Treating Depression with Ultrasound: An Exploratory Study

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Abstract

Depression is a common, recurrent and frequently chronic mental health disorder that has already become one of the leading health-care burdens. Despite the wide clinical application of antidepressants, a significant portion of patients does not benefit sufficiently from the standard medication therapies. Therefore, developing alternative safe and effective treatment options is required. Over the years, studies have shown that transcranial ultrasound has the ability to noninvasively and remotely modulate neurons and neural network activity. However, the prospect of using ultrasound as a treatment for depression still needs to be elucidated.

The current study investigated the potential application of low-intensity pulsed ultrasound (LIPUS), a specific type of ultrasound, as a therapeutic option for depression. It was found that LIPUS stimulation significantly increased the viability of both neuron-like SH-SY5Y cells and primary glia cells *in vitro*. Further protein analysis revealed that LIPUS promoted the phosphorylation of β-catenin in primary glia cells and increased the level of brain-derived neurotrophic factor (BDNF) in both cell types. Animal studies using the repetitive restraint stress (RRS) model showed that LIPUS administration significantly alleviated the depression-like behaviors of mice in the sucrose preference test (SPT), tail suspension test (TST), forced swimming test (FST) and Y-maze test (YMT). Further testing indicated potential mechanisms for the beneficial effects of LIPUS on depression are associated with the promotion of neurogenesis and elevation of BDNF levels, which were consistent with the results of the *in vitro* study. The cuprizone (CPZ) induced demyelination animal model was used to determine the role of myelin and oligodendrocytes (OLs) both in pathogenesis and as therapeutic targets. It was found that LIPUS did not significantly attenuate the depressive-like behaviors in the TST, FST, and YMT in

mice exposed to CPZ, although additional testing revealed that LIPUS alleviated CPZ-induced damage to both mature myelin and oligodendrocyte progenitor cells (OPCs).

Taken together, the findings from the *in vitro* and *in vivo* studies indicate that LIPUS has potential as a promising therapeutic option for depression, not only by promoting neurogenesis and elevating BDNF levels, but also through protection and promotion of myelin and OLs. In addition to offering a new possible treatment for depression, this study also highlights the importance of neurogenesis and neurotrophy and the role of myelin and OLs in the pathogenesis of depression.

Preface

This research was mainly conducted in collaboration with Dr. Xin-Min Li in Department of Psychiatry and Dr. Jie Chen in Department of Electrical and Computer Engineering at the University of Alberta. Dr. Jie Chen's lab provided the low-intensity pulsed ultrasound (LIPUS) device and technical support for the whole project, and I conducted my work in Dr. Xin-Min Li's lab. This included animal model induction, behavioral tests, euthanasia and brain tissue collection, tissue processing, western blotting, immunohistochemistry staining, data collection and subsequent analysis. All the animals were housed in the Health Sciences Laboratory Animal Services (HSLAS) facility at the University of Alberta. All animal studies were performed in accordance with the guidelines set by the Canadian Council on Animal Care and under ethical approval of the University of Alberta Animal Care and Use Committee in AUP #1101, protocol name "Ultrasound Treatment of Depression", approved on June 11, 2014. For *in vitro* studies, cell samples were provided courtesy of Dr. Ian Winship in Department of Psychiatry at the University of Alberta, and I conducted the subsequent tests in Dr. Xin-Min Li's lab and collected the results. Writing and preparation of the thesis were conducted on my own accord, with edits suggested by the examining committee.

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Abbreviations

LIPUS - Low-Intensity Pulsed Ultrasound

mW - Milliwatt

BDNF - Brain-Derived Neurotrophic Factor

pβ-catenin - Phosphorylated β-catenin

RRS - Repetitive Restraint Stress

SPT - Sucrose Preference Test

TST - Tail Suspension Test

FST - Forced Swimming Test

YMT - Y-Maze Test

DCX - Doublecortin

CPZ - Cuprizone

OL - Oligodendrocyte

MBP - Myelin Basic Protein

NG-2 - Neural/Glial Antigen 2

OPC - Oligodendrocyte Progenitor Cell

1. Introduction

1.1 Depression

1.1.1 Introduction to Depression

Depression, officially termed major depressive disorder (MDD), is a state of low mood and aversion to activity that can affect a person's thoughts, feelings and behavior. The core symptoms of depression include persistent negative mood, anhedonia, a lack of energy, abnormalities in appetite and sleep and fatigue, combined with cognitive deficiencies including difficulties in concentration, feelings of worthlessness and suicidal thoughts, all of which can cause significant distress and functional impairment (American Psychiatric Association, 2013). Depression is a common, recurrent and frequently chronic disorder, which has been identified as the leading source of disease burden globally, and especially in North America (World Health Organization, 2010). Data from the Mood Disorders Society of Canada showed that the lifetime prevalence of depression in Canadians is 8.2%, and that 4.7% of Canadians are experiencing a major depressive episode at any point in time (Mood Disorders Society of Canada, 2009). Depression is associated with a high mortality rate, with 10-15% of depressed individuals committing suicide (Wulsin et al., 1999). The estimated annual cost attributed to depression in Canada is around 20 billion dollars (Mood Disorders Society of Canada, 2009), placing a substantial strain on patients, their families, and society.

1.1.2 Treatment for Depression

Current treatments for MDD involve pharmacological and psychological interventions, as well as emerging neurostimulation interventions. Since the discovery of the first antidepressant in the 1950s (Freis, 1954), a variety of antidepressants have been developed and used widely in clinical settings. Among these antidepressants, the selective serotonin reuptake inhibitors (SSRIs), together with serotonin and norepinephrine (NE) reuptake inhibitors (SNRIs), both of which were launched during the 1980s and 1990s, are considered as the most effective medications for psychiatric disorders and remain first-line treatment recommendations for depression (Lam et al., 2009; Mann, 2005). However, conventional wisdom suggests that the therapeutic effects of antidepressant agents often take two to three weeks or longer to become evident (Taylor et al., 2006). Moreover, despite the extensive clinical application of antidepressants, a significant proportion of patients (20%–40%) do not benefit sufficiently from the standard medication therapies (Aguirre et al., 2011; Trivedi et al., 2006). In addition, about 20% of patients are refractory to any antidepressant medication (Rush et al., 2006). Therefore additional safe and efficient treatment options have been developed, particularly for patients with antidepressant-resistant depression (Rachid & Bertschy, 2006).

The most commonly used psychological intervention for depression is cognitive therapy, more specifically, the cognitive behavior therapy (CBT) (Cuijpers et al., 2013; Hundt et al., 2013). CBT is a form of psychotherapy based on the principle that emotional distress and maladaptive

behaviors in depression are induced by dysfunctional patterns of cognition (Beck & Haigh, 2014). Therefore CBT focuses on changing unhelpful thinking and behaviors based upon a combination of basic behavioral and cognitive principles (Amick et al., 2015; Coull & Morris, 2011). The goal of CBT is to help patients identify, evaluate, challenge and modify their dysfunctional beliefs and alter the maladaptive behaviors for better self-being (Cuijpers et al., 2008). The efficacy of CBT for treating (Cuijpers et al., 2013; Ma et al., 2014) and preventing (van Zoonen et al., 2014) mild to moderate depression is well-established, while the relative effectiveness of CBT, especially in patients with moderate to severe depression, is still a controversial issue (Siddique et al., 2012; Thase et al., 2014).

Over the past two decades, neurostimulation treatment has gained attention as a promising therapeutic intervention for treating depression. The Food and Drug Administration (FDA) approved neurostimulation, including electroconvulsive therapy (ECT) (Abrams, 1991; Weiner et al., 2013), repetitive transcranial magnetic stimulation (rTMS) (George et al., 2013), and vagus nerve stimulation (VNS) (Cristancho et al., 2011) for the treatment of depression. Investigations into deep brain stimulation (DBS) are still on going (Wani et al., 2013).

ECT is usually used in cases of antidepressant-refractory depression. It induces a seizure in the patient monitored by electroencephalogram (EEG) and is widely considered to be the most effective antidepressant treatment (Kellner et al., 2006; Matthews et al., 2013). In fact, it is considered as first-line for MDD with severe suicidal ideation or severe depression with psychotic

features due to its fast onset of action and repeatedly proven efficacy. In almost all cases it is utilized after failure of at least one, and usually more antidepressants; a meta-analysis showed that the overall remission rate after ECT was 48.0% for patients with prior failure of pharmacotherapy and 64.9% for patients without the failure (Heijnen et al., 2010). The requirement for general anesthesia in ECT and the administration of a muscle-paralyzing agent can prevent an overt seizure, making it a relatively safe procedure. Memory impairment after ECT procedure is a major drawback (Squire, 1977), as well as the stigma associated with its use.

rTMS is FDA approved for depression in patients who have failed to respond to only one antidepressant trial. In the rTMS procedure, a magnetic field is produced around the brain, and then electrical currents will be generated and delivered through the cortex. It is currently the least invasive of the brain stimulation techniques and most frequently targets the dorsolateral prefrontal cortex (DLPFC) (Pascual-Leone et al., 1996). Several studies have demonstrated that rTMS is as effective as both an augmenting agent and monotherapy for the treatment of major depression (Berlim et al., 2014; Fitzgerald et al., 2006). A recent meta-analysis showed that 38.2% and 15.1% of subjects receiving active rTMS and sham rTMS were responders, respectively, whereas 34.6% and 9.7% of subjects were classified as remitters (Berlim et al., 2013). The problem regarding rTMS is the lack of standardization of parameters with regard to frequencies, durations, number of pulses applied per treatment session, and efficacy (Kedzior & Reitz, 2014). Clearer guidelines are necessary for this reason.

DBS involves implanting electrodes into the deep brain structure bilaterally and then delivering an electrical pulse that alters the activity of targeted circuits. Investigations on the efficacy for this procedure as the treatment for depression are still ongoing. A study including 7 patients with refractory depression had DBS delivered to the brain; 6 of the patients achieved response criteria seven days after stimulation with all 6 still continuing to meet response at 6 months and 4 of them meeting remission (Bewernick et al., 2010).

VNS was first performed in the 1980s and then approved by FDA for the treatment of medication-resistant epilepsy (Schachter, 2002). Epilepsy patients treated with VNS were noted to exhibit improvements in their mood. It was later approved by FDA in 2005 for the treatment of depression in patients who failed more than two antidepressant treatment regimens (Cristancho et al., 2011). In a VNS procedure, an electrode is surgically attached to the left vagus nerve and a generator that delivers the electrical pulse is later implanted into the chest wall (Rush et al., 2000). A meta-analysis showed a significant reduction in depression symptoms, with 31.8% of subjects becoming responders after a VNS procedure (Martin & Martin-Sanchez, 2012).

Both DBS and VNS are invasive procedures, with risk factors including surgical infection, anesthesia risks, and postoperative complications (Voges et al., 2007). Part of the criticism of the DBS and VNS data is lack of randomized controlled trials (RCTs). In an effort to develop an efficacious treatment with little to no unwanted side effects, the search for other therapeutic

options must be widened. And understanding the pathogenesis of depression will certainly facilitate this approach.

1.1.3 Pathogenesis of Depression

Monoamine Hypothesis of Depression

The first antidepressant was unintentionally discovered in the 1950's through the observation that the symptoms of depression were improved by agents that acted through various mechanisms to increase the synaptic concentrations of monoamines (Delgado, 2000; Hirschfeld, 2000). This finding led to the adoption of the monoamine hypothesis as the accepted explanation for the pathogenesis of depression. The monoamine hypothesis asserts that depression is triggered by the underactivity of monoamine neurotransmitters in the brain, which include serotonin (5-HT), norepinephrine (NE), and dopamine (DA) (Ruhe et al., 2007). The hypothesis received considerable support over the last few decades since it provides a pathophysiological explanation for the actions of antidepressants (Tatsumi et al., 1997). Most antidepressants currently available have been discovered and developed based on this hypothesis, including, but not limited to tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), and norepinephrine–dopamine reuptake inhibitors (NDRIs) (Stahl et al., 2013). Despite the support the monoamine hypothesis has received, it still does not provide a complete explanation for the pathogenesis of depression (Hindmarch, 2002) and the mechanisms of action of antidepressants

(Li et al., 2006; Sawynok et al., 2001). Additionally, genetic studies have shown that monoamine depletion alone does not produce depressive symptoms in healthy individuals (Krishnan & Nestler, 2010; Salomon et al., 1997). These findings indicate that there must be other pathological changes underlying the development of depression.

Neurogenesis Hypothesis of Depression

Numerous studies have suggested hippocampal degeneration as the principal source of pathogenesis in depression (Eisch & Petrik, 2012; Hanson et al., 2011; Kempermann & Kronenberg, 2003). Hippocampal neurogenesis, the production of new neurons in the hippocampus, may play a role in the ability of antidepressants to bring about a remission of depressive symptoms (Boldrini et al., 2012; Boldrini et al., 2009; Malberg et al., 2000; Marcussen et al., 2008; Surget et al., 2008). External stress, the major psychological risk factor for developing depression, has been reported to induce impairment of hippocampal neurogenesis in many aspects, including but not limited to decreasing the proliferation rate of neural progenitor cells (NPCs) (Hitoshi et al., 2007), suppressing the survival of neuroblasts and immature neurons (Gonzalez-Perez et al., 2011), and reducing the growth and development of newly-born neurons (Pham et al., 2003). Animal studies have demonstrated that stressful experiences strongly inhibit adult neurogenesis in the hippocampus (Dranovsky & Hen, 2006; Mirescu & Gould, 2006; Schoenfeld & Gould, 2012), and this deficiency could be rescued by antidepressant treatment (Malberg, 2004; Morais et al., 2014; Pereira et al., 2007). Previous research from our lab has also

found that repetitive restraint stress (RRS) leads to suppressed neurogenesis in rats' hippocampus, as demonstrated by the reduced number of bromodeoxyuridine (BrdU)-positive cells with RRS exposure in the subgranular zone (SGZ) of the dentate gyrus, one of the two major sites of neurogenesis in the adult central nervous system (CNS) (Hagg, 2005; Ming & Song, 2005; Santarelli et al., 2003). This RRS-induced reduction in neurogenesis was reversed by chronic treatment with the antidepressant venlafaxine (Xu et al., 2004).

Neurotrophic Hypothesis of Depression

Studies have shown that brain-derived neurotrophic factor (BDNF), the most robust neurotrophic factor in the brain, also plays an important role in the pathogenesis of depression and in the mechanisms underlying antidepressant action (Duman & Monteggia, 2006; Hurley et al., 2013; Martinowich et al., 2007). Clinical studies have reported that the level of BDNF was increased in patients treated with antidepressants, compared with untreated subjects (Chen et al., 2001; Karege et al., 2005). The serum level of BDNF in depressed patients was found to be significantly lower than in controls and was negatively correlated with depression severity (Karege et al., 2002). Animal studies have shown that administration of antidepressants, as well as repetitive ECT, increased BDNF expression in the brain (Bocchio-Chiavetto et al., 2006; Piccinni et al., 2008; Russo-Neustadt et al., 1999). A previous study from our group also found that RRS significantly decreased the level of BDNF in rats' hippocampus, and this change was rescued by chronic administration of venlafaxine (Xu et al., 2004). BDNF has also been shown to play a role in

regulating neurogenesis in the hippocampus. Studies have found that both direct central infusion of BDNF into the hippocampus and chronic peripheral administration of BDNF can dramatically increase the survival of newborn cells in the dentate gyrus through an unknown, but expected indirect mechanism (Scharfman et al., 2005; Schmidt & Duman, 2010). A study in healthy human populations has found that the hippocampal and parahippocampal volumes of subjects with the met allele of the val66met BDNF gene polymorphism are smaller than those with the more common Val/Val genotype, giving rise to further support for BDNF's importance in maintaining normal hippocampal cell populations (Gatt et al., 2009).

Glucocorticoids and Depression

An extensive literature has demonstrated the role of corticosteroids in depression, in which stress has always been considered as a major risk factor. Despite the fact that stress-induced hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis results in hypersecretion of corticosteroids, it has been shown that corticosteroids influence neurotransmitter tone and that corticosteroid secretion is also regulated by the neurotransmitters implicated in depression (Pariante, 2003; Plotsky et al., 1998). The hippocampus and prefrontal cortex are the two brain regions that exert inhibitory neural control over the HPA axis, therefore reducing excess corticosteroid secretion in response to stress (Diorio et al., 1993; Holsboer, 2001; Jacobson & Sapolsky, 1991). Depending on the intensity and duration of the stress as well as individual resilience, the endocrine response to stress could become dysfunctional if the system loses its

ability to switch the HPA axis off. Due to the impaired endocrine response to stress, hypersecretion of glucocorticoids would continue unabated, which is a hallmark of depression (Martinac et al., 2014; Yu et al., 2008; Zunszain et al., 2011).

White Matter Hypothesis of Depression

There is also considerable evidence to support a close relationship between depression and white matter dysfunction. Studies with depressed patients have found evidence of abnormalities in myelin and oligodendrocytes (OLs), the myelin-forming cells in the CNS (Hamidi et al., 2004; Yamazaki et al., 2007). A lower density of OLs was found in the prefrontal cortex (PFC) of depressed patients (Uranova et al., 2004). A post-mortem study found ischemic demyelination in the PFC of depression patients (Thomas et al., 2003). Dorsolateral PFC and ventromedial PFC displayed less intense myelin staining in depressed patients compared to healthy controls (Regenold et al., 2007). A decrease in myelin basic protein (MBP), a mature component of myelin, was found in multiple brain regions of patients with depression (Regenold et al., 2007). Neuroimaging studies have found subcortical white matter hyper-intensities (WMHI) in patients suffering from depression (Iosifescu et al., 2005) and it seemed to be correlated with more severe illness and poorer treatment outcomes (Heiden et al., 2005). Magnetic resonance imaging (MRI) studies revealed that patients with depression demonstrated apparent reductions in white matter integrity in the PFC and the limbic system (Cotter et al., 2001; Zuo et al., 2012). Fractional anisotropy (FA) was also reported to be lower in the cortical and subcortical regions of patients

with depression (Kieseppa et al., 2010; Zou et al., 2008). Genetic studies also found myelin and OL-associated genes were significantly decreased in their transcripts in depression (Frodl et al., 2008; Hashimoto et al., 2006). Specific genes involved included those that are essential for axon growth, signal transduction and synaptic function (Aston et al., 2005; Novak & Tallerico, 2006). Downregulation of both OL development-related genes and myelin formation-related genes were observed in depressed patients' temporal cortex (Aston et al., 2005). In addition, longitudinal studies revealed that the abnormalities in white matter of depressed patients could be attenuated or even rescued by antidepressant treatment (Zeng et al., 2012; Zhang et al., 2015) as well as neurostimulation therapies (Lyden et al., 2014; Peng et al., 2012).

