canada) weighing 175-200 grams upon arrival, were individually housed (cages made of polycarbonate with standard bedding and environmental enrichment) and maintained on a reverse light-dark schedule (lights off at 7:00 AM, on at 7:00 PM). Water and rat chow (Purina rat chow 18% protein) was available ad-libitum. Before experimental procedures began animals were given a week of acclimatization to the facility, adequate handling and habituation to experimental apparatuses. Experiments were approved by the Animal Care Committee of the University of Guelph and were carried out with the recommendations of the Canadian Council on Animal Care.

2.2 Apparatus

2.2.1 Escape and Avoidance Training

These procedures were carried out using the commercially available Gemini Avoidance Chambers. These chambers (composed of acrylic and aluminum) consisted of two compartments divided by a retractable stainless-steel gate (7 cm wide x 10 cm high), with 28 stainless-steel floor grids. The dimensions for the chambers on the outside were: 66 cm wide x 33 cm long x 44 cm high, and inside: 24 cm wide x 20 cm long x 20 cm high. The shock was delivered to the grid floor with a solid-state feedback controlled, constant current shocker. A screen on each box allowed a real-time visualization of the current being administered to the subject. Eight infrared photobeams in each compartment allowed detection of subject location. (Gemini Avoidance System: Microsoft Access, San Diego Instruments, SD USA).
2.2.2. Light-Dark Emergence

The light-dark apparatus consisted of an opaque white plastic rectangular box, divided into two compartments: a small (25 cm wide × 20.5 cm long × 20.5 cm high) enclosed dark box built of opaque black plastic with an opening (8 cm wide × 10 cm high) leading to a larger (25 cm wide × 39.5 cm long) open illuminated box. The open box was lit by one lamp (60-W bulb, 180 lux) positioned 115 cm above the center of the lit box. A video camera mounted over the top of apparatus recorded movement. Videotapes were analyzed by Ethovision software (Noldus Information Technology, Leesburg, VA, USA).

2.2.3. Locomotion

Twelve, custom made (University of Guelph, ON), activity chambers were used to measure locomotion. The chambers (30 cm wide × 40 cm long × 26 cm high) were made of gray polyvinyl chloride and were covered by black wire mesh. Movement was recorded using EthoVision tracking software (version 3, Noldus Information Technology, Netherlands).

2.3 Biomarkers

2.3.1. BDNF Plasma Enzyme-Linked Immunosorbent Assay (ELISA)

Trunk blood was collected, transferred into anticoagulant tubes and centrifuged at 2500xg for 15 minutes. Plasma was extracted with a pipette, placed into a new tube, and stored at -80°C until used for analyses. Plasma BDNF was measured using the human/rat/mouse BDNF DuoSet ELISA kit (R&D Systems, Minneapolis, MN) and samples were diluted 10x (R&D Systems). ELISA samples and standards were run in duplicate. Absorbance was measured at 450 nm (with a reference of 540 nm), using a
Multiskan GO UV/Vis microplate spectrophotometer and SKANIT 3.2 software (Thermo-Fisher Scientific, Waltham, MA).

2.3.2. Preparation of RNA Extracts and Solution Hybridization Ribonuclease Protection–Trichloroacetic Acid (TCA) Precipitation Assay

Immediately following the final avoidance test and Delayed light-dark emergence test, animals were euthanized using carbon dioxide (CO2), brains were collected and stored at −80°C for mRNA analyses. BDNF mRNA in the hippocampus (DG+CA1), the NAcc core (NAcc) and basolateral amygdala (BLA) were quantified (attomoles/µg) using a solution hybridization RNase protection TCA precipitation assay using the protocol described in Zhou et al. (2011).

3. Procedures

3.1. Experiment 1

ES and IES groups (n=18 each) underwent four days of escape training (100 trials, 30-second foot-shock, 0.8mA, inter-trial interval of 22-38 seconds). The first five trials were carried out using a fixed ratio 1 schedule (where one cross to the adjacent compartment of the chamber terminated the shock), while the remaining trials employed a fixed ratio 2 schedule (where two crosses were required to terminate the shock). The IES group received the same duration and intensity of shock as the ES group but were not able to terminate it (yoked). The NS controls (n=12) experienced the same environmental conditions but no foot-shock (0 mA).

Following a one-week delay, subjects underwent six days of avoidance training. ES and IES groups were trained on 30 trials where a 6-second tone preceded and terminated with a 30-second foot shock (0.8 mA, inter-trial interval between 22-38
seconds). Subjects were given the opportunity to avoid the stressor by crossing to the adjacent compartment during the 6-second tone presentation, escape the stressor during the 30-second shock administration, or failed to escape the stressor. NS controls did not receive any foot-shock (0 mA). Upon completion of the last avoidance training session, subjects were sacrificed, and blood and brain collection took place. See Figure 1A for visual timeline depiction of Experiment 1 procedures.

