NOVEL APPROACHES TO THE NEUROPHARMACOLOGY OF MOOD DISORDERS

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NOVEL APPROACHES TO THE NEUROPHARMACOLOGY OF MOOD DISORDERS

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EDITORIAL
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Editorial: Novel Approaches to the Neuropharmacology of Mood Disorders

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Editorial on the Research Topic

Novel Approaches to the Neuropharmacology of Mood Disorders

These are exciting times for research on the pharmacology of mood disorders. The "omics" revolutions, the development of noninvasive neuroimaging techniques, the definition of biomarkers, and studies in animal models have all partially redefined the field and contributed to the boom in research articles focusing on novel approaches to the neuropharmacology of both major depressive disorder and bipolar disorder. Recent studies on the fast antidepressant effects of ketamine have also brought this topic to the mainline press, where the effectiveness (or lack of) of psychopharmacological drugs targeting mood disorders has been discussed multiple times.

Although management of mood disorders does not solely imply the use of a psychopharmacological approach, the prescription of antidepressants and mood stabilizers represents the mainstay of the standard of care for the treatment of mood disorders.

This research topic is a collection of reviews and original research articles that focus on the study of mechanistic approaches to decipher specific actions of currently used drugs, and on evaluating possible therapeutic interventions by acting on novel pharmacological targets and analyzing signal transduction pathways that may be involved in mediating the effects of drugs acting on those targets. The topic also collects a series of articles devoted to the analysis of different biomarkers that could be used to predict the therapeutic efficacy of specific pharmacologic treatments particularly in the case of treatment-resistant depression.

The effects of inflammatory processes in mood and anxiety disorders and the possible efficacy of current and novel psychopharmacological approaches in tackling mood disorders symptoms by acting on peripheral and/or central inflammatory events are evaluated in three contributions to the present topic: First, Zhang et al. study the effects of the dual serotonin and norepinephrine reuptake inhibitor venlafaxine in reversing the deficits in cognition and depressive-like behavior induced by cuprisone treatment in rodents, by attenuating demyelination and neuroinflammation. Then, review by Brymer et al. examines the putative antidepressant mechanisms of anti-inflammatory drugs that target tumor necrosis factor alpha (TNFα) and explains how both peripheral and central anti-inflammatory mechanisms may be operative in fostering the antidepressant effects of these drugs. Finally, Nisbett and Pinna contribute an opinion article on how fostering the function of the peroxisome proliferator-activated receptor alpha (PPARα) brings about a decrease in proinflammatory cytokines, and focus on the effects of cannabinoids on PPARα in the context of posttraumatic stress disorder (PTSD). These three contributions emphasize how the anti-inflammatory effects of current antidepressants, like venlafaxine, or of anti-inflammatory drugs, like etanercept [an antagonist of tumor necrosis factor alpha (TNFα)], that have been shown to exert antidepressant effects, or those related to

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novel targets, like PPARα, may be essential for their therapeutic effects on mood disorders and underline how understanding the mechanistic implications of inflammatory processes in mood disorders may give some clues both to better understand the neurobiology of these disorders and to develop novel and more efficacious drugs.

Another two contributions center on the analysis of circuit and/or molecular mechanisms that can relate to the therapeutic actions of psychopharmacological interventions on mood and anxiety disorders: An original research article by Zhang et al. describes how overexpression in the hippocampal dentate gyrus of the translocator protein of 18 kDa results in anxiolytic effects in an animal model of PTSD and discusses the roles of hippocampal neurogenesis in the formation and maintenance of emotional memories that also pertain to the neurobiology of major depression and bipolar disorder, as it has been proposed in multiple occasions that rescuing of hippocampal neurogenesis may be a mechanism by which antidepressant drugs may reverse some key symptoms in depression. A second original report, authored by Park et al., investigates the actions of liraglutide (a glucogen-like peptide 1 receptor agonist) on mammalian target of rapamycin (mTOR)-mediated signal transduction pathways and on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activity in hippocampal cell cultures treated with dexamethasone, and its impact on brain-derived neurotrophic factor (BDNF) expression, dendritic outgrowth, and spine formation, which results of clear interest when considering that the fast antidepressant actions of ketamine appear to be based on its effects on all these factors.

A review by Senese et al. recapitulates their results on the direct effects of antidepressant drugs on regulating specific components of G-protein coupled receptors systems, particularly their effects on reversing the increase in G protein coupled receptor (GPCR) subunit localization and clustering into lipid-raft microdomains observed in depression, which they propose are essential for the elucidation of the antidepressant effects of current antidepressant drugs. Interestingly, an original report by Romay-Tallon et al. also analyzes the clustering of GPCRs and other proteins in the plasma membrane of lymphocytes in the repeated-corticosterone model of depression, and shows how analysis of this clustering patterns resembles those observed in depression patients and could be considered as putative biomarkers of therapeutic efficacy in major depression, an aspect that is further discussed and analyzed in a review article by Caruncho et al. These three contributions thereby point toward the importance of the patterns on membrane proteins distribution within specific membrane domains as putative key issues in the neurobiology of depression, and foster the design of additional studies on membrane protein clustering not only to develop novel biomarkers but also as potential drug targets.

The last two contributions to the topic describe original research on biomarkers of therapeutic efficacy for treatment-resistant depression: Veldic et al. describe a pharmacogenomic approach to the analysis of cytochrome P450 2C19 variants in relation to their metabolizer phenotype in treatment-resistant depression and evaluate the implications of their results in terms of differential diagnosis between major depression and bipolar disorder, as well as their implications for pharmacological therapeutics in depression. Finally, Shalbaf et al. evaluate how non-linear entropy analysis of electroencephalography (EEG) can be developed as a biomarker to predict the therapeutic response to repetitive transcranial magnetic evaluation in treatment-resistant depression. Their results indicate the interest of additional EEG studies to validate this approach and thereby contribute to a better management of the use of nonpharmacological therapeutic strategies for treatment-resistant depression.

Overall, the contributions to this topic present a wide-scope and multidisciplinary approach that is essential when evaluating novel pharmacological strategies for the treatment of mood disorders.

AUTHOR CONTRIBUTIONS

HC wrote the editorial. LK, ML, and JO read and approved the text.

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ORIGINAL RESEARCH
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Venlafaxine Improves the Cognitive Impairment and Depression-Like Behaviors in a Cuprizone Mouse Model by Alleviating Demyelination and Neuroinflammation in the Brain

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Growing evidence has implicated that myelin deficits and neuroinflammation are the coexisted pathological features that contribute to the mood swing and cognitive decline in major depressive disorder (MDD) and multiple sclerosis (MS). Therefore, attenuation of neuroinflammation and reduction of demyelination became newly emerging treatment strategies for the mood and cognitive symptoms. Antidepressant venlafaxine has been used in depression and anxiety through its multiple neuroprotective effects. However, it is unclear whether venlafaxine can improve myelin integrity and alter inflammation status in the brain. By using a well-established cuprizone-induced acute mouse model of demyelination, we investigated the protective effects of venlafaxine on these facets. The cuprizone-fed animals exhibited cognitive impairment and mood disturbances together with myelin loss and prominent neuroinflammation in the brain. Our present study showed that a high dose of venlafaxine alleviated the loss of myelin and oligodendrocytes (OLs), mitigated depression-like behaviors, and improved cognitive function in cuprizone-fed animals. Data from the present study also showed that venlafaxine reduced microglia-mediated inflammation in the brains of cuprizone-fed animals. These findings suggest that venlafaxine may exert its therapeutic effects *via* facilitating myelin integrity and controlling neuroinflammation, which may provide extra benefits to MS patients with depression and anxiety beyond the symptom management.

Keywords: venlafaxine, cuprizone, demyelination, cognition, major depressive disorder, oligodendrocyte, microglia

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INTRODUCTION

Major depressive disorder (MDD) affects around 300 million people worldwide and becomes a leading burden of the economy (Kassebaum et al., 2016). The core symptoms of MDD include depressed mood, lack of interest, difficulties in concentration, changes in appetite and sleep, and cognitive impairment (APA, 2013). The treatment for depression is challenging as approximately 30% of MDD patients develop treatment-resistant depression (TRD) despite subsequent antidepressant augmentations or switches (Rush et al., 2006). The development of TRD is mainly due to our limited understanding of the neurobiology of MDD and the action mechanism of antidepressant treatment (Huezo-Diaz et al., 2005). It was supposed that antidepressants exert their acute effects primarily by increasing the availability of serotonin (5-HT), norepinephrine (NE), or both in the synaptic cleft (Berton and Nestler, 2006). Although antidepressants immediately increase the monoamine levels in the brain, it takes at least 2–3 weeks for the occurrence of mood-enhancing effects, thereby indicating other mechanisms may contribute to its efficiency.

MDD is considered a complex brain disorder resulting from abnormal brain structural and functional connectivity (Gong and He, 2015). White matter tracts are critical for interconnecting brain regions and transferring the neural activities essential for organizing human behavior, emotions, and cognition (Filley and Fields, 2016). Neuroimaging studies have shown white matter abnormalities in the brains of patients with MDD (Yamada et al., 2015; Cyprien et al., 2016). Oligodendrocytes (OLs) are residential glial cells responsible for generating myelin sheaths and white matter tracts in the central nervous system (CNS) (Nave and Werner, 2014). Abnormalities in myelin and OL are associated with cognitive impairment and increased suicide attempts in MDD patients (Yamada et al., 2015; Cyprien et al., 2016).

Multiple sclerosis (MS) is a demyelinating inflammatory disorder. Over half of MS patients exhibit MDD comorbidity (Feinstein, 2004), thereby suggesting some biological abnormalities (e.g., deficits of myelin and OL and neuroinflammation) may coexist in MDD and MS (Morris et al., 2018). While a variety of treatments including antidepressants, psychotherapy, and neuromodulation have shown therapeutic effects due to the improvement of the white matter structure and function (Mostert et al., 2006; Zeng et al., 2012; Wang et al., 2013; Anderson et al., 2016), cases with severe white matter changes showed poor responses to antidepressant treatment (Peng et al., 2013; Serafini et al., 2015). Together, these findings suggest white matter/myelin integrity is a new treatment target for MS and MDD, especially for treatment-resistant depression (Serafini et al., 2015).

Venlafaxine, a serotonin and noradrenaline reuptake inhibitor (SNRI) antidepressant, has been widely used for MDD, anxiety, and neuropathy (Tundo et al., 2015; Waldfogel et al., 2017). Venlafaxine is also a preferred choice for monotherapy or combination treatment for TRD (Tundo et al., 2015). Studies found that venlafaxine possesses the neuroprotective effects *via* its anti-inflammatory activities (Xu et al., 2006; Chen et al., 2018). Our previous research showed that desvenlafaxine, a major active metabolite of venlafaxine, prevented stress-induced white matter injuries in mice (Wang et al., 2014). However, it is unknown whether venlafaxine can provide neural protections by acting on OLs and preserve myelin integrity. The cuprizone-induced demyelinated mice showing white matter deficits and cognitive and emotional impairments with minimal motor function deficits (Zhang et al., 2012; Yan et al., 2015) are a suitable animal model in our present study to explore the effects of venlafaxine on behavioral abnormalities, myelination/OL deficits, and neuroinflammation.

MATERIALS AND METHODS

Animals

Seven-week-old female C57BL/6 mice were purchased from Charles River (Montreal, Canada) and hosted in the animal facility maintained at a 12-h/12-h light–dark cycle, at 22 ± 0.5°C and 60% humidity, with *ad lib* to food and water. All animal procedures were performed under the Canadian Council on Animal Care (CCAC) guidelines and were approved by the University Committee on Animal Care and Supply (UCACS) of the University of Saskatchewan. Mice develop selective central demyelination and inflammation in the prefrontal cortex, hippocampus, and the corpus callosum (CC) after 5 weeks of CPZ treatment (Zhang et al., 2008).

Drug Treatment

Cuprizone (CPZ, Sigma-Aldrich, St. Louis, MO, USA) was mixed into the milled Lab Diet rodent chow (PMI Nutrition International LLC, Brentwood, MO, USA) with a final concentration of 0.2% (w/w), as previously described (Zhang et al., 2008). Venlafaxine (Pfizer, Montreal, Canada) was dissolved in distilled water. After acclimatization with the regular rodent diet for 1 week, mice were divided into six groups (16 mice per group). Mice in the first three groups received regular chows plus daily treatment with either water (CTL), or 5 mg/kg/day of venlafaxine (VEN5), or 20 mg/kg/day of venlafaxine (VEN20) for 5 weeks. The remaining groups received rodent chows containing 0.2% CPZ (w/w) plus daily treatment with either water (CPZ), or venlafaxine 5 mg/kg/day (CPZ + VEN5), or venlafaxine 20 mg/kg/day (CPZ + VEN20) for 5 weeks. Body weight was measured twice weekly. Behavioral tests were performed to evaluate depression-like behaviors and working memory during the fifth week.

Behavioral Tests

The locomotor activity, spatial working memory (Y-maze spontaneous alternation), and depression-like behaviors such as tail suspension test (TST) and forced swim test (FST) were performed during the fifth week after treatment. Only one behavioral test was carried out in each day with the order like this: (1) locomotor activity, (2) Y-Maze, (3) the first FST, (4) TST, and (5) the second FST.

Locomotor Activity Test

The spontaneous locomotor activity was measured using a light beam system as described previously (Zhang et al., 2012). Briefly, each mouse was put in a transparent cage (40 cm × 40 cm × 25 cm) equipped with photo beams near the bottom of the cage. After 1-min adaptation, the frequency of photo-beam interruptions during the subsequent 5-min period was recorded to measure total movements, including both horizontal and vertical movements.

Y-Maze Test

The working memory was assessed by recording spontaneous alternation in a Y-maze apparatus. It is the natural tendency of rodents to explore a novel environment (Lamberty et al., 1992). The normal mice will remember the arm they have already explored and will enter one of the other arms of the maze. The Y-maze has three arms, named A, B, and C. Mice were placed individually onto the end of one arm (A) and allowed to explore all three arms freely for 8 min. After the first 2 min for habituation, the alternations between arms were recorded during the remained 6 min using Anymaze™ behavioral analyzing software (Hughes, 2004). The alternation defined as the overlapping entrance sequence (e.g., ABC, BCA) was calculated as the percentage: alternation % = (number of alternations)/(total number of arm entries − 2) × 100 (Zhang et al., 2008). Total arm entries were used as an index of ambulatory activity. Mouse with five or fewer arm entries in 6 min was excluded from the data analysis.

TST and FST

FST and TST have been commonly employed to evaluate the depression-like behaviors and antidepressant efficacy in the rodent models (Porsolt et al., 1977). In these tests, a mouse was submitted to an inescapable aversive situation, alternating periods of activity and immobility to reflect "behavioral despair" (Steru et al., 1985). In FST, mice were placed in a Plexiglas cylinder (10 cm internal diameter, 20 cm in height) filled with water (25–26°C temperature and 10 cm in height). Each mouse was allowed to swim for a 6-min session. The total immobility period during the last 4 min of the session was recorded. The immobility period was defined as the time while mice were utterly inactive or made movements only necessary to keep their heads above water. In TST, mice were suspended for 6 min in the air by taping their tails on a hanging bar. The immobility time during the 6 min was recorded. The immobility period was defined as the time while mice displayed no movement when suspended on the bar. All behaviors were recorded using a digital camcorder. The videos were analyzed by an observer blind to treatments using Anymaze™ behavioral software.

Histological and Immunohistochemical Staining

After behavioral tests, all mice were euthanized with sodium pentobarbital (50 mg/kg, i.p.) and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were then fixed overnight in 4% PFA. The fixed brains were subsequently rinsed three with PBS and cryoprotected in 30% sucrose in PBS at 4°C for 36 h. Three coronal sections (30 and 300 μm apart) between levels 1 and −1 mm from bregma were used for histological or immunohistochemistry staining. Luxol fast blue periodic acid-Schiff (LFB-PAS) (Sigma, St. Louis, MO, USA) staining was used to detect the severity of white matter demyelination in the CC (Pappas, 1981; Lin et al., 2005). For immunohistochemical staining, floating brain sections were quenched for half hour in PBS with 0.3% hydroperoxide, followed by 1-h incubation in blocking solution containing 10% host serum in PBS at room temperature (RT). The sections were subsequently incubated overnight with the primary antibodies diluted in the blocking solution and incubated with the anti-goat or anti-rabbit biotin-conjugated secondary antibody (1:1,000; Vector Laboratories, Burlingame, CA) for 2 h at RT. The avidin-biotin complex kit (Vector Laboratories, Burlingame, CA) and 3,3-diaminobenzidine (DAB) chromogen (Sigma-Aldrich, St. Louis, MO) were used to visualize the staining. The brain sections were incubated at RT with the Alexa Fluor fluorescence secondary antibodies for immunofluorescent staining.

Antibodies

Goat polyclonal antibody directed against myelin basic protein (MBP) (1:250; Santa Cruz Biotechnology, CA) was used to detect the myelin protein component (Zhang et al., 2008). Rabbit anti-Olig2 (1:200) and anti-nerve/glial antigen 2 (NG2) antibodies (1:200) (Millipore, Temecula, CA) were used as markers for cells in OL lineage and oligodendrocyte progenitor cells (OPCs), respectively (Zhang et al., 2012). Rat anti-glutathione S-transferase isoform π (GST-π, 1:500; Stressgen, Victoria, BC, Canada) was used to identify the mature OLs (Zhang et al., 2012). Olig2 and GST-π double labeling enabled to identify a subgroup of OL lineage cells with negative GST-π staining; these cells are considered either OPCs or immature OLs (Zhang et al., 2008). Rabbit polyclonal anti-CD11b (1:500; AbD Serotec, Raleigh, NC, USA) and goat anti-glial fibrillary acidic protein (GFAP) antibodies (1:1,000; Sigma, St. Louis, MO, USA) were used to identify activated microglia and astrocytes, respectively (Zhang et al., 2008).

Image Analysis

All images were obtained using an Olympus BX-51 light microscope or Olympus Confocal Laser Microscope 510 Meta. For each immunostaining analysis, digital images from three coronal sections were analyzed by two researchers blinded to the treatment using ImageJ software (version 6.1, Media Cybernetics, Inc., Silver Spring, MD). The software settings for imaging were kept identical among brain sections in each immunostaining. Demyelination in the CC was determined using a modified semiquantitative scale system with 0–4 points (Das Sarma et al., 2009): 0 (no demyelination), 1 (rare and focal demyelination), 2 (multiple focal demyelination), 3 (large or confluent demyelination), and 4 (large and confluent demyelination over 75% of CC). Demyelination in the cortical area was evaluated and expressed as the percentage of MBP-positive staining in a selected area vs. the staining in the

corresponding area from control groups. GST-π and Olig2 immunostainings were measured from randomly selected areas within the CC in each section to quantify mature OLs and OL lineage cells, respectively. The cell numbers of CD11b-, GFAP-, and NG2-positive cells in the CC were evaluated using ImageJ software. Results were presented as the mean number of positive cells per square millimeter. Results from each animal were counted in three coronal sections, and the data are presented as the average of 16 mice per group.

Statistical Analysis

Group differences were determined using two-way analyses of variance (ANOVA). Bonferroni *post hoc* analyses assessed statistical significance between groups. The nonparametric data were analyzed by the Kruskal-Wallis test and follow-up Dunn's multiple comparison test. The results were expressed as mean ± SEM. $p < 0.05$ was considered statistically significant.