In addition to research on human populations, an animal study demonstrated that depressive-like symptoms were induced by exposure to a toxin that caused a selective loss of white matter in the PFC of rats (Banasr & Duman, 2008). Previous research from our group found that unpredictable chronic mild stress (UCMS) induced depressive-like behaviors in mice and significant damage to myelin and OLs in terms of decreased levels of MBP and platelet-derived growth factor alpha-receptor (PDGFRα), a protein expressed predominantly by oligodendrocytes progenitor cells (OPCs). These behavioral and pathological changes were attenuated by chronic administration of the antidepressant desvenlafaxine (Wang et al., 2014).

The many potential theories on the pathogenesis of depression and the relatively unsatisfactory response rate to all current available antidepressant treatments clearly suggest that depression is an

etiologically and clinically heterogeneous disorder. There is no clear evidence for one hypothesis being central to the etiology of depression. A variety of emotional, behavioral and cognitive components make up the complex, multifaceted nature of depression. It is even possible that each of these components may involve some different neurobiological substrates (Hasler, 2010; Hindmarch, 2001). This encourages research for therapeutic interventions targeted at different biomarkers rooted in different hypotheses. So far a variety of different animal models have been established to validate the neurobiological underpinnings of depression, and have proven extremely useful concerning the identification and improvement of therapeutic strategies.

1.1.4 Animal Models of Depression

Most animal models used in depression research are based on two principles: actions of known antidepressants and response to stress (Yan et al., 2010). In these animal models, certain symptoms that are inferred to be "depression-like" can be evoked in animals by lesions, pharmaceuticals, genetic approaches, or HPA axis alterations. Currently, animal models of depression can be categorized as aligned with these triggers, including lesion models which involve removal of specific regions of the brain to induce neurological and behavioral changes that are parallel to that observed in depressed patients and can be reversed by chronic antidepressant treatment (Krishnan & Nestler, 2011); pharmacological models involving the administration of specific chemicals to induce behavioral changes and treatment responses partially similar to depression (Nutt, 2006); and genetic alteration models that employ forward and reverse genetics to study candidate genes

that participate in depression to demonstrate depressive or anti-depressive phenotypes (Pogorelov et al., 2005).

For the HPA axis alterations, converging studies have implicated environmental alterations, especially stressful events, in the development of affective disorders (Aldridge et al., 2005; Becker et al., 2008; Henn & Vollmayr, 2005). The HPA axis is a key endocrine adaptor against stressors and therefore plays an essential role in the pathogenesis of stress-related psychiatric disorders, such as depression and anxiety (Gillespie & Nemeroff, 2005; McEwen, 2008). In this way, depression can also be regarded as a stress-related disorder. Many of the animal models of depression are developed based on this theory, in which animals are exposed to various types of acute or chronic external stressors including but not limited to, early life stress (Mirescu et al., 2004), isolation stress (Fone & Porkess, 2008), social defeat stress (Keeney & Hogg, 1999), repetitive restraint stress (Murakami et al., 2005), and unpredictable chronic mild stress (Mineur et al., 2006) to induce depression.

Different studies have shown that repetitive restraint stress (RRS) is a reliable animal model of depression. To induce RRS, mice are restrained in acrylic cylinders with air vents at the nasal end, for 2 to 6 hours every day continuously for around 3 weeks. Mice exposed to RRS showed decreased body weight and food intake, increased immobility in the forced swimming test and decreased sucrose solution preference (Gregus et al., 2005; Hageman et al., 2009; Kim et al., 2006), indicating exacerbated despair- and anhedonia-like behaviors, respectively, which can be

alleviated by treatment with typical antidepressants (Duman et al., 1999; Warner-Schmidt & Duman, 2006). There is evidence that exposure to RRS progressively suppresses the generation of new neurons and their survival in the hippocampus of adult animals (Pham et al., 2003) and increases the activity of antioxidant enzymes directly involved in the neutralization of reactive oxygen species (Budni et al., 2013). Interestingly, these effects are still distinguishable after the completion of the restraint procedure, suggesting that long-term pathological changes in the mood-regulating system might be induced by exposure to RRS (Chiba et al., 2012).

In the CNS, OLs synthesize massive amounts of cellular membrane to form multiple myelin internodes with a particular set of tightly packed lipids and proteins (Aggarwal et al., 2011). Therefore normal functional maintenance of OLs is crucial for myelin repair in the CNS. Cuprizone (CPZ) is a copper chelator that alters the copper content of tissue and interferes with enzyme functions that take copper as a cofactor, which has detrimental effects on the anti-oxidative system and may lead to oxidative damage (Biancotti et al., 2008). OLs are very vulnerable to oxidative damage as evident in some neurological diseases (Dewar et al., 2003; Goldbaum & Richter-Landsberg, 2001). Administration of CPZ to animals is able to induce consistent demyelination in the CNS through specifically damaging the OLs (Ludwin, 1978; Matsushima & Morell, 2001) and has been widely applied as an animal model for multiple sclerosis (MS) (Liebetanz & Merkler, 2006; Praet et al., 2014). In addition, the myelin and OL pathology in the brains exposed to CPZ provide a valuable tool in attempting to understand the

possible pathogenesis of depression. CPZ-exposed mice displayed depressive-like behaviors mimicking certain depression phenotypes (Kondo et al., 2016). In addition, mice exposed to CPZ showed changes reminiscent of those observed in neuroimaging studies of patients with depression, including reduced myelin integrity in cortical areas and the corpus callosum (Chandran et al., 2012; Thiessen et al., 2013). Based on previous studies from our lab, chronic exposure of mice to CPZ in food at a low dose of 0.2% (w/w) daily for 6 continuous weeks caused damage primarily to OLs in the brain, particularly in the PFC and the striatum, in addition to the large white matter bundles in the brain such as corpus callosum (Xu et al., 2010; Xu et al., 2009). Chronic exposure to CPZ results in a period of acute demyelination followed by spontaneous remyelination during subsequent weeks after withdrawal (Lindner et al., 2009). CPZ exposure also leads to behavioral changes including more anxiety- and depression-like behaviors, affected working memory, and impaired social function, all of which can be considered as animal behavioral phenotypes of depression. All these behavioral deficits induced by CPZ exposure can be rescued by treatment with quetiapine (QTP) (Zhang et al., 2008), an antipsychotic also recommended as a frontline treatment option for depressive episodes in bipolar disorder (Calabrese et al., 2005; Suppes et al., 2010), as well as fluoxetine (data not published), and neurostimulation rTMS (manuscript in preparation). Follow-up pathological tests showed that all these therapeutic interventions significantly alleviated the damage to myelin and OLs caused by CPZ administration. This pharmaceutical model provides an opportunity to manipulate the timing

of the therapeutic intervention and creates a suitable platform for testing promising therapeutic applications in the study of depression.

1.2 Low-Intensity Pulsed Ultrasound (LIPUS)

1.2.1 Introduction to Ultrasound

Ultrasound is an oscillating sound pressure wave with a frequency greater than the upper limit of the range of human hearing (20kHz). It has shown great potential in the processing of liquids and slurries and also introduces an obvious cavitation effect on liquid-solid interfaces (Shankar & Pagel, 2011). Therapeutic ultrasound refers to any procedure that uses ultrasound for therapeutic benefits (Baker et al., 2001). There are three primary benefits of ultrasound: speeding up the healing process by increasing blood flow (Baker & Bell, 1991; Morishita et al., 2014), decreasing pain through the reduction of swelling and edema (Balzarini et al., 1993; ElHag et al., 1985), and delivering a gentle massage to the treated area (Draper et al., 2010; Gan et al., 1995). These benefits are mainly achieved through the thermal and cavitational effects of ultrasound (Kennedy et al., 2003). Different studies with therapeutic ultrasound have shown that it can improve local blood perfusion as well as induce angiogenesis (Paliwal & Mitragotri, 2008), increase fibroblast production (Farcic et al., 2013), and impact on osteoblasts (El-Bialy et al., 2004; Ikai et al., 2008). It can also induce an anti-inflammatory effect by regulating the expression of inflammatory factors (Chung et al., 2012; Ying et al., 2012) and protect target tissue from oxidative injury (Freitas et al., 2007). Research has also shown that ultrasound has the ability to promote the metabolic activities

of cells, thus aiding in tissue repair, particularly in soft tissue injuries (Baker et al., 2001). Various past and current studies have been focusing on the potential application of ultrasound in different areas of medical research.

1.2.2 Research on Low-Intensity Pulsed Ultrasound (LIPUS)

Low-intensity pulsed ultrasound (LIPUS) is a specific type of ultrasound with a unique intensity (0-100 mW/cm^2), frequency (1.5 MHz), pulse repetition rate (1.0 KHz), and duty cycle (20%, i.e. 800 microseconds "off" and 200 microseconds "on"). Previous studies have shown that LIPUS can enhance the healing of orthodontically induced tooth-root resorption in humans through the positive stimulatory effects in cellular differentiation and functional activation of bone formation (Leung et al., 2004). LIPUS has also been shown to promote the proliferation of fresh hematopoietic stem/progenitor cells (HSPC), stimulate the viability, proliferation, and differentiation of these progenitor cells (Xu et al., 2012). Daily LIPUS treatment increased the production of human anti-Interleukin-8 monoclonal antibody in Chinese Hamster Ovary (CHO) cells by more than 30%, which can be attributed to both elevated cell count and the increase in cell permeability after LIPUS stimulation (Zhao et al., 2014). A US patent (No. 8292834) entitled "Ultrasound Stimulation Devices and Techniques" with LIPUS was granted to Dr. Jie Chen in the year 2011. The *ad-hoc* research was carried out at the University of Alberta for LIPUS on engineering knee meniscus cartilage, accelerating wound healing, producing cultured skin substitutes, and enhancing enzyme activities (programs in progress, data not shown).

1.3 Present Study: Treating Depression with Ultrasound: An Exploratory Study

Emerging studies focusing on the effects of ultrasound on the CNS have produced promising results. Low-intensity, low frequency ultrasound has been reported to be able to noninvasively and remotely excite neurons and network activity by triggering voltage-gated sodium and calcium channels. These ultrasound-induced changes were sufficient to stimulate exocytosis and synaptic transmission in hippocampal circuits (Tyler et al., 2008). Short-term of transcranial pulsed ultrasound stimulation has been shown to induce a significant increase in the density of BDNF-positive puncta in the hippocampus after 30 minutes stimulation, indicating ultrasound can be employed to excite neuronal activity in the intact mouse hippocampus and to promote endogenous brain plasticity (Tufail et al., 2010). Transcranial focused ultrasound has also been shown to induce a significant increase in the proliferation of newborn cells and later in their differentiation and survival as mature neurons after one-time stimulation (Scarcelli et al., 2014). As the pathogenesis of depression may involve deficits of neurogenesis (McKinnon et al., 2009), loss of neurotrophic support (Otsuki et al., 2008) and alteration of neuroplasticity (Hayley et al., 2005), it can be inferred that therapeutic ultrasound might serve as an alternative strategy to treat depression due to its stimulatory effects on neural systems.

The current study was designed to explore the application of LIPUS as a therapeutic option for depression. We hypothesized that LIPUS could work as a treatment for depression, with neurogenesis and neurotrophy serving as the underlying mechanism for the therapeutic effects of

LIPUS on depression. We also hypothesized that the protection and promotion of myelin and OLs might also be the therapeutic targets for LIPUS. To verify these hypotheses, we started with *in vitro* cell study to investigate whether LIPUS stimulation could increase neural cell viability through the activation of the Wnt signaling pathway and elevate the levels of BDNF, with two types of cultured cells, neuron-like SH-SY5Y cells and primary glia cells. With the results from *in vitro* study confirming the simulative effects of LIPUS in neural cells, we then expanded the application of LIPUS treatment to an *in vivo* animal study with two models of depression, a repetitive restraint stress (RRS) animal model and a cuprizone (CPZ)-induced demyelination animal model. In the RRS study, after 3 weeks administration of restraint stress and LIPUS treatment, behavioral tests were carried out to evaluate whether LIPUS could ameliorate animals' depression-like behaviors, as measured by the sucrose preference test (SPT), tail suspension test (TST), forced swimming test (FST) and Y-maze test (YMT). Followup protein analysis with Western blotting was then performed to reveal whether the beneficial effects of LIPUS were associated with the promotion of neurogenesis and elevation of the levels of BDNF. In the CPZ study, after 5 weeks administration of CPZ and LIPUS, the efficacy of LIPUS treatment on white matter deficits in depression was tested through behavioral tests and followup protein analysis with Western blotting and immunohistochemistry staining (refer to Figure 1 as the study flowchart). To the best of our knowledge, the present study is the first focusing on the long-term effects of ultrasound in CNS as a therapeutic option for depression employing both *in vitro* cell studies and i*n vivo* animal studies with two different animal models. The novel findings produced

in the current study provide some new insight into furthering the application of LIPUS as a potential non-invasive treatment option for depression, and also serve to highlight the importance of neurogenesis, neurotrophy, and myelin and OLs in the pathophysiology of depression.

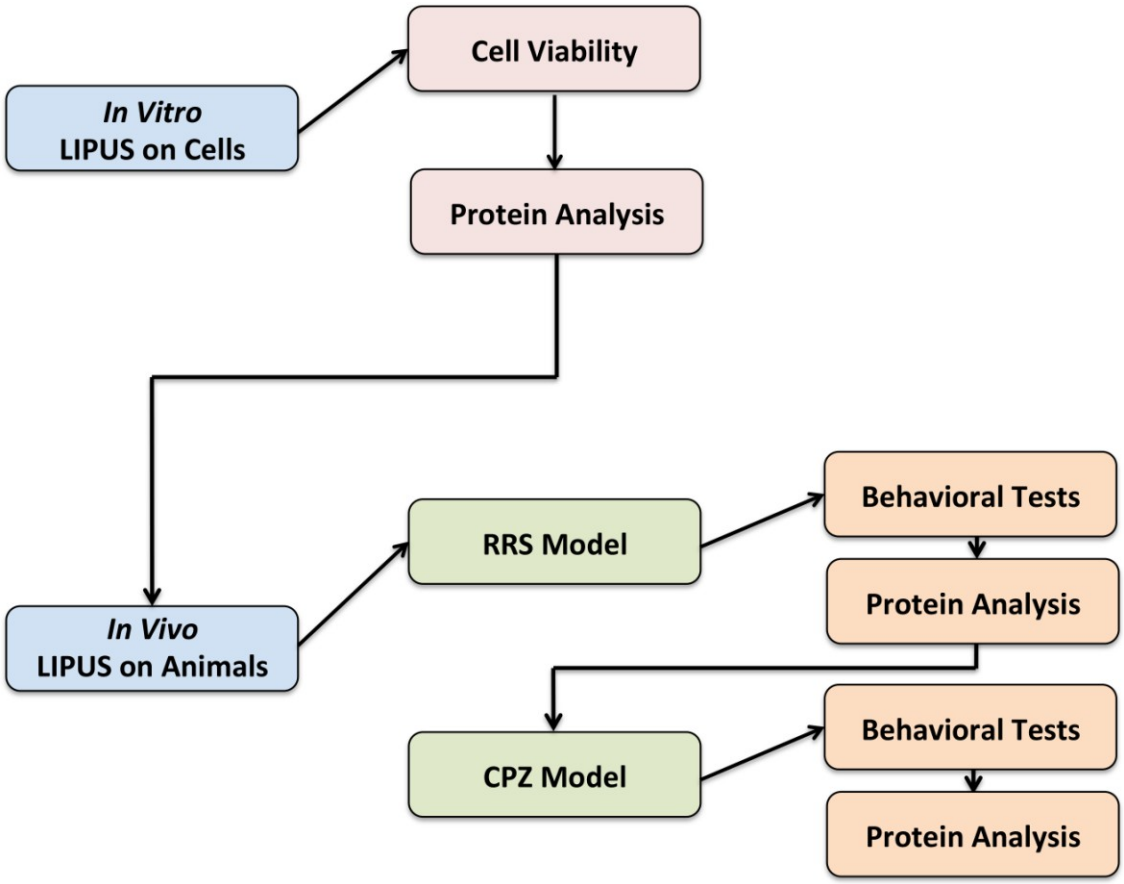


Figure 1. Flowchart for the current study. For the *in vitro* cell study, effects of LIPUS stimulation on cell viability were tested, followed by protein analysis on neurogenesis and levels of BDNF. For the *in vivo* animal study, first in the RRS model, behavioral tests were performed to evaluate effects of LIPUS on depression-like behaviors, followed by protein analysis on markers of neurogenesis and BDNF. Second, in the CPZ model, the same process was followed to evaluate effects of LIPUS on animal behaviors and changes in the levels of protein markers for myelin and OLs.

2. Materials and Methods

2.1 LIPUS Device

LIPUS was generated using SonaCell^{TM} (IntelligentNano Inc., Edmonton, Canada) (Figure 2). The device has the capability to drive 5 ultrasound transducers. Under the control of SonaCell^{TM} software, it automatically generated ultrasound with the designated power intensity required for the experiments. SonaCell^{TM} operated at a frequency of 1.5 MHz and a pulse repetition rate of 1.0 kHz, and the pulse duty cycle used was 20%. For the cell study, LIPUS was applied via a SonaCell^{TM} ultrasound device, by stimulating the cells in an enclosed sterile conventional 12-well cell culture plate in an incubator (Figure 2). Ultrasound was transmitted through the bottom of the wells via transmission gel between the transducer and the plate (Figure 2). The intensity was adjusted to 15 or 30 mW/cm^2 (refer to Supplementary Figure 1 for intensity adjustment). The treatment time was 5 minutes per 24 hours, 3 treatment sessions in total.

The intensity of LIPUS used in the animal study was adjusted to 25 mW/cm^2 (refer to Supplementary Figure 2 for intensity adjustment). The treatment time was 20 minutes per day. Mice were restrained in plastic tubes with the ultrasound transducer fixed near the end of the tube directly above the vertex of the mouse's head. Transmission gel was placed between the skin and the transducer (Figure 2).