3.2. Experiment 2

ES, IES and NS groups (n=12 each) underwent a 2-hour baseline locomotion test (Pre-Escape). Each subject was placed in a chamber and movement was recorded. The following day, subjects underwent four days of escape training (as described in Experiment 1). The day following the last escape training session, subjects underwent another 2-hour locomotion test (Post-Escape). No tissue samples were taken from these subjects. See Figure 1B for visual timeline depiction of this experimental procedure.

In addition, separate ES, IES and NS groups (n=12 each) underwent a baseline light-dark emergence test (Pre-Escape). Subjects were placed in the corner of the dark chamber, facing away from the opening between the light and dark chamber. Movement was tracked during the 5-minute test, allowing a quantification of time spent in the lit area. The following day, subjects underwent four days of escape training (as described in Experiment 1). Post-Escape and seven days later (Delayed), subjects were tested on light-dark emergence. Upon completion of the Delayed test, subjects were sacrificed, and blood and brain collection took place. See Figure 1C for visual timeline depiction of this experimental procedure.
3.3. Statistical Analyses

For all data, appropriate repeated or one-way analyses of variance (ANOVA) were conducted. Significant main effects and interactions were further analyzed using Student Neuman-Keuls and planned comparisons to test specific predictions were conducted using t-tests. To explore individual differences, high shock (HS) and low shock (LS) sub-groups were created by performing a median split on total shock exposure on the last day of escape training. All data is presented as the mean ± standard error of the mean (±SEM), with the significance criterion of $p<0.05$. Details of non-significant tests are not reported. All analyses were computed using SigmaStat (v. 12.5, Windows, SPSS Inc) or SPSS (v. 25, Windows, SPSS Inc).

4. Results

Panel A of Figure 2 represents mean (±SEM) total shock exposure across the four days of escape training in the ES group. The ANOVA revealed a significant main effect of Training Day [$F(17, 51) = 3.01, p<0.05$]). Multiple comparisons further indicated that shock duration significantly decreased from training day 1 to training day 4 ($p<0.05$). Panel B represents mean (±SEM) total shock exposure across four days of escape training in ES HS and ES LS groups. The ANOVA revealed a significant main effect of Group [$F(1, 28) = 43.68, p<0.001$] and a significant main effect of Training Day [$F(3, 28) = 3.80, p= 0.02$]. Multiple comparisons on marginal means further indicated that overall, the ES HS sub-group received significantly more shock exposure than the ES LS sub-group ($p<0.05$), and that shock exposure overall decreased from training day 1 to training day 4 ($p<0.05$).
Panel A of Figure 3 represents mean (±SEM) percent shocks avoided across the six days of avoidance training in the ES and IES groups. The ANOVA revealed a significant main effect of Training Day \( [F (5, 170) = 2.31, \ p<0.05] \) only. Multiple comparisons on marginal means further indicated that percent shocks avoided increased from training day 3 to training day 6 \( (p<0.05) \). Panel B represents mean (±SEM) percent shocks avoided across 6 days of avoidance training in ES HS and ES LS sub-groups, while Panel C represents mean (±SEM) percent shocks avoided across 6 days of avoidance in IES HS and IES LS sub-groups. The ANOVA revealed a significant Training Day x Shock Escapability x Shock Duration interaction \( [F (5, 160) = 3.33, \ p<0.01] \), as well as a significant main effect of Training Day \( [F (5, 160) = 2.61, \ p<0.05] \). In the ES group, multiple comparisons further indicated that the HS sub-group initially had significantly less avoidances when compared to the LS sub-group \( (p<0.05) \), but this difference dissipated with training, and no significant differences were detected on the last day. The IES group displayed an opposite pattern of results: no significant differences in avoidance responding were detected between HS and LS sub-groups initially, but by the last day, the HS sub-group displayed significantly less avoidances than the LS sub-group \( (p<0.05) \).