RESULTS

Effects of Venlafaxine on CPZ-Induced Behavioral Deficits

Locomotor Activity

As previously reported, mice receiving 5-week CPZ diet exhibited locomotor hyperactivity in a novel environment (Xu et al., 2009; Bustillo et al., 2010). We further examined whether venlafaxine treatment would moderate the behavioral abnormality. Locomotor activity level was measured by the frequency of photo-beam interruptions in a 5-min test period. Two-way ANOVA showed a main effect of CPZ ($F = 15.76$, $p = 0.0002$); CPZ exposure significantly increased the frequency of photo-beam interruption compared to regular diet (**Figure 1A**). Mice exposed to CPZ also had higher total arm entries in the Y-maze test compared to those with the regular diet ($F = 15.57$, $p = 0.00021$). Importantly, two-way ANOVA yielded a model-by-treatment interaction ($F = 4.04$, $p = 0.02$). Venlafaxine showed no effects on the frequencies of photo-beam interruption or arm entries ($p > 0.05$) (**Figures 1A,B**).

Working Memory

There was a significant model-by-treatment interaction ($F = 4.54$, $p = 0.015$) in the Y-maze spontaneous alternation test. CPZ mice displayed a substantial spatial memory deficit compared to the CTL group ($p < 0.003$) (Xu et al., 2009). The *post hoc* Scheffe's test showed a significantly lower alternation in the CPZ group (54.0%) compared to the CTL group (80.7%). Notably, there was a dose-dependent effect of venlafaxine on CPZ-induced spatial memory deficit, showing that only a higher dose was effective (CPZ + VEN20, $p = 0.02$) (**Figure 1C**).

Depression-Like Behaviors

In the first FST (FST-1), there was no significant difference in immobility time among groups (**Figure 1D**), suggesting that neither venlafaxine nor CPZ elicited "despair behaviors" after 5-week treatment. Two-way ANOVA revealed significant model-by-treatment interaction in the TST on day 2 ($F = 3.41$, $p = 0.03$). The CPZ group had a significantly prolonged immobility time compared to the CTL group ($p = 0.0137$). There were no differences among CTL and venlafaxine suggesting that venlafaxine reversed the prolonged immobility time caused by CPZ (**Figure 1E**). Interestingly, there was a significant model-by-treatment interaction ($F = 4.24$, $p = 0.02$) and a main effect for CPZ ($F = 8.84$, $p = 0.005$) in the second FST (FST-2). The CPZ groups had a significantly longer immobility time compared to CTL group ($p = 0.00063$); alternatively, venlafaxine treatments decreased the immobility times compared to CPZ group (CPZ + VEN5 vs. CPZ, $p = 0.015$, CPZ + VEN20 vs. CPZ, $p = 0.035$) (**Figure 1F**).

Effects of Venlafaxine on CPZ-Induced Demyelination in the CC and the Cerebral Cortex

Five-week CPZ treatment causes extensive demyelination in the CC and the cerebral cortex of C57BL/6 mice (Xu et al., 2009) (**Figure 2A**). The LFB-PAS staining showed significantly higher demyelination scores in the CC in CPZ compared to CTL groups in the CC (**Figure 2B,** $p < 0.0001$). Venlafaxine in the regular diet mice had no effect on the myelination status. On the contrary, a high dose of venlafaxine (20 mg/kg/day) significantly alleviated CPZ-induced demyelination in the CC, while lower dose of venlafaxine (5 mg/kg/day) failed to show any improvement (**Figure 2B**). The percentage of MBP-positive stained area showed that mice exposed to CPZ had a significant loss of MBP compared to the CTL mice in the cerebral cortex; venlafaxine treatment did not change the percentage of MBP staining in mice on the regular diet. High dose of venlafaxine (20 mg/kg/day) significantly reduced the cortical demyelination caused by CPZ ($p < 0.05$). Altogether, these findings suggest that venlafaxine exerted neuroprotective effects on myelin sheaths in both the CC and cortical areas against CPZ-induced demyelination in a dose-dependent fashion (**Figure 2C**).

Effects of Venlafaxine on Oligodendrocytes in the CPZ-Induced Demyelination

The number of total OL lineage cells (Olig2+) in the CC was significantly reduced in the CPZ and CPZ + VEN5 groups, compared to the CTL groups (**Figures 3A–C**); however, CPZ + VEN20 groups demonstrated a significantly higher number of Olig2+ cells compared to the CPZ and CPZ + VEN5 groups (**Figures 3A–C**). The Olig2+ cells were further categorized into GST-π– and GST-π+ cells using Olgi2 and GST-π double labeling. The majority of Olig2+ cells (~80%) were mature OLs (GST-π+) in the CC in CTL groups; there was only 15% of Olig2+ cells were GST-π+ in CPZ group. A high dose of venlafaxine significantly increased the percentage of the GST-π+ cells to 35% (**Figure 3D**). The Olig2+/GST-π– cells were significantly increased in all CPZ groups, and venlafaxine seemed did not alter the cell numbers in CPZ groups (**Figure 3E**). The number of NG2+ OPCs was significantly increased in

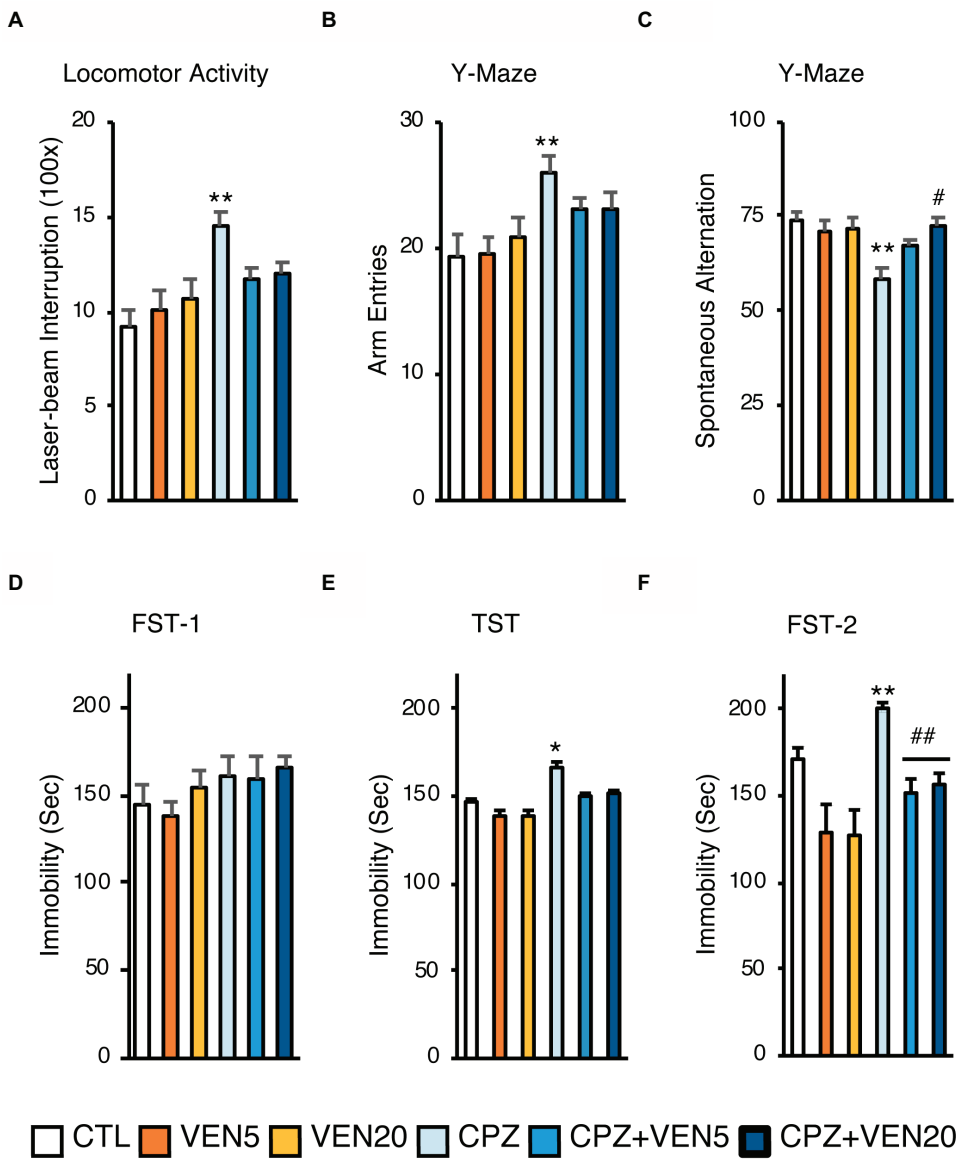

FIGURE 1 | The effects of venlafaxine on behaviors in normal and demyelinated mice. All mice were tested for **(A)** locomotor scores in the open field; **(B)** arm entries in the Y-maze test; **(C)** spontaneous alternative in the Y-maze test; **(D)** immobile time during the first trial of forced swim tests (FST-1); **(E)** immobile time during tail suspension test (TST); **(F)** immobile time during the second trial of forced swim tests (FST-2). Data were presented as means ± SEM (n = 16 in each group). $^{*}p < 0.05$ or $^{**}p < 0.01$ compared to the CTL; $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ compared to CPZ. CTL, control; CPZ, cuprizone; VEN5, venlafaxine 5 mg/kg/day; VEN20, venlafaxine 20 mg/kg/day.

CPZ-exposed mice compared to CTL groups ($p < 0.001$). Conversely, venlafaxine treatment significantly reduced the number of NG2+ in the CC in the CPZ-exposed mice (**Figure 4A**). A high dose of venlafaxine was more efficient compared to a low dose of venlafaxine (**Figure 4B**).

Venlafaxine Reduces Inflammatory Changes in the CC

In the present study, we used CD11b and GFAP immunostainings to identify activated microglia and astrocytes, respectively (**Figure 5A**). There was a significantly increased number of CD11b+ cells in the CC of CPZ-exposed mice compared to the CTL mice ($p < 0.001$) (**Figure 5B**). Alternatively, venlafaxine produced a dramatic dose-dependent reduction in the number of CD11b+ cells in the CPZ-exposed mice ($p < 0.001$). The number of GFAP+ cells was also significantly higher in the CPZ-exposed mice than in the CTL mice, whereas venlafaxine counteracted to the CPZ effect ($p < 0.001$) (**Figure 5C**). No dose-dependent effect was observed.

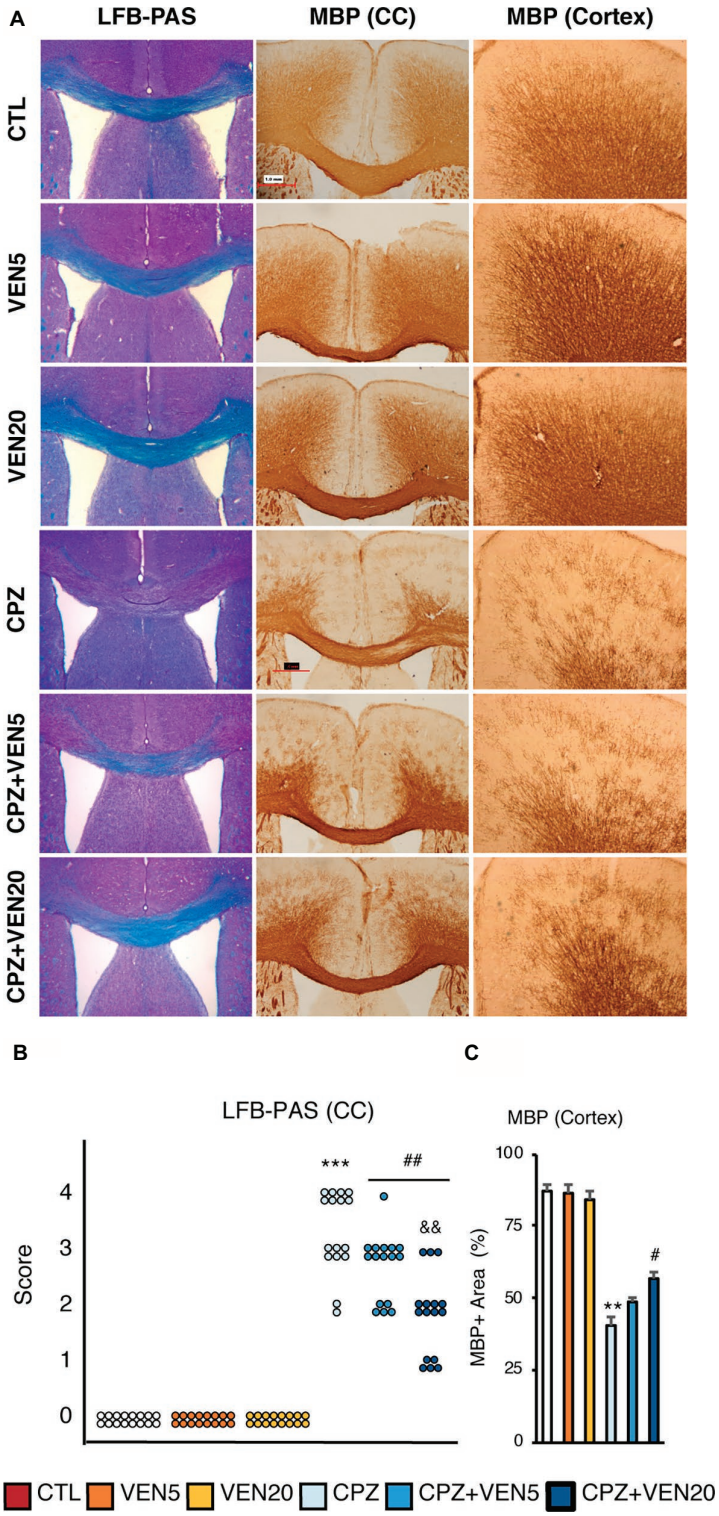

FIGURE 2 | The effects of venlafaxine on myelin integrity in normal and demyelinated mice. **(A)** Representative images of Luxol fast blue and periodic acid-Schiff (LFB-PAS) staining in the corpus callosum (CC) and myelin basic protein (MBP) immunostaining in the CC and cortex; **(B)** demyelination score based on LFB-PAS staining in the CC; **(C)** Quantitative analysis of cortical demyelination measured by percentage of MBP immunostaining area in the frontal cortex in each group. Data are presented as mean ± SEM (n = 16 mice per group). The LFB-PAS score was analyzed by the Kruskal-Wallis test (Dunn's multiple comparison test). MBP staining data were analyzed by two-way ANOVA followed by Bonferroni *post hoc* analyses. Significances among different conditions are indicated as below: $^{**}p < 0.01$ or $^{***}p < 0.001$ compared to the CTL; $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ compared to CPZ. $^{\&\&}p < 0.01$ compared to CPZ + VEN5. Scale bar represents 100 μm; CTL, control; CPZ, cuprizone; VEN5, venlafaxine 5 mg/kg/day; VEN20, venlafaxine 20 mg/kg/day.

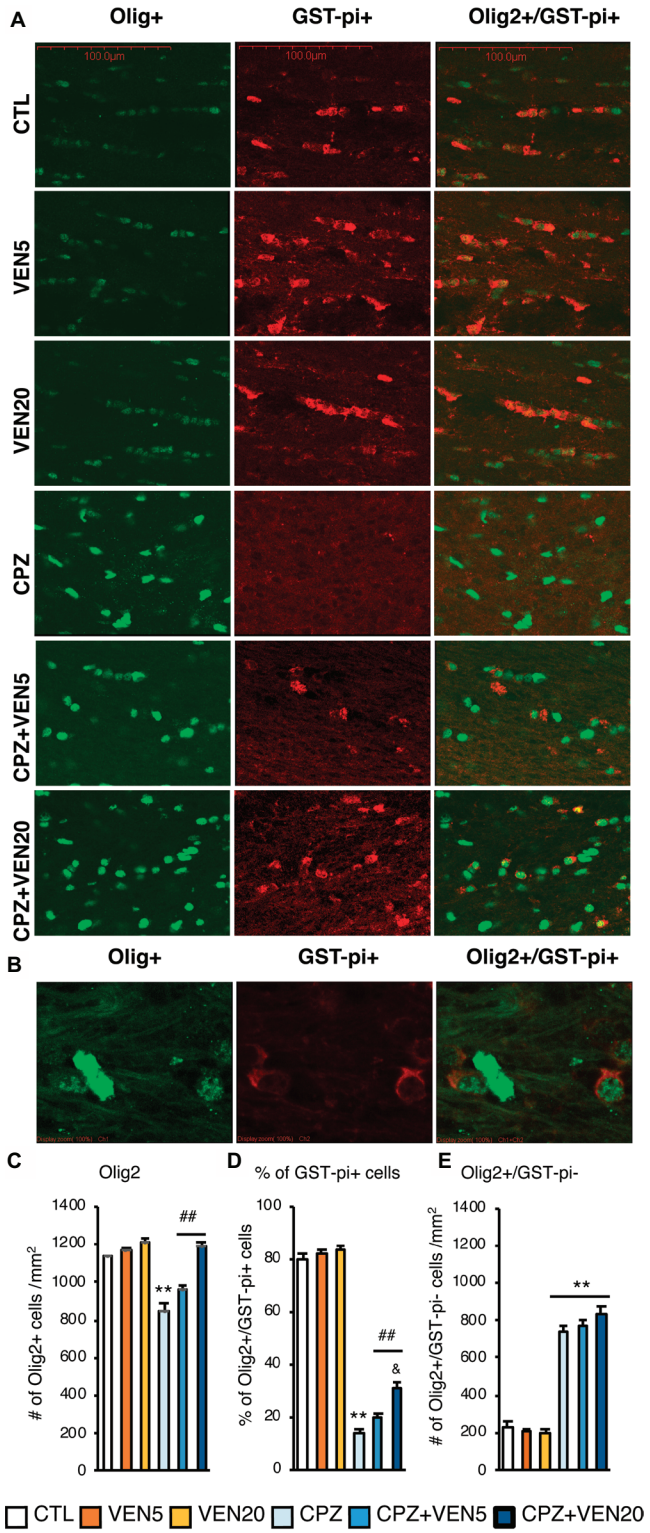

FIGURE 3 | Venlafaxine reduced mature OL loss in the corpus callosum (CC) of cuprizone-exposed C57BL/6 mice. **(A)** Representative images of Olig2, GST-π, and Olig2/GST-π double labeling in the CC areas; **(B)** the double labeling of Olig2 and GST-π at high magnification; **(C)** quantitative analysis of the total number of Olig2+ cells; **(D)** the percentage of Olig2+/GST-π+ cells in the CC in each group; **(E)** quantitative analysis of the Olig2+/GST-π– cells in the CC in each group. Data are presented as mean ± SEM (n = 16 mice per group). The data were analyzed by two-way ANOVA followed by Bonferroni *post hoc* analyses. Significances among different conditions are indicated as below: $^{**}p < 0.01$ compared to the CTL; $^{\#\#}p < 0.01$ compared to CPZ and $^{\&}p < 0.05$ compared to CPZ + VEN5. Scale bar represents 100 μm; CTL, control; CPZ, cuprizone; VEN5, venlafaxine 5 mg/kg/day; VEN20, venlafaxine 20 mg/kg/day.