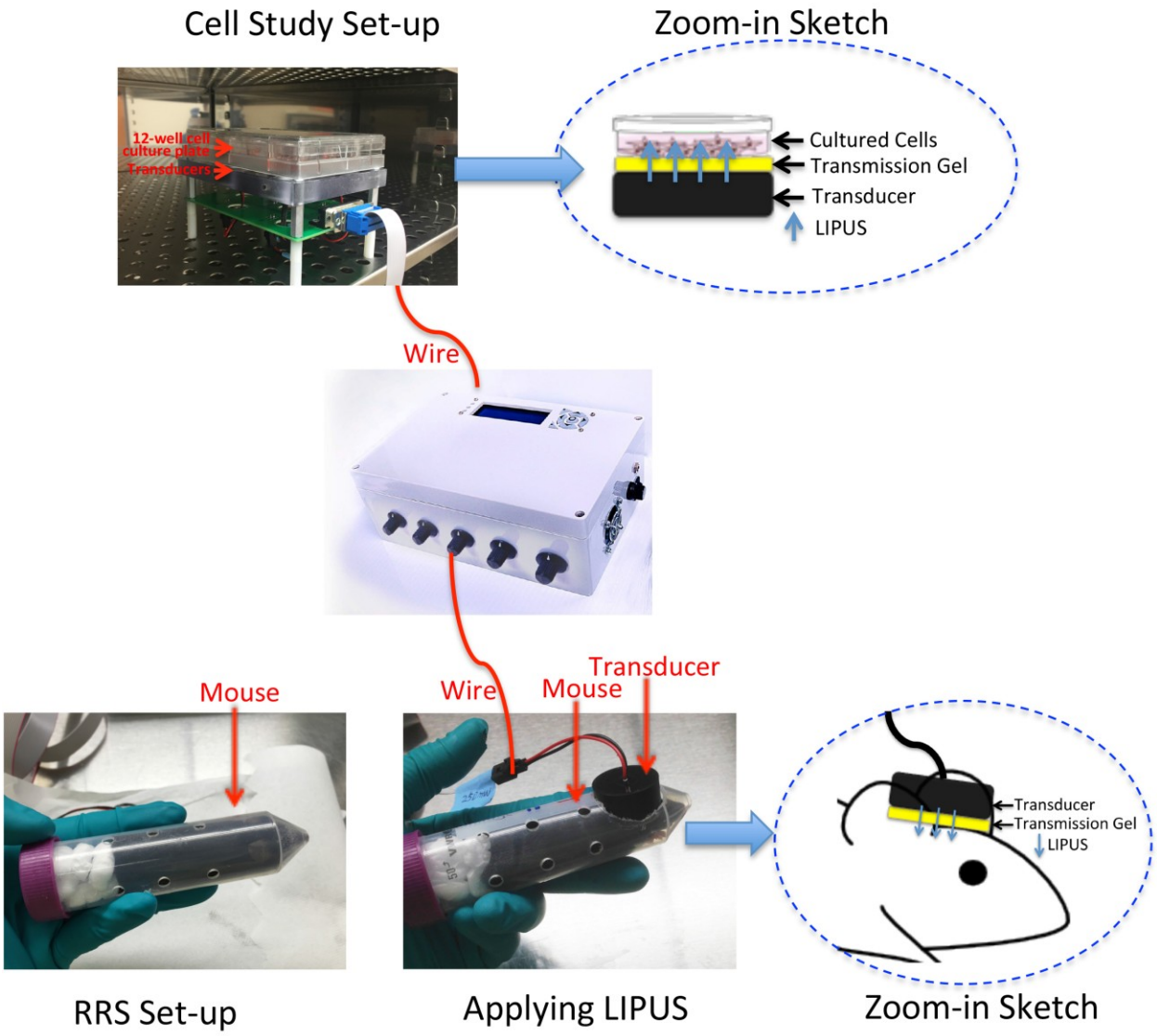


Figure 2. LIPUS device and experimental set-up. The center image is the LIPUS generating box; the image in the upper left shows the cell experiment set-up; the image in the upper right shows a zoom-in sketch for the cell experiment; the image in the lower left shows the set-up of RRS on a mouse; the image in the lower middle shows the set-up of LIPUS treatment on a mouse; the image in the lower right shows a zoom-in sketch for the LIPUS treatment on a mouse.

2.2 Cell Cultures

2.2.1 SH-SY5Y Cell Cultures

SH-SY5Y is a human neuroblastoma-derived cell line possessing neuron-like characteristics and is often used as *in vitro* model of neuronal function. The cell line was purchased from Sigma-Aldrich (St. Louis, USA) and the cells were then cultured on a poly-D-lysine-coated surface at 37°C with 5% CO_2 and 95% air. The growth medium was Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 Nutrient Mixture (modified, GE Healthcare Life Science, Pittsburgh, USA) plus 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, USA). Cells were seeded at $1x10^5$ cells/well in 12-well plates with complete FBS-containing medium and then switched to serum-free DMEM/F-12 medium for 18 hours. The cells were treated with LIPUS at an intensity of 15 mW/cm^2 or 30 mW/cm^2 for 5 minutes per 24 hours for 3 sessions. Twenty-four hours after the last treatment, cell viability tests were carried out, or cell proteins were collected (Figure 3a).

2.2.2 Primary Glia Cell Cultures

Primary glia cell cultures were prepared using cerebral cortical glia cells collected from 1-day-old rat pups as previously described (Qiao et al., 2016), with some minor modifications. The glia cells were then cultured on a poly-D-lysine-coated surface at 37°C with 10% CO_2 and 90% air. The cells were fed with DMEM/F12 medium (Life Technologies, Carlsbad, USA) plus 10% FBS twice

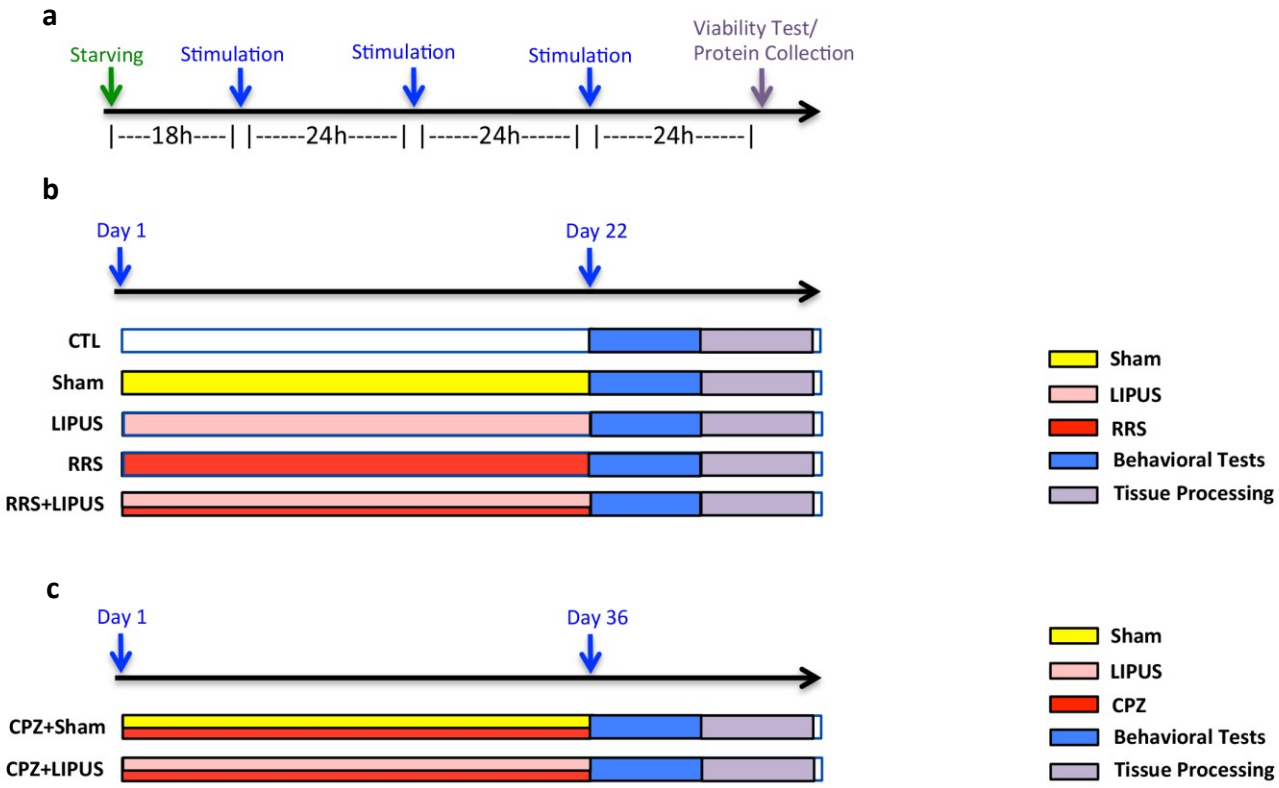


Figure 3. Experimental design. a) Experimental design for *in vitro* cell study. b) Experimental design for *in vivo* animal study with the RRS model. c) Experimental design for *in vivo* animal study with the CPZ model.

a week during the first two weeks. Then the cells were seeded at $2x10^5$ cells/well in a 12-well plate with the FBS-containing DMEM/F12 medium. Later the medium was replaced with serum-free DMEM/F12 medium for 18 hours. Then the cells were treated with LIPUS at an intensity of 15 mW/cm^2 or 30 mW/cm^2 for 5 minutes per 24 hours for 3 consecutive sessions. Twenty-four hours after the last stimulation, cell viability tests were carried out, or cell proteins were collected (Figure 3a).

2.3 Cell Viability Tests

Cell viability tests were performed with a cell proliferation reagent colorimetric assay, the Water Soluble Tetrazolium-1 (WST-1) Kit (Roche, Basel, Switzerland), based on manufacturer's instructions. Briefly, 60 μL/well of cell proliferation reagent WST-1 was added to the cells that were already cultured with the original medium at 540 μL/well (1:10 final dilution) in a 12-well plate. Cells were then incubated for 30 minutes at 37°C. After mildly shaking on a shaker, the absorbance of the samples against a background control as blank was measured at 450nm with a micro-plate reader. The relative viability of cells stimulated by different intensities of LIPUS was calculated based on the absorbance.

2.4 Cell Protein Analysis

Cell protein samples were collected after LIPUS stimulation using a Tris-ethylenediaminetetraacetic acid (EDTA) lysis buffer (1% Triton X-100, 20 mM Tris, 2 mM

EDTA, pH 7.6), with freshly added Protease Inhibitors Cocktail (Sigma-Aldrich, St. Louis, USA). Then Western blots were carried out and results analyzed (as stated in 2.5). The following primary antibodies were used: rabbit anti-BDNF, mouse anti-phosphorylated β-catenin (pβ-catenin), rabbit anti-β-catenin (refer to Table 1 for detailed information and dilution on primary antibodies).

2.5 Western Blots

After protein concentration determination with a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, USA), protein samples were boiled and loaded (15 μL per well) on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) mini-gels (5% stacking, 10% separation, 10 wells). Proteins were then separated by electrophoresis at 80 V for 1.5 hours at room temperature and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes at 120 mA for 2 hours in ice-cold transfer buffer. Membranes were blocked with 5% (w/v) skim milk in tris-buffered saline plus Tween-20 (TBST) for 1 hour at room temperature and then incubated with a series of primary antibodies at 4°C overnight. An antibody against β-actin was used as an internal loading control. Membranes were then rinsed three times with TBST and then incubated with specific horseradish peroxidase (HRP)-conjugated secondary antibodies at a dilution of 1:5000 (goat anti-rabbit, Abcam, Cambridge, UK; goat anti-mouse, Cedarlane, Burlington, Canada) for 2 hours at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) substrate kit (Thermo Fisher Scientific, Waltham, USA). Quantification of immunoblots was carried out by densitometric analysis of

chemiluminescence-exposed films, using software ImageJ (version 64, National Institutes of Health, Bethesda, USA), and the results were expressed as a ratio of the target protein to β-actin.

2.6 Animals and Housing

Seven-week old female C57BL/6 mice were purchased from Charles River (Montréal, Canada). The animal facility was maintained a 12-hour/12-hour light-dark cycle, a constant temperature of 22 ± 0.5°C, and 60% humidity. All procedures involving animals were performed in accordance with the guidelines set by the Canadian Council on Animal Care and approved by the University of Alberta Animal Care and Use Committee.

2.7 Repetitive Restraint Stress (RRS) Administration

In the RRS study, after acclimatization in the housing facility for 2 weeks, mice were randomly divided into 5 groups: control (CTL), sham (with the apparatus but transducer not connected to LIPUS generating box), RRS, LIPUS, RRS plus LIPUS (RRS + LIPUS). All mice were continuously fed with normal food chow *ad libitum*. Body weight was measured weekly for health condition monitoring. For the RRS groups, RRS was induced by placing the mice in plastic tubes with air holes in the nasal end (Figure 2), for 3 hours every day continuously for 3 weeks (Figure 3b). This procedure is similar to the ultrasound administration in the present study (see Figure 2 for details), providing a suitable experimental set-up to initiate the investigation on the effects of LIPUS, with no extra disturbance induced by the ultrasound treatment procedure alone. A battery

of behavioral tests was then performed to evaluate the depression-like behaviors of mice assigned to each group.

2.8 Cuprizone (CPZ) Administration

In the CPZ study, after acclimatization with normal food chow for 2 weeks, mice were divided into 2 groups: CPZ + sham, CPZ + LIPUS. CPZ was purchased from Sigma-Aldrich (St. Louis, USA), and mixed into milled LabDiet (St. Louis, USA) rodent chow at a final concentration of 0.2% (w/w). All mice were continuously fed with CPZ food chows *ad libitum* for 5 weeks (Figure 3c). Body weight was measured weekly for health condition monitoring. For the LIPUS group, mice were administered with LIPUS treatment for 20 minutes every day. The sham mice were set up in the LIPUS apparatus for 20 minutes every day, but the cable was not connected to the generating box. Behavioral tests were then performed to evaluate depression-like behaviors of mice in treatment and sham groups.

2.9 Animal Behavioral Tests

2.9.1 Sucrose Preference Test (SPT)

The SPT was carried out as previously described (Zhu et al., 2014), with minor modifications. Mice were first trained with two identical bottles for 3 hours every day for 2 days while group-housed before the test. The mice were exposed to a palatable sucrose solution (2%, w/w) as well as plain water during the training. On the testing day, after 4 hours of water deprivation, mice

were exposed to two identical bottles for 1 hour, one filled with 2% sucrose solution and the other with plain water. Liquid consumption during this testing session was determined by measuring changes in the weight of the bottles. A sucrose preference index was defined as the ratio of sucrose solution to total fluid consumed during the 1-hour testing period. Food was withdrawn during the test.

2.9.2 Tail Suspension Test (TST)

The TST was carried out as previously described by our group (Yan et al., 2007), with minor modifications. Mice were suspended by a small metal hook fixed with adhesive tape wrapped around the tail. The total time mice spent immobile during the 6-minute testing period was recorded with a radio camera connected to ANY-MAZE tracking system software (Stoelting Company, Wood Dale, USA). The last 4 minutes of the testing period were scored for immobility, which was automatically analyzed by the software. After each test, the mouse was checked for tail condition and then put back into its home cage.

2.9.3 Forced Swimming Test (FST)

The FST was performed as previously described (Wang et al., 2014), with some minor modifications. Each mouse was placed in a glass beaker filled with 15 cm of water at room temperature. The total time mice spent immobile during the 15-minute testing period was recorded by ANY-MAZE and the last 6 minutes were scored for immobility, which was automatically

analyzed by the software. After each test, the wet mouse was dried with a towel and then put back into its home cage.

2.9.4 Y-Maze Test (YMT)

Spatial working memory was assessed with the YMT as previously described by our group (Zhang et al., 2013). Mice were placed at the end of the start arm and allowed to move freely through the maze during an 8 minutes session. Total arm entries were recorded by ANY-MAZE and spontaneous alterations were calculated accordingly.

2.10 Tissue Processing

Mice were deeply anesthetized with isofluorane and then perfused intracardially with 0.01 M phosphate-buffered saline (PBS, pH 7.4). The brain (excluding cerebellum, pons, and medulla oblongata) was then removed. The right hemisphere was separated into frontal, medial and hind cortex and hippocampus, snap frozen and stored at -80°C for Western blotting. The left hemisphere was post-fixed in 4% paraformaldehyde (PFA) in PBS for 48 hours, followed by cryoprotection in 30% sucrose at 4°C for 72 hours. Serial coronal sections (30 μm) were cut using a sliding cryostat (Leica Biosystems, Wetzlar, Germany) and collected in 24-well plates containing 0.01 M PBS for immunohistochemistry (IHC) staining.

2.11. Animal Protein Analysis

Four to six mice were randomly selected from each group, and protein samples from the medial cortex or hippocampus were collected by using EDTA lysis buffer with freshly added Protease Inhibitors Cocktail. Then Western blots were carried out and results analyzed (as stated in 2.5). The following primary antibodies were used: guinea pig anti-doublecortin (DCX), rabbit anti-BDNF, chicken anti-myelin basic protein (MBP), rabbit anti-neural/glial antigen 2 (NG2) (refer to Table 1 for detailed information and dilution on primary antibodies).

2.12 Immunohistochemistry (IHC) Staining

Free floating brain sections were washed three times with PBS and then quenched in 3% hydrogen peroxide in PBS for 20 minutes to block endogenous peroxidase activity. Sections were blocked at room temperature for 60 minutes in 3% normal goat serum, 1% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS and incubated overnight at 4°C with the following primary antibodies: chicken anti-MBP, rabbit anti- NG2 (refer to Table 1 for detailed information and dilution on primary antibodies). Specific secondary biotinylated antibody was incubated at a dilution of 1:400 (goat anti-chicken & goat anti-rabbit, Vector Laboratories, Burlingame, USA) for 1.5 hours at room temperature. After washing three times with PBS containing Tween-20 (PBST), the slides were incubated with avidin-biotin complex (ABC) reagents for 30 minutes. The antigen-antibody complexes were then visualized with a diaminobenzidine (DAB) kit (Sigma-Aldrich, St. Louis, USA). Slides were then air-dried in the dark, mounted and viewed with a Leica DMI6000B Microscope (Wetzlar, Germany) for bright and dark fields and captured with LAS AF computer

software. Four or five random fields at 16X (MBP) or 100X (NG2) magnification from each animal of the CPZ + Sham and CPZ + LIPUS groups were examined, and optical densities of the staining were measured by ImageJ. These fields of interest were those parts of the brain containing the corpus callosum (CC). The same conditions were maintained in ImageJ when performing measurements on all selected brain regions. Measurement values were automatically calculated by the software. The data of optical density of MBP-positive and NG2-positive IHC staining were expressed as relative density, which is equal to the area average optical density.