Panel A of Figure 4 represents mean (±SEM) total shock exposure across six days of avoidance training in ES and IES groups. The ANOVA revealed a significant Group x Training Day interaction, \( [F (5, 170) = 7.27, \ p<0.001] \), as well as a main effect of Group \( [F (1, 170) = 4.62, \ p<0.05] \) and a main effect of Training Day \( [F (5, 170) = 21.19, \ p<0.001] \). Multiple comparisons further indicated that the IES group received significantly more shocks on days 1 and 2 in comparison to the ES group \( (p<0.05) \).
Furthermore, there was a significant decrease in shock exposure in groups over the course of avoidance training ($p<0.05$). Panel B represents mean ($\pm$SEM) total shock exposure across the six days of avoidance training in ES HS and ES LS sub-groups, while Panel C represents mean ($\pm$SEM) total shock exposure across the six days of avoidance training in IES HS and IES LS sub-groups. The ANOVA revealed a significant Training Day x Shock Escapability interaction [$F(5, 160) = 6.37, p<0.001$], as well as a main effect of Time [$F(5, 160) = 16.81, p<0.001$], a main effect of Escapability [$F(1, 32) = 5.60, p<0.05$], and a main effect of Shock Duration [$F(1, 32) = 9.41, p<0.01$]. Multiple comparisons on marginal means revealed that all groups differed from the ES LS sub-group ($p<0.05$), that the IES group received significantly more shocks than the ES group ($p<0.05$), and that HS groups received significantly more shocks than the LS group ($p<0.01$). Interestingly, except for the ES LS sub-group, all other groups displayed a progressive reduction of shock exposure across training days ($p<0.05$).

An ANOVA was performed on mean plasma BDNF in NS (Mean= 2.14, $\pm$SEM = 0.19), ES (Mean=2.23, $\pm$SEM = 0.13) and IES (Mean=2.32, $\pm$SEM = 0.10) groups, but no significant group difference was found. It was also explored whether this effect was influenced by amount of shock exposure (HS & LS sub-groups), but no significant effect was found (data not shown).

Panel A of Figure 5 represents mean ($\pm$SEM) BDNF mRNA in the hippocampus in NS, ES and IES groups. The ANOVA revealed a significant main effect of Group [$F(2, 39) = 10.40, p<0.001$], and multiple comparisons further indicated that both ES and IES shock groups displayed lower expression when compared to the NS group ($p<0.05$). Panel B represents mean ($\pm$SEM) BDNF mRNA in the NAcc core of NS, ES
and IES groups. The ANOVA revealed a significant main effect of Group \[F (2, 38) = 5.88, p=0.01\], and multiple comparisons further indicated that both ES and IES groups had significantly higher expression when compared to the NS group \(p<0.05\). It was also explored whether these effects were influenced by amount of shock exposure (HS & LS sub-groups), but no significant effects were found (data not shown).

Table 1 represents mean (±SEM) total distance moved Pre-Escape and Post-Escape training in the NS, ES and IES groups. The ANOVA failed to reveal significant group differences. Table 1 also represents mean (±SEM) time in lit area Pre-Escape, Post-Escape and Delayed in NS, ES and IES groups. The ANOVA revealed a significant Group x Test interaction \[F (4, 66) = 3.63, p=0.01\] and multiple comparisons further indicated that on the Post-Escape test, the IES group entered the lit area significantly less than the other groups \(p<0.05\). However, on the Delayed test, no group differences were found and the IES group entered the lit area significantly more than they did Post-Escape, \(p<0.05\). It was also explored whether this effect was influenced by amount of shock exposure (HS & LS sub-groups), but no significant effect was found (data not shown).

Panel A of Figure 6 represents mean (±SEM) BDNF mRNA in the hippocampus in NS, IES and ES groups. The ANOVA revealed a significant main effect of group \[F (2, 33) = 11.48, p<0.001\]. Multiple comparisons further indicated that both ES and IES shock groups had lower expression when compared to the NS group. Panel B represents mean (±SEM) BDNF mRNA in the NAcc core of NS, ES and IES groups. The ANOVA revealed a significant main effect of group \[F (2, 34) = 8.53, p<0.001\] and multiple comparisons indicated that both ES and IES groups had significantly higher
expression when compared to the NS group ($p<0.05$) Finally, Panel C represents mean (±SEM) BDNF mRNA in the BLA of NS, ES and IES groups. The ANOVA revealed a significant effect of group on expression [$F(2, 35) = 9.47, p<0.001$]. In this region, the pattern of results were different: multiple comparisons indicated that the IES group had significantly higher expression when compared to both AC and NS groups ($p<0.05$). It was also explored whether these effects were influenced by amount of shock exposure (HS & LS sub-groups), but no significant effects were found (data not shown).