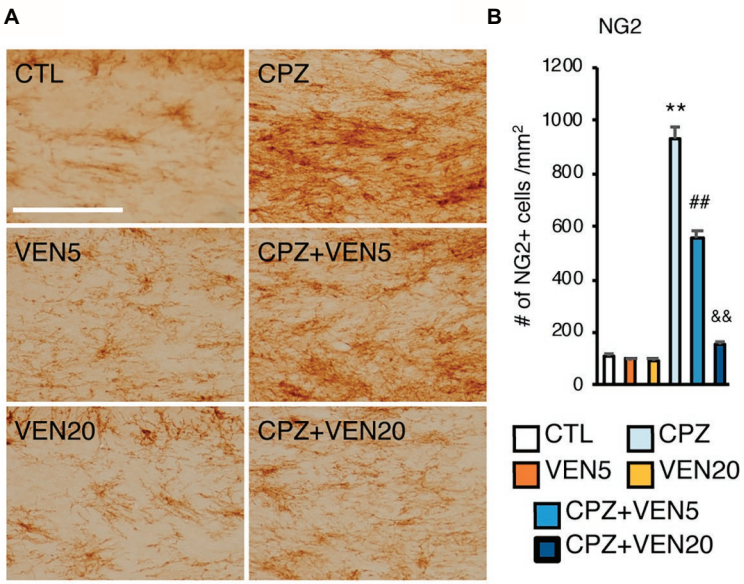

FIGURE 4 | Venlafaxine decreased oligodendrocyte (OL) progenitor cell (OPC) accumulation in the corpus callosum (CC) of cuprizone-exposed C57BL/6 mice. **(A)** Representative images of NG2 immunostaining in the CC areas; **(B)** quantitative analysis of the number of NG2+ cells in the CC in each group. The results are presented as mean ± SEM (n = 16 mice per group). The data were analyzed by two-way ANOVA followed by Bonferroni *post hoc* analyses. Significances among different conditions are indicated as below: $^{**}p < 0.01$ compared to the CTL; $^{\#\#}p < 0.01$ compared to CPZ, and $^{\&\&}p < 0.01$ compared to CPZ + VEN5. Scale bar represents 100 μm; CTL, control; CPZ, cuprizone; VEN5, venlafaxine 5 mg/kg/day; VEN20, venlafaxine 20 mg/kg/day.

DISCUSSION

Venlafaxine is a SNRI antidepressant that increases the equimolar concentrations of 5-HT and NE at neuronal terminals (Polluzi et al., 2013; Magalhães et al., 2014). Venlafaxine also has neuroprotective and anti-inflammatory effects in the CNS and peripheral nervous system (Tynan et al., 2012; Galecki et al., 2018). Moreover, a few clinical studies reported the cognitive effect of venlafaxine in patients with MDD (Tian et al., 2016; Cristancho et al., 2018). Nevertheless, it is difficult to determine whether cognitive improvements due to venlafaxine in MDD are due to the improvement of cognitive processes *per se* or because of improvement of depressive symptoms as well, because the severity of cognitive impairment is highly associated with the severity and subtypes of MDD (Snyder, 2013). Thus, the purpose of this research is to explore venlafaxine effect on cognitive and depression-like processes with respect to demyelination and inflammatory processes in the brain. The CPZ-induced demyelination mouse model was utilized to investigate cognitive impairments and mood symptoms associated with OL cell death and acute demyelination and associated neuroinflammation (Sun et al., 2017). Y-maze testing evaluated the cognitive effects of venlafaxine on CPZ-induced demyelination; a series of FST and TST behavioral tests assessed depression-like behaviors.

Notably, no depression-like behaviors were observed in all groups in the first FST following Y-maze test, suggesting that CPZ-induced cognitive impairment occurred in the absence of depression (Borsoi et al., 2015). Venlafaxine was found to improve working memory deficits in mice exposed to CPZ in a dose-specific manner. A high dose of venlafaxine attenuated the cognitive impairment of CPZ-treated mice; a lower dose of venlafaxine failed to reduce abnormalities in working memory. Venlafaxine's effect on the cognitive process is likely independent to its effect on spontaneous depression. As it is accepted that carrying out FST or TST imposes significant stress to the testing animals, repeated expose to FST or TST is considered to generate a chronic depression model (Serchov et al., 2015). Mice receiving regular diet showed consistent immobility time across FST1-TST-FST2 processes, while CPZ-fed groups exhibited increased immobile time on TST and FST2. Venlafaxine treatment reversed the immobile time in the second FST. The results indicate that CPZ exposure for 5 weeks may not lead to an early depression-like behavior. However, demyelination and neuroinflammation caused by CPZ may alter the neurobiology of resilience and make animals more vulnerable to psychosocial stress (Osorio et al., 2017). Venlafaxine's cognitive benefit may be partially due to its protective effects against chronic psychosocial stress (i.e., repeated exposure to stressors – FST and TST).

Venlafaxine also improved myelin integrity on CPZ-induced demyelination. Again, the effect was dose specific, a higher dose more beneficial than a lower one. In consistent with previous findings (Matsushima and Morell, 2001), CPZ feeding for 5 weeks induced OLs apoptosis and acute demyelination. On the contrary, high dose of venlafaxine reduced demyelination in the CC in the cortical areas. Venlafaxine's effect on myelination mirrored the Y-maze results, suggesting that cognitive improvement can be associated with venlafaxine's

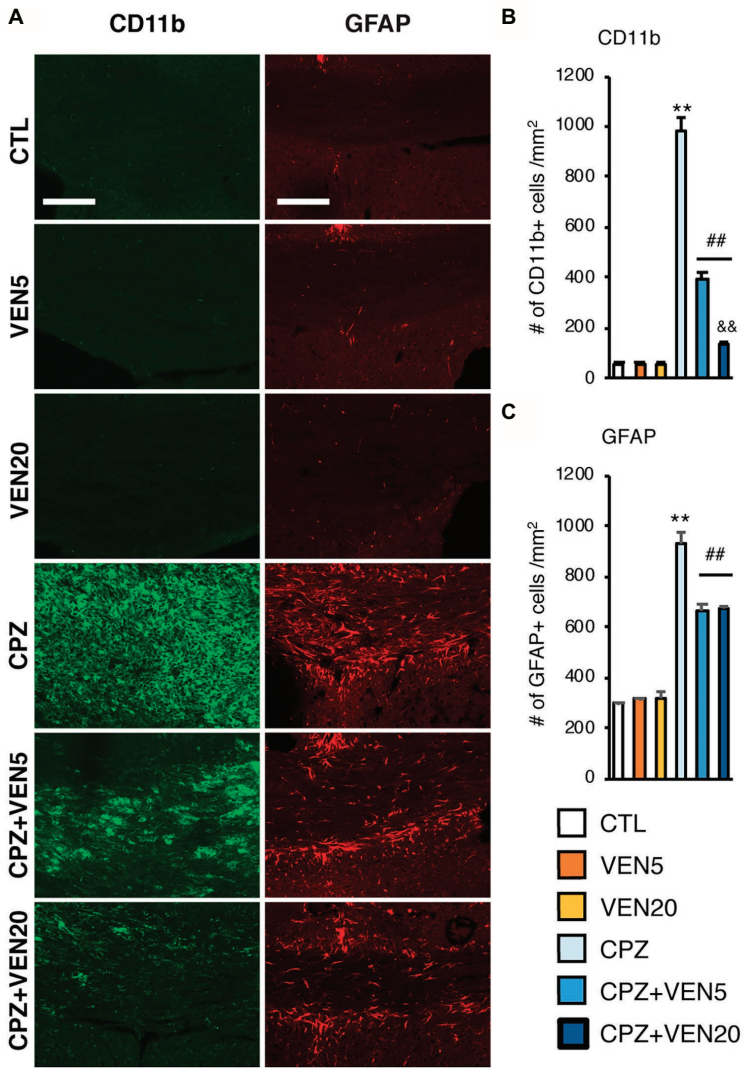

FIGURE 5 | Venlafaxine inhibited microglia and astrocyte activation in the corpus callosum (CC) of cuprizone-exposed C57BL/6 mice. **(A)** Representative images of CD11b and GFAP immunostaining in the CC areas; **(B)** quantitative analysis of the cell number of CD11b+ cells in the CC in each group. **(C)** Quantitative analysis of the cell number of GFAP+ cells in the CC in each group. Data are expressed as mean ± SEM (n = 16 mice per group). The data were analyzed by two-way ANOVA followed by Bonferroni *post hoc* analyses. Significances among different conditions are indicated as below: $^{**}p < 0.01$ compared to the CTL; $^{\#\#}p < 0.01$ compared to CPZ, and $^{\&\&}p < 0.01$ compared to CPZ + VEN5. Scale bar represents 100 μm; CTL, control; CPZ, cuprizone; VEN5, venlafaxine 5 mg/kg/day; VEN20, venlafaxine 20 mg/kg/day.

therapeutic effect on myelin integrity. Venlafaxine's antidepressant effect seems independent to its myelin protection and anti-inflammatory effect.

Both mature, myelin-producing OLs and immature cell lines (OPC) belong to OL lineage and have a crucial role in myelination. OPCs proliferate and migrate to repair demyelinated lesion sites in MS and MDD (Kotter et al., 2006). Remyelination can be affected due to a hindered differentiation of these immature cells into myelin-producing, mature OLs (Lampron et al., 2015). Additionally, OPCs are capable of differentiating into mature OLs and astrocytes (Dimou and Gallo, 2015). These cells are the primary source of myelin repair and are upregulated following OL cell death (Matsushima and Morell, 2001) (VonDran et al., 2011).

Venlafaxine protected OL lineage cells in the present study. Specifically, a high dose of venlafaxine attenuated the depletion of mature OLs (i.e., Olig2+/GST-π+ cells) observed in the CPZ group. A high dose of venlafaxine also normalized compensatory increase of immature (i.e., NG2+) cells. Thus, the attenuated NG2+ cell numbers in CPZ + VEN20 groups may be due to preventing mature OL death as opposed to promoting the differentiation of OPCs.

A protective role of venlafaxine may also be attributed to reducing neuroinflammatory processes. Microglia, resident immune cells of the CNS, may polarize into a pro-inflammatory M1 phenotype or regenerative M2 phenotype (Karamita et al., 2017). Increased microglial expression in the CPZ model acts as a marker for neuroinflammation (Zhang et al., 2008). It is

no surprise that a high dose of venlafaxine significantly reduced the number of CD11b (i.e., microglia) and GFAP (i.e. astrocytes) signal in the present study. Venlafaxine was reported to possess strong immunoregulatory activities (Galecki et al., 2018). Its ability to modulate microglia and macrophage activation in animal models of neurological disorders was also reported (Zychowska et al., 2015). Venlafaxine's anti-inflammatory effect found that this study aligns with earlier reports (Mansouri et al., 2018). Further work is required to elucidate this mechanistic relationship; however, in the present study, we provide original evidence regarding the role of venlafaxine on attenuating microglia-mediated inflammation and demyelination.

SUMMARY

Here, we provide the evidence, for the first time, about a comparable protective effect of venlafaxine on myelin integrity and cognitive function in a demyelination mouse model. This study suggests that venlafaxine can be a medication of choice for depression and anxiety in MS. Venlafaxine's strong neuroprotective and anti-inflammatory effects on myelin and OLs may provide extra therapeutic benefits to MS patients by altering the inflammation and demyelination process. Clinical studies with MS patients are needed to validate the findings and assess psychological and pathological progress.

ETHICS STATEMENT

All animal procedures were performed in accordance with Canadian Council on Animal Care (CCAC) guidelines and were approved by the University Committee on Animal Care and Supply (UCACS), University of Saskatchewan.

AUTHOR CONTRIBUTIONS

All authors have participated and made substantial contributions to this paper. YZ and XB contributed in designing the study, conducting the experiments, and collecting the data. OA contributed to the data analysis and interpretation and writing of the manuscript. JW, AM, JC, and ZW performed part of the data analysis. FW, X-ML, and YZ contributed to the study design and manuscript revisions. All authors contributed to and had approved the final manuscript.

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REVIEW
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Patterns of Membrane Protein Clustering in Peripheral Lymphocytes as Predictors of Therapeutic Outcomes in Major Depressive Disorder

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There is an utmost necessity of developing novel biomarkers of depression that result in a more efficacious use of current antidepressant drugs. The present report reviews and discusses a recent series of experiments that focused on analysis of membrane protein clustering in peripheral lymphocytes as putative biomarkers of therapeutic efficacy for major depressive disorder. This review recapitulates how the ideas were originated, and the main findings demonstrated that analysis of serotonin transporter and serotonin 2 A receptor clustering in peripheral lymphocytes of naïve depression patients resulted in a discrimination of two subpopulations of depressed patients that showed a differential response upon 8 weeks of antidepressant treatment. The paper also reviews the usefulness of animal models of depression for an initial evaluation of membrane protein clustering in lymphocytes, which provides a screening tool to determine additional proteins to be further evaluated in depression patients. Finally, the present review provides a brief discussion of the general field of biomarkers of depression in relation to therapeutic outcomes and suggests additional ideas to provide extra value to the reviewed studies.

Keywords: biomarkers, depression, membrane protein clustering, therapeutic efficacy, antidepressants

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CLINICAL INTEREST AND GENERAL APPROACHES FOR DEVELOPING NOVEL BIOMARKERS OF DEPRESSION

There is a pressing necessity to provide a better way for diagnosis, prognosis, and therapeutic treatment of psychiatric disorders. This is because in contrast to other medical specialties, psychiatry has lacked clear biological indicators (i.e., biomarkers) to help guide a proper diagnosis or prognosis or to ascertain the best therapeutic approach for individuals suffering mental disorders (for a general review of the neurobiology, physiopathology, and treatment of depression, see Otte et al., 2016). This is of particular interest in the context of major depressive disorder (MDD) when considering that, although antidepressants are clearly efficacious to treat MDD (Cipriani et al., 2018), a high percentage of patients fail to show a proper therapeutic response upon the first antidepressant treatment (Rush et al., 2006). Accordingly, there has been an exponential increase in the number of publications focusing

on biomarkers of depression (almost 1,400 hits when searching for the term "biomarkers of depression" in the past 3 years in a recent PubMed search) and on biomarkers of antidepressant response (almost 200 hits when searching for the term "biomarkers of antidepressant response" in the past 3 years in a recent PubMed search). Although this blooming of publications indicates a keen interest by researchers in evaluating the efficacy of multiple biomarkers of depression, it seems that most of these biomarkers may not be specific for MDD, and at the same time, there is a need of additional studies and clinical trials to validate the efficacy of these putative biomarkers (Quevedo and Yatham, 2018).

Current approaches to the discovery of novel biomarkers of diagnosis and/or therapeutic efficacy for MDD are mostly based on technological advances in neuroimaging or on the use of "Omics" technologies (i.e., genomics or other "Omics" approaches primarily used to define peripheral biomarkers of MDD) (see Gururajan et al., 2016; Voegeli et al., 2017; Busch and Menke, 2018). Recently, a series of systematic reviews have evaluated the effectiveness of multiple biomarkers of depression using genomics (Menezes et al., 2019), epigenomics (Goud Alladi et al., 2018), metabolomics (MacDonald et al., 2018), antidepressant pharmacologic treatment response (Voegeli et al., 2017), inflammatory biomarkers (Smith et al., 2018; Yang et al., 2018), and neuroimaging biomarkers (Drago et al., 2018; Levy et al., 2019; Suh et al., 2019).

Hypotheses-based approaches can also help to define novel biomarkers of depression and complement the information obtained from neuroimaging and "Omics" studies, with the final purpose of finding specific combinations of biomarkers (including "Omics," neuroimaging, and hypotheses-based approaches) that can be translated to the clinical and public health settings. In fact, some of the most replicated studies on biomarkers of depression are based on hypotheses-based approaches, such as alterations on serotonin transporter (SERT) binding in platelets or alterations in serum proinflammatory cytokines that may relate to specific inflammatory events underlying the pathophysiology of depression (reviewed in Gadad et al., 2018).

Following that line of thinking, during the past few years, we carried out an experimental approach to develop and test the hypothesis that alterations in the patterns of membrane protein clustering in peripheral lymphocytes can predict the therapeutic outcomes of psychopharmacological treatment in MDD. The present scientific review summarizes and discusses our findings, providing a proper context on how the studies were developed and points out toward additional experimental approaches designed for the validation and clinical translation of this approach.

DEVELOPMENT OF THE HYPOTHESIS THAT ALTERATIONS IN MEMBRANE PROTEIN CLUSTERING MAY BE A PUTATIVE BIOMARKER OF MDD

During the second half of the 1990s, a series of reports from the laboratory of Drs. Erminio Costa and Alessandro Guidotti (University of Illinois at Chicago) provided the first demonstrations that the extracellular matrix protein reelin was heavily downregulated (about 50%) in multiple brain areas from schizophrenia post-mortem brain samples (Impagnatiello et al., 1998) and in the cerebral cortex of bipolar patients with psychotic episodes (Guidotti et al., 2000). These findings were followed by other laboratories that not only were able to replicate them but also demonstrated a downregulation of reelin levels in the hippocampus of schizophrenia, bipolar disorder, and major depression (Fatemi et al., 2000).

Reelin is an extracellular matrix protein involved in developmental regulation of neuronal migration and in regulation of neural plasticity in the adult brain, which (as mentioned above) is downregulated in multiple psychiatric disorders (see Ishii et al., 2016, as a review). Reelin primarily binds to the membrane receptors apolipoprotein receptor 2 and the very low density lipoprotein receptor in a heterodimeric combination and brings about the phosphorylation of the cytoplasmic adaptor protein DAB1, resulting in the activation of multiple signaling pathways resulting in the control of neural migration and cortical layer formation, promotion of protein translation, dendrite outgrowth and development of dendritic spines, and in regulation of glutamatergic synaptic plasticity (reviewed in Lee and D'Arcangelo, 2016).

From there on, studies focusing on the origin of reelin downregulation observed in psychiatric disorders (primarily in schizophrenia) pointed toward epigenetic alterations involving a hypermethylation of CpG islands in the reelin gene promoter as the cause of brain reelin deficits (recently reviewed in Guidotti et al., 2016). At the same time, studies on animal models of depression provided numerous evidences that reelin downregulation in the subgranular zone of the dentate gyrus may affect the maturation of dentate newborn neurons (Lussier et al., 2009, 2013a) and dysregulate the glutamatergic-GABAergic systems crosstalk in limbic brain areas (Lussier et al., 2013b). These alterations could be reversed by conventional antidepressants (Fenton et al., 2015) or by anti-inflammatory drugs with an antidepressant effect such as etanercept (Brymer et al., 2018). Reelin haploinsufficient heterozygous reeler mice show some subtle alterations in neurochemistry and behavior, but otherwise are almost indistinguishable from wild-type mice; however, we have found that these animals are extremely susceptible to the depressogenic effects of repeated subcutaneous injections of the stress hormone corticosterone and show depressive-like behavioral paradigms at corticosterone doses that fail to induce such behaviors in wild-type mice, which prompted us to consider reelin downregulation as a putative vulnerability factor for depression (Lussier et al., 2011). In summary, all these studies indicated a possible important role for reelin in the pathophysiology of depression (reviewed in Caruncho et al., 2016).

Concomitantly, other reports provided evidence indicating that reelin induces protein translation in synaptosome preparations (Dong et al., 2003), increases the number and clustering of synaptosomal membrane proteins (Caruncho et al., 2004), and promotes the clustering of the canonical reelin receptors ApoER2 and VLDLR (Strasser et al., 2004). For recent reviews of the

canonical and non-canonical reelin signaling pathways, see Bock and May (2016) and Lee and D'Arcangelo (2016).

Reelin is also expressed in blood plasma (Smalheiser et al., 2000) where it is secreted by hepatocytes (Smalheiser et al., 2000) and platelets (Tseng et al., 2010), and plasma reelin levels are also altered in neuropsychiatric disorders (Fatemi et al., 2001).