2.13 Statistical Analysis

All statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, version 20, IBM, New York, USA). All results are expressed as mean ± standard error of the mean (S.E.M.). Differences across experimental groups were determined by one- or two-way analysis of variance (ANOVA), followed by Newman–Keuls *post-hoc* tests for multiple comparisons. A two-tailed *t*-test for independent samples was used for two-group comparisons. A p-value of less than 0.05 was considered statistically significant.

Table 1. Details of the primary antibodies used in this study

1.1 Primary antibodies used for Western blots

Antibody	Type	Host	Western Blot Dilution	Manufacturer	Catalog No.
BDNF	Polyclonal	Rabbit	1:500	EMD Millipore Corporation	AB1534
pβ-catenin	Polyclonal	Mouse	1:1000	EMD Millipore Corporation	05-665
β-catenin	Polyclonal	Rabbit	1:500	EMD Millipore Corporation	AB19022
DCX	Polyclonal	Guinea Pig	1:1000	EMD Millipore Corporation	AB2253
MBP	Polyclonal	Chicken	1:2000	Aves Labs	MBP
NG2	Polyclonal	Rabbit	1:1000	EMD Millipore Corporation	AB5320
β-actin	Monoclonal	Mouse	1:5000	Novus Biologicals	NB600-501
β-actin	Polyclonal	Rabbit	1:5000	Novus Biologicals	NB600-532H

1.2 Primary antibody used for immunohistochemistry staining

Antibody	Type	Host	Immunohistochemistry Staining Dilution	Manufacturer	Catalog No.
MBP	Polyclonal	Chicken	1:1000	Aves Labs	MBP
NG2	Polyclonal	Rabbit	1:200	EMD Millipore Corporation	AB5320

3. Results

3.1 Effects of LIPUS on Cell Viability

Cell viability tests were performed to evaluate the effects of LIPUS on the proliferation of neuron-like SH-SY5Y cells and primary glia cells. For SH-SY5Y cells, one-way ANOVA demonstrated changes in cell viability, $F(2, 6) = 4.478$, $p = 0.065$. *Post-hoc* analysis indicated that compared to the un-stimulated control group, LIPUS at the intensity of 15 mW/cm^2 significantly increased cell viability, while 30 mW/cm^2 did not produce such change (Figure 4a). For primary glia cells, one-way ANOVA showed significant changes in cell viability, $F(2, 15) = 10.783$, $p = 0.001$, indicating that LIPUS was able to substantially influence the viability of this cell type. *Post-hoc* analysis showed that compared to un-stimulated controls, LIPUS at an intensity of 15 mW/cm^2 significantly increased cell viability, while 30 mW/cm^2 stimulation led to a significant decrease in cell viability than 15 mW/cm^2 (Figure 4b). In summary, it was found that LIPUS at a lower intensity was able to significantly accelerate cell viability in both neuron-like SH-SY5Y cells and primary glia cells.

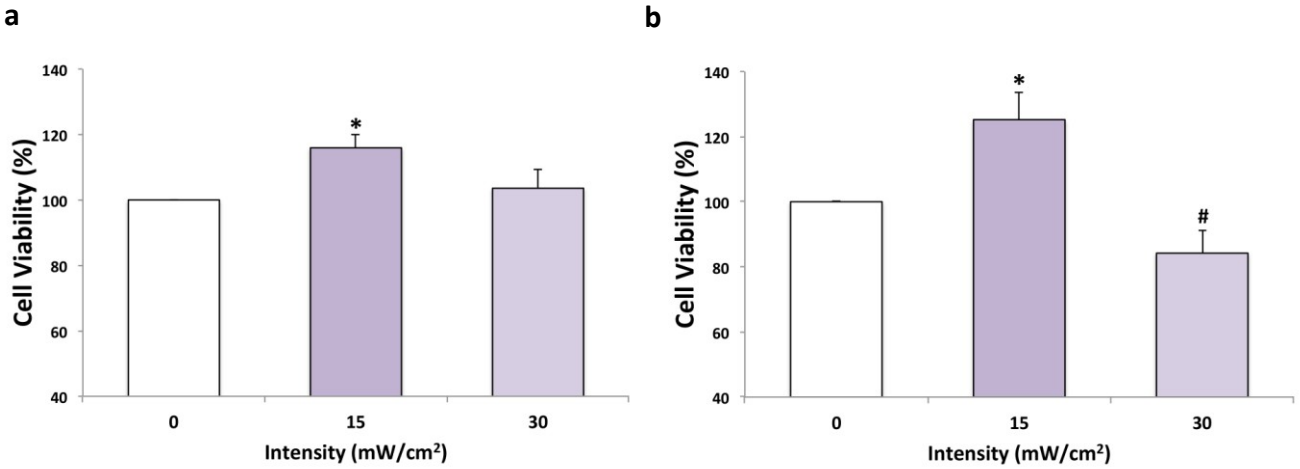


Figure 4. LIPUS influenced cell viability of both neuron-like SH-SY5Y cells and primary glia cells. a) LIPUS at the intensity of 15mW/cm^2 significantly increased the viability of SH-SY5Y cells, while LIPUS at the intensity of 30mW/cm^2 had no significant effect on cell viability. b) LIPUS at the intensity of 15mW/cm^2 significantly increased the viability of primary glia cells, while LIPUS at the intensity of 30mW/cm^2 significantly decreased the cell viability than LIPUS at 15 mW/cm^2. Values represented group mean values ± S.E.M.. *$p < 0.05$ vs. 0 mW/cm^2 (control), #$p < 0.05$ vs. 15 mW/cm^2.

3.2 Effects of LIPUS on Cell Signaling Pathways and Cytokines

3.2.1 Effects of LIPUS on Wnt Signaling Pathway

The Wnt-signaling pathway plays an important role in adult hippocampal neurogenesis. Research has shown that Wnt signaling mediates neuroblast proliferation and neuronal differentiation in adult hippocampal progenitor cells via β-catenin, an intracellular signal transducer in the Wnt pathway (Lie et al., 2005). For neuron-like SH-SY5Y cells, one-way ANOVA showed no changes in the level of pβ-catenin, $F(2, 5) = 0.798$, $p = 0.500$. *Post-hoc* analysis indicated that compared to the un-stimulated control group, LIPUS at the intensity of 15 mW/cm^2 showed a non-significant tendency to increase the level of pβ-catenin, while 30 mW/cm^2 did not produce any change in the level of pβ-catenin (Figure 5a). For primary glia cells, one-way ANOVA showed changes in the level of pβ-catenin, $F(2, 5) = 4.946$, $p = 0.065$. *Post-hoc* analysis showed that compared to un-stimulated controls, LIPUS at the intensity of 15 mW/cm^2 significantly increased the level of pβ-catenin, while 30 mW/cm^2 stimulation did not induce such change (Figure 5b). In summary, LIPUS at a lower intensity was found to activate the phosphorylation of β-catenin in primary glia cells, and showed a non-significant tendency to promote the phosphorylation of β-catenin in neuron-like SH-SY5Y cells.

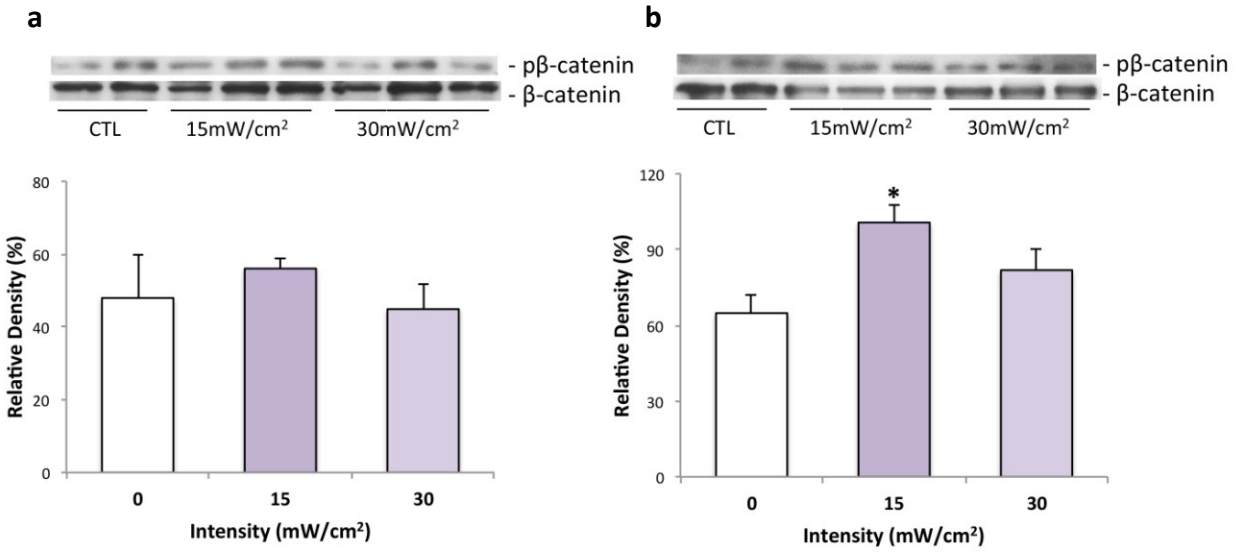


Figure 5. LIPUS increased the level of pβ-catenin in primary glia cells. a) A representative Western blot image showing that LIPUS at the intensity of both 15mW/cm^2 and 30mW/cm^2 had no significant effect on the level of pβ-catenin in SH-SY5Y cells. The relative amounts of pβ-catenin protein in all groups are presented in the bar graph below. b) A representative Western blot image showing that LIPUS at the intensity of 15mW/cm^2 significantly increased the level of pβ-catenin in primary glia cells, while LIPUS at the intensity of 30mW/cm^2 had no such significant effect on the level of pβ-catenin. The relative amounts of pβ-catenin protein in all groups are presented in the bar graph below. Values represented group mean values ± S.E.M.. *$p < 0.05$ vs. 0 mW/cm^2 (control).

3.2.2 Effects of LIPUS on BDNF

Neurotrophic factors such as BDNF not only help to support the survival of existing neurons and encourage the growth and differentiation of new neurons and synapses, but also play important roles in the development and maintenance of the CNS as extracellular signaling proteins (Zweifel et al., 2005). For SH-SY5Y cells, one-way ANOVA showed significant differences in the level of BDNF, $F(2, 5) = 8.262$, $p = 0.026$, indicating that LIPUS treatment was able to influence the expression of BDNF. *Post-hoc* analysis indicated that compared to the un-stimulated control group, LIPUS at the intensity of both 15 mW/cm^2 and 30 mW/cm^2 significantly increased the level of BDNF (Figure 6a). In primary glia cells, one-way ANOVA showed a trend towards significant differences in the level of BDNF, $F(2, 5) = 5.091$, $p = 0.062$. *Post-hoc* analysis showed that compared to un-stimulated controls, LIPUS at the intensity of 15 mW/cm^2 significantly increased the level of BDNF, while 30 mW/cm^2 stimulation did not induce obvious change (Figure 6b). In summary, it was found that LIPUS at a lower intensity was able to significantly increase BDNF expression in both neuron-like SH-SY5Y cells and primary glia cells.

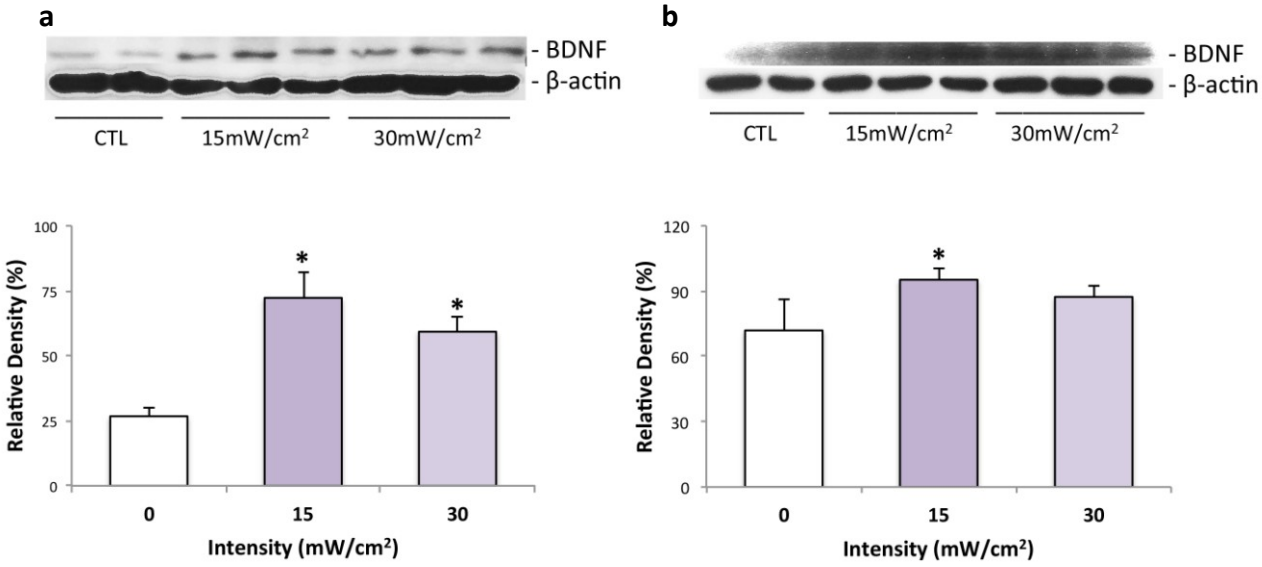


Figure 6. LIPUS increased the level of BDNF in both neuron-like SH-SY5Y cells and primary glia cells. a) A representative Western blot image showing LIPUS at the intensity of 15mW/cm^2 and 30mW/cm^2 significantly increased the level of BDNF in SH-SY5Y cells. The level of BDNF at the intensity of 15mW/cm^2 was observed to be relative higher than that at 30mW/cm^2. The relative amounts of BDNF protein in all groups are presented in the bar graph below. b) A representative Western blot image showing that LIPUS at the intensity of 15mW/cm^2 significantly increased the level of BDNF in primary glia cells, while LIPUS at the intensity of 30mW/cm^2 had no significant effect on the level of BDNF. The relative amounts of BDNF protein in all groups are presented in the bar graph below. Values represent group mean values ± S.E.M.. *$p < 0.05$ vs. 0 mW/cm^2 (control).

3.3 Effects of LIPUS on Animal Behaviors after Exposed to RRS

3.3.1 Effects of LIPUS on Depressive-like Behaviors in Mice Exposed to RRS

Assessment of depressive-like behavior in mice was tested with the SPT, TST, and FST. For the SPT, two-way ANOVA showed that the changes in preference index were $F(1, 30) = 0.011$, $p = 0.917$ for RRS group, $F(1, 30) = 7.196$, $p = 0.012$ for LIPUS group and $F(1, 30) = 8.231$, $p = 0.007$ for the interaction between RRS and LIPUS. A *post-hoc* analysis indicated the RRS mice had a significantly lower preference index than the control group and LIPUS treatment significantly increased the preference index in RRS mice (Figure 7a). Two-way ANOVA showed that changes in the total fluid consumption were $F(1, 30) = 0.979$, $p = 0.330$ for RRS group, $F(1, 30) = 0.000$, $p = 0.993$ for LIPUS group and $F(1, 30) = 0.006$, $p = 0.938$ for the interaction between RRS and LIPUS. These results indicate there were no significant changes in fluid consumption across groups (Figure 7b).

For the TST, two-way ANOVA showed that the changes in the total time of immobility were $F(1, 30) = 0.438$, $p = 0.513$ for RRS group, $F(1, 30) = 6.445$, $p = 0.017$ for LIPUS group and $F(1, 30) = 0.254$, $p = 0.618$ for the interaction between RRS and LIPUS. A *post-hoc* analysis indicated that the RRS + LIPUS mice had a significant decrease of total immobile time compared to the RRS mice (Figure 7c).

For the FST, two-way ANOVA showed the changes in total immobile time were $F(1, 30) = 0.415$, $p = 0.525$ for RRS group, $F(1, 30) = 9.298$, $p = 0.005$ for LIPUS group and $F(1, 30) = 2.006$, $p =$

0.167 for the interaction between RRS and LIPUS. *Post-hoc* analysis indicated that LIPUS significantly decreased the total time of immobility in the RRS groups (Figure 7d).

3.3.2 Effects of LIPUS on Working Memory in Mice Exposed to RRS

Considering the fact that cognitive deficiency is also fairly common in depression (Papakostas, 2014), spatial working memory, a feature of cognitive function, was also evaluated using the YMT. Two-way ANOVA showed changes in alternation performance, $F(1, 30) = 0.698$, $p = 0.410$ for RRS group, $F(1, 30) = 7.314$, $p = 0.011$ for LIPUS group and $F(1, 30) = 0.603$, $p = 0.443$ for the interaction between RRS and LIPUS. *Post-hoc* analysis indicated that LIPUS treatment significantly increased the spontaneous alternations in the RRS mice (Figure 8a). Two-way ANOVA on changes in total arm entries showed, $F(1, 30) = 3.127$, $p = 0.087$ for RRS group, $F(1, 30) = 0.001$, $p = 0.973$ for LIPUS group and $F(1, 30) = 0.008$, $p = 0.930$ for the interaction between RRS and LIPUS. These results indicated there were no significant differences in total arm entries across groups (Figure 8b).