5. Discussion

Inescapable stress plays a role in the development of LH (Abramson et al., 1978), a behavioural component of depression (Beck et al., 1988; Grote et al., 2007). As well, abnormalities of brain and plasma BDNF are present in depressed patients (Matrisciano et al., 2009; Molendijk et al., 2014 and Sen et al., 2008). Hence, this study investigated whether BDNF is altered in regions of the hippocampus (DG+CA1), NAcc and BLA and plasma of male Sprague-Dawley rats trained in a LH procedure induced by exposure to foot-shock stress. Further, this study investigated the role of anxiety and motor functions on LH testing. Finally, individual differences in LH were studied to explore two related aspects of stress: amount (i.e., number of foot-shocks) and escapability (i.e., yoked LH procedure). The LH procedure in Experiment 1 involved training inescapable (IES) and escapable (ES) foot-shock groups. One week later, the groups were tested on an avoidance task whereby shocks could be avoided or escaped. On this test, it was found that the IES group displayed a transient impairment in comparison to the ES group. Group differences in anxiety-like behaviour could not explain the observed escape deficit. Furthermore, subjects in each group were sub-
divided into HS and LS sub-groups by a median split, and it was found that number of foot-shocks received during initial escape training contributed to group differences on the avoidance test. In comparison to a control group of animals never exposed to foot-shock stress (NS), BDNF mRNA was lower in the DG+CA1 and higher in the NAcc, in subjects exposed to shock (ES and IES), regardless of number of shocks received, or whether they could escape. In the BLA however, BDNF mRNA was elevated only in subjects that could not escape the foot-shocks (IES group). Plasma BDNF was not affected by any manipulation. Taken together, these data in rats suggest that stress inescapability and stress intensity interact to influence LH, and that BDNF alterations in limbic regions are sensitive to different aspects of stress induced LH.

In Experiment 1, rats were first trained to cross between compartments to escape foot-shocks delivered every 22-38 seconds (ES group) on 4 consecutive days. Another group of animals were yoked to the ES group: that is, they could also cross between compartments, but their behavior had no consequences on the termination of foot-shocks. Hence, although this group could not escape (IES group), it received the same number, pattern and duration of foot-shock that was received by the ES group. A week later, when both groups were tested on avoidance learning (animals could avoid shocks by crossing during a warning stimulus, or escape shocks during delivery), it was found that the IES group displayed escape impairments, but only on days 1 and 2 of testing (See Figure 4A). Although this basic result supports the idea that inescapable stress contributes to LH (Overmier & Seligman, 1967), to our knowledge, this is the first study to examine delayed effects of inescapable and escapable shocks over multiple and consecutive avoidance training sessions. While current results corroborate past LH
experiments that reported LH effects on a single test session (Yang et al., 2015; Shirayama et al., 2015; Overmier & Seligman, 1967; Altenor et al., 1977; Anisman et al., 1980; Cotton et al., 1982; Telner & Singhal, 1980), our results revealed that some of these effects can be transient and dissipate with additional training. Therefore, the “learned helplessness” effect in rodents may, in some cases, not be a stable learning impairment, but rather a temporary “retardation of acquisition” (Rescorla, 1969) that is observed when inhibitory conditioning precedes excitatory conditioning.

Two potential behavioural correlates of LH were also explored: anxiety-like behaviour and motor functions. Before (Pre-Escape) and after escape training (Post-Escape) IES, ES and NS groups underwent a light-dark emergence test (test of anxiety-like behaviour) or locomotion test. In the Pre-Escape test of anxiety, no group differences were observed, but Post-Escape the IES group displayed increased anxiety (i.e., avoidance for the lit compartment). This is consistent with the observation of others (Maier, 1990; Minor et al., 1994; Christianson et al., 2009) and suggests anxiety is elevated soon after exposure to inescapable, but not escapable shock. A week later subjects that underwent anxiety testing were placed in a third, identical test (Delayed). Interestingly, even this effect dissipated with time (See Table 1) and therefore, anxiety may not account for slowed acquisition in the IES group in Experiment 1. On locomotion, no group differences were found at any time point, suggesting that differences in anxiety were not due to motor deficits.

Corroborating past research (Yang et al., 2015; Minor et al., 1994), there were individual differences in escape responding, with some subjects readily learning the escape response, and others displaying a flat learning curve. These differences lead
some subjects in the ES group, and consequently the yoked IES group, to be exposed to high shock amounts, and others lower amounts. Hence, although the primary objective of this study was not to investigate individual differences, differences allowed a relative weighing of stress escapability and stress amount on subsequent behaviour. HS and LS sub-groups were created by a median split on escape training day 4 total shock exposure. This revealed group differences: HS sub-groups performed worse than LS sub-groups, and of HS groups, only the escapable group acquired the appropriate avoidance response (See Figure 3B and 3C). On the escape component, shock amount and inescapability also had summative, deleterious effects on responding (See Figure 3B and 3C). While within these sub-groups differences in LH behaviour was observed, no differences in anxiety-like behaviour or motor functions were present before or after escape training.

In the original LH experiment, amount of inescapable shock was varied, and it was found that HS groups exhibited less escapes than the LS groups (Overmier & Seligman, 1967). The results are consistent with these findings, but also suggest that high amounts of escapable shock can result in responding comparable to low amounts of inescapable shock exposure. In the current study, subjects that were exposed to high amounts of escapable shock received high shock intensity during training and subsequently, high intensity during testing. Indeed, maximal LH effects have been observed when training and testing intensities are both high (Rosellini & Seligman, 1978). Perhaps then, since subjects had very low escapes, they are experiencing a form of shock inescapability, which then in combination with the high shock intensities, leads to the observed LH. In sum, shock intensity and shock amount may play a large
role, interacting with shock inescapability to influence LH.