ALTERATIONS IN SEROTONIN TRANSPORTER (SERT) AND SEROTONIN RECEPTOR 2A (5HT2A) PERIPHERAL LYMPHOCYTES FROM ANIMALS WITH REELIN DEFICITS

The observations of reelin alterations in depression summarized above are the evidence that reelin induces membrane protein clustering (Dong et al., 2003; Caruncho et al., 2004), the finding that animals with reelin deficits (i.e., heterozygous reeler mice) are quite susceptible to the depressogenic effects of corticosterone (Lussier et al., 2011), and the demonstration of reelin expression in blood plasma and its alterations in psychiatric disorders (Smalheiser et al., 2000; Fatemi et al., 2001), prompted us to evaluate the possibility that animals expressing low reelin levels (such as heterozygous reeler mice) might show alterations in membrane protein clustering in peripheral blood cells.

We primarily centered our studies on analyzing the patterns of membrane clustering of two proteins pertaining serotonergic neurotransmission (SERT and 5HT2A) that are also expressed in lymphocytes and may be involved in the regulation of inflammatory processes (recently reviewed by Wu et al., 2018; also see Ahern, 2011). Alterations in SERT and 5HT2A are directly involved in the pathophysiology of depression and represent some of the targets of antidepressant medication, which is not surprising when considering the essential roles that the serotonergic system plays in the regulation of behavioral patterns directly affected in depression, such as mood, emotion, or sleep (for a recent review of the serotonergic hypothesis of depression, see Fakboury, 2016).

Our studies analyzing the pattern of membrane clustering of the serotonin transporter protein (SERT) in peripheral lymphocytes from heterozygous reeler mice, null reeler mice, and wild-type mice showed a patchy pattern of expression of SERT immunolabeling in lymphocyte membranes that becomes disrupted in animals with reelin deficits (Rivera-Baltanas et al., 2010). In fact, heterozygous reeler mice showed a significant increase in SERT cluster size, while in null reeler mice SERT immunolabeling was mostly evidenced as a diffuse staining and was difficult to demonstrate well-detailed patches (Rivera-Baltanas et al., 2010). A schematic representation of alterations in SERT clustering in reeler mice lymphocytes is illustrated in **Figure 1A**.

Additionally, we also evaluated possibly alterations in SERT and 5HT2A protein clustering in the repeated-corticosterone model of depression (Romay-Tallon et al., 2018). This is a

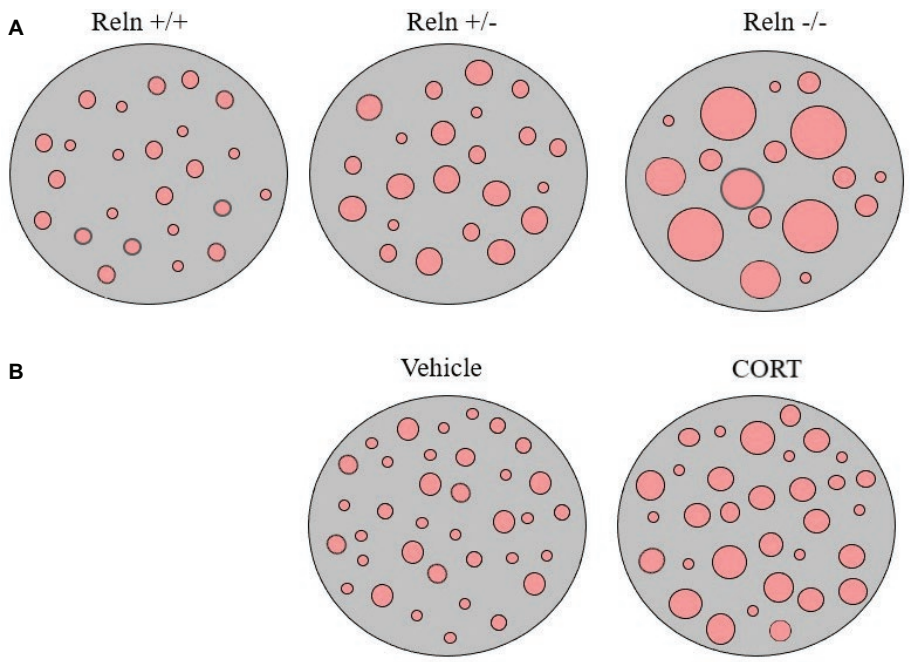

FIGURE 1 | Schematic representation of serotonin transporter (SERT) clusters in the plasma membrane of one representative lymphocyte in reeler mice **(A)** and in rats treated with corticosterone **(B)**. Note that heterozygous reeler mice (Reln +/–) have an increase in SERT cluster number and size in comparison to wild-type mice (Reln +/+) and that homozygous reeler mice (Reln –/–) show much larger clusters that sometimes are difficult to differentiate [see Panel **(A)**, and Rivera-Baltanas et al., 2010]. After repeated-corticosterone treatment, a well-defined animal model of depression, SERT clusters also appear to increase in size [see Panel **(B)**, and Romay-Tallon et al., 2018].

well-characterized animal model of depression (see Sterner and Kalynchuk, 2010, as a review) that we have used in many of our studies pertaining reelin and depression (see above). We found that repeated-corticosterone induced an increase in cluster size but not in cluster number for both SERT and 5HT2A (Romay-Tallon et al., 2018). A schematic representation of alterations in SERT clustering in lymphocytes from animals treated with corticosterone is illustrated in **Figure 1B**.

The results of these studies, together with evidence of alterations in SERT binding in lymphocytes of depression patients (Urbina et al., 1999; Lima and Urbina, 2002; Lima et al., 2005; Pena et al., 2005), brought us to develop the idea that perhaps the pattern of SERT clustering in lymphocytes might be disrupted in depression patients and if so to study if those alterations correlate with scores on psychological scales.

TABLE 1 | Differential improvement in scores in the Hamilton Depression Rating Scale (HDRS) passed to D-I and D-II patients after 8 weeks of antidepressant treatment.

HDRS after treatment	Non-responders and partial responders (%)	Responders without remission (%)	Remission of symptoms (%)
Overall depression	36	32	32
D-I	45	33	22
D-II	0	25	75

Non-responders: HDRS scores improvement of less than 25%.
Partial responders: HDRS scores improvement between 25 and 50%.
Responders without remission: HDRS scores improvement of more than 50%, but absolute value of 7 or more.
Remission of symptoms: HDRS scores improvement of more than 50%, and absolute value below 7.

ALTERATIONS IN SERT AND SEROTONIN 2A RECEPTOR (5HT2A) IN PERIPHERAL LYMPHOCYTES FROM DEPRESSION PATIENTS

We hypothesized that in naïve depression patients (i.e., patients who had not taken antidepressants at least for several months) the pattern of SERT clustering in lymphocytes would follow the lines of that observed in heterozygous reeler mice (i.e., the average SERT cluster size would be larger in depression patients than in normal controls) and that response to antidepressant medication would be followed by a reversal of the alterations in the pattern of SERT clustering. Our findings generally showed that this was the case (i.e., naïve depression patients showed a similar number of SERT clusters per lymphocytes but they were of a larger size than those observed in samples from control non-psychiatric patients, for example, the average size of SERT clusters in the control population was about 0.11 and 0.14 μm^2 for naïve depression patients). However, the analysis of the distribution of SERT cluster size allowed us to differentiate two subpopulations of naïve depression patients that we named D-I and D-II, the D-I subpopulation represented about ¾ of the patients and showed more than 40% of SERT clusters being between 0.05 and 0.010 μm^2 (the modal peak of cluster size), while the DII subpopulation showed around 25% of SERT clusters between 0.05 and 0.010 μm^2 (Rivera-Baltanas et al., 2012). Although we thought that these two subpopulations perhaps might reflect differential scores in psychological scales (i.e., in the Hamilton Depression Rating Scale, HDRS), when we proceeded to check the scores we observed that this was not the case, so that naïve depression D-I and D-II patients had similar HDRS scores (Rivera-Baltanas et al., 2012).

Interestingly, upon 8 weeks of psychopharmacological treatment, there was a differential response between D-I and D-II patients, as about half D-I patients showed no-response or a partial response to antidepressant medication, while the whole group of D-II patients responded to treatment and 75% of them showed remission of symptoms (see **Table 1**, and also Rivera-Baltanas et al., 2012, 2014, 2015). When analyzing the patterns of SERT clustering in lymphocytes after treatment, we found a significant increase in the number of SERT clusters within the size modal peak and a general increase in SERT cluster number in D-II patients, while there were no observable changes in the pattern of SERT clusters in D-I patients. A schematic representation of alterations in SERT clustering in lymphocytes from patients with depression is illustrated in **Figure 2**. In addition, the changes observed in D-II patients correlated with the amelioration of depression symptoms in these patients. These findings allowed us to suggest that analysis of the pattern of SERT protein clustering in lymphocytes could be considered a putative biomarker of therapeutic efficacy in MDD (Rivera-Baltanas et al., 2012) and prompted us to analyze the pattern of SERT clustering in relation to additional psychological scales like Self-Assessment Anhedonia Scale (SAAS) (Olivares et al., 2005; Rivera-Baltanas et al., 2015) and to study possible alterations in the pattern of clustering of other proteins like 5HT2A receptor (Rivera-Baltanas et al., 2014).

We focused on the study of the patterns of SERT clustering in depression patients in relation to a scale that measures anhedonia symptoms, because anhedonia is not only considered a key symptom of depression (APA, 2013) but is also conceptualized as a specific endophenotype of MDD (Pizzagalli, 2014) and a predictor of treatment response (Spijker et al., 2001). The use of the SAAS scale to provide a possible correlation between anhedonia scores and SERT clusters was also motivated by the issue that the HDRS does not properly evaluate anhedonia symptoms (Berrios and Olivares, 1995; Olivares and Berrios, 1998). When we evaluated the SAAS scores of D-I and D-II naïve depression patients, we did not found any difference between the two groups (Rivera-Baltanas et al., 2015). However, the post-treatment analysis evidenced differences in response between the D-I and D-II groups that were higher than those observed in the SERT-HDRS study, as the population of treated D-I patients did not improve at all SAAS scores, while D-II patients showed a remarkable improvement after 8 weeks of psychopharmacological treatment (Rivera-Baltanas et al., 2015). These findings may have some implications in developing proper strategies for the treatment

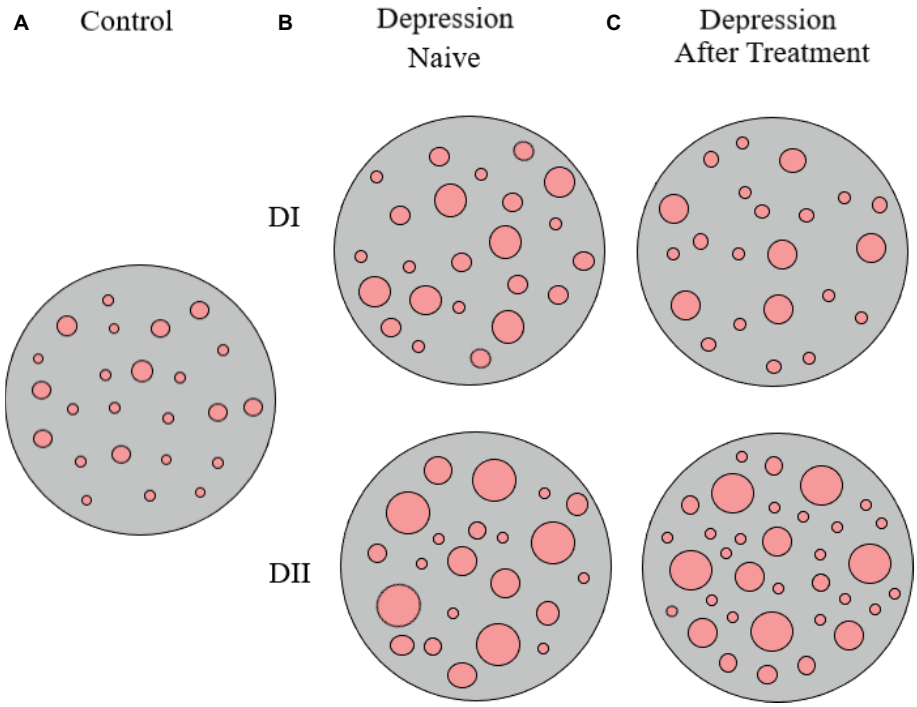

FIGURE 2 | Schematic representation of SERT clusters in the membrane of lymphocytes from control **(A)** and depression patients **(B,C)**. Analysis of SERT clustering in naïve depression patients allowed the differentiation of two subpopulations of depression patients (D-I and D-II) that primarily differ in the average size of SERT clusters. After 8 weeks of antidepressant treatment, there were no changes in SERT cluster number and size for D-I patients, but there was an increase in the number of SERT clusters in D-II patients (for more details see text, and Rivera-Baltanas et al., 2012).

of anhedonia symptoms in MDD, as anhedonia seems to be particularly refractory to treatment with current first-line antidepressant drugs (Shelton and Tomarken, 2001; Nutt et al., 2007; McCabe et al., 2009).

In a follow-up study, we studied possible alterations in the pattern on 5HT2A receptor clustering in lymphocytes in MDD (Rivera-Baltanas et al., 2014). We analyzed alterations in 5HT2A clustering in samples from a subset of the same population that we studied for SERT clustering (see above). We were surprised to find that measurements of 5HT2A clustering patterns in lymphocytes from naive MDD patients not only allowed us to again differentiate two patient subpopulations according to the distribution of 5HT2A cluster size, but that the same patients who were characterized as D-I and D-II when studying SERT clustering parameters (see above) were similarly shown as D-I or D-II when analyzing the characteristics of 5HT2A clusters and logically gave rise to a similar differential response to treatment (Rivera-Baltanas et al., 2014). In fact, these data made us think that perhaps there would be a general disturbance in membrane protein clustering in lymphocytes that may be operative in depression. Although logically additional studies on clustering patterns of other membrane proteins would be necessary to prove or falsify that hypothesis, we indicated that patterns of clustering of both SERT and 5HT2A receptor could be considered as putative biomarkers of therapeutic efficacy for MDD (Rivera-Baltanas et al., 2014).

As a possible limitation, we acknowledge that all these studies were carried out in a relatively small number of patients and thereby should be replicated in larger cohorts and also tested in a properly designed clinical trial.

ADDITIONAL STUDIES ON MEMBRANE PROTEIN CLUSTERING (MPC) IN LYMPHOCYTES IN AN ANIMAL MODEL OF DEPRESSION

In a very recent report, we evaluated the patterns of clustering of multiple proteins in the repeated-corticosterone model of depression (Romay-Tallon et al., 2018).

We centered our studies on proteins that tend to cluster into lipid rafts as alterations in G-protein-coupled receptor (GPCR) subunits integration into lipid rafts have been recently proposed as a putative mechanism of antidepressant actions (reviewed by Senese et al., 2018). As mentioned above, our analyses indicated that changes in SERT and 5HT2A receptor MPC in the repeated-corticosterone model of depression paralleled those observed in depression patients (Romay-Tallon et al., 2018). We also demonstrated that MPC patterns of SERT, 5HT2AR, dopamine transporter, and NMDA receptor 2B subunit, indicate an increase in cluster size but not in cluster number, while MPC analysis of beta-adrenergic receptor 2 gives rise to

a decrease in receptor cluster size but no changes in numbers, and MPC study of pannexin 1 and prion cellular protein indicates that both the number and size of clusters are increased in the repeated-corticosterone model of depression (Romay-Tallon et al., 2018). Thereby, this study indicated the feasibility of using animal models of depression both to study alterations in MPC in lymphocytes (including the design of novel studies focusing on mechanistic approaches) and to screen for additional patterns of MPC to be further studied in MDD patients.

OTHER STUDIES DESIGNED TO FACILITATE TRANSLATION OF MPC STUDIES TO THE CLINICAL SETTING

Thinking about fostering the validation of MPC studies as a biomarker of therapeutic efficacy for MDD and how to bring those studies closer to the clinical setting, we came to the realization that most diagnoses of MDD are first determined by family physicians in a family clinic and that collection of samples for MPC studies will be much facilitated and also be cheaper if those analyses could be performed directly on blood smears instead that having a trained nurse drawing blood samples and then performing MPC studies on extracted lymphocytes as it was done for the studies mentioned in this review. Accordingly, we designed a comparative study of immunolabeling and analysis of MPC in extracted lymphocytes and in blood smears (Romay-Tallon et al., 2017) and were able to demonstrate that altering some parameters in fixation, incubation, and image analysis setting can result in similar measurements in MPC in whole blood drawn samples and in blood smears (Romay-Tallon et al., 2017). One should also consider that proper establishment of fixation, incubation temperature and time, and image analysis protocols are essential for an adequate standardization of MPC analysis technologies (for a more detailed discussion see Rivera-Baltanas et al., 2010 and Romay-Tallon et al., 2017).

ADDITIONAL IDEAS AND EXPERIMENTS

The set of experiments discussed here clearly points to the interest of the MPC approach for developing of novel biomarkers of depression. Logically, this set of studies is complementary to the use of other technologies (i.e., "Omics," neuroimaging, etc.) with the final intention of providing novel tools that result in a more precise approach to the use of the therapeutic arsenal for MDD treatment from a personalized medicine viewpoint.

We already mentioned the necessity of validating these studies in larger cohorts and in proper clinical trials. It is also a necessity to develop a way to perform automatic analyses of MPC that will facilitate the standardization for the technology and provide more accurate and faster measurements.

Next steps will logically involve the analysis in MPC patients of proteins that have been screened in animal models, as explained above, as this will also increase the efficacy of the test and the validation of the biomarker. It is also logic to think in using a similar approach for studies of other psychiatric disorders not only in terms of evaluating MPC as biomarkers of therapeutic efficacy but also of differential diagnoses.

Independently of research focusing on fostering MPC analyses as biomarkers of psychiatric disorders, there is also the necessity of developing mechanistic studies designed to evaluate how stress and depression bring about alterations in MPC in immune cells, what are the functional/pathological consequences of these alterations from a psychoneuroimmunology perspective, what are the mechanisms on antidepressant actions on MPC, and to evaluate if similar MPC alterations are also prevalent in the CNS and what would be the consequences of such changes. Finally, we can also surmise that these set of data may foster the idea of using MPC as a process to screen for antidepressant efficacy of novel compounds.

CONCLUSIONS

The data discussed in this review indicate the feasibility of analysis of MPC as a technology to develop novel biomarkers of therapeutic efficacy for MDD, which hopefully together with other biomarker technologies can result in a more efficacious use of current antidepressant drugs.

AUTHOR CONTRIBUTIONS

HC wrote the initial draft of the review. All authors contributed to the original experiments and to the discussion, writing, and approval of the final version.

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Cytochrome P450 2C19 Poor Metabolizer Phenotype in Treatment Resistant Depression: Treatment and Diagnostic Implications

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Background: Pharmacogenomic testing, specifically for pharmacokinetic (PK) and pharmacodynamic (PD) genetic variation, may contribute to a better understanding of baseline genetic differences in patients seeking treatment for depression, which may further impact clinical antidepressant treatment recommendations. This study evaluated PK and PD genetic variation and the clinical use of such testing in treatment seeking patients with bipolar disorder (BP) and major depressive disorder (MDD) and history of multiple drug failures/treatment resistance.