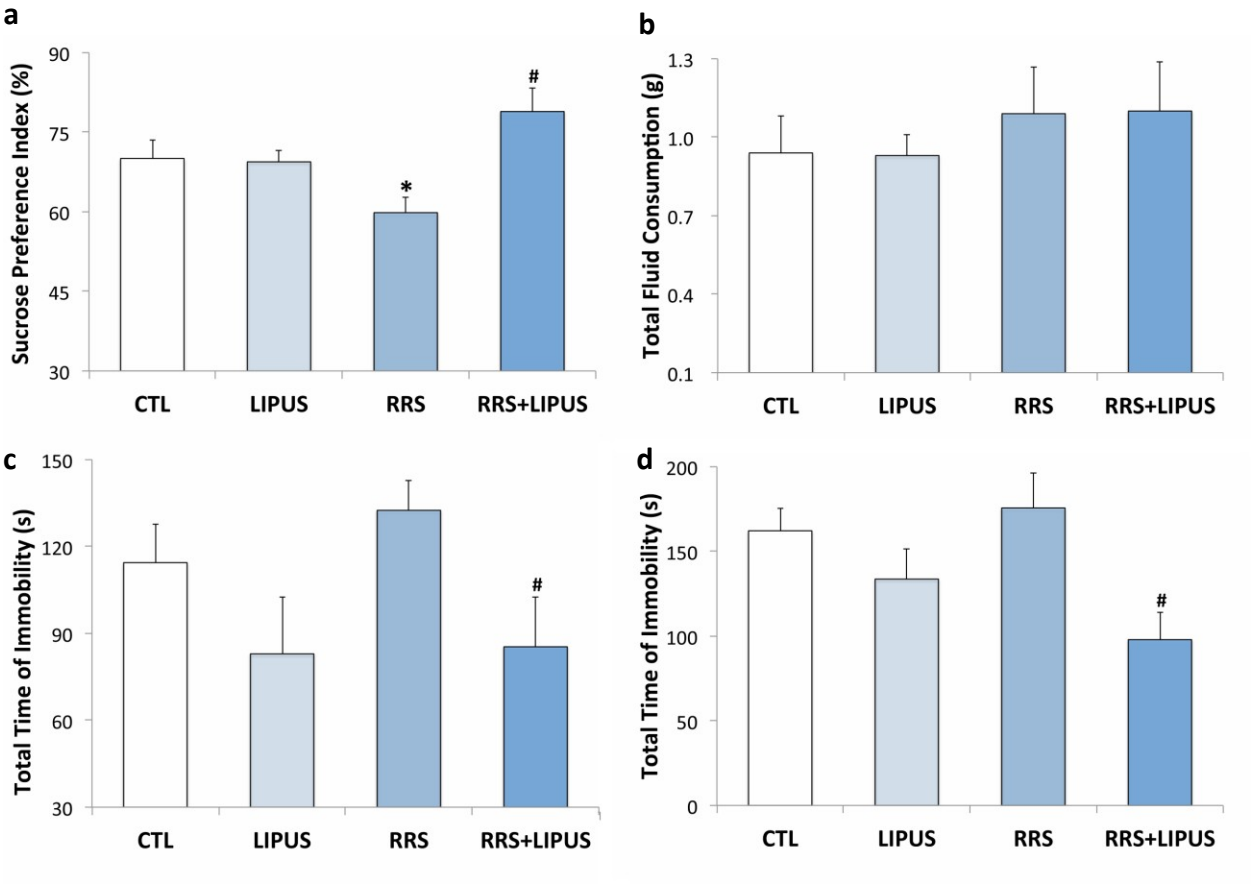


Figure 7. LIPUS improved mice performance in tests of depressive-like behaviors. a) Sucrose preference was decreased by RRS exposure and reversed by LIPUS treatment in the sucrose preference test. b) Total fluid consumption was not significantly influenced by treatment in the sucrose preference test. c) There was a non-significant trend to an increase in total time of immobility by RRS exposure and this immobility time was significantly decreased by LIPUS treatment in the tail suspension test. d) There was a non-significant trend to an increase in total time of immobility by RRS exposure and this immobility time was significantly decreased by LIPUS treatment in the forced swimming test. Values represent group mean values ± S.E.M.; n = 8-9 mice per group. *$p < 0.05$ vs. CTL; #$p < 0.05$ vs. RRS.

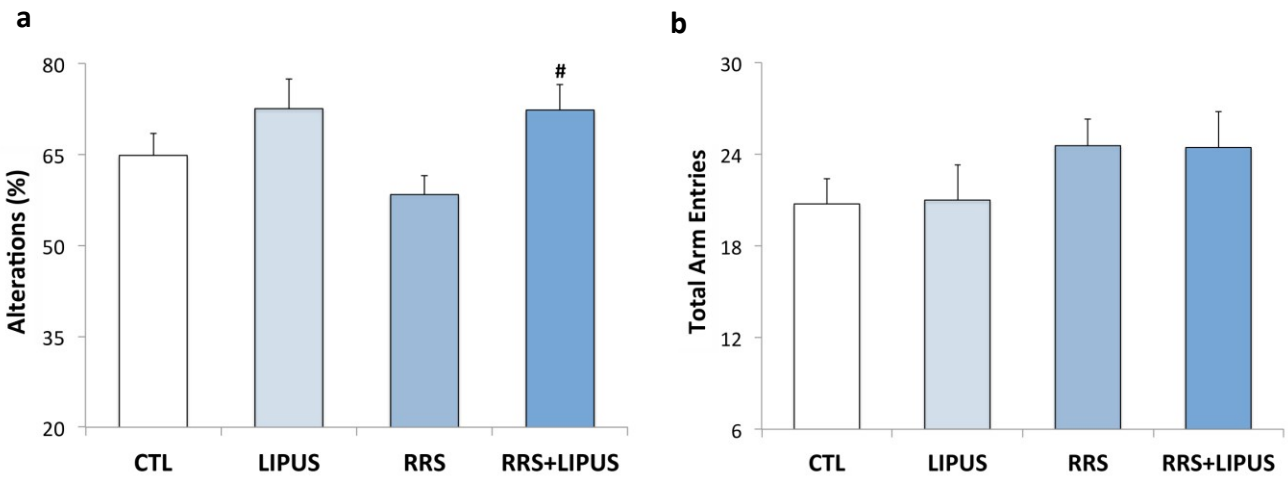


Figure 8. LIPUS improved spatial working memory of mice exposed to RRS. a) There was a non-significant trend to a decrease in spontaneous alterations by RRS exposure and this decrease was significantly reversed by LIPUS treatment in Y-maze test. b) No significant differences were observed in total number of arm entries as tested by Y-maze test. Values represent group mean values ± S.E.M.; n = 8-9 mice per group. #$p < 0.05$ vs. RRS.

3.3.3 No Difference of Behavioral Performance between CTL and Sham Groups

The behavioral performance of CTL and Sham groups were compared to exclude the possible effects of the LIPUS apparatus on animal behaviors. For the SPT, one-way ANOVA showed $F(1, 14) = 0.014$, $p = 0.906$, indicating no significant difference in the sucrose preference index between these two groups (Figure 9a). For the TST, one-way ANOVA showed $F(1, 14) = 2.752$, $p = 0.119$, indicating no significant difference in total time of immobility between these two groups (Figure 9b). For the FST, one-way ANOVA showed $F(1, 14) = 0.250$, $p = 0.625$, indicating no significant difference in total time of immobility between CTL and Sham (Figure 9c). For the YMT, one-way ANOVA showed $F(1, 14) = 0.005$, $p = 0.944$, indicating no significant difference in spontaneous alterations between these two groups (Figure 9d).

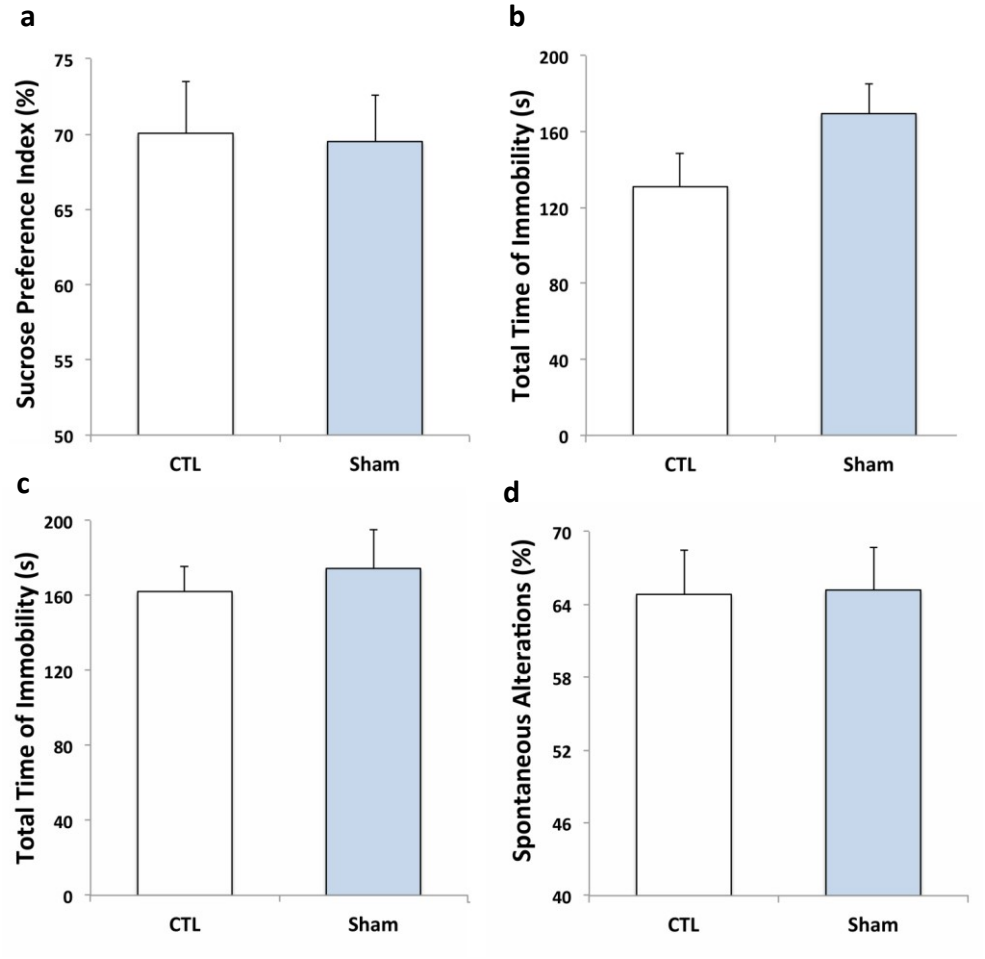


Figure 9. Comparison of CTL and Sham groups on performance in behavioral tests. a) No significant difference was found for sucrose preference index in the SPT. b) No significant difference was found for total time of immobility in the TST. c) No significant difference was found for total time of immobility in the FST. d) No significant difference was found for spontaneous alterations in the YMT. Values represent group mean values ± S.E.M.; n = 8 mice per group.

3.4 Effects of LIPUS on Brain Pathological Changes after Exposure to RRS

3.4.1 Effects of LIPUS on DCX in Mice Exposed to RRS

DCX is a microtubule-associated protein expressed by neuronal precursor cells and immature neurons and is widely considered a marker for neurogenesis (Magavi et al., 2000). Analysis of the hippocampus proteins of mice from different groups using a two-way ANOVA showed that changes in the level of DCX, $F(1, 20) = 19.142$, $p = 0.000$ for RRS group, $F(1, 20) = 2.830$, $p = 0.108$ for LIPUS group and $F(1, 20) = 0.706$, $p = 0.411$ for the interaction between RRS and LIPUS. *Post-hoc* analysis indicated the level of DCX in RRS mice showed a trend towards significant decrease compared to control mice ($p = 0.09$) and that LIPUS treatment significantly alleviated the decrease of DCX in RRS mice (Figure 10a). LIPUS also significantly increased the level of DCX in control animals (Figure 10a). Analysis of CTL and Sham groups using a one-way ANOVA showed $F(1, 6) = 0.130$, $p = 0.730$, indicating no significant differences in the level of DCX between these two groups (Figure 10b).

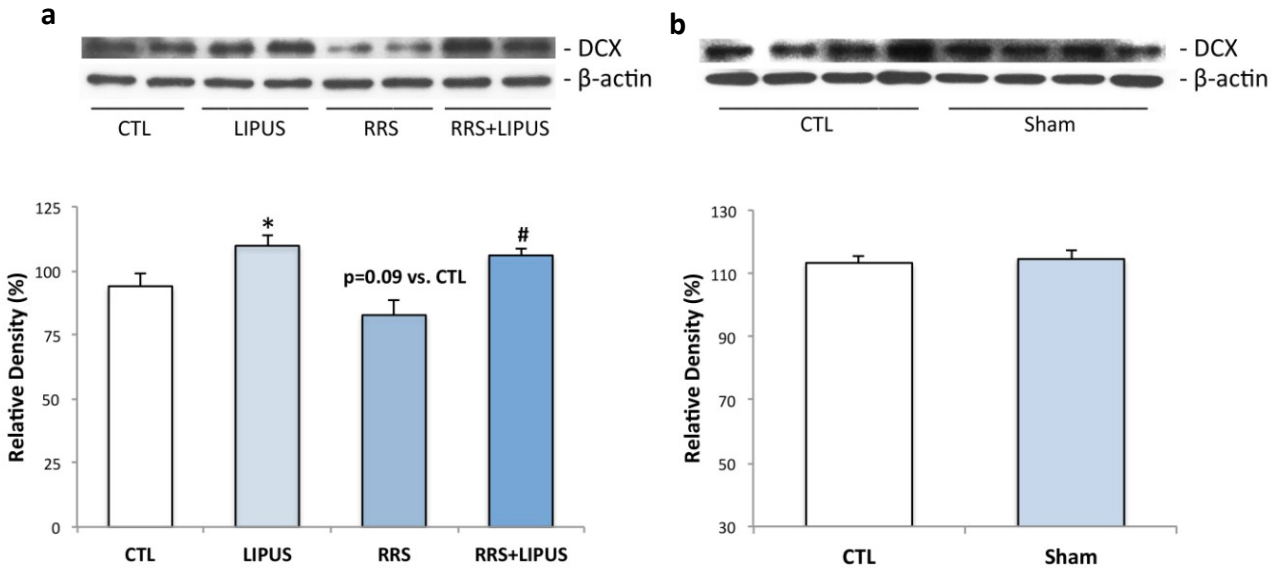


Figure 10. LIPUS increased the level of DCX in mice exposed to RRS. a) A representative Western blot image showing the level of DCX showed a trend towards a decrease by RRS exposure, which was significantly reversed by LIPUS treatment. LIPUS treatment also significantly increased the level of DCX in mice without exposure to RRS. Relative amounts of DCX protein in all groups are presented in the bar graph below. b) A representative Western blot image showing no obvious difference in the level of DCX between CTL and Sham groups. Relative amounts of DCX protein in both groups are presented in the bar graph below. Values represent group mean values ± S.E.M.; n = 6 (for a) or 4 (for b) mice per group. *$p < 0.05$ vs. CTL; #$p < 0.05$ vs. RRS.

3.4.2 No Difference in the Level of pβ-catenin in Mice Exposed to RRS

Analysis of the hippocampus from mice in different experimental groups using a two-way ANOVA showed no changes in the level of pβ-catenin, $F(1, 20) = 1.105$, $p = 0.306$ for RRS group, $F(1, 20) = 0.578$, $p = 0.456$ for LIPUS group and $F(1, 20) = 0.130$, $p = 0.722$ for the interaction between RRS and LIPUS. *Post-hoc* analysis indicated there were no significant differences in the level of pβ-catenin across these groups (Figure 11).

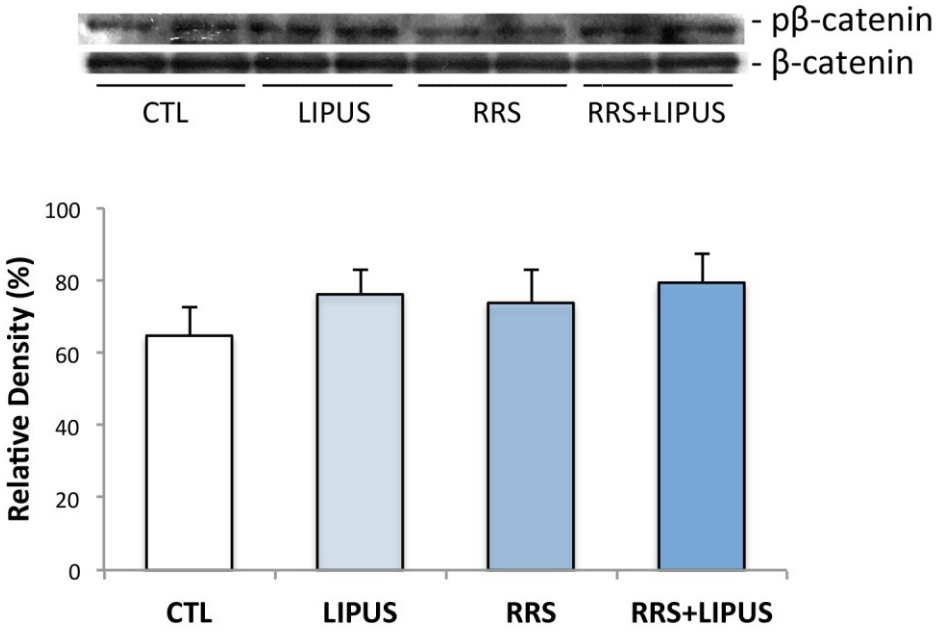


Figure 11. No significant difference in the level of pβ-catenin in the hippocampus was observed across experimental groups. A representative Western blot image showing no significant difference was found across groups. Relative amounts of pβ-catenin protein in all groups are presented in the bar graph below. Values represent group mean values ± S.E.M.; n = 6 mice per group.

3.4.3 Effects of LIPUS on the Level of BDNF in Mice Exposed to RRS

Analysis of the hippocampus proteins of mice from different experimental groups using a two-way ANOVA showed that changes in the level of BDNF, $F(1, 20) = 6.158$, $p = 0.025$ for RRS group, $F(1, 20) = 0.836$, $p = 0.374$ for LIPUS group and $F(1, 20) = 0.003$, $p = 0.955$ for the interaction between RRS and LIPUS. *Post-hoc* analysis indicated that LIPUS treatment significantly increased the level of BDNF in both RRS and CTL mice, and that the RRS + LIPUS group had a non-significant tendency of a higher level of BDNF than the RRS only group (Figure 12a). Comparison of CTL and Sham groups using one-way ANOVA showed $F(1, 6) = 0.080$, $p = 0.786$, indicating no significant difference in the level of BDNF between these two groups (Figure 12b).