The analyses of individual differences and identification of HS and LS sub-groups was particularly useful in interpreting BDNF mRNA data. While BDNF mRNA was differentially expressed in limbic regions of rodents exposed to inescapable, escapable and no shock, differences were not found between HS and LS sub-groups. That is, in the DG+CA1, BDNF mRNA expression was lower in both the ES and IES groups, when compared to the NS group. However, in the NAcc, BDNF mRNA expression was higher in ES and IES groups, when compared to the NS group. No differences were found between the ES and IES groups in either of these regions. Interestingly, despite clear training differences, these results were duplicated in Experiment 1 (See Figure 5A and 5B) and Experiment 2 (See Figure 6A and 6B). Last, BDNF mRNA in the BLA was higher in the IES group when compared to the ES and NS groups (See Figure 6C). All mRNA data should be interpreted with caution because they reflect mRNA levels at a single time point and observed behaviour cannot be directly related to expression.

BDNF mRNA in the DG+CA1 and NAcc appears sensitive to stress exposure, but not stress escapability or amount. It has been previously shown that low BDNF in the DG is associated with depressive-like symptoms (Yang et al., 2015) and LH subjects have high BDNF expression in the NAcc (Yang et al., 2015). While the overall pattern of results corroborates past findings, analyses were unable to detect differences between ES and IES groups. It is possible that differences would have been detected if BDNF protein and phosphorylated TrkB levels (high affinity receptor for BDNF) were quantified, or if mRNA was quantified immediately after escape training (Shirayama et al., 2015; Alboni et al., 2010; Yang et al., 2015; Hellweg et al., 2006).
BDNF mRNA in the BLA could be a biomarker of LH, as expression was altered only after inescapable stress. Although BDNF in the BLA has not been extensively studied in the context of LH, serotonin neurotransmission has received attention (Maier & Watkins, 1998; Maier & Watkins, 2005). That is, inescapable stress results in the inhibition of ventral-medial prefrontal cortex glutamatergic output to GABAergic interneurons in the dorsal raphe nucleus and therefore, an acute serotonergic response and synaptic release into the mesocorticolimbic pathway (i.e., into the amygdala, NAcc and hippocampus) (Amat et al., 2005; Amat et al., 2008; Pryce et al., 2011). Fittingly, BDNF and serotonin interact: the upregulation of synaptic serotonin increases levels of BDNF, and BDNF has demonstrated a role in modulating serotonergic transmission and function (See Homberg et al., 2014 for review). So, it is possible that the high BDNF expression could be a result or cause of serotonergic release into mesocorticolimbic regions. Finally, it is also possible that different forms of BDNF protein (i.e., pro and mature BDNF) are differentially altered in the mesocorticolimbic pathway under conditions of escapable and inescapable shock (Shirayama et al., 2015), hence the observed pattern of results in the DG+CA1, NAcc and BLA. Broadly, mature BDNF binding to TrkB promotes neuronal survival and plasticity, whereas, pro-BDNF interacts with the p75 receptor to increase apoptosis and eliminate synapses (Buduni et al., 2016). Therefore, this possibility would be important to investigate further because the functional effects are dependent on the receptor (i.e., TrkB or p75), form of protein (i.e, pre or mature) and the relative ratio of both receptors in limbic regions.

No group differences in plasma BDNF were detected. However, the predominant view is that serum BDNF levels are decreased in depressed individuals (Molendijk et
al., 2014 and Sen et al., 2008). It is possible that no differences were detected here because plasma BDNF may be a biomarker of depressive symptoms, that exclude LH. Also, serum BDNF levels are reported to be higher than plasma (Radka et al., 1996), coinciding with the finding that BDNF is released from platelets during clotting (Fujimura et al., 2002). So, differences may have been detected if serum BDNF was quantified because what may be altered in depression is not the concentration of BDNF in blood, but the ability for the platelets to release BDNF. Further, although BDNF is unable to efficiently cross the blood-brain barrier (Björkholm & Monteggia, 2016), a debate remains as to whether serum levels are associated with brain levels (Karege et al., 2002; Luo et al., 2010). Notably however, with differential region-specific expression in the brain of rodents displaying depressive-like symptoms (Shirayama et al., 2015; Yang et al., 2015), it is not surprising that blood levels do not appear to associate with LH, as they may be representative of overall circulating protein levels.