Methods: Consecutive depressed patients evaluated at the Mayo Clinic Depression Center over a 10-year study time frame (2003–2013) were included in this retrospective analysis. Diagnoses of BP or MDD were confirmed using a semi-structured diagnostic interview. Clinical rating scales included the Hamilton Rating Scale for Depression ($HRSD_{24}$), Generalized Anxiety Disorder 7-item scale (GAD-7), Patient Health Questionnaire-9 (PHQ-9), and Adverse Childhood Experiences (ACE) Questionnaire. Clinically selected patients underwent genotyping of cytochrome P450 *CYP2D6/CYP2C19* and the serotonin transporter *SLC6A4*. PK and PD differences and whether clinicians incorporated test results in providing recommendations were compared between the two patient groups.

Results: Of the 1795 patients, 167/523 (31.9%) with BP and 446/1272 (35.1%) with MDD were genotyped. Genotyped patients had significantly higher self-report measures of depression and anxiety compared to non-genotyped patients. There were significantly more *CYP2C19* poor metabolizer (PM) phenotypes in BP (9.3%) vs. MDD patients (1.7%, $p = 0.003$); among participants with an S-allele, the rate of *CYP2C19* PM phenotype was even higher in the BP (9.8%) vs. MDD (0.6%, $p = 0.003$). There was a significant difference in the distribution of *SLC6A4* genotypes between BP (*l/l* = 28.1%, *s/l* = 59.3%, *s/s* = 12.6%) and MDD (*l/l* = 31.4%, *s/l* = 46.1%, *s/s* = 22.7%) patients ($p < 0.01$).

Conclusion: There may be underlying pharmacogenomic differences in treatment seeking depressed patients that potentially have impact on serum levels of *CYP2C19* metabolized antidepressants (i.e., citalopram / escitalopram) contributing to rates of efficacy vs. side effect burden with additional potential risk of antidepressant response vs. induced mania. The evidence for utilizing pharmacogenomics-guided therapy in MDD and BP is still developing with a much needed focus on drug safety, side effect burden, and treatment adherence.

Keywords: pharmacogenomics, cytochrome P450, *CYP2C19*, *SLC6A4*, bipolar disorder

INTRODUCTION

In 2018, the World Health Organization (WHO) identified major depressive disorder (MDD) and bipolar disorder (BP) as leading causes of disability worldwide, negatively impacting over 360 million people (Whiteford et al., 2013; Vos et al., 2015; Ferrari et al., 2016; World Health Organization, 2018). While genetic factors are thought to contribute 59–85% to BP risk (McGuffin et al., 2003; Lichtenstein et al., 2009), and 31–42% to MDD risk (Sullivan et al., 2000) or shared genetic risk related to overlapping symptoms of bipolar and major depressive disorder (Lee et al., 2013; Doherty and Owen, 2014), there is less systematic research focused on pharmacokinetic (PK) and/or pharmacodynamic (PD) genetic variation in these two distinct patient groups. This may be of potential interest recognizing marked differences in rates of antidepressant response and antidepressant induced mania (AIM+) by diagnostic group (Frye et al., 2015).

Selective serotonin reuptake inhibitors (SSRIs) are now considered first-line treatment for MDD (Crismon et al., 1999; Anderson et al., 2008), but only an approximate 50% of patients with MDD achieve partial remission, and only 30% complete remission, with SSRI therapy (Rush et al., 2006). However, antidepressants have less evidence base in bipolar depression and may in fact contribute to mood destabilization (Frye, 2011; Sidor and Macqueen, 2011). PK and PD genetic variation (i.e., pharmacogenomics) may contribute to BP and MDD treatment-resistance (Porcelli et al., 2012).

The use of pharmacogenomics testing for mental illnesses therapy selection has increased (Drozda et al., 2014). Thus, the implementing of pharmacogenomics-guided recommendations may improve treatment outcomes for patient with treatment-resistance depression (Kung and Li, 2010; Rundell et al., 2011b). Several studies have shown improvement in antidepressant response rates associated with the use of pharmacogenomic testing in clinical settings (Hall-Flavin et al., 2012; Hall-Flavin et al., 2013; Winner et al., 2013) and a recent meta-analysis of four randomized controlled trials and two open label trials have shown the same results (Rosenblat et al., 2018). However, several other reports (Rosenblat et al., 2017; Zeier et al., 2018; Zubenko et al., 2018) have identified potential limitations of industry support and lack of blinding and control groups (Hall-Flavin et al., 2012, 2013).

The goal of this study was to assess the outcomes of PK and PD genetic variation in treatment seeking depressed patients with history of multiple drug failures/treatment resistance and assess results of genomic testing on subsequent treatment recommendations. We assessed the clinical value of pharmacogenomic testing examining the differences in psychometrics mean scores at baseline between genotyped and non-genotyped patients; and assessed the relationships between PK (*CYP2D6* and *CYP2C19*) or PD (*SLC6A4*) genetic variations, and MDD/BP severity scales in pharmacogenomically-tested vs. not tested patients.

MATERIALS AND METHODS

This study was approved by the Mayo Clinic Institutional Board. All participants provided written informed consent prior to enrollment, evaluation and blood draw in the Mayo Clinic Depression Center.

Subjects

This was a naturalistic study. A consecutive sample of treatment-seeking adults (age 18–65) with a clinical diagnosis of MDD or BP, currently in a depressive episode, was recruited from the Mayo Clinic Depression Center between February 26, 2003 and March 27, 2013. Clinical diagnoses were confirmed by DSM-IV-TR Structured Clinical Diagnostic Interview (SCID). Inclusion criteria were based on patients who presented with long history of multiple drug failures or treatment resistance. Exclusion criteria were inability to provide written informed consent, other Axis I or II diagnoses that by clinical judgment were the main reason for seeking treatment, substance use disorder determined by clinical interview, and (+) drug screen (except nicotine and caffeine) were excluded. Data were abstracted from Electronic health record (EHR) by two reviewers (Caren J. Blacker and Kristin L. Borreggine). For data abstraction validation, 10% of the abstracted data was reviewed by Marin Veldic.

Clinical Ratings

Psychometrics utilized in the consultation included the 24 item Hamilton Rating Scale for Depression ($HRSD_{24}$) (Hamilton, 1960), Patient Health Questionnaire-9 (PHQ-9) (Spitzer et al., 1999), Generalized Anxiety Disorder 7 item scale (GAD-7) (Spitzer et al., 2006), and Adverse Childhood Experiences (ACE) questionnaire (Felitti et al., 1998); however, not all patients had all the scales completed at the time of consultation. Clinical demographics included age, gender, and treatment. The EHR data was reviewed to assess relevance of genotyping which was quantified as: (1) clinician providing genotype-guided

recommendations (GGR), or (2) clinician providing treatment as usual (TAU), where genotyping was or was not acknowledged, but treatment was guided based on the discretion of the treating clinician.

Genotyping

Subjects were evaluated for the clinical treatment decision impact of genetic testing for PK [cytochrome P450 *2D6* (*CYP2D6*) and *2C19* (*CYP2C19*)] and PD [serotonin transporter (*SLC6A4*)] genetic variation on treatment as usual in MDD or BP depressed patients. Testing was completed either with the AssureX Health GeneSight® platform or individual testing of PK or PD genes by Mayo Medical Laboratory. *CYP2D6* phenotypes were defined pharmacokinetically as extensive metabolizer (EM), intermediate metabolizer (IM), poor metabolizer (PM), or ultrarapid metabolizer (URM). *CYP2C19* phenotypes were defined pharmacokinetically as EM, IM, or PM. Detailed *CYP2D6* and *CYP2C19* allele variants are showed in **Supplementary Table S1**. *SLC6A4,* phenotypes were defined as [long/long (*l*/*l*)], [short/long (*s*/*l*)], or [short/short (*s*/*s*)]. *SLC6A4* has other genetic variations that may be relevant for the analysis (Hu et al., 2006). As reviewed by Frye and colleagues, in addition to the L allele and the *SLC6A4* and the SNP rs2553, known to influence the association of the 5-HTTLPR alleles with expression of the *SLC6A4* gene, there is a second intron variable number of tandem repeats (VNTR) identified that would be of interest in subsequent analysis. However, these variants were not identified in earlier samples of this study (i.e., from 2003) (Frye et al., 2015). The simultaneous determination of the long and short form of *SLC6A4* was performed by polymerase chain reaction (PCR) amplification of the promoter region of 5-HTT followed by Haemophilus parainfluenzae II digestion of the resulting amplicon, as described by Wendland et al. (2006). *CYP2C19* and *CYP2D6* genotyping was performed on genomic DNA extracted from whole blood using the xTAG Assay for P450-2C19 v2, which incorporates multiplex PCR and multiplex allele-specific primer extension (ASPE) with Luminex Molecular Diagnostics' proprietary Universal Tag sorting system on the Luminex 100 xMAP platform. Detailed genotyping laboratory methodology is highlighted in our previous work (Mrazek et al., 2009; Mrazek et al., 2011; Frye et al., 2015).

Statistical Analysis

Means and standard deviations are presented for continuous variables, and were compared by genotyping status and recommendation group using *t*-test or Wilcoxon Rank Sum tests. Chi-squared test and Fisher's exact test were used to describe the differences in proportions between the genotyping status and recommendation group. The level of statistical significance was set at $p < 0.05$ (two-sided).

RESULTS

Patient Characteristics

Using the SCID, 523 of the 1795 patients were diagnosed with BP and 1272 were diagnosed with MDD. 167/523 (31.9%) with BP and 446/1272 (35.1%) with MDD were genotyped. 317 subjects (18%) and 510 subjects (28%) underwent *CYP2D6/CYP2C19* and *SLC6A4* genotyping, respectively. Genotyped patients were less prescribed antidepressants ($p = 0.009$) versus other medication classes, and had significantly higher measures of self-reported anxiety (GAD-7 = 12.9 (5.6), $p < 0.016$) and depression (PHQ-9 = 18 (6.1), $p < 0.001$) in comparison to non-genotyped patients (**Table 1**). PK and PD genotype-guided recommendations were associated with significantly higher measures of anxiety and depression [(GAD-7 = 13.2 (5.6), $p = 0.02$) and (PHQ-9 = 18.1 (6.1), $p = 0.005$), (GAD-7 = 13.2 (5.63), $p = 0.009$) and (PHQ-9 = 18.0 (6.1), $p < 0.001$), respectively] (**Table 2**). There was no significant association between different genotypes and the measures of anxiety and depression.

CYP2C19

The pharmacogenomic profiles of *CYP2C19* were: PM (3.5%), IM (27.4%), and EM (69.1%). There was a higher rate of *CYP2C19* poor metabolizer phenotype in BP (9.3%) vs. MDD patients (1.7%, $p = 0.003$) (**Table 3**). Among those participants with an S-allele, the rate of *CYP2C19* PM phenotype was even higher in the BP (9.8%) vs. MDD (0.6%, $p = 0.003$). There was no significant difference in distribution of treatment guided recommendations groups between *CYP2C19* phenotypes [EM (GGR = 68.85%,

TABLE 1 | Demographics and Clinical Characteristics of Patients with Genotyped *vs.* Not Genotyped.

	Genotyped (*n* = 613)	Not Genotyped (*n* = 1182)	*p*
Demographic			
Female	66.7%	61.6%	0.032
Age, years	43.3 (12.8)	42.0 (14.2)	< 0.001
MDD	72.8%	69.9%	0.202
Race/Ethnicity			
White (Caucasian)	89.2%	89.8%	0.282
Black/African American	1.1%	0.7%	
Asian	0.7%	1.8%	
American Indian/Alaskan Native	0.7%	0.3%	
Native Hawaiian/Pacific Islander	0.2%	0.1%	
Others/ Unknown	8.2%	7.3%	
Rating scales			
$HRSD_{24}$	31.6 (8.5)	30.3 (9)	0.671
PHQ-9	18 (6.1)	15.5 (6.8)	< 0.001
GAD 7	12.9 (5.6)	10.4 (6.2)	< 0.016
ACE	2.0 (2.3)	2.2 (2.2)	0.412
Medications			
Mood stabilizers	12.4%	11.6%	0.617
Benzodiazepines	24.8%	24.1%	0.749
Antidepressants	33.1%	39.3%	0.009
Stimulants	4.7%	3.1%	0.093

Values are expressed as Mean (SD) unless otherwise indicated; MDD, major depressive disorder; $HRSD_{24}$, 24 item Hamilton Rating Scale for Depression; PHQ-9, item scale Patient Health Questionnaire-9; GAD 7, Generalized Anxiety Disorder 7; ACE, Adverse Childhood Experiences score. p-value, Chi-squared test.

TABLE 2 | Demographics and Clinical Characteristics of Patients with Genotype-Guided Recommendations (GGR) *vs.* Treatment as usual (TAU).

	CYP2D6 / CYP2C19			***SLC6A4***		
	GGR (*n* = 317)	**TAU (*n* = 1478)**	***p***	**GGR (*n* = 510)**	**TAU(*n* = 1285)**	***p***
Demographic						
Female	63.4%	63.4%	0.979	66.3%	62.2%	0.104
Age, years	44.2 (13.1)	42.1 (13.9)	0.152	43.4 (12.8)	42.1 (14.1)	0.003
MDD	76.3%	69.7%	0.018	73.5%	69.8%	0.117
Rating scales						
$HRSD_{24}$	32.4 (8.7)	30.6 (8.9)	0.867	32.2 (8.3)	30.2 (9.1)	0.240
PHQ-9	18.1 (6.1)	15.8 (6.7)	0.005	18.0 (6.1)	15.6 (6.8)	< 0.001
GAD 7	13.2 (5.6)	10.5 (6.2)	0.02	13.2 (5.63)	10.4 (6.2)	0.009
ACE	2.0 (2.3)	2.2 (2.2)	0.648	2.0 (2.2)	2.2 (2.2)	0.720
Medications						
Mood stabilizers	12.3%	11.8%	0.791	12.9%	11.4%	0.375
Benzodiazepines	28.7%	23.4%	0.046	26.5%	23.5%	0.186
Antidepressants	33.8%	38.0%	0.160	35.1%	38.1%	0.242
Stimulants	5.1%	3.4%	0.153	5.1%	3.1%	0.044

Values are expressed as SD, mean unless otherwise indicated; MDD, major depressive disorder; GGR, Genotype-Guided Recommendations; TAU, treatment as usual. $HRSD_{24}$, 24 item Hamilton Rating Scale for Depression; PHQ-9, item scale Patient Health Questionnaire-9; GAD 7, Generalized Anxiety Disorder 7; ACE, Adverse Childhood Experiences score. p-value, Chi-squared test.

TABLE 3 | Phenotype results by diagnose and Genotype-Guided Recommendations (GGR) *vs.* Treatment as usual (TAU).

Gene	**Phenotype**	**MDD**	**BP**	***p***	**Phenotype**	**GGR**	**TAU**	***p***
CYP2D6	PM (*n* = 45)	13.2 %	17.3%	0.41	PM (*n* = 42)	16.0%	5.1%	0.087
	IM/EM (*n* = 242)	78.1 %	70.7%		IM/EM (*n* = 232)	75.0%	83.1%	
	URM (*n* = 30)	8.7 %	12.0%		URM (*n* = 29)	9.0%	11.9%	
CYP2C19	PM (*n* = 11)	1.7%	9.3%	**0.003**	PM (*n* = 11)	4.1%	1.7%	0.67
	IM (*n* = 87)	29.3%	21.3%		IM (*n* = 83)	27.1%	28.8%	
	EM (*n* = 219)	69.0%	69.3%		EM (*n* = 209)	68.9%	69.5%	
SLC6A4	*s/s* (*n* = 102)	22.7%	12.6%	**0.012**	*s/s* (*n* = 96)	20.9%	15.9%	0.13
	s/l (*n* = 253)	46.1%	59.3%		*s/l* (*n* = 246)	47.2%	32.1%	
	l/l (*n* = 155)	31.2%	28.2%		*l/l* (*n* = 150)	31.9%	26.8%	

MDD, major depressive disorder; BP, bipolar depression. GGR, Genotype-Guided Recommendations; TAU, treatment as usual. PM, poor metabolizer; IM/EM, intermediate metabolizer/extensive metabolizer; URM, ultra-rapid metabolizer; l, long; s, short allele variants. p-value, Chi-squared test.

TAU = 69.49%), IM (GGR = 27.05%, TAU = 28.81%), PM (GGR = 4.10%, TAU = 1.69%), ($p = 0.67$)] (**Table 3**).

CYP2D6

The pharmacogenomic profiles of *CYP2D6* were: IM/EM (76.3%), PM (14.2%), and URM (9.5%). There was no significant difference in distribution of *CYP2D6* phenotypes by diagnosis ($p = 0.41$) (**Table 2**). There was no significant difference in distribution of treatment guided recommendations groups between *CYP2D6* phenotypes [PM (GGR = 16%, TAU = 5.1%), EM/IM (GGR = 75%, TAU = 83.1%), URM (GGR = 9%, TAU = 11.9%), ($p = 0.087$)] (**Table 3**).

SLC6A4

The pharmacogenomic profiles of *SLC6A4* were: *l/l* (30.4%), *s/l* (49.6%), and *s/s* (20.0%). There was a statistically significant difference in distribution of *SLC6A4* genotypes between BP (*l/l* = 28.2%, *s/l* = 59.3%, and *s/s* = 12.6%) and MDD (*l/l* = 31.2%, *s/l* = 46.1%, and *s/s* = 22.7%) patients ($p = 0.012$) (**Table 3**). Among S-allele carries, in comparison to MDD patients, there was a significantly higher rate of BP patients with PM in either *CYP2D6* or *CYP2C19*. There was no significant difference in distribution of treatment guided recommendations groups between *SLC6A4* phenotypes [*l/l* (GGR = 31.9%, TAU = 26.8%), *s/l* (GGR = 47.2%, TAU = 32.1%), and *s/s* (GGR = 20.9%, TAU = 15.9%) ($p = 0.13$)] (**Table 3**).

DISCUSSION

This study assessed the relationship between symptom severity, demographics, and pharmacokinetic / pharmacodynamics genetic variation among diagnostic mood disorder subgroups. There was a significant difference in *CYP2C19* and *SLC6A4* PK and PD phenotype distribution between BP and MDD patients with history of multiple drug failures/treatment resistance. Specifically, there were significantly higher rates of *CYP2C19* PM in BP patients in comparison to MDD

patients; among those participants with an S-allele, the rate of *CYP2C19* PM phenotype was more than 10X higher in the BP vs. MDD.

The clinical implications of *CYP2C19* and serotonin transporter genetic variation are not fully understood. It is known, however, that poor metabolizer phenotype is associated with high blood levels and increased risk of side effects. As suggested by the Clinical Pharmacogenetics Implementation Consortium (CPIC) Guideline and Nassan et al. (2016), individuals on citalopram / escitalopram with *CYP2C19* PM phenotype should reduce dose by 50% and /or use an alternative antidepressant (Hicks et al., 2015). There has been little investigation as to metabolizer status, blood level, and risk of antidepressant induced mania in either bipolar or unipolar depression. There is evidence to suggest that the *s/s* genotype is associated with increased antidepressant side-effects, including antidepressant-induced mania (Frye et al., 2015); further studies should investigate risk of Antidepressant Induced Mania (AIM+) as a function of PK-PD interaction as is being done with other antidepressant pharmacogenomic antidepressant analyses (Ahmed et al., 2018). The genotype-guided recommendations of *CYP2D6*, *CYP2C19*, and *SLC6A4* were associated with significantly higher measures of anxiety and depression in comparison to treatment as usual. Like Rundell et al., 2011a, our study has found significantly higher baseline self-reported scores of depression in GGR individuals' possible indicative of increase symptom burden and greater treatment resistance.