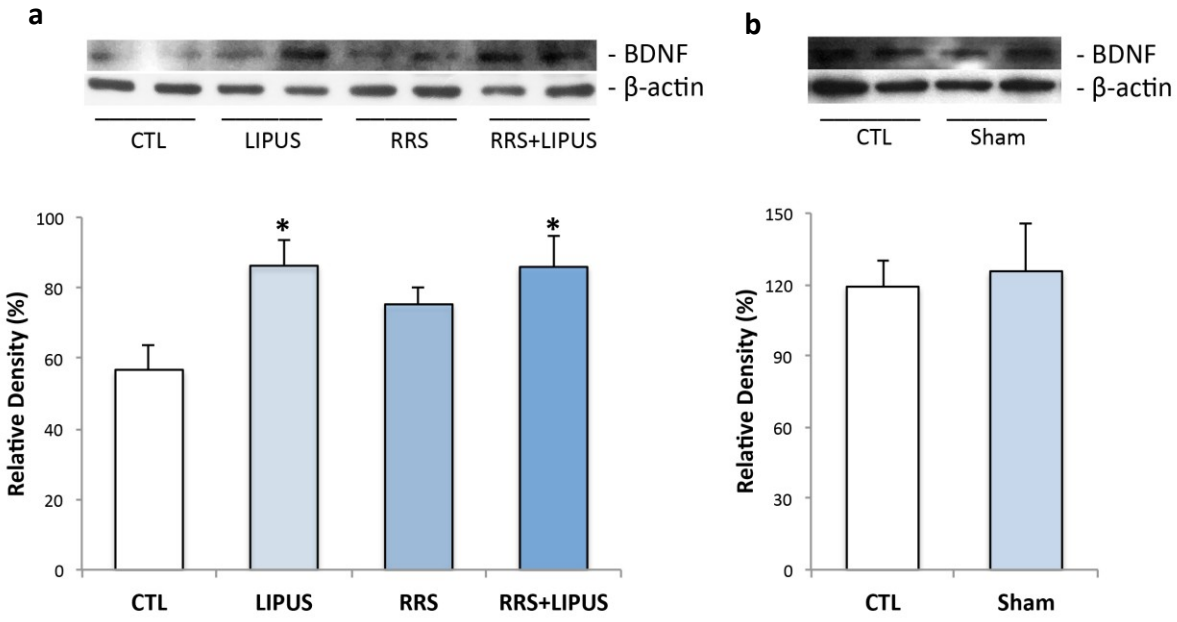


Figure 12. LIPUS elevated the level of BDNF in mice. a) A representative Western blot image showing the level of BDNF was significantly increased by LIPUS treatment in mice with or without exposure to RRS. Relative amounts of BDNF protein in all groups are presented in the bar graph below. b) A representative Western blot image showing no significant difference in the level of BDNF between CTL and Sham groups. Relative amounts of BDNF protein in all groups are presented in the bar graph below. Values represent group mean values ± S.E.M.; n = 5 (for a) or 4 (for b) mice per group. *$p < 0.05$ vs. CTL.

3.5 No Difference of Animal Behaviors Induced by LIPUS after Exposed to CPZ

Depression-like behavior of mice exposed to CPZ with or without LIPUS treatment was also tested with the TST, FST and YMT. For the TST, one-way ANOVA showed $F(1, 25) = 0.229$, $p = 0.636$, indicating no significant difference in total time of immobility between these two groups (Figure 13a). For the FST, one-way ANOVA showed $F(1, 25) = 0.334$, $p = 0.569$, indicating no significant difference in total time of immobility between these two groups (Figure 13b). For the YMT, one-way ANOVA showed $F(1, 25) = 0.120$, $p = 0.732$, indicating no significant difference in spontaneous alterations between these two groups (Figure 13c). Also one-way ANOVA showed $F(1, 25) = 0.819$, $p = 0.374$, indicating no significant difference in total arm entries between these two groups (Figure 13d).

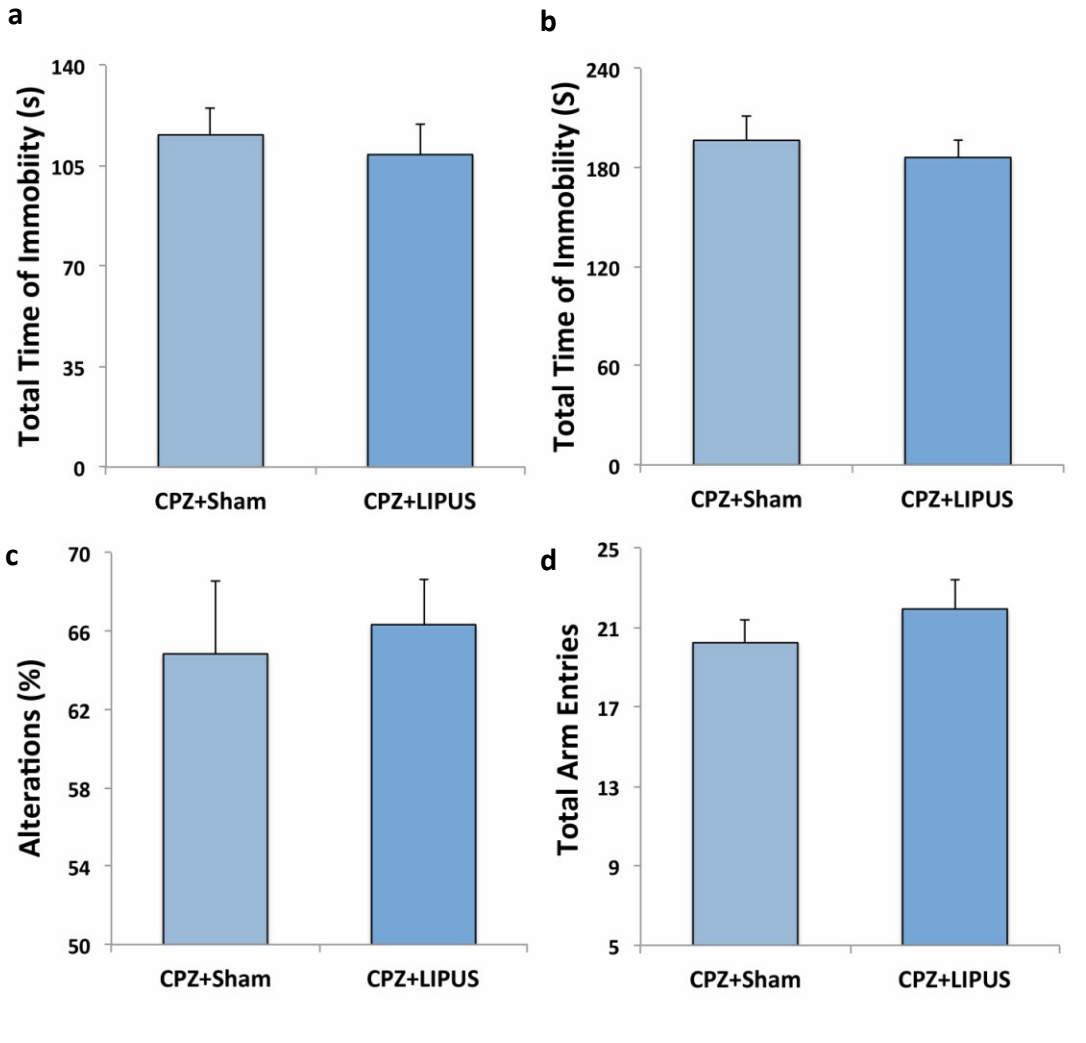


Figure 13. LIPUS did not improve mice depression-like behaviors after exposure to CPZ. a) No significant difference was found for total time of immobility in the TST between CPZ+ Sham and CPZ + LIPUS groups. b) No significant difference was found for total time of immobility in the FST between CPZ+ Sham and CPZ + LIPUS groups. c) No significant difference was found for spontaneous alterations in the YMT between CPZ+ Sham and CPZ + LIPUS groups. d) No significant difference was found for total arm entries in the YMT between CPZ+ Sham and CPZ + LIPUS groups. Values represent group mean values ± S.E.M.; n = 13-14 mice per group.

3.6 Effects of LIPUS on Brain Pathological Changes after Exposed to CPZ

3.6.1 Effects of LIPUS on the Level of MBP in Mice Exposed to CPZ

MBP is a protein marker for mature oligodendrocytes and is essential for the process of myelination (Mei et al., 2012). In the cortex of mice from CPZ + Sham and CPZ + LIPUS groups, one-way ANOVA showed changes in the level of MBP from Western blotting, $F(1, 6) = 6.589$, $p = 0.043$, indicating that LIPUS significantly increased the level of MBP in mice exposed to CPZ (Figure 14a). In subsequent IHC staining, one-way ANOVA also indicated that LIPUS significantly increased MBP-positive staining in the corpus callosum of animals exposed to CPZ (Figure 14b).

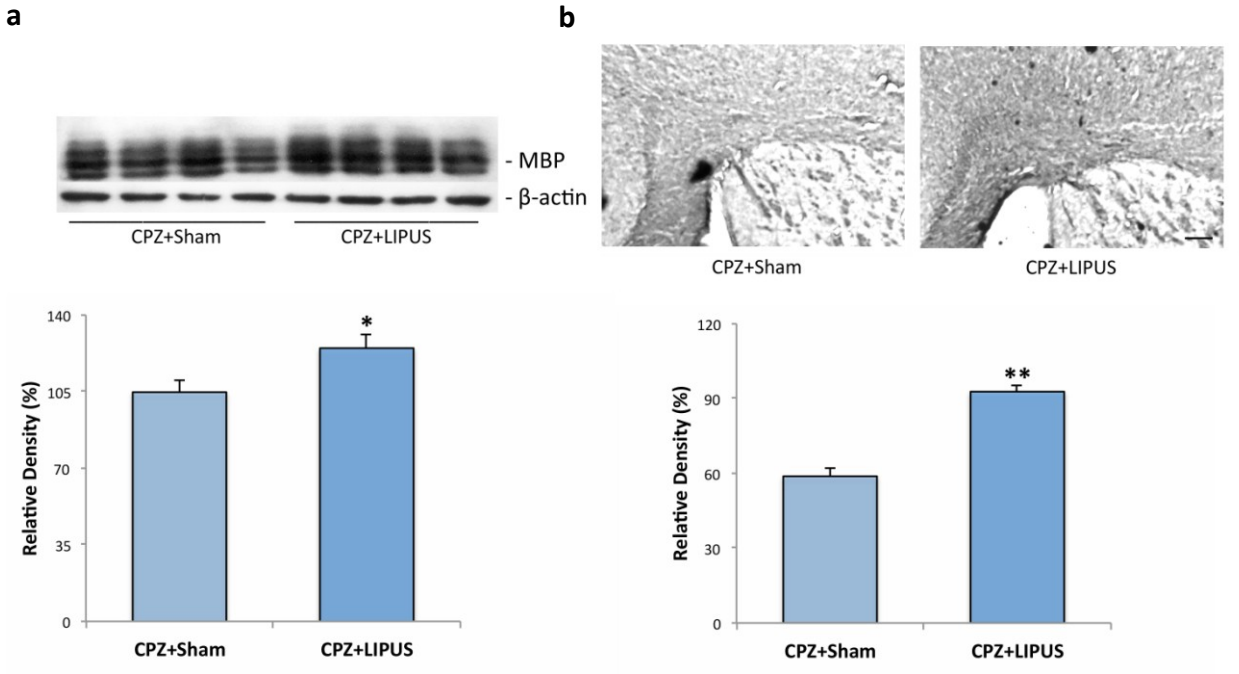


Figure 14. LIPUS increased level of MBP in mice exposed to CPZ. a) A representative Western blot image showing that the level of MBP was increased by LIPUS treatment after exposure to CPZ. Relative amounts of MBP protein in both groups are presented in the bar graph below. b) Representative immunohistochemistry staining images showing the level of MBP was increased by LIPUS treatment after CPZ exposure. Relative amounts of MBP-positive labeling in corpus callosum in both groups are presented in the bar graph below. Values represent group mean values ± S.E.M.; n = 3-4 mice per group. *$p < 0.05$ vs. CPZ+Sham; **$p < 0.01$ vs. CPZ+Sham. Bar, 150 μm.

3.6.2 Effects of LIPUS on the Level of NG2 in Mice Exposed to CPZ

NG2 is a protein marker for OPCs that are capable of giving rise to new OLs under both normal and demyelinating conditions (Wang et al., 2010). In the cortex of mice from these two groups, one-way ANOVA showed changes in the level of NG2 from Western blotting, $F(1, 5) = 11.786$, $p = 0.019$, indicating that LIPUS significantly increased the level of NG2 in mice exposed to CPZ (Figure 15a). In subsequent IHC staining, one-way ANOVA also showed that LIPUS significantly increased the positive staining of NG2 in the corpus callosum of animals exposed to CPZ (Figure 15b).

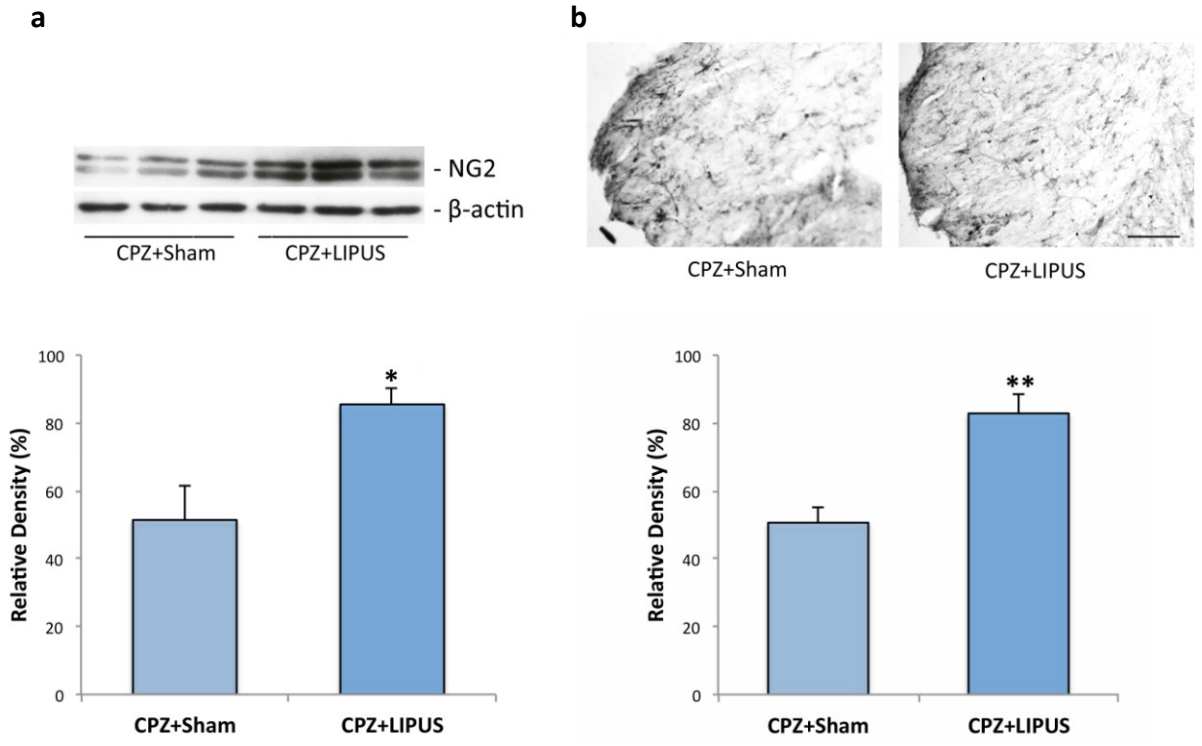


Figure 15. LIPUS increased level of NG2 in mice exposed to CPZ. a) A representative Western blot image showing the level of NG2 was increased by LIPUS treatment after exposure to CPZ. Relative amounts of NG2 protein in both groups are presented in the bar graph below. b) Representative immunohistochemistry staining images showing the level of NG2 was increased by LIPUS treatment after CPZ exposure. Relative amounts of NG2 positive labeling in corpus callosum in both groups are presented in the bar graph below. Values represent group mean values ± S.E.M.; n = 3-4 mice per group. *$p < 0.05$ vs. CPZ+Sham; **$p < 0.01$ vs. CPZ+Sham. Bar, 100 μm.

4. Discussion

The present study provides insights into the potential application of LIPUS as a non-invasive therapeutic option for depression. To the best of our knowledge, this is the first study to demonstrate that LIPUS has the ability to increase the proliferation of neuron-like SH-SY5Y cells and primary glia cells *in vitro*, as well as alleviate depression-like symptoms in mice exposed to RRS, and attenuate neurobiological changes in mice exposed to both RRS and CPZ.

Transcranial ultrasound is attracting research attention as a promising option for neuromodulation. Ultrasound was first shown to be able to excite nerve fibers in frog and turtle muscle preparations in 1929 (Harvey, 1929). Increasing evidence has shown that ultrasound can directly modulate neuronal activity in hippocampus (Tyler et al., 2008), elicit action potentials in neurons (Bachtold et al., 1998), and stimulate the motor cortex in mice (King et al., 2013) and the somatosensory cortex in humans (Legon et al., 2014). LIPUS, a specific type of ultrasound, has been shown to have an arousing effect on several types of progenitor cells, including HSP cells (Xu et al., 2012), CHO cells (Zhao et al., 2014), and osteoblast cells (Leung et al., 2004). Combined with the prompt effects of ultrasound on neurons and neural network activity (Legon et al., 2014), these studies imply that LIPUS might provide a stimulatory effect on CNS activity and thus might serve as a therapeutic option for depression, the pathogenesis of which is thought be closely associated with a decline in neuronal activity. A recent pilot study showed a potential association between ultrasound administration and elevated mood levels (Hameroff et al., 2013), and the behavior

testing results in the present study with the RRS model confirmed this causality. In the present study, histological testing revealed changes in neurogenesis, neurotrophic factors, and myelin integrity as a function of the presence of LIPUS treatment, further demonstrating the potential beneficial effects of LIPUS in depression.