Due to the prevalence of depression and low treatment response rates, identifying biomarkers is critical. This study investigated whether BDNF, an implicated biomarker of depression, is altered in the brain and plasma of rodents trained in LH. Results suggest stress inescapability transiently contributes to LH and may interact with stress amount to influence LH. Plasma BDNF may not be a feasible biomarker of LH, and limbic BDNF seems to have a heterogeneous and region-specific role.
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Figure Legend

Figure 1


Figure 2

Panel A: Mean (±SEM) total shock exposure across the four days of escape training in the ES group. The # indicates a significant difference between days 1 and 4, p<0.05.

Panel B: Mean (±SEM) total shock exposure across four days of escape training in ES HS and ES LS sub-groups. The # indicates a significant difference between days 1 and 4, p<0.05. The *** indicates a group difference between ES HS and ES LS sub-groups, p<0.001.

Figure 3

Panel A: Mean (±SEM) percent shocks avoided across the six days of avoidance training in the ES and IES groups. The # indicates a significant difference between days 3 and 6, p<0.05. Panel B: Mean (±SEM) percent shocks avoided across 6 days of avoidance training in ES LS and ES HS sub-groups. The * indicates a significant difference between ES HS and ES LS sub-groups on day 1 of training, p<0.05. Panel C: Mean (±SEM) percent shocks avoided across 6 days of avoidance training in IES LS and IES HS sub-groups. The * indicates a significant difference between IES LS and IES HS sub-groups on day 6 of training, p<0.05.
**Figure 4**

**Panel A:** Mean (±SEM) total shock exposure across six days of avoidance training in ES and IES groups. The ^ indicates a significant difference between ES and IES groups on days 1 and 2 of training, *p*<0.05. The # indicates a significant difference between day 1 and day 6, *p*<0.05. **Panel B:** Mean (±SEM) total shock exposure across the six days of avoidance training in ES LS, and ES HS sub-groups. The ** indicates a group difference between HS and LS groups, *p*<0.01. The # indicates a significant difference between ES HS day 1 and day 6, *p*<0.05. **Panel C:** Mean (±SEM) total shock exposure across the six days of avoidance training in ES LS, and ES HS sub-groups. The ** indicates a group difference between HS and LS groups, *p*<0.01. The # indicates a significant difference between day 1 and day 6 in IES LS and IES HS sub-groups, *p*<0.05.

**Figure 5**

**Panel A:** Mean (±SEM) BDNF mRNA levels in the hippocampus (DG+CA1) in NS, IES and ES groups. The * indicates a significant difference between the ES and NS group and the IES and NS group, *p*<0.05. **Panel B:** Mean (±SEM) BDNF mRNA in the NAcc of NS, ES and IES groups. The * indicates a significant difference between the ES and NS group and the IES and NS group, *p*<0.05.

**Figure 6**

**Panel A:** Mean (±SEM) BDNF mRNA levels in the hippocampus (DG+CA1) in NS, IES and ES groups. The * indicates a significant difference between ES and NS groups and the IES and NS group, *p*<0.05. **Panel B:** Mean (±SEM) BDNF mRNA in the NAcc of NS,
ES and IES groups. The * indicates a significant difference between ES and NS group, and the IES and NS group, $p<0.05$. **Panel C:** Mean (±SEM) BDNF mRNA in the amygdala of NS, ES and IES groups. The * indicates a significant difference between IES and NS group, and the IES and ES group, $p<0.05$. 
Table Legend

Table 1. Mean (±SEM) total distance moved and mean (±SEM) total time in lit area of the light-dark box in NS, ES and IES groups. The * indicates a significant difference between IES Pre-Escape and Post-Escape training, \( p<0.05 \). The ^ indicates a significant difference between IES and all other groups Post-Escape, \( p<0.05 \). The # indicates a significant difference between IES Post-Escape and Delayed, \( p<0.05 \).
Figure 1

A

**ESCAPE TASK**
100 trials, 30 sec 0.8 mA foot-shock
4 TRAINING DAYS

**AVOIDANCE TASK**
30 trials, 30 sec 0.8 mA foot-shock
6 TRAINING DAYS

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B

**PRE-ESCAPE**
5 minute test of anxiety

**ESCAPE TASK**
100 trials, 30 sec 0.8 mA foot-shock
4 TRAINING DAYS

**POST-ESCAPE**
5 minute test of anxiety
7 DAYS

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C

**PRE-ESCAPE**
2 hour locomotion test

**ESCAPE TASK**
100 trials, 30 sec 0.8 mA foot-shock
4 TRAINING DAYS

**POST-ESCAPE**
2 hour locomotion test
Figure 2

(A) Escapable Shock

(B) Escapable Low Shock

Escapable High Shock

Mean (SEM) Total Shock Exposure (min)

Training Day

***

#
Figure 3

(A) Escapable Shock vs. Inescapable Shock

(B) Escapable Low Shock vs. Escapable High Shock

(C) Inescapable Low Shock vs. Inescapable High Shock

Mean (SEM) % Shocks Avoided vs. Training Day
Figure 4

(A) Escapable Shock vs. Inescapable Shock

(B) Escapable Low Shock vs. Escapable High Shock

(C) Inescapable Low Shock vs. Inescapable High Shock

Mean (SEM) Total Shock Exposure (min)

Training Day
Figure 5

(A) Mean (SEM) BDNF mRNA in the Dentate Gyrus+CA1 region across different conditions: NS, ES, IES.