The results of *CYP2D6* / *CYP2C19* genotyping were more commonly used to make treatment recommendations in MDD than in BP. This study is limited by it cross sectional design with no longitudinal mood outcome data based on GGT vs. TAU. However, there is increasing interest and investigation identifying PK *CYP2D6* and *CYP2C19* genetic variants associated with clinical response to several SSRIs (Tsai et al., 2010; Mrazek et al., 2011; Gressier et al., 2015; Hicks et al., 2015). Several studies have investigated GGR vs. TAU in treatment of MDD patients. However, none have included BP patients. Studying ACE score in relationship with *SLC6A4* S-allele and depression severity is also important, as there are gene and environment interactions (Caspi et al., 2003).

Limitations

The decision to genotype was based on clinical factors and not pre-determined systematic criteria. Typically, patients who received genotyping might also have been self-selected and more interested in receiving it. Thus, there is inherent selection bias affecting the comparison between the two diagnostic groups. Even though the sample size was large, given the lower prevalence of *CYP2C19* PM and *SLC6A4* S-allele, the final number of patients with these findings were (n = 6) and, ideally, the initial sample size should be larger. This study did not have systematic follow-up to look at outcome measures of efficacy and side effects/tolerability based on these recommendations; these are important prospective studies to complete and such studies are currently underway. Our outcomes data have lacked the statistical power to accurately analyze the ancestry data; due to 89% of our population being white Caucasians, this may have affected the interpretation of our findings, this study was conducted in a clinical setting with a naturalistic study design, and is lacking standard criteria for the selection of patients for pharmacogenetic testing (Gelernter et al., 1997; Mrazek et al., 2009; Strom et al., 2012). Although, this type of design has the advantage of mimicking "real life" clinical practice, it has significant limitations when it comes to controlling for confounding. This is an issue that needs to be addressed in the future through longitudinal prospective studies with systematic genetic screening. Finally, our sample data was deficient of medication blood levels, which would clarify some of the study findings.

CONCLUSION

There may be underlying pharmacogenomic differences in treatment seeking depressed patients that potentially have impact on serum levels of *CYP2C19* metabolized antidepressants (i.e., citalopram / escitalopram) contributing to rates of efficacy vs. side effect burden with additional potential risk of antidepressant response vs. induced mania. The evidence for utilizing pharmacogenomics-guided therapy in MDD and BP is still developing with a much needed focus on drug safety, side effect burden, and treatment adherence. Future work is essential; scientific and logistic barriers still exist before there can be widespread implementation of clinical genomics. Genomic science has a profound potential to individualize the drug therapy for depression.

ETHICS STATEMENT

This study was carried out in accordance with the recommendation of the Mayo Clinic Institutional Review Board with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Mayo Clinic Institutional Review Board.

AUTHOR CONTRIBUTIONS

ATA and JRG performed all the data analysis. CJB, KLB, and MV contributed to the acquisition of data. JMB assisted with data analysis and interpretation of findings. All co-authors provided critical revision of the manuscript for important intellectual content. MV and MAF were responsible for the study concept and design. MV an ATA drafted the manuscript. All authors critically reviewed content and approved the final version for publication.

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SUPPLEMENTARY MATERIAL

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frontiers in Neuroscience

REVIEW
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Exploring the Potential Antidepressant Mechanisms of TNFα Antagonists

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Human and animal studies suggest an intriguing relationship between the immune system and the development of depression. Some peripherally produced cytokines, such as TNF-α, can cross the blood brain barrier and result in activation of brain microglia which produces additional TNF-α and fosters a cascade of events including decreases in markers of synaptic plasticity and increases in neurodegenerative events. This is exemplified by preclinical studies, which show that peripheral administration of pro-inflammatory cytokines can elicit depression-like behavior. Importantly, this depression-like behavior can be ameliorated by anti-cytokine therapies. Work in our laboratory suggests that TNF-α is particularly important for the development of a depressive phenotype and that TNF-α antagonists might have promise as novel antidepressant drugs. Future research should examine rates of inflammation at baseline in depressed patients and whether anti-inflammatory agents could be included as part of the treatment regimen for depressive disorders.

Keywords: depression, inflammation, cytokines, stress, TNF-α, rat, antidepressant, hippocampus

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DEPRESSION

Depression remains the most common psychiatric disorder, affecting approximately 350 million people worldwide. Accordingly, depression now ranks as the top cause of global disability in terms of years lost due to disability. The defining characteristics of depression include anhedonia, a loss of interest in pleasurable activities, and lowered mood (Nestler et al., 2002). Depression is further characterized by alterations in cognition, weight, sleep, irritability, thoughts of suicide, and decreased sexual function/interest (Nemeroff, 1998). Medications that target the monoaminergic system provide the primary course of treatment for depressed patients. These medications include monoamine oxidase inhibitors (MOAI's), tricyclic antidepressants (TCAs), and selective serotonin reuptake inhibitors (SSRI's). Despite their widespread use, currently available antidepressants are frequently ineffective, with the percentage of patients experiencing remission as low as 45% (Thase et al., 2001). This suggests that factors other than disruptions in monoamines are at play in the development of depressive symptoms. Indeed, recent evidence has implicated astroglial pathology (Wang et al., 2017), mitochondrial dysfunction (Allen et al., 2018), and deficient hippocampal neurogenesis and neuronal maturation (Bessa et al., 2009; Eisch and Petrik, 2012; Lussier et al., 2013; Mateus-Pinheiro et al., 2013) as potential causal factors in depression. In addition, it has long been thought that neuroinflammation plays a key role in the etiology of depression

(Brites and Fernandes, 2015; Furtado and Katzman, 2015). In this short review, we focus on new data suggesting that elevations in the inflammatory cytokine TNF-α might instigate depressive symptoms and that therapies aimed at reducing TNF-α levels hold therapeutic promise.

THE ROLE OF INFLAMMATION IN DEPRESSION

The notion that inflammation could be a contributing factor in the pathogenesis of depression is not a new one. Work in the 1980s's first revealed that some patients with heightened immune activity (e.g., a patient with an intense cold) displayed the hallmark features of depression (i.e., lethargy, depressed mood, anhedonia) (Miller and Raison, 2016). This in turn led to the cytokine sickness hypothesis of depression, which posits that sustained increases in circulating levels of pro-inflammatory cytokines can produce depressive symptoms (Dantzer, 2009). This hypothesis is summarized in **Figure 1**. Interestingly, high circulating levels of the pro-inflammatory cytokine IL-6 are strongly associated with feelings of guilt and suicidal ideation (Alesci et al., 2005; O'Donovan et al., 2013). If the cytokine sickness hypothesis of depression is correct, then one would expect that disorders characterized by high circulating levels of cytokines would share a high comorbidity with depression, and this turns out to be the case. For example, rheumatoid arthritis, which is a disorder in which the immune system targets bodily tissues and instigates widespread inflammation, shares a 13–42% comorbidity rate with depression (Margaretten et al., 2011). Furthermore, cancer patients treated with cytokines experience a significant reduction in plasma tryptophan levels that coincides with depression (Capuron et al., 2002). This tryptophan reduction reduces the bioavailability of serotonin, which is a known risk factor for the development of depression according to the monoamine hypothesis. It was later discovered that the culprit for this decrease in plasma tryptophan levels in cancer patients receiving immunotherapy is indoleamine 2,3-dioxygenase (IDO). Activation of IDO decreases tryptophan bioavailability, creating a net decrease in monoamines (Dantzer, 2009). Immuno-activation in healthy control subjects creates depressive-like behavior and impairments in cognition (Reichenberg et al., 2001), and serum concentrations of interleukin-6 at 9 years of age is positively correlated with depressive symptomology at 18 years of age (Khandaker et al., 2014). Finally, high circulating levels of cytokines are associated with treatment-resistant depression (Carvalho et al., 2013).

Stress is also known to transiently elevate the expression of pro-inflammatory cytokines. Unsurprisingly, pro-inflammatory cytokines are efficient activators of the hypothalamic-pituitary-adrenal (HPA) axis (Kenis and Maes, 2002), which often becomes dysregulated in depression. However, as not all depressed patients display a dysregulated HPA axis, it is tempting to speculate that elevated levels of pro-inflammatory cytokines occur in a subset of depression patients, possibly those patients who develop symptoms after a period of major life stressors leading to HPA dysfunction.

INFLAMMATION AND CYTOKINES: THE ROLES OF TNF-α

Cytokines are increasingly being recognized as having a contributive role in the development of depressive symptoms. Treatment with proinflammatory cytokines, including IL-1, IL-6, or TNF-α, or lipopolysaccharide (LPS), induces sickness behavior and corresponding depression-like behavior on the forced swim test (Dantzer, 2004; Dunn and Swiergiel, 2005; Henry et al., 2008). Yang et al. (2015) showed that chronic mild stress (CMS) resulted in an increased expression of proinflammatory cytokines, particularly IL-1, IL-6, and TNF-α, and lower immunoreactivity of myelin protein and decreased numbers of oligodendrocytes in the prefrontal cortex that coincided with the development of depression-like behavior. Maldonado-Bouchard et al. (2015) showed that the stress associated with lesioning the spinal cord in rodents resulted in increased levels of hippocampal cytokines and increased depression and anxiety-like behaviors. This suggests that the inflammation and increased cytokine release *per se* produced by a spinal cord injury can lead to the development of depression-like behaviors. Dunn and Swiergiel (2005) demonstrated that mice treated with IL-1 spent significantly more time immobile on both the forced swim test and tail suspension test, which are two classic rodent indices of depression-like behavior. Mice that lack certain cytokines or cytokine receptors do not display stress-induced depression-like behavior (Chourbaji et al., 2006), which suggests that lower levels of cytokines confer a protective effect on the development of depression-like behavior. The idea that low levels of cytokines could protect against the development of depression-like behavior is an interesting one and one that will be explored in greater detail in subsequent sections of this review.

Although the release of pro-inflammatory cytokines can contribute to the development of depression-like behavior, TNF-α in particular is receiving considerable attention due to its prominent roles in promoting inflammation and its dampening effects on synaptic plasticity (Khairova et al., 2009; Pribiag and Stellwagen, 2014; Lewitus et al., 2016). It is important to differentiate between TNF-α in the periphery and TNF-α in the brain. Recent findings suggest TNF-α is produced peripherally by leukocytes, lymphoid cells, mast cells, endothelial cells, and adipose tissue and is involved in functions of host defense including the stimulation of protective granuloma formation incurred during mycobacterial infections and the promotion of liver and spleen function (Kruglov et al., 2008). However, when TNF-α signaling is not tightly controlled, dysregulation of peripheral TNF-α signaling can contribute to the development of inflammatory and autoimmune disorders including septic shock and rheumatoid arthritis (Kruglov et al., 2008).

TNF-α is a protein that is initially released as a soluble cytokine (sTNF-α) after being enzymatically cleaved by its cell surface bound precursor (tmTNF-α) by TNF-α converting enzyme

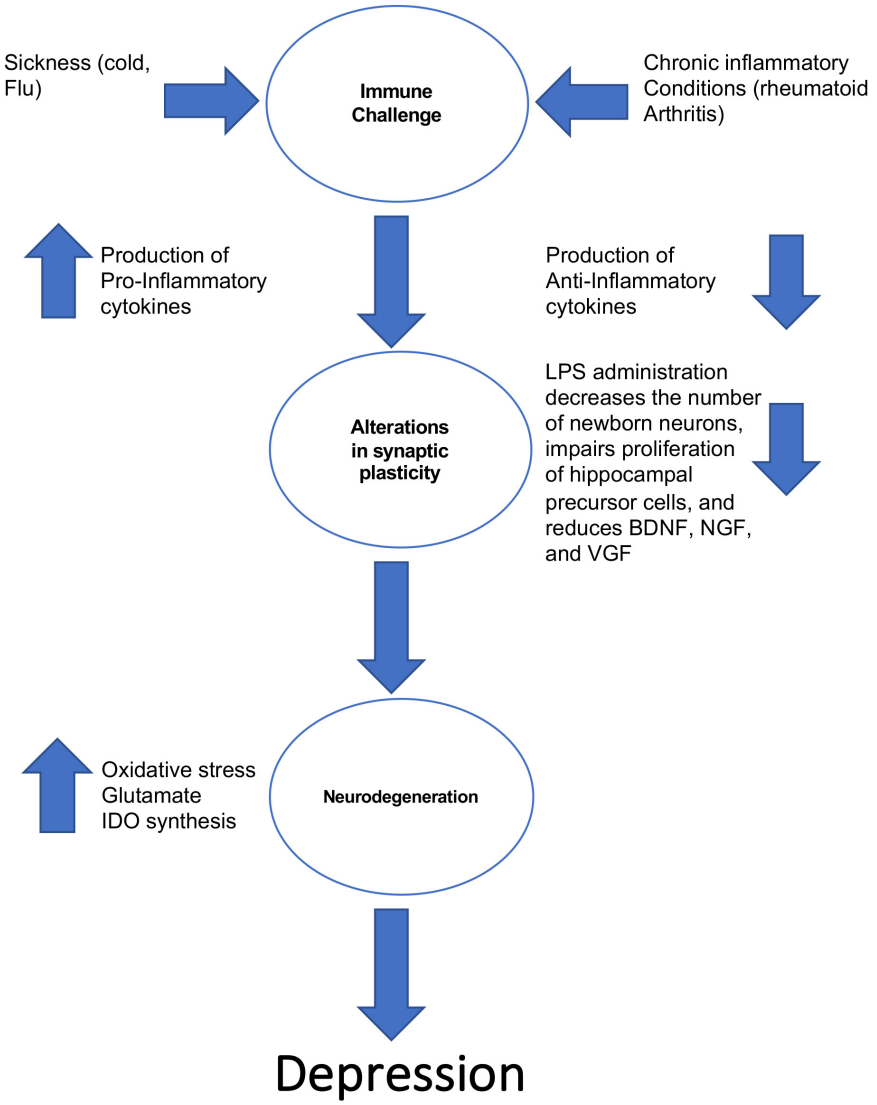

FIGURE 1 | The ways in which inflammation can lead to the development of depressive-like behavior.

(TACE) (Bortolato et al., 2015) and is therefore expressed as a transmembrane protein. TNF-α binds to one of two receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). TNFR1 is activated by soluble and transmembrane TNF-α, and promotes inflammation and tissue degeneration (Kalliolias and Ivashkiv, 2016). TNFR2's expression is restricted to neurons, endothelial cells, and immune cells, and is involved in mediating cell survival and tissue regeneration (Kalliolias and Ivashkiv, 2016). The sTNF-α possesses a higher affinity for binding with TNFR1. When TNF-α binds to TNFRs, complex 1 is assembled at the plasma membrane and includes the TNF-α associated death domain protein (TRADD) among other complexes, resulting in the creation of a scaffolding ubiquitin network (Kalliolias and Ivashkiv, 2016). This scaffolding ubiquitin creates the recruitment and activation of two signaling complexes: transforming growth factor (TGF) -β activated kinase 1 (TAK1) complex and the inhibitor of kB (Ikkβ) kinase complex (Kalliolias and Ivashkiv, 2016).

One of the main roles of TNF-α is in maintaining inflammation during times of proinflammatory conditions. During proinflammatory events, TNF-α production is induced by other cytokines (e.g., IL-1) and microglia. Once released, TNF-α stimulates the production of other proinflammatory cytokines, including IL-1 and 6, and it increases the production of reactive oxygen intermediates, including nitric oxide (Bortolato et al., 2015). It is easy to conceptualize this process as a positive feedback loop, whereby an initial stressful or inflammatory event triggers the release of TNF- α, which in turn triggers the release of other pro-inflammatory cytokines, creating a state of prolonged inflammation. This helps explain, why autoimmune diseases are among the hardest disorders to treat. Perhaps it is not surprising that increased inflammation as a result

of sustained TNF-α production and release results in altered glutamatergic signaling and excitotoxicity. Mechanistically, TNF-α upregulates glutaminase (the enzyme responsible for the conversion of glutamate from glutamine) expression, resulting in the transportation of glutaminase from the mitochondria into the extracellular space. This in turn leads to elevated concentrations of glutamate both intracellularly and extracellularly, eventually causing cell death through excitotoxicity (Ye et al., 2013). This fits in line with the reported elevations of plasma glutamate levels seen in depressed populations (Inoshita et al., 2018). Interestingly, proinflammatory cytokines (TNF-α) trigger the release of kidney type glutaminase (KGA) from mitochondria, which then travels to the cytosolic compartment of neurons (Ye et al., 2013), increasing glutamate content. This is of interest as we have recently published a report outlining a link between mitochondrial function and depression (see Allen et al., 2018).

TNF-α AND DEPRESSION: ANIMAL MODELS AND CLINICAL STUDIES

Preclinical studies corroborate the role of TNF-α in depression-like behavior. Peripheral administration of TNF-α can produce anhedonic behavior in rodents (van Heesch et al., 2013). Likewise, deletion of TNFR1 or TNFR2 creates an antidepressant phenotype on measures of depressive-like behavior (Simen et al., 2006). Yamada et al. (2000) showed that TNF-α knockout mice display a mild antidepressant phenotype. Along these same lines, administration of the TNF-α inhibitor infliximab during chronic mild stress significantly decreased immobility time in the forced swim test, increased sucrose consumption during the sucrose preference test, and decreased anxiety-like behavior in the elevated plus maze (Karson et al., 2013).

Given that cytokine treatment produces depression-like behavior and reducing cytokine levels alleviates this, the question that arises is whether or not antidepressants can influence inflammation. Interestingly, SSRIs and tricyclic antidepressants are known to reduce levels of TNF-α and other proinflammatory cytokines and increase anti-inflammatory cytokines, including IL-10 (Song et al., 1994). Two potential explanations for how antidepressant drugs might reduce pro-inflammatory cytokine levels have been offered. The first explanation posits that higher levels of activation of serotonergic receptors located on immune cells dynamically regulate the production of pro-and anti-inflammatory cytokines, and this could be influenced by a higher availability of serotonin upon antidepressant treatment, although the pro- or anti- inflammatory effects of peripheral serotonin is still under debate (recently reviewed in Herr et al., 2017; see also Kenis and Maes, 2002). The second explanation sees the increased production of cyclic adenosine monophosphate (cAMP) by antidepressants as a mechanism by which antidepressants might reduce cytokine levels. Specifically, cAMP activates protein kinase A (PKA), increasing the production of cAMP responsive element binding protein (CREB), both of which act to decrease pro-inflammatory cytokine production (Kenis and Maes, 2002).

Considerable attention has been dedicated to the antidepressant potential of ketamine. Ketamine rapidly reverses depression-like behavior in both clinical and preclinical subjects, in a timeframe of hours (Yoshi and Constantine-Paton, 2010; Cusin et al., 2012). An important question asked within the literature is whether ketamine is altering neuroinflammation? Although this question is relatively recent, it does appear to be the case that at least a component of the antidepressant properties of ketamine involve reductions in neuroinflammation, in particular TNF-α. For example, Wang et al. (2015) have shown that in chronically-stressed rats, the rapid antidepressant effects of ketamine are accompanied by a reduction of hippocampal TNF-α levels. Moreover, a reduction in depressive symptoms 40 min post-ketamine infusion in depressed patients has been shown to be correlated with reductions in serum TNF-α levels (Chen et al., 2018). These findings suggest that rapid changes in neuroinflammation, in particular TNF-α, are perhaps one of the mechanisms underlying the antidepressant actions of ketamine.