4.1 Effects of LIPUS in vitro

Based on pilot tests (Supplementary Figure 1), we found that only LIPUS at intensity lower than 40 mW/cm^2 was able to promote cell growth. In the present study, we selected LIPUS at an intensity of 15 and 30 mW/cm^2 to test whether either intensity was able to exert a positive effect on the cell viability of neuron-like SH-SY5Y cells and primary glia cells. As shown in Figure 4a, LIPUS at the intensity of 15 mW/cm^2 accelerated SH-SY5Y cell growth after 3 sessions of stimulation each lasting 5 minutes over a 24-hour period. When treated with an intensity of 30 mW/cm^2, the number of cells still increased, but not as much as that of the 15 mW/cm^2 group. It can be inferred that the growth of SH-SY5Y cells can only be affected by LIPUS within a certain range of intensities, with around 15 mW/cm^2 resulting in the most significant impact. Figure 4b showed how LIPUS increased the viability of primary glia cells. With the administration of 15 mW/cm^2 for 5 minutes per 24 hours, a significant increase in cell viability occurred after 3 sessions of stimulation. When treated with 30 mW/cm^2, the number of cells still increased compared to un-stimulated control, but the increase was significantly lower than the group under 15 mW/cm^2. This phenomenon suggests that ultrasound has the ability to significantly promote primary glia cell

viability within a certain range of intensities. This is consistent with previous studies that showed LIPUS was able to influence the proliferation of several cell types (Xu et al., 2012; Zhao et al., 2014). It is important to note that the pro-proliferation effects of LIPUS stimulation are highly dependent on the cell type in terms of the intensity and duration that is required. Taking into consideration the fact that each cell type has its own individual properties with different growth environments both *in vivo* and *in vitro*, it can be concluded that LIPUS did exert a positive effect on neuron-like and glia cell viability, but parameters including stimulation intensity and duration should be appropriately tailored according to the specific cell type being stimulated.

In order to examine the potential mechanisms underlying the pro-proliferative effects of LIPUS, we tested the effects of LIPUS on the Wnt signaling pathway. The Wnt signaling pathway is a highly conserved signaling pathway implicated in nervous system development (McMahon & Bradley, 1990; Zechner et al., 2003). Disruption of this signaling pathway has been proven to be associated with several psychiatric disorders, including autism, schizophrenia, and depression (De Ferrari & Moon, 2006; Inkster et al., 2010; Lovestone et al., 2007). Studies have examined the essential role of Wnt signaling in adult neurogenesis. It has been demonstrated that Wnt is highly expressed in DG hilar cells and in cultured hippocampal astrocytes (Lie et al., 2005) and that β-catenin signaling, the intracellular signal transducer of the Wnt signaling pathway, was active in the adult SGZ and dentate granule cell layer (Mu et al., 2010). Another study showed that the activation of the Wnt/β-catenin pathway promoted the proliferation of adult neural stem cells

(NSCs) (Shi et al., 2004). Directly inducing transcription of Wnt in the neurogenesis regions of the adult brain was found to promote the proliferation of adult NSCs (Qu et al., 2010). In the present study, it was found that LIPUS at an intensity that significantly increased cell viability (15 mW/cm^2) was also able to elevate the phosphorylation of β-catenin in primary glia cells (Figure 5b), and showed a non-significant tendency to increase the phosphorylation of β-catenin in neuron-like SH-SY5Y cells (Figure 5a). This finding suggested that the positive effects of LIPUS on cell viability might be associated with the activation of Wnt pathway in these neural cells.

To further investigate the underlying mechanisms of LIPUS on cell proliferation, tests were carried out to determine whether LIPUS treatment altered the level of BDNF in cultured neuron-like SH-SY5Y cells and primary glia cells. In addition to providing neurotrophic support, BDNF has been shown to play important roles in both the developing and adult central nervous system (Emsley et al., 2005; Fawcett et al., 1998). BDNF infusion directly into the adult DG resulted in the increased neurogenesis of granule cells (Scharfman et al., 2005). An *in vitro* study showed that BDNF administration enhanced the excitatory synaptic activity of hippocampal pyramidal cells (Pozzo-Miller, 2006). Studies have also shown that increased activated astrocytes (Qu et al., 2010; Saha et al., 2006) and microglia (Miwa et al., 1997; Mizuno et al., 2005) led to an increased production of BDNF, which provides essential support for neural stem cell proliferation and differentiation. These findings suggest that BDNF plays an important role in maintaining normal cell populations in the brain. In our *in vitro* cell tests, LIPUS at the specific intensity of 15

mW/cm^2 significantly increased the level of BDNF in both neuron-like SH-SY5Y cells (Figure 6a) and primary glia cells (Figure 6b), while LIPUS at 30 mW/cm^2 also induced a significant difference in SH-SY5Y cells but not in primary glia cells, indicating a different pattern of response to LIPUS stimulation between these two cell types. Consistent with our findings, a recent study found that LIPUS at an intensity of 110 mW/cm^2 given over 2 sessions each lasting 5 minutes significantly increased the expression of BDNF in cultured astrocytes (Yang et al., 2015). In summary, the results of the present study taken together with the findings of previous research provide evidence to support the idea that LIPUS promotes cell proliferation through regulating the Wnt signaling pathway and BDNF.

4.2 Effects of LIPUS in vivo

Based on the *in vitro* findings, the study was then extended to include an examination of the effects of LIPUS on animal behavior and potential underlying mechanisms *in vivo*. A common side effect of ultrasound is over-heating the treated area due to its thermal effects (Hynynen & McDannold, 2004; Tung et al., 2006), particularly at higher intensities. HIFU, which is an abbreviation for high intensity focused ultrasound, has already been developed as a medical procedure that applies high intensity focused ultrasonic energy (commonly with an intensity over 100 W/cm^2) to locally heat and destroy diseased tissue through ablation (Kennedy et al., 2003; McDannold et al., 1998). Compared to HIFU, the thermal effects of LIPUS are far lower with very little potential for over-heating the targeted area even after a considerable time of treatment. In a pilot study with

pulsed ultrasound at intensities higher than 100 mW/cm^2, we did notice a range of side effects varying from severe to mild in the animals that received the treatment (Supplementary Figure 3). Systematic testing was then undertaken to find the intensity that both offered therapeutic effects and did not cause bodily harm to the animals. An intensity of 25 mW/cm^2 was ultimately selected to administer to the animals in the present study, with the treatment duration set as 20 minutes per day, lasting for 3 weeks. No obvious side effects were observed over the course of the entire LIPUS treatment process in the present animal study. Previous research has also found no evidence that transcranial ultrasound produced damage to the blood-brain barrier (BBB) or a change in the density of apoptotic neural cells (Tufail et al., 2010). It was also found in the same study that transcranial ultrasound did not alter brain ultrastructure or produce any gross impairment in mice behavior. Another study showed that the rate of tissue heating was slower and the likelihood of transient cavitation was reduced using pulsed ultrasound compared with continuous ultrasound (Sun & Ye, 2013). The findings of the current study combined with studies from the ultrasound literature indicate that low-intensity, pulsed, transcranial ultrasound, which can be summarized as transcranial LIPUS, is a safe and noninvasive method for animals when setting at specific parameters. However, further evaluation is still required on whether similar levels of safety and effectiveness would be observed in other animal species.

4.2.1 Effects of LIPUS in the RRS Model

RRS was employed in the present study to induce depressive-like behaviors in mice. A battery of behavioral tests was conducted to investigate whether LIPUS was able to alleviate any observed depressive symptoms. In animal studies, the key symptom for depression is anhedonia, which is defined as the inability to experience pleasure from activities normally found enjoyable. The most widely accepted behavioral test to measure anhedonia is the sucrose consumption and preference test, also known as the sucrose preference test (SPT). The decreased intake of sucrose solution in this test is regarded as a behavioral measure of anhedonia (Willner, 2005), making it the gold standard for the evaluation of depression-like behaviors (Mateus-Pinheiro et al., 2014). In the present study, mice exposed to RRS had a decreased sucrose preference index in SPT, while LIPUS treatment significantly reversed this decrease (Figure 7a). The total fluid consumption across groups showed no differences (Figure 7b). Consistent with our findings, previous studies have shown that chronic restraint stress (2 to 6 hours every day for 4 weeks) induced a decrease in sucrose preference in animals (Hageman et al., 2009; Plaznik et al., 1989) which could be reversed by daily administration of the antidepressants fluoxetine and reboxetine (Ampuero et al., 2015). Therefore LIPUS showed a comparable anti-depressive effect to these antidepressants in terms of increased anhedonia evaluated by SPT. Similar to what was found in the SPT, LIPUS also had an effect on lack of escape-related behavior associated with depression in the tail suspension test (TST) and the forced swimming test (FST) by decreasing the total time animals stayed immobile during these tests (Figure 7c and Figure 7d). Both TST and FST were designed to evaluate behavioral despair associated with depression (Bai et al., 2001; Castagne et al., 2011) and are

widely used for screening antidepressant medications. In accordance with what we found, previous studies have showed that most antidepressants, such as fluoxetine (Griebel et al., 1999; Page et al., 1999), venlafaxine (Berrocoso & Mico, 2009) and bupropion (Bourin et al., 2005), quetiapine (Kotagale et al., 2013), and neurostimulation therapies, including ECT (Teste et al., 1990), rTMS (Gur et al., 2004; Tsutsumi et al., 2002) and DBS (Hamani et al., 2010), all effectively reduce animals' total time of immobility in TST or FST. Taken together with the results of the behavioral tests in the present study showing that LIPUS decreased mice immobility time in both the TST and FST, compelling evidence has been provided for the anti-depressive effects of LIPUS treatment.

Cognitive symptoms are also quite common in patients with depression (Schiffer et al., 2009; Trivedi & Greer, 2014). The majority of effective neurostimulation therapies can improve cognitive functions as well (Guse et al., 2010; Schachter, 2004; Stoudemire et al., 1995). In the present study, spatial working memory, which represents an aspect of cognitive function, was evaluated by the YMT. It was found that mice exposed to RRS had a non-significant trend of lower spontaneous alterations (Figure 8a), which indicated poorer spatial memory, while LIPUS treatment significantly improved performance in this test, suggesting that in addition to attenuating depressive-like behaviors, LIPUS also exerted a beneficial effect on cognitive dysfunction related to depression.

To further explore the possible mechanisms underlying LIPUS effects on behaviors, we investigated several important mechanisms involved in the pathogenesis of depression. There are substantial studies implicating deficits in neurogenesis in the development of depression (Czeh et al., 2001; Petrik et al., 2012). Almost all major types of antidepressant medications (Jaako-Movits et al., 2006; Peng et al., 2008), as well as neurostimulation therapies like ECT and rTMS (Madsen et al., 2000; Ueyama et al., 2011) have been found to induce hippocampal neurogenesis in animals. To evaluate the role of neurogenesis in the present study, the level of DCX, a neuronal migration protein expressed by neuronal precursor cells and immature neurons that is considered a marker of neurogenesis (Couillard-Despres et al., 2005; Snyder et al., 2011) was examined in the *in vivo* experiment. Western blot analysis was used to compare DCX expression in the hippocampus of mice from different experimental groups. It was found that the mice exposed to RRS had a trend towards a significant decrease in levels of DCX ($p = 0.090$) compared with the control group, while LIPUS treatment significantly reversed this decrease. This finding was further confirmed by the quantitative protein analysis of DCX (Figure 10a). The results also showed that the LIPUS only group had a significantly higher level of DCX than normal controls, indicating that LIPUS also promoted neurogenesis in regular animals (Figure 10a). The potential mechanism of this phenomenon still needs to be elucidated, but could lead to potential applications of LIPUS to facilitate neurogenesis in other neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Winner et al., 2011).

Similar to what was found in the present study, other research has shown that most antidepressants are also able to increase hippocampal neurogenesis by promoting proliferation of adult hippocampal neural stem cells. A study using chronic daily administration of fluoxetine observed an increase in neurogenesis and dendritic spine density in the mouse hippocampus (Ohira et al., 2013). Another study revealed that the decreased hippocampal neurogenesis following olfactory bulbectomy, a lesion animal model of depression, was reversed by repeated citalopram administration (Jaako-Movits et al., 2006). Studies examining alternative depression treatments found that both consecutive administration of ECT (Madsen et al., 2000) and rTMS (Ueyama et al., 2011) increased the number and dendritic complexity of adult-born migrating neuroblasts. Results of the present study demonstrated that LIPUS also has the capacity to promote hippocampal neurogenesis. These results suggest that the beneficial effects of LIPUS treatment on animal behavior are associated with promotion of adult hippocampal neurogenesis.

A subsequent analysis of hippocampal proteins from mice exposed to RRS with or without LIPUS did not yield any significant differences in the level of pβ-catenin across experimental groups (Figure 11), compared with the *in vitro* tests. It could be speculated from these results that the activation of the Wnt signaling pathway by LIPUS is a short-term effect (Olkku et al., 2010) that could lead to a long lasting consequences with respect to neurogenesis after RRS exposure.

Emerging evidence has suggested that BDNF plays an important role in the pathophysiology of depression (Altar, 1999; Angelucci et al., 2005; Bocchio-Chiavetto et al., 2010). Several classes of

antidepressants have been shown to be able to increase BDNF expression in animal brains (Matrisciano et al., 2009; Molteni et al., 2009). Similar effects were also observed in studies on ECT and rTMS (Marano et al., 2007; Zanardini et al., 2006). To investigate the involvement of BDNF in the present study, we examined the levels of BDNF in mice from different experimental groups. The results showed LIPUS treatment significantly elevated the level of BDNF in the brains of the mice in both control animals and animals exposed to RRS (Figure 12a). Compared to the RRS group, the expression of BDNF in RRS + LIPUS group was not significantly higher, although there was a non-significant tendency for an increase. These results support the idea that the beneficial effects of LIPUS on mice exposed to RRS should be associated with the elevation of BDNF. This is in line with our *in vitro* findings that LIPUS increased the level of BDNF in both neuron-like SH-SY5Y cells and primary glia cells. Similar to our findings, BDNF levels in the hippocampus of mice have been reported to be increased after treatment with chronic administration of the antidepressant escitalopram (Doron et al., 2014). Chronic administration of the antidepressants duloxetine and mirtazapine was shown to up-regulate BDNF expression in the mouse hippocampus (Engel et al., 2013). Studies have also shown that the remission of depression following ECT was associated with higher levels of BDNF (Freire et al., 2016) and there was a strong association between the effectiveness of rTMS and elevated BDNF (Gedge et al., 2012). The evidence provided by the literature in combination with our findings indicate that LIPUS exerts a positive effect on BDNF expression in depression, which is comparable to that observed after administration of classic antidepressants and neurostimulation therapies.

Interestingly, we did not observe a significant reduction of BDNF levels in mice exposed to RRS (Figure 12a). Contradictory evidence exists regarding the change of BDNF expression after restraint stress. Previous studies have shown that chronic restraint stress for 6 hours a day, continuously for 3 weeks did not change the level of BDNF in hippocampus (Kuroda & McEwen, 1998; Yamaura et al., 2013). Nevertheless, another study found that 1-hour daily restraint for 3 weeks led to a relative increase of plasma BDNF in animals, in spite of the high level of corticosterone (Maghsoudi et al., 2014), indicating incongruity in the relationship between repetitive restraint stress and BDNF expression. The results of the current study seem to fit into this inconsistency. Considering the fact that RRS is not as severe as other acute stressors, differences between the applied stress paradigms in duration or type could be the explanation for the discrepancy. Further studies examining the effect of LIPUS on BDNF expression using other animal models of depression induced by chronic stress, such as unpredictable chronic mild stress (UCMS), which is more correlated with actual human patients' experience than the predicted stress model (Forbes et al., 1996; Gumuslu et al., 2014; Willner, 1997), need to be carried out to better understand the relationship.

4.2.2 Effects of LIPUS in the CPZ Model

The CPZ model was introduced in the present study to evaluate the effects of LIPUS on myelin and OLs in depression. The results of our *in vivo* study showed that LIPUS did not significantly attenuate the depression-like behaviors of mice after exposure to CPZ, as tested by the TST, the

FST, and the YMT. A possible explanation for the lack of significant differences observed here is that LIPUS could most likely be considered as a mild treatment, while CPZ is capable of producing extensive demyelination in the CNS and inducing severe behavioral and neurobiological dysfunction that LIPUS may not be able to ameliorate appreciably (Hibbits et al., 2009; Torkildsen et al., 2008). Future studies should compare the anti-depressive efficacy of LIPUS with other standardized therapeutic options, including medications and neurostimulations.

MBP plays a crucial role in the process of myelination and is a marker for mature OLs. From the Western blotting protein analysis and subsequent immunohistochemistry staining in the present study, it was found that LIPUS significantly increased the level of MBP after CPZ exposure (Figure 14a and 14b), indicating that LIPUS exerted a protective effect on mature myelin and OLs against CPZ toxicity. Previous studies from our group found that MBP was significantly decreased in animals exposed to CPZ for 6 weeks (Xiao et al., 2008). This decrease was reversed by chronic treatment with quetiapine, an antipsychotic that also has antidepressant properties. Quetiapine has been found to be effective both when administered as a preventative measure introduced at the same time as CPZ exposure has begun (Zhang et al., 2008), and as a treatment administered after ongoing CPZ exposure (Zhang et al., 2012). Another study showed the CPZ-induced decrease in MBP was attenuated by chronic treatment with rolipram, a selective phosphodiesterase-4 inhibitor discovered and developed as a potential antidepressant (Sun et al., 2012). Rolipram was also found to attenuate deficits in MBP in the experimental autoimmune encephalomyelitis (EAE) animal

model (Jung et al., 1996), a popular model of demyelination in the CNS (Gold et al., 2000). The findings of the present study fit in well with those in the literature, suggesting that the beneficial effects of LIPUS treatment are, in part, associated with the protection of myelin and OLs.

NG2 is an integral membrane proteoglycan found in several progenitor cell populations including OPCs. The loss or lack of OPCs, and consequent lack of differentiated OLs, is associated with loss of myelination, less support of axons, and subsequent impairment of neurological functions. Studies have shown the expression of NG2 was significantly disturbed in demyelinating animal models including CPZ exposure (Tsiperson et al., 2015) and EAE (Reynolds et al., 2002; Tripathi et al., 2010). Previous studies from our group found that this disruption in NG2 expression was attenuated by treatment with quetiapine (Zhang et al., 2012), fluoxetine (data not published) and rTMS (manuscript in preparation). Similarly, the present study found that LIPUS significantly increased the level of NG2 in the brains of mice exposed to CPZ (Figure 15a). The subsequent immunohistochemistry staining revealed that NG2 positive cells in mice after LIPUS administration significantly out-numbered those in mice without LIPUS (Figure 15b). Previous studies have found that NG2-positive cells were altered in depression (Elsayed et al., 2012) and that consecutive ECT treatment dramatically increased the proliferation of NG2-expressing glia cells in animals' hippocampus (Wennstrom et al., 2003) and amygdala (Wennstrom et al., 2004). The results from previous studies in combination with those from the present study provide support for the theory that promotion of OPCs plays a part in the beneficial effects of LIPUS.