(B) Mean (SEM) BDNF mRNA in the Nucleus Accumbens Core region across different conditions: NS, ES, IES.

* Indicates statistically significant differences.
Figure 6

**A**

Mean (SEM) BDNF mRNA
Dentate Gyrus+CA1

<table>
<thead>
<tr>
<th></th>
<th>NS</th>
<th>ES</th>
<th>IES</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>2.0</td>
<td>1.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**B**

Mean (SEM) BDNF mRNA
Nucleus Accumbens Core

<table>
<thead>
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<th>IES</th>
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<tbody>
<tr>
<td>NS</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**C**

Mean (SEM) BDNF mRNA
Basolateral Amygdala

<table>
<thead>
<tr>
<th></th>
<th>NS</th>
<th>ES</th>
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<tbody>
<tr>
<td>NS</td>
<td>1.0</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Table 1. Mean (±SEM) total distance moved Pre-Escape and Post-Escape training and Mean (±SEM) Time in Lit Area Pre-Escape, Post-Escape and Delayed in NS, ES and IES Groups.

<table>
<thead>
<tr>
<th></th>
<th>Pre-Escape</th>
<th></th>
<th>Post-Escape</th>
<th></th>
<th>Delayed</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>NS</td>
<td>ES</td>
<td>IES</td>
<td>NS</td>
<td>ES</td>
<td>IES</td>
</tr>
<tr>
<td>Total distance moved</td>
<td>13831.47</td>
<td>13774.56</td>
<td>13442.23</td>
<td>13882.78</td>
<td>11434.49</td>
<td>12130.54</td>
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<tr>
<td></td>
<td>±1116.44</td>
<td>±1083.4</td>
<td>±904.53</td>
<td>±1047.22</td>
<td>±855.92</td>
<td>±1653.90</td>
</tr>
<tr>
<td>Total time in lit area</td>
<td>71.14</td>
<td>94.36</td>
<td>96.02</td>
<td>104.43</td>
<td>88.233</td>
<td>26.20</td>
</tr>
<tr>
<td></td>
<td>±13.68</td>
<td>±12.56</td>
<td>±13.91</td>
<td>±21.52</td>
<td>±28.35</td>
<td>±13.40**</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

*^ denotes a significant difference from the corresponding NS group (p < 0.05). 
** denotes a significant difference from the corresponding ES group (p < 0.05). 
# denotes a significant difference from the corresponding IES group (p < 0.05).
General Discussion

Summary
Due to the high prevalence of depression and low treatment response rates, identifying biomarkers is critical. This study investigated whether brained-derived neurotrophic factor (BDNF), a putative biomarker of depression, is altered in the brain and plasma of rodents trained in a learned helplessness (LH) procedure. LH was explored because it is a critical feature of human depression that can be studied in rodents. This is the first known document to report the following differences in BDNF mRNA: 1) low expression in the hippocampus (DG+CA1) after stress exposure; 2) high expression in the nucleus accumbens core (NAcc) after stress exposure and 3) high expression in the baso-lateral amygdala (BLA) of subjects exposed to inescapable stress, but not escapable stress. Further stress inescapability transiently contributed to LH and anxiety-like symptoms. Stress amount however, only contributed to LH and not anxiety-like symptoms. In sum, stress amount may interact with stress inescapability to influence LH, plasma BDNF may not be a useful biomarker of LH, and limbic BDNF seems to have a heterogeneous and region-specific role.

Limitations to Address
In the current study, there are several limitations to address. First, these experiments were conducted in only male rats. Sex differences in depressive symptomology (Diehl, 2018; Bromet et al., 2011; Cyranowski et al., 2000; Hyde, Mezulis & Abramson, 2008) and BDNF expression (Wei, Wang & Xu, 2017) is observed. Therefore, results from the current study may have been different in male and female rats. Additionally, although emphasis was intentionally placed on the symptom of LH, it
would have been interesting to look at other depressive-like symptoms to achieve a full symptom profile in the rodents (i.e., a battery of behavioural tests). That is, additional behavioural tests could be implemented after exposure to inescapable stress to look at other domains affected by depression such as: sleep, food consumption, anhedonia (sucrose preference), social behaviour and despair (forced swim test). This would reveal if inescapable stress can lead to not only helplessness, but other depressive-like symptoms (i.e., generalized helplessness).