In terms of clinical studies, a large body of evidence supports the role of TNF-α in depression. Endotoxin administration in control subjects produces an increase in TNF-α in addition to depressed mood and cognitive impairment (Della and Hannestad, 2010). Microarray mRNA studies demonstrate increased expression of tmTNF-α in the prefrontal cortex (PFC) of suicide victims (Pandey et al., 2012). In fact, higher suicidal ideation itself is associated with an increased cytokine profile, including elevated TNF-α (O'Donovan et al., 2013), and high circulating levels of TNF-α are found in peripheral tissues of suicide victims (Lindqvist et al., 2009). Several lines of evidence support the efficacy of TNF-α inhibitors in the treatment of depression. Patients with rheumatoid arthritis and plaque psoriasis taking prescribed etanercept, which is a TNF-α antagonist, reported significant reductions in depressive symptoms (Gelfand et al., 2008; Kekow et al., 2011). Similarly, patients with Crohns Disease receiving infusions of infliximab experienced significant reductions in depressive symptoms and this decrease was associated with corresponding reductions in proinflammatory cytokines (Guloksuz et al., 2013). Finally, psoriasis patients with comorbid psychiatric conditions report improvement in mood and overall well-being when taking infliximab (Bassukas et al., 2008). Interestingly, inflammation itself is associated with anhedonia, and one of the first symptoms to be alleviated in depressed patients receiving anti-inflammatory compounds is anhedonia (Felger et al., 2016).

Some interesting extensions of these clinical studies have been observed in patients with treatment-resistant depression. Raison et al. (2013) reported that patients with treatment - resistant depression with a high baseline level of inflammation as indicated by elevated high sensitivity C-reactive protein expression responded favorably to infusions of the TNF-α inhibitor infliximab. However, in patients with a low baseline level of high sensitivity C-reactive protein infliximab was not more effective than placebo. This pattern of results suggests that subsets of treatment-resistant patients experience high levels of inflammation, and therapies aimed at reducing inflammation might be particularly effective in these patients. However, other patients may have a different physiological profile such that factors other than inflammation are at play. This conclusion is consistent with the observation that two depressed patients

can present with a different cluster of symptoms. For example, patient A could present with agitation, weight loss, an inability to sleep, and lowered mood, whereas patient B presents with weight gain, excess sleep, psychomotor retardation, and anhedonia. This raises the question (asked elsewhere; see Nestler and Hyman, 2010) of whether depression is actually part of a constellation of different disorders. It is tempting to suggest that depressed patients with inflammation might represent a subset of depressed patients who require a different course of treatment compared to their non-inflamed counterparts. Kappelmann et al. (2016) conducted a meta-analysis of the effectiveness of anti-cytokine treatments in depression. They found that across seven double-blind clinical trials involving 1309 subjects, anti-cytokine treatment was generally more efficacious than treatment with placebo. Of interest is the fact that several research groups have reported that currently available non-cytokine antidepressant medications are not more effective than placebo, at least in the treatment of non-severe depression (Garland, 2004; Kirsch et al., 2008; Fournier et al., 2010). Therefore, treatments that target inflammation might represent a more viable approach to the treatment of depression, as the monoamine hypothesis of depression largely does not align with what is currently known about the biological causes of depression. Of the anti-cytokine treatments analyzed, Kappelmann et al. (2016) reported that anti-TNF-α drugs were the most commonly used option. The picture that emerges is that depression is associated with elevations in TNF-α, and treatments aimed at reducing circulating TNF-α produce significant normalization of depressive symptoms. In the next section, we elaborate on some putative mechanisms underlying the antidepressant effect of TNF-α antagonists.

TARGETING TNF-α IN DEPRESSION

Peripheral injection of TNF-α antagonists (i.e., etanercept) causes a functional decrease in peripheral TNF-α with only an indirect effect on central TNF-α expression, as drugs like etanercept cannot cross the blood brain barrier (Boado et al., 2010). Therefore, it has been assumed that drugs like etanercept are only able to indirectly reduce central inflammation as a consequence of reduced peripheral TNF-α activity (Kerfoot et al., 2006). However, TNF-α *per se* can cross the blood brain barrier by a receptor mediated mechanism (Pan and Kastin, 2001, 2002; Pan et al., 2006), and when this occurs, it instigates an increase of both TNF-α protein and mRNA by stimulating central expression of TNF-α by microglial cells (Qin et al., 2007; McCoy and Tansey, 2008). Peripheral TNF-α also can stimulate secretion of TNF-α from circumventricular organs and choroid plexus, and TNF-α secreted by these organs can then induce the activation of microglia and a subsequent increase in TNF-α secretion by microglial cells (Qin et al., 2007; McCoy and Tansey, 2008). As stated, etanercept does not cross the blood brain barrier but it binds peripheral TNF-α, and in doing so, it reduces the effect of peripheral TNF-α in promoting the activation of microglia, which results in decreased secretion of central TNF-α.

One should therefore expect that etanercept injections would prevent some of the central effects of protracted release of central TNF-α, such as its effects on hippocampal activity and neurogenesis (see McCoy and Tansey, 2008; and Bortolato et al., 2015). In line with this observation, we have recently shown that semi-weekly peripheral injections of etanercept (0.8 mg/kg) can normalize the depression-like behavior produced by 21 days of exogenous corticosterone injections in rats (Brymer et al., 2018). Etanercept can also restore performance on object-location and object-in-place recognition memory tests of hippocampal functioning. Moreover, etanercept restores the number and complexity of dentate subgranular/granular neurons expressing doublecortin, which is a marker of immature newborn neurons, and perhaps this action of etanercept may be underlying its antidepressant effects (Brymer et al., 2018). It should in any case be noted that the role of adult hippocampal neurogenesis in depression remains a contentious issue. Antidepressant effects can be achieved without increases in neurogenesis, and ablation of neurogenesis is not sufficient to create a depression-like phenotype (Hanson et al., 2011). Recently, Sorrels et al. (2018) found that human hippocampal neurogenesis sharply drops from childhood to near undetectable levels in adulthood, boldly suggesting that hippocampal neurogenesis does not occur past childhood. However, counter to this report, Boldrini et al. (2018) report that human hippocampal neurogenesis persists throughout the lifespan, even into the 70th year of life. Moreover, recent reports have found reductions in hippocampal neurogenesis in post-mortem tissue from patients with depression (Boldrini et al., 2012). In an elegant study, Hill et al. (2015) showed that increasing hippocampal neurogenesis through transgenic methods alone is sufficient to create an antidepressant phenotype. While the debate seems far from over, the picture that emerges is that neurogenesis is associated with depression, however, the degree of causality in human populations is still unknown.

Another intriguing hypothesis about the mechanism by which etanercept might enhance hippocampal neurogenesis comes from a separate set of studies we conducted that focus on the extracellular matrix protein reelin. Reelin has been extensively studied for its role in guiding cell migration during development, but in the adult brain it is involved in the promotion of synaptic plasticity. Reelin binds to two receptors, the very-low-density lipoprotein receptor (VLDR) and apolipoprotein receptor 2 (ApoER2). Activation of these receptors by reelin ultimately excites downstream targets including mTOR and P13K (Jossin and Goffinet, 2007). Importantly, inactivation of either PI3K or mTOR has been shown to reduce dendritic complexity in neuronal cultures (Jaworski et al., 2005). On a more direct level, reelin overexpression accelerates dendritic growth within adult-generated neurons, and inactivation of the reelin signaling pathway impairs adult hippocampal neurogenesis (Teixeira et al., 2012). In addition to our findings that peripheral etanercept injections can normalize neurogenesis after a period of corticosterone administration, we also showed that etanercept rescues reelin expression in GABAergic interneurons located in the proliferative subgranular zone of the dentate gyrus (Brymer et al., 2018). We have previously hypothesized about

the important role that reelin could play in the neurobiology of depression (Caruncho et al., 2016), as revealed by our observations that depression-like behavior is associated with a significant decrease in the number of reelin+ cells in the subgranular zone (Lussier et al., 2009; Fenton et al., 2015). We found that the timecourse for the emergence of depression-like behavior after corticosterone administration parallels the timecourse for dampened neurogenesis and the loss of reelin-positive cells (Lussier et al., 2013). We also found that heterozygous reeler mice, with 50% normal levels of reelin, were more susceptible to the depressogenic effects of corticosterone than wild type mice (Lussier et al., 2011). Taking all these observations into account, we believe that the antidepressant effects of etanercept in rats treated with corticosterone could occur through a normalization of hippocampal reelin expression. This is an important area for future studies as it could help explain the mechanism by which TNF-α antagonists exert their antidepressant effects.

It is therefore worth considering how etanercept might interact with the reelin signaling system in the dentate gyrus. Very little direct research has been done on this topic to date, but there could be a link through neuronal nitric oxide. TNF-α promotes the expression of nitric oxide synthase, and there are reports that some neuronal subtypes might reflect nitric oxide-mediated oxidative damage in response to increased levels of TNF-α (see as example Thomas et al., 2005). There is also evidence that the nitric oxide system has multiple effects in modulating adult neurogenesis (reviewed in Carreira et al., 2013). Overproduction of nitric oxide and accumulation of nitric oxide metabolites has been linked to mitochondrial dysfunction and oxidative stress in depression (recently reviewed by Allen et al., 2018). Interestingly, we have found that heterozygous reeler mice, which as mentioned above are highly susceptible to the depressogenic effects of repeated corticosterone (Lussier et al., 2011), show a decrease in the number of neurons co-expressing reelin and neuronal nitric oxide synthase (nNOS) specifically in the proliferative subgranular zone (Romay-Tallon et al., 2010). We also know that repeated corticosterone has differential effects on the co-expression of reelin and nNOS in wild type and heterozygous reeler mice (Romay-Tallon et al., 2015). We have discussed these data as an indication that nitric oxide-mitochondria-mediated excitotoxic events in reelin expressing neurons in the subgranular zone may instigate a decrease in reelin secretion by these neurons, resulting in deficits in dendritic maturation within newborn granule cells and dampened hippocampal plasticity that may be a key event in the pathophysiology of depression (see Caruncho et al., 2016; Allen et al., 2018). One could therefore surmise that the effects of etanercept in antagonizing the actions of TNF-α could result in a reversal of its effects in activating the NO system, and a subsequent neuroprotective action on reelin expressing neurons in the dentate subgranular zone. Additional research is necessary to properly assess these hypotheses, but they could open the door to the identification of novel targets for new antidepressant drug development.

One should also consider that there seems to be considerable cross-talk between ApoE and TNF-α in that low levels of ApoE result in increased cytokine production, in particular TNF- α and that in a similar vein, release of proinflammatory cytokines (TNF-α) downregulates ApoE production (Zhang et al., 2011). It is therefore likely that under conditions of stress, such as during administration of exogenous corticosterone, peripheral production and release of TNF-α is enhanced, which in turn increases central expression of TNF-α through the actions of microglia. This increase in TNF-α levels in the brain would then downregulate ApoE expression, and as both ApoE and reelin work through ApoE receptors and may have a synergistic effect on regulating neural plasticity, one could speculate that alterations in ApoE might also indirectly affect reelin binding to ApoE receptors and as a result, reelin functionality would be altered. This process would be reversed upon treatment with TNF-α antagonists.

Another question that remains to be answered is *when* etanercept actually interacts with reelin. In the Brymer et al. (2018) study mentioned above, etanercept was given to the rats semi-weekly during the 21-day period of corticosterone administration. Therefore, the rats received etanercept early on, presumably before any significant stress-related pathology had been created by the corticosterone injections. This experimental design leaves open the question of whether etanercept might have neuroprotective effects rather than antidepressant effects *per se*. It will be important to conduct further studies on this issue, with etanercept given in different animal models of depression at different time periods during and after periods of stress, so that a more complete understanding of the beneficial effects of etanercept can emerge.

CONCLUSION

Numerous reports point toward the effectiveness of anti-TNFα drugs for some depression patients. Use of TNF-α antagonists as antidepressants may be particularly important for subpopulations of patients with treatment resistant depression that show high levels of expression of proinflammatory cytokines.

AUTHOR CONTRIBUTIONS

All authors contributed to the ideas presented in this review and contributed to editing the manuscript. KB wrote the first draft and developed the figure. LK and HC finalized the manuscript.

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The 18 kDa Translocator Protein (TSPO) Overexpression in Hippocampal Dentate Gyrus Elicits Anxiolytic-Like Effects in a Mouse Model of Post-traumatic Stress Disorder

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The translocator protein (18 kDa) (TSPO) recently attracted increasing attention in the pathogenesis of post-traumatic stress disorder (PTSD). This study is testing the hypothesis that the overexpression of TSPO in hippocampus dentate gyrus (DG) could alleviate the anxiogenic-like response in the mice model of PTSD induced by foot-shock. In this study, hippocampal DG overexpression of TSPO significantly reversed the increase of the contextual freezing response, the decrease of the percentage of both entries into and time spent in the open arms in elevated plus maze test and the decrease of the account of crossings from the dark to light compartments in light–dark transition test induced by electric foot-shocks procedure. It was further showed that the behavioral effects of TSPO overexpression were blocked by PK11195, a selective TSPO antagonist. In addition, the expression of TSPO and level of allopregnanolone (Allo) decreased in the mouse model of PTSD, which was blocked by overexpression of TSPO in hippocampal dentate gyrus. The difference of neurogenesis among groups was consistent with the changes of TSPO and Allo, as evidenced by bromodeoxyuridine (BrdU)- positive cells in the hippocampal dentate gyrus. These results firstly suggested that TSPO in hippocampal dentate gyrus could exert a great effect on the occurrence and recovery of PTSD in this animal model, and the anti-PTSD-like effect of hippocampal TSPO over-expression could be at least partially mediated by up-regulation of Allo and subsequent stimulation of the adult hippocampal neurogenesis.

Keywords: TSPO, post-traumatic stress disorder, neurosteroid, neurogenesis, hippocampus

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INTRODUCTION

Post-traumatic stress disorder (PTSD) is a chronic and debilitating mental disorder that develops in survivors of traumatic events and PTSD can cause disturbing thoughts, helpless, and the attempt to avoid trauma-related cues (Bisson et al., 2015). However, the precise mechanisms of the intricate biological and psychological symptoms of PTSD remain unclear. Over the last two decades,the down-regulation of neurosteroid biosynthesis has been shown in several mental disorders including PTSD (Ceremuga et al., 2013; Locci and Pinna, 2017). Additionally, quite many clinical trials have shown that the decreased level of neuroactive steroids allopregnanolone (Allo) may play an important role in the pathology of PTSD (Rasmusson et al., 2006; Pinna, 2010; Pinna and Rasmusson, 2012). Pre-clinical studies have also found that corticolimbic Allo content remarkably reduced in patients of anxiety and aggression and the Allo decrease was positively related with the impaired behavioral performance (Pibiri et al., 2008; Pinna and Rasmusson, 2012). Previous results showed that the infusion of Allo into the dorsal hippocampus induced vigorous anxiolytic-like behavior in the elevated plus-maze test (Modol et al., 2011). These interesting studies gave reason to the hypothesis that downregulation of neurosteroids could contribute to the etiology of PTSD.

In the central nervous system, the translocator protein (18 kDa) (TSPO) are mainly expressed in glial cells. It mediates the translocation of cholesterol from the outer to the inner mitochondrial membrane, and thus regulates the synthesis of neurosteriods (Nothdurfter et al., 2012; Hatty and Banati, 2015). Studies demonstrated that long-term stress induced a decrease in the TSPO expression in the central nervous system in rodents (Milenkovic et al., 2015; Wang et al., 2015; Zhang et al., 2016). Interestingly, our group was the first to show that oral administration of certain TSPO ligands, including AC-5216 and YL-IPA08, enhanced synthesis of neurosteroids (such as Allo) in the brain and exerted anti-PTSD-like effect in some PTSD animal models with a favorable side effect (Qiu et al., 2013; Zhang et al., 2014a, 2016). Hence, TSPO protein might provide a promising target for novel anti-PTSD drug, but the specific mechanism remains to be determined.

The prefrontal cortex, the amygdala and the hippocampus are three brain regions in the limbic system which have been identified as the most clearly involved regions in PTSD (Wingenfeld and Wolf, 2014). Among them, the hippocampus plays an important role in remembrance of traumatic events and correlation of learned responses to contextual cues. Indeed, hippocampal reduction was reported to happen in patients with PTSD in many structural neuroimaging studies (Rodrigues et al., 2011). We therefore proposed that an up-regulation of TSPO expression in hippocampus, which could then enhance neurosteroidogenesis (such as Allo), may contribute to the behavioral adaptation to PTSD. To address our hypothesis, we used foot-shock procedure, an established mice model of PTSD to specifically examine the role of TSPO in PTSD (Bali and Jaggi, 2015). Furthermore, we examined a possible interference in PTSD-like behavior after application of a lentivirus-mediated overexpression of TSPO into the dentate gyrus (DG) of hippocampus. To explore the possible mechanisms in mediating the anti-PTSD effect of hippocampal TSPO overexpression, we then tested the changes of Allo and the hippocampal neurogenesis after behavioral tests.

MATERIALS AND METHODS

Animals

Adult male ICR mice (18–22 g) were obtained from the Beijing SPF Animal Technology Company (Animal License No. SCXK 2016-0002; Beijing, China). All animals were housed in groups of 3 to 5 per plastic cage (320 mm × 220 mm × 160 mm) in an air-conditioned room of controlled temperature (23 ± 1°C) and a 12-h light/dark circle (lights on at 6:00 AM). Mice had access to food and water *ad libitum*. All procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition). The experimental procedures were approved by the Institutional Committee on Animal Care and Use of Academy of Military Medical Sciences (No. IACUC.20094).

Drugs and Treatments

Lentiviral vectors containing the non-targeting negative control (Lv-NC) or TSPO (Lv-TSPO) sequence were generated by Genechem Company (Genechem, Co., Ltd., Shanghai, China). Recombinant and packaging lentiviruses encoding the TSPO gene was constructed as our previous studies by the Genechem Company (Genechem, Co., Ltd., Shanghai, China) (Wang et al., 2016; Li et al., 2017). EGFP was added to all viral vectors to track lentivirus-mediated target gene as a marker expression. TSPO is expressed in different organs, including the adrenal cortex, luteal cells, the testis, ovarian granulosa cells, the placenta and glial cells in the brain. Specifically, we overexpressed TSPO in the bilateral hippocampus using a lentivirus to study the important role of TSPO in the hippocampal dentate gyrus and confirm the overexpression site by tracking the lentivirus-mediated target gene under an inverted fluorescence microscope.

Bromodeoxyuridine (BrdU), Sertraline (Ser, a serotonin reuptake inhibitor) and PK11195 (a TSPO antagonist) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Ser (15 mg/kg, Sigma, St. Louis, MO, United States) was administered by intragastric gavage (*i.g.*) and PK11195 (3 mg/kg) was administrated intraperitoneally (*i.p.*, suspended in saline containing 2% DMSO and 0.8% Tween 80 for injection). Both drugs were given daily from day 1 to day 30 as **Figure 1A** shows. Behavioral tests were performed 1 h after the Ser or PK11195 administration.

Experiment Design

Sixty mice were randomly assigned to five groups: Lv-negative control (NC), Lv-NC + foot-shock (FS), Lv-NC + Ser + FS, Lv-TSPO + FS and Lv-TSPO + PK11195 + FS (n = 12 for each). A schematic overview of the experiment is depicted in **Figure 1A**. First, BrdU (100 mg/kg, i.p.) was administered for 3

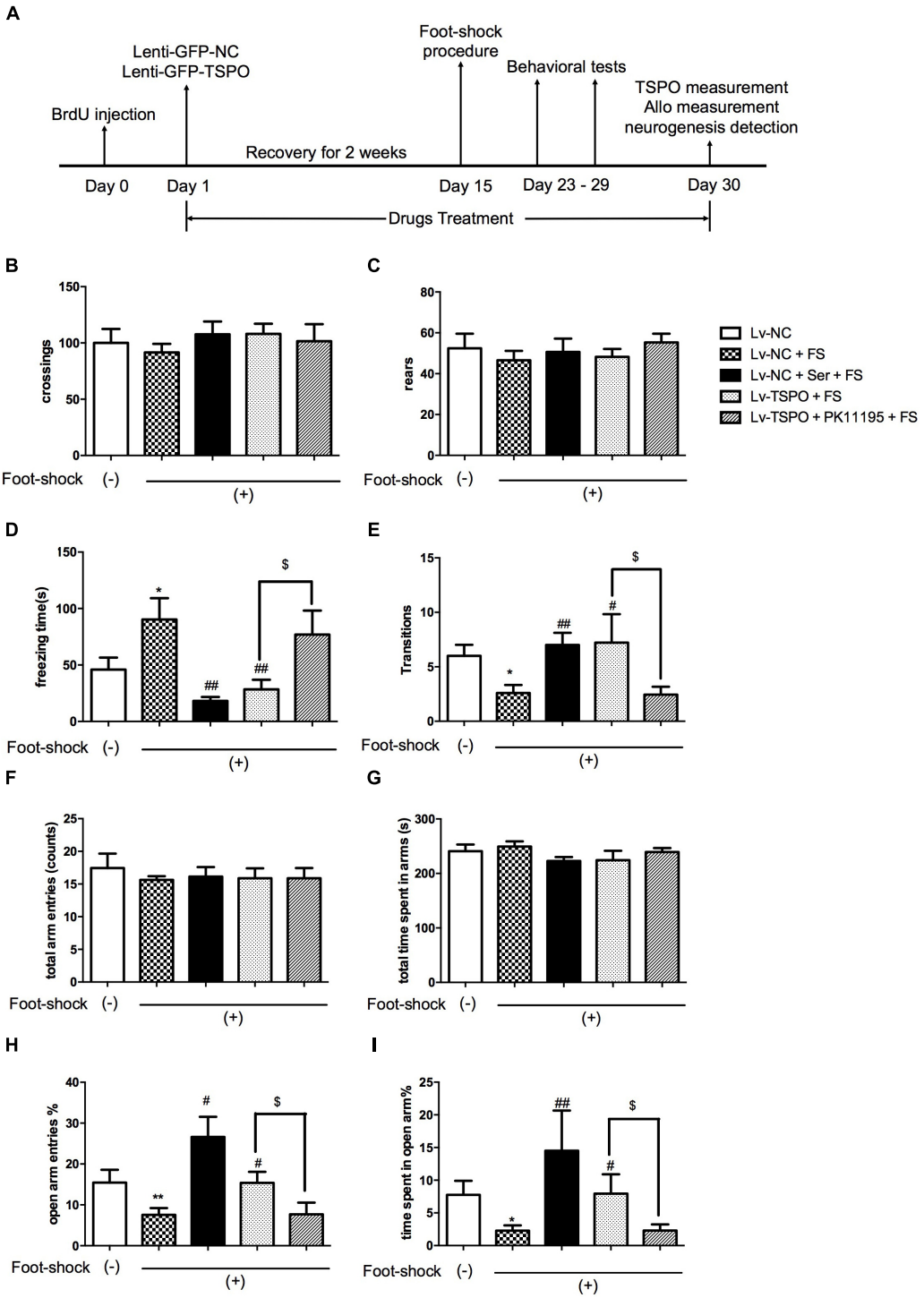

FIGURE 1 | TSPO overexpression in the DG elicited anxiolytic-like effect in the mouse exposed to electric foot-shocks. **(A)** Design of the experiment. Results of spontaneous locomotor activity showed no difference among groups for the number of line crossings **(B)** or rears **(C)** in the open field test. **(D)** The contextual freezing time was increased in electric foot-shock model group (LV-NC + FS). The freezing behavior was significantly reduced in both Lv-NC + Ser + FS and Lv-TSPO + FS groups on day 23. **(E)** Exposure to foot-shock resulted in less transitions in the Light–dark transition test, which was attenuated by Ser or Lv-TSPO on day 29. **(F–I)** No differences were observed between groups for total arm entries **(F)** or total time spent in all arms **(G)** in the EPM test on day 27. Compared to the control group, the Lv-NC + Foot Shock group showed decreased open arm entries percentage **(H)** and time percentage in open arms **(I)**. And the effects were reversed by either Ser administration or Lv-TSPO injection. All the effects of Lv-TSPO in **(D,E,H,I)** was blocked by PK11195. Data were presented as the means ± SEM (n = 8–11). $^{*}P < 0.05$ compared with the Lv-NC+foot-shock (–) group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the Lv-NC+FS group; $^{\$}P < 0.05$ compared with the Lv-TSPO+FS group.

times at a 3 h interval 24 h before lentiviral vector administration. Then animals were subjected to microinjection of lentiviral vectors containing the non-targeting negative control (Lv-NC) or TSPO (Lv-TSPO) into the DG of hippocampus. Following a recovery period of 2 weeks, we conducted the electric foot-shock procedures and assessed the behavioral effects of over-expression of TSPO on anxiety-like behaviors induced by the inescapable electric foot shock, an established mouse model of PTSD.

To observe and confirm the microinjection sites, three vector-treated mice in each group were randomly chosen and perfused transcardially following the behavioral experiments. The brains were removed, post-fixed and dehydrated. Serial coronal brain sections (30 μm thick) were cut. The microinjection sites and infected zones were defined by direct visualization with a fluorescence microscope (Olympus AX70 Provis, Center Valley, PA, United States) for the benefit of the green fluorescent protein (GFP) tag as described previously (Li et al., 2009). To detect the TSPO protein expression and allopregnanolone (Allo) level after hippocampus injection of Lv-NC or Lv-TSPO, hippocampal tissues (3 mm in diameter around the injection site on both sides) were removed and Western blot analysis (n = 3) and enzyme-linked immunosorbent assay (ELISA) (n = 3) were performed respectively as described previously. The neurogenesis in hippocampus DG was evaluated by the immunohistochemistry of BrdU/NeuN-positive cells in DG (n = 3).

Mouse Surgery and Lentiviral Microinjections

After 2-week acclimatization period and the following BrdU administration, mice received lentiviral microinjection under anesthesia with chloral hydrate (400 mg/kg, *i.p.*). The craniotomy was created aimed for bilateral DG according to the coordinates of the mouse brain atlas (Franklin and Paxinos, 2008): (AP, −1.7 mm; ML, ±1.8 mm; DV, −2.0 mm). The bilateral DG were both injected by a 10 μl microsyringe on the stereotaxic apparatus. The 30-gauge-needle was lowered into the dorsal DG. Lentiviral vectors containing NC or TSPO (2×10^8 TU/μl, 1 μl/side) were infused at a rate of 0.2 μl/min by a microsyringe injector and Micro4 controller (World Precision, Ins., Sarasota, FL, United States). The amount of lentivirus and the duration of infusion were determined by the repeated preliminary experiments and previous studies. The needle stayed in place after injection for 5 min to guarantee proper diffusion of the vectors. From the day (Day 1) of lentiviral microinjection, Ser (15 mg/kg, *i.g.*) or PK-11195 (3 mg/kg, *i.p.*) was administered once per day. The dosages were selected according to our previous studies (Miao et al., 2014; Zhang et al., 2014b; Qiu et al., 2015).

Behavioral Experiments

Electric Foot-Shock Procedures

Two weeks after lentiviral vectors injection, the electric foot-shock procedure was conducted as per Zhang et al. (2012) and Qiu et al. (2013). Electric foot-shocks were delivered through a stainless-steel grid floor (9 mm interval) by an isolated shock generator (Med Associates, Inc., United States) in a Plexiglas chamber (20 cm × 10 cm × 10 cm). Each mouse received 15 intermittent inescapable foot-shocks (intensity: 0.8 mA, interval: 10 s, and duration: 10 s) for 5 min following a 5-min adaptation period. Mice in the control group were placed in the same chambers without electric foot-shocks for total 10 min to adapt to and remember the same circumstance without trauma. Ethyl alcohol was used to wipe the chamber to avoid the effect of feces and smell between mice.

Contextual Freezing Measurement

The rodents of electric foot-shock model will freeze intermittently when re-exposed to the shock context and the freezing behavior is associated with the fear memory induced by the trauma-related cues, as the symptoms of PTSD patients. Thus, contextual freezing measurement was reported as one of the effective methods to evaluate PTSD (Maier, 1990; Siegmund and Wotjak, 2007; Zhang et al., 2012). All mice were re-exposed to the same chamber, which is the reminder situation of foot-shocks for 5 min 8 days after electric foot-shock procedures (Day 23; **Figure 1A**). The total cumulative freezing time was recorded and analyzed by computer (Med Associates, Inc., Video Freeze SOF-843, United States).

Open Field Test

To evaluate whether the reversion of PTSD-like behavior by over-expression of TSPO depends on locomotor activity change in mice, the number of line crossings and rears were assessed 10 days after electric foot-shock procedures (Day 25; **Figure 1A**) as in our previous study (Qiu et al., 2013; Zhang et al., 2016). Mice were placed in the center of a transparent box (36 cm × 29 cm × 23 cm) of which the base were divided into nice equal section. And then an observer who was blind to the study design counted and recorded the number of crossings (all four paws placed into a new square) and rears (both front paws raised from the floor) for 5 min. Ethyl alcohol was used to wipe the interior wall and floor to avoid the effect of feces and smell between mice.

Light–Dark Transition Test

Twelve days after electric foot-shock procedures (Day 27; **Figure 1A**), each mouse started in the dark side of the light–dark chamber and was free to cross through an opening between the light compartment and the dark one (30 cm × 23 cm for each side). The illumination of the light compartment was 720 lux. This test pits mice' innate aversion to bright areas against their natural drive to explore in response to mild stressors such as a novel environment. The transitions were only defined by the number of crossings from the dark to the light compartments. An observer who was blind to the study design counted and recorded the transitions for 5 min.

Elevated Plus Maze Test

Fourteen days after electric foot-shock procedures (Day 29; **Figure 1A**), the elevated plus maze test was performed. It is a widely-applied method to assess the anxiogenic-like behavior of PTSD model rodents (Li et al., 2009; Zhang et al., 2012). The four branching arms (30 cm × 5 cm) composed the cross-shaped maze. The maze was set at 40 cm above the ground. The opposite

two open arms without wall and two closed arms with dark walls (10 cm high) were connected by a central platform (5 cm × 5 cm). Each mouse was placed on the central platform and recorded by video for the time spent on the open/closed arms and number of entries into any arm. The entrance was defined by all four paws on the arm base. We calculated the ratio of time spent in the open arms to the total time spent in all arms and the ratio of the number of entries into open arms to that into any arm. The maze was cleaned with 5% ethanol between tests to avoid the effect of feces and smell between mice.

Western Blot Analysis

The western blot analysis was conducted as described previously (Wang et al., 2016). Briefly, hippocampal tissues (3 mm in diameter around both injection sites) were removed and then extracted by RIPA lysis buffer (Applygen, China). Fifty microgram of protein were separated by SDS-PAGE, measured and analyzed by western blot with primary antibody rabbit anti-TSPO (1:1000; Abcam, Cambridge, MA, United States) and β-actin (1:3000; Santa Cruz, CA, United States). The expression of protein was measured by Gel-Pro Analyzer software, Version 3.1 (Media Cybernetics, Rockville, MD, United States) and the TSPO expression was normalized to β-actin. Every experiment was independently repeated no less than four times.

Enzyme-Linked Immunosorbent Assay (ELISA)

The removed hippocampal tissues (3 mm in diameter around both injection sites) were extracted on ice with the lysis buffer containing 137 mM NaCl, 0.5 mM sodium vanadate, 10 μg/mL aprotinin, 1 μg/mL leupeptin1% NP40, 10% glycerol, 1 mM PMSF, 20 mM Tris-HCl (pH 8.0). Allo concentrations were quantified using ELISA kits according to the manufacturer's protocol (Arbor Assays, United States), and the density values were detected by the spectrophotometer at a wavelength of 450 nm and a reference wavelength of 650 nm.

Immunohistochemistry

Immunohistochemistry was conducted as Li et al. (2009). Mice were anesthetized deeply with chloral hydrate (500 mg/kg, *i.p.*), transcardially perfused with ice-cold 0.9% NaCl and then 4% buffered formalin. Brains of mice that received intraperitoneal injections of BrdU were carefully and quickly removed and fixed in 4% paraformaldehyde at 22°C for 48 h for histochemistry of BrdU. Coronal 12-μm sections were cut and incubated free-floating for 24 h at 4°C in PBS containing both rat anti-BrdU antibody (1:200; Abcam, Cambridge, MA, United States) and mouse anti-NeuN antibody (1:1000; Chemicon, Temecula, CA, United States). After rinsing with PBS for three times, the sections were then incubated with Red-X-conjugated goat anti-rat IgG and FITC- conjugated goat anti-mouse IgG (1:200 for both; Jackson, MS, United States) to react to the corresponding primary antibody in PBS for 2 h at 22°C before mounting. The sections were photographed and analyzed by confocal microscope (Zeiss LSM510, Thornwood, NY, United States). The BrdU-positive cells were counted as described previously (Li et al., 2009). Briefly, the BrdU immunohistochemistry was performed for every sixth section throughout the entire hippocampus. All BrdU-positive cells in the hippocampus DG were counted by a blind observer and multiplied by 6, recorded as the total number of labeled cells in the DG.

Statistical Analysis

Data were analyzed using GraphPad Prism 6 software (Graphpad Prism Institute, Inc., La Jolla, CA, United States). Results are expressed as means ± SEM. Outliers were removed according to the interquartile range (IQR) test (Zijlmans et al., 2018). Outliers here were defined as observations that fall below data set median - 1.5 IQR or above data set median + 1.5 IQR. Data were analyzed by Mann–Whitney *U*-test for multiple comparisons, followed by the Holm–Sidak test as *post hoc* analyses to adjust. Values of $P < 0.05$ were considered statistically significant.

RESULTS

TSPO Overexpression in the DG Elicited Anxiolytic-Like Effect in the Mice Exposed to Electric Foot-Shocks

There was no significant difference in the line crossings and rears between groups in the open field test. These results indicated that none of Lenti, Ser (15 mg/kg) or PK11195 (3 mg/kg) significantly did harm to locomotor activity in this animal model (**Figures 1B,C**).

A significant increase in the contextual freezing time was observed in Lv-NC + Foot Shock group compared to the non-shocked Lv-NC group, indicating that the anxiogenic-like mouse model of PTSD was successfully established. The freezing behavior was alleviated in the Lv-NC + Ser + FS group as the positive control compared with Lv-NC + FS group. After Holm–Sidak correction was used to calibrate the error from multiple tests, the significant difference remained, demonstrating that the validity of this model ($P = 0.0272$ for Lv-NC+FS vs. Lv-NC; $P = 0.0019$ for Lv-NC+Ser+FS vs. Lv-NC+FS; **Figure 1D**). The contextual freezing response was also decreased in mice that received an intra-hippocampal injection Lv-TSPO compared with foot-shock vehicle group ($P = 0.0038$ for Lv-TSPO+FS vs. Lv-NC+FS; **Figure 1D**). These results demonstrated that TSPO overexpression in DG of hippocampus attenuated the contextual freezing behavior in post-shocked mice.

In the light–dark transition test, the number of crossings from dark to light compartment decreased in the foot-shock-exposed Lv-NC + FS mice compared to the non-shocked Lv-NC mice ($P = 0.0134$ for Lv-NC+FS vs. Lv-NC; **Figure 1E**). It was also shown that either repeated administrations of Ser or Lv-TSPO injection increased the number of transitions ($P = 0.0018$ for Lv-NC+Ser+FS vs. Lv-NC+FS; $P = 0.0461$ for Lv-TSPO+FS vs. Lv-NC+FS; **Figure 1E**), suggesting that TSPO overexpression in DG of hippocampus significantly ameliorated PTSD-associated anxiogenic-like behaviors.

As shown in **Figures 1F,G**, no significant differences were observed between groups for total arm entries or total time spent in all arms in the EPM test. Compared to the control group, both open arm entries percentage and time percentage in open arms decreased in the Lv-NC + FS group ($P = 0.0249$ for the number of entries into open arms; $P = 0.0189$ for percentage of time spent in open arms for Lv-NC+FS vs. Lv-NC). Compared with Lv-NC + FS group, Lv-TSPO treatment significantly increased the percentage of entries into open arms and the percentage of time spent in open arms ($P = 0.0156$ for the number of entries into open arms; and $P = 0.0496$ for percentage of time spent in open arms for Lv-TSPO+FS vs. Lv-NC+FS), as did repeated administration of Ser ($P = 0.0013$ for the number of entries into open arms; $P = 0.0044$ for percentage of time spent in open arms for Lv-NC+Ser+FS vs. Lv-NC+FS).

It was further showed that these above anti-PTSD behavioral effects of TSPO overexpression was reversed by PK11195 administration ($P = 0.0412$ for freezing time in contextual freezing test, **Figure 1D**; $P = 0.0486$ for dark-to-light transition in light–dark transition test, **Figure 1E**; $P = 0.0355$ for the percentage of entries into open arms, $P = 0.0432$ for percentage of time spent in open arms in EPM test, **Figures 1H,I**). These results indicated that TSPO overexpression in DG of hippocampus attenuated the anxiogenic-like behavior induced by electric foot-shock procedures in mouse model of PTSD, but these effects could be blocked by the TSPO ligand PK11195, suggesting that these effects might be at least partially attributed to TSPO activation.

Targeted Overexpression of TSPO in the Hippocampus

To confirm the overexpression of TSPO *in vivo*, the lentivirus-mediated overexpression was traced by the expression of GFP using fluorescence microscopy (**Figures 2A,B**). The results showed that fluorescence obtained with this lentiviral vector was localized to the subgranular layer and hilus of the DG, as indicated by GFP-positive cells (green), while no expression was detected outside the hippocampus. Combined with the immunoblots of TSPO in punched hippocampus tissue, the results showed that foot-shock procedure significantly reduced the expression of TSPO ($P = 0.0143$), and chronic administration of Ser or Lv-TSPO injection clearly increased the TSPO expression ($P = 0.0286$ for Lv-NC+Ser+FS vs. Lv-NC+FS; $P = 0.0143$ for Lv-TSPO+FS vs. Lv-NC+FS) at the same time of exerting the anti-PTSD-like effect. The overexpression of TSPO by administration of LV-TSPO was blocked by TSPO antagonist PK11195 ($P = 0.0143$; **Figure 2C**).

Effects of Hippocampal TSPO Overexpression on the Level Allo After Electric Foot-Shock

The endogenous Allo level in the hippocampus tissues (3 mm in diameter around both injection sites) of post-shock mice were measured at the end of the behavioral tests to further confirm the role of Allo in the anti-PTSD-like behavior effect of Lv-TSPO. As shown in **Figure 2D**, the foot-shock procedure significantly