Furthermore, since OLs serve a multitude of functions in the CNS, including providing structural support, supplying nutrients to neurons, aiding in myelin formation, and maintaining homeostasis (Beasley et al., 2009; Tham et al., 2011), follow-up specific investigations into the detailed changes of OLs after certain external stressors like CPZ and the effects of treatments like LIPUS should be carried out in an effort to better understand the pathophysiology of myelin changes in depression.

4.3 Potential Mechanisms for the Beneficial Effects of LIPUS

The potential mechanisms underlying LIPUS stimulation of the brain remain to be clarified. However, two major categories of mechanisms have been implicated: thermal and mechanical. With respect to thermal effects, ultrasound stimulation is known to be able to heat the treated area (O'Brien, 2007), and the increased temperature should be capable of activating neurons with temperature-sensitive ion channels (Benham et al., 2003; Patapoutian et al., 2003). However, measurement of temperature in neural tissue during the low-intensity ultrasound administration has shown a negligible temperature increase (Tufail et al., 2010; Yoo et al., 2011). Although we did not carry out temperature measurements in the present study, the effects of temperature change after low-intensity ultrasound stimulation have been calculated using the standard equations of ultrasound physics (O'Brien, 2007; Tufail et al., 2010), and the temperature changes generated with LIPUS should have been below 0.1°C (King et al., 2013), which are considered as negligible to produce changes in neuronal activity.

Therefore, the alternative explanation regarding the possible effects of ultrasound must be a mechanical process. This processes involved in ultrasound include mainly cavitation, which entails the formation and collapse of micro-bubbles created by ultrasound in the liquid-solid interface (Khraiche et al., 2008; Krasovitski et al., 2011) and acoustic radiation force, a non-cavitation phenomenon associated with the spread of acoustic waves through an attenuating medium (Wood & Loomis, 1927). These acoustic waves could act on the neuronal cell membrane and proteins embedded in the membrane, impacting the ion channel kinetics and membrane dynamics, which could then promote depolarization and alter resulting action potentials (Tyler, 2011). Thermodynamic research has shown that mechanical waves like ultrasound can be propagated through neuronal membranes, to influence fluidity and excitability of the cells (Heimburg, 2010; Heimburg & Jackson, 2005). Such sound waves have been estimated to be able to produce depolarizing potentials from 1 to 50 mV, due to the viscosity of the monolayers and their surrounding environment (Griesbauer et al., 2009). In this way, ultrasound may be able to initiate mechanical waves into the neuronal membranes to sufficiently depolarize the neurons and activate ion channels triggering action potentials (Tufail et al., 2011).

Low-intensity ultrasound is also highly correlated with cavitational effects (Edmonds & Ross, 1988; Feril & Kondo, 2004). Cavitation is a complex phenomenon with two major forms: inertial and non-inertial. Inertial cavitation requires higher energy and is known to be associated with tissue damage (Miller, 2007; Miller et al., 1996). Non-inertial cavitation that occurs in mammalian

tissue at intensities lower than 3 W/cm^2 can lead to stable micro-bubble production (ter Haar et al., 1982; ter Haar & Daniels, 1981; Ter Harr et al., 1986), which has potential for impacting on substrate transmission and membrane permeability. The intensities of LIPUS used in the present study were within this range, indicating that cavitation could be playing a role in the neurostimulative effects of LIPUS that were observed.

In addition, LIPUS stimulation was able to transfer momentum into the tissue and cellular fluid, which could result in nutrient redistribution, influence homeostasis, and trigger a biological response in the treated area (Padilla et al., 2014). The cavitational micro-bubbles can cause circulatory movement of the fluid and modulate the extracellular matrix, activating mechanoreceptors on the cell membrane (Krasovitski et al., 2011). Studies have also suggested that LIPUS could potentially initiate intracellular displacements (Or & Kimmel, 2009), interacting with signaling peptides and proteins, leading to accelerated cytoskeleton-remodeling events (Mizrahi et al., 2012). At lower intensities like LIPUS, intracellular molecular changes including molecule-to-molecule or molecule-to-solvent interactions can be promoted by ultrasound stimulation, which could then modify the biological functions of the cytoskeletal proteins (Johns, 2002). The possible explanation for this phenomenon is thought to be due to the force induced by LIPUS that is large enough to disrupt the weak nonspecific intracellular bonds, alter conformation of proteins and trigger cytoskeleton remodeling (Padilla et al., 2014).

Although ultrasound has been shown to have beneficial effects, it is important to set standards and safety margins for its application. Repeated ultrasound exposure has previously been shown to disrupt normal neuronal function (Ang et al., 2006). Therefore the potential damage caused by repeated, long-term LIPUS administration should be evaluated. In terms of further application of LIPUS in human patients, careful consideration needs to be taken regarding such factors as the size of the human brain and the thickness of the skull compared to that of a mouse, as they might present an obstacle for effective ultrasound transmission. The parameters of LIPUS should be adjusted to harness the positive biological effects without causing tissue damage (Leinenga & Gotz, 2015). One of the several cranial windows, for example, the temporal window, could be considered as a way for ultrasound to access the human brain (Deckers & Moonen, 2010). This approach could facilitate the development of an advanced delivery method for ultrasound treatment (Jolesz & McDannold, 2014; Rajasethupathy et al., 2016), but the safety of which should be monitored in real time.

4.4 Future Study Plan

The major limitation of the current study lies in its design as an exploratory study. Therefore the results obtained during the study were still preliminary. Followup experiments can be further processed in each aspect of the current study, for more thorough and detailed demonstration of the effects of LIPUS on cellular testing, animal behaviors and changes of neurogenesis, neurotrophy, and white matter. Despite the unanswered questions in the present study regarding its exact

mechanisms of action, LIPUS still represents a promising new therapeutic option for depression. Further studies are necessary to first explore in detail the potential mechanisms underlying the capability of LIPUS to stimulate neuronal activity and induce anti-depressive effects. A detailed explanation of why LIPUS can produce such neurobiological change should be provided from more detailed *in vitro* cell studies. Meanwhile, the positive effects of LIPUS on depression in the RRS model presented in the current study should be validated in other animal models of depression, such as UCMS, to provide extra pre-clinical data that further confirm the potential of LIPUS as a treatment for depression. In addition, the antidepressive efficacy of LIPUS should be systematically evaluated through the comparison with other standardized treatment options for depression. Moreover, considering the stimulatory effects of LIPUS on neurogenesis and gliogenesis, additional studies on the application of LIPUS in other psychiatric and neurological disorders like Alzheimer's disease, multiple sclerosis, and schizophrenia, should be carried out to explore whether LIPUS can work as a therapeutic option for these diseases as well. The ultimate goal for a therapeutic intervention is for clinical use. With the development of advanced delivery methods for LIPUS in the human population, the efficacy of LIPUS treatment in patients with depression should be thoroughly investigated. And this eventual clinical study should be accompanied with more innovative LIPUS devices, for example wearable, intensity adjustable devices, and with real-time feedback during the treatment.

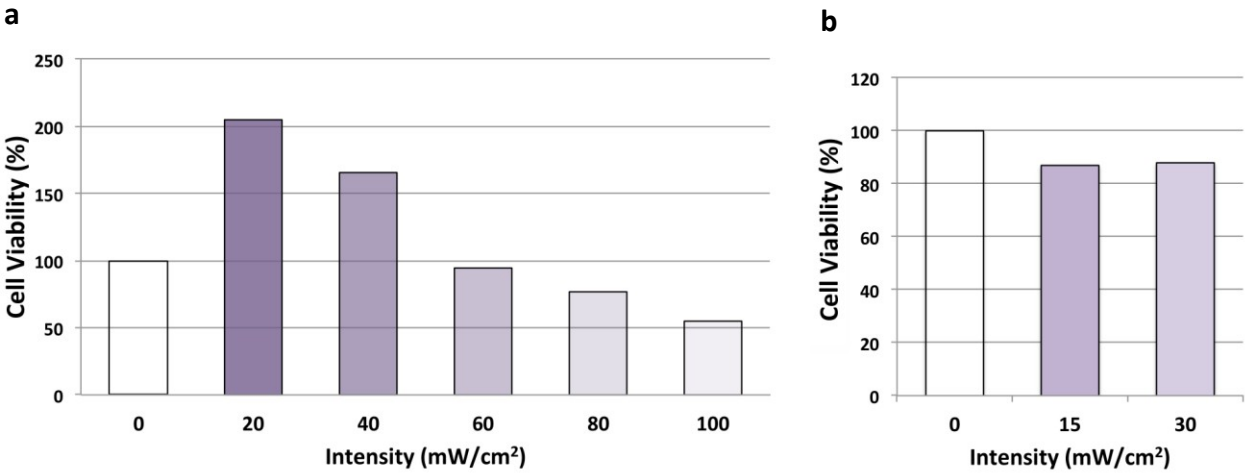
Conclusion

The present study provided evidence that LIPUS was able to significantly alleviate depressive behaviors in mice after exposure to RRS, indicating that LIPUS could potentially work as a treatment for depression. Results from *in vivo* and *in vitro* experiments suggested the possible mechanisms for the beneficial effects of LIPUS on depression were the promotion of neurogenesis and the increase of BDNF. Pathological tests revealed that the protection and promotion of myelin and OLs might also be the therapeutic targets for LIPUS. Although the results were unable to provide further information on the exact mechanisms through which LIPUS was working, they did offer support for LIPUS as a promising therapeutic option for depression. The results of the present study also served to highlight the importance of neurogenesis and neurotrophy, myelin and OLs in the pathogenesis of depression.

Supplementary

Dose-response for Proper LIPUS Administration in Cell Study

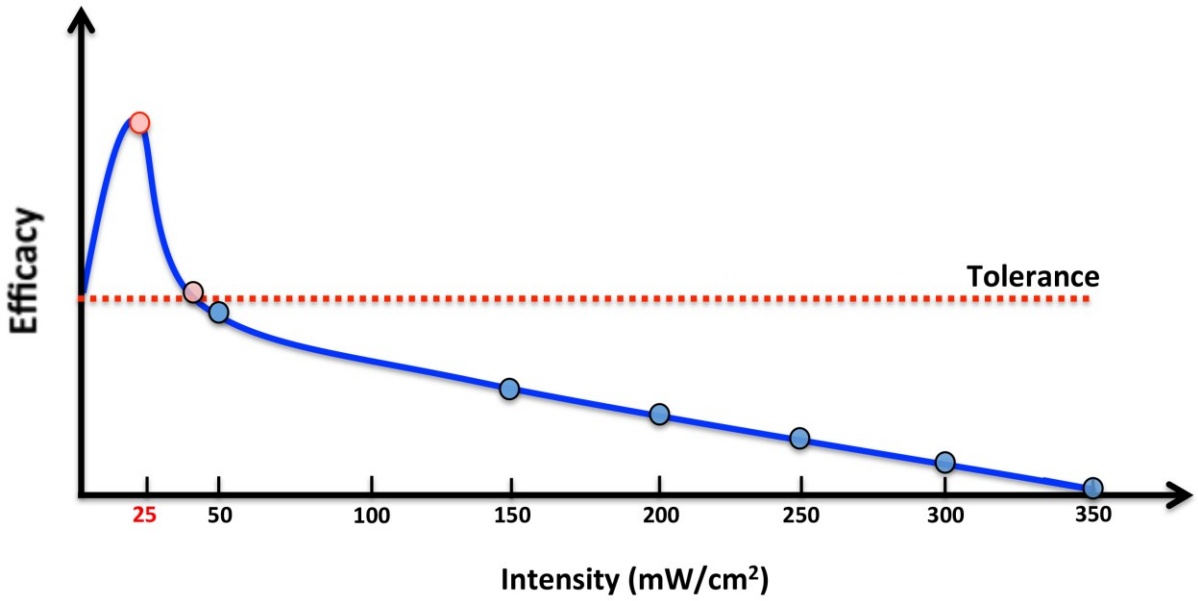
Pilot trials for the cell study were performed to determine the appropriate intensities and duration of LIPUS stimulation. Tests with LIPUS at variable intensities for 5 minutes stimulation per 24 hours showed that LIPUS at an intensity higher than 40 mW/cm^2 induced a reduction in cell viability in SH-SY5Y cells (Supplementary Figure 1a), while LIPUS at an intensity lower than 40 mW/cm^2 relatively increased cell viability. Taking into consideration the range of responses of cells to LIPUS stimulation, 15 mW/cm^2 and 30 mW/cm^2 were selected for further cell testing. Longer durations of LIPUS treatment showed that stimulation for 15 minutes (a relatively long stimulation session) per 24 hours, for 3 sessions in total, did not produce positive effects on cell viability in SH-SY5Y cells (Supplementary Figure 1b). Combined with the positive effects of LIPUS stimulation for 5 minutes (see Figure 4), the treatment duration of 5 minutes per 24 hours, for 3 sessions in total was ultimately decided upon for further cell testing.



Supplementary Figure 1. LIPUS at higher intensity or longer treatment did not increase cell viability of SH-SY5Y cells. a) LIPUS at the intensity of 20 mW/cm^2 and 40 mW/cm^2 for 5 minutes per 24 hours increased cell viability, while LIPUS at the intensity of higher than 40 mW/cm^2 decreased cell viability. b) LIPUS treatment at both 15 mW/cm^2 and 30 mW/cm^2 for 15 minutes per 24 hours did not increase cell viability.

Dose-response Curve for Proper LIPUS Intensity in Animal Studies

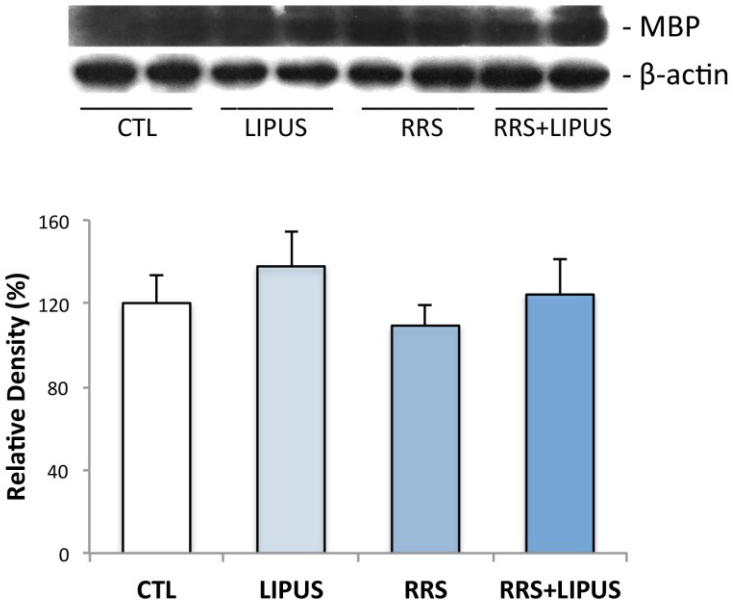
Pilot trials in animal studies were performed to determine the appropriate intensity of LIPUS stimulation. Tests of LIPUS at variable higher intensities showed that LIPUS at an intensity of 350 mW/cm^2 for 5 minutes directly resulted in the death of the animal and was obviously way beyond the animal's tolerance level. LIPUS at 300 mW/cm^2 for 5 minutes induced a moribund state of the mouse that received the stimulation and later required euthanasia of the animal. LIPUS at 250 mW/cm^2 for 5 minutes caused damage to the outer ear of the mouse. LIPUS at the intensity of 200 mW/cm^2 for 5 minutes induced a 3-day-delayed ear-wound after administration. LIPUS at 150 mW/cm^2 for 5 minutes caused a 7-day-delayed ear wound after one session of administration. All of these intensities were considered well above the animals tolerance range (Supplementary Figure 2). Taking the findings from the high intensity testing into account, tests with much lower intensities of 25 mW/cm^2 and 50 mW/cm^2 were then carried out. The results showed LIPUS at 50 mW/cm^2 for 20 minutes every day caused mouse hair loss around the area where the transducer was attached. After reducing the intensity to 40 mW/cm^2, the hair-loss problem was still existent but to a lesser degree. All mice treated with 25 mW/cm^2, for 20 minutes every day were in good condition during the pilot test, with no obvious side effects. Therefore LIPUS at 25 mW/cm^2 and 20 minutes per day was selected for further animal studies (Supplementary Figure 2).



Supplementary Figure 2. LIPUS at higher intensities were not tolerated by animals. LIPUS at the intensity not exceeding 50 mW/cm^2 could be tolerated by animals. LIPUS at the intensity of 25 mW/cm^2 was ultimately selected for further animal studies. The intensity-efficacy curve is shown in the graph, with blue and red dots representing treatment outcomes at different intensities.

Changes in the Level of MBP in RRS Mice with or without LIPUS

With cortical tissue from mice in different groups in the RRS study, two-way ANOVA showed that no changes in the level of MBP, $F(1, 10) = 1.157$, $p = 0.307$ for RRS group, $F(1, 10) = 0.646$, $p = 0.440$ for LIPUS group and $F(1, 10) = 0.008$, $p = 0.931$ for the interaction between RRS and LIPUS. *Post-hoc* analysis indicated that there was no obvious difference in the level of MBP among these groups (Supplementary Figure 3). But mice exposed to RRS did show a non-significant tendency of decrease in MBP, while RRS + LIPUS group had a non-significant tendency of increase (Supplementary Figure 3). These results identify the necessity to proceed with the CPZ-induced demyelination model to further investigate the potential effects of LIPUS on myelin and OLs as therapeutic targets, through exaggerating myelin and OL deficits induced by CPZ.



Supplementary Figure 3. No obvious difference in the level of MBP was found in mice exposed to RRS and that received LIPUS. A representative Western blot image showing no obvious difference was found across experimental groups. Relative amounts of MBP protein in all groups are presented in the bar graph below. Values represented group mean values ± S.E.M.; n = 3-4 mice per group.

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