Another limitation to address is the quantification of BDNF. Specifically, BDNF mRNA was quantified and not BDNF protein or TrkB phosphorylation. While mRNA is telling and interesting to examine, if protein levels were quantified it is possible that differences would have been detected between shock groups and a better understanding of functional protein levels would be obtained (Shirayama et al., 2015; Alboni et al., 2010; Yang et al., 2015; Hellweg et al., 2006). Further, as mentioned plasma BDNF was measured, rather than serum BDNF. Serum BDNF levels are higher than plasma (Radka, Holst, Fritsche, & Altar, 1996) and as a result, serum is more commonly measured in clinical and preclinical research. Therefore, measuring serum may have been more in line with research to date and may have provided more biomarker insight.

The final limitation to address is that although individual differences were apparent, the cause of the differences is not known and could have been explored more thoroughly. This limitation requires some elaboration. HS and LS groups were chosen by conducting a median split on escape day 4 total shock exposure but interestingly, individual differences in responding were present on day 1 of training. Current results
suggest that these initial differences are unlikely caused by motor impairments or anxiety-like behaviour, as there were no differences in these measures Pre-Escape. However, there are other conceivable reasons for the differences. First, it is possible that subjects varied in pain sensitivity or some could have developed stress induced analgesia. Upon exposure to painful situations, an adaptive decrease in pain sensitivity is seen through activation of endogenous opioid systems (termed stress induced analgesia; Amit & Galina, 1986; Akil, Madden, Patrick & Barchas, 1976; Maier, 1986). Also, subjects exposed to escapable shock (Maier, Drugan & Grau, 1982; Drugan, Ader & Maier, 1985) have developed (and not developed, Maier, 1986) stress induced analgesia after both high and low amounts of shock exposure (Drugan, Ader & Maier, 1985). So, it is possible that some ES subjects had adopted this mechanism, decreasing their escape responding. Importantly after blocking stress induced analgesic effects, LH effects are still observed (Maier, 1986). Therefore, it is possible that initial differences in escape responding are caused by stress induced analgesia, whereas LH is not. Second, since a direct measure of learning ability was not measured, individual differences could be caused by differences in task learning. Third, although anxiety-like behaviour in the light-dark emergence test and locomotion was examined, individual differences could have been caused by freezing behaviours that were not directly measured (Bannerman, Grubb, Deacon, Yee, Feldon & Rawlins, 2003; Conti, Maciver, Fekany & Abreu, 1990). Finally, motivation and emotionality have been shown to influence avoidance-escape responding (Kaplan, Kaplan & Walker, 1960). Therefore, this in combination with other factors could be underlying observed individual differences and could have been investigated more thoroughly.
Future Directions

The current research raises questions to be addressed by future research. For example, can the observed LH effects be alleviated with pharmacological intervention? Future studies could address this by administering drug treatment within the 7-day time interval between escape and avoidance training. This would reveal if this particular LH procedure is sensitive to treatment. Further, are pre-shock levels of plasma or serum BDNF predictive of individual differences in LH and/or treatment response? Future experiments could measure levels of plasma or serum BDNF both pre- and post-shock, as well as pre- and post-treatment. This would permit an investigation into the predictive validity of pre-treatment BDNF levels in not only stress induced LH, but treatment response as well. Finally, future studies could investigate what happens to treatment response in LH, with the modification of BDNF-TrkB in limbic regions. Specifically, future experiments should take into consideration what is known about the DG and NAcc, while placing more emphasis on BDNF and TrkB signalling in the BLA, as this was the region that seemed most sensitive to LH effects. This would help solidify and expand on the current understanding of the region-specific role of BDNF-TrkB in depressive-like symptoms and treatment response.

This research has implications for not only pre-clinical research modelling depressive symptoms, but also human depression. Specifically, this study elucidates the deleterious and summative effects that stress inescapability and stress amount can have on the presentation of LH. Therefore, clinicians may want to take this into consideration if they have depressed patients presenting with LH symptoms. It would be essential to teach these depressed patients an internal locus of control, so that they feel
they have control over stressful events in their lives. This could decrease the level of helplessness patients feel and essentially reduce this component of depression. Further, the individual differences in escape responding speak to the importance of investigating vulnerability and resiliencies in stress-related disorders. Finally, while plasma BDNF as a biomarker of LH may not be valid, the importance of BDNF in LH was revealed in the BLA. Therefore, post-mortem studies should investigate the possible role of BDNF in this region and the possible interaction with serotonin further.
References


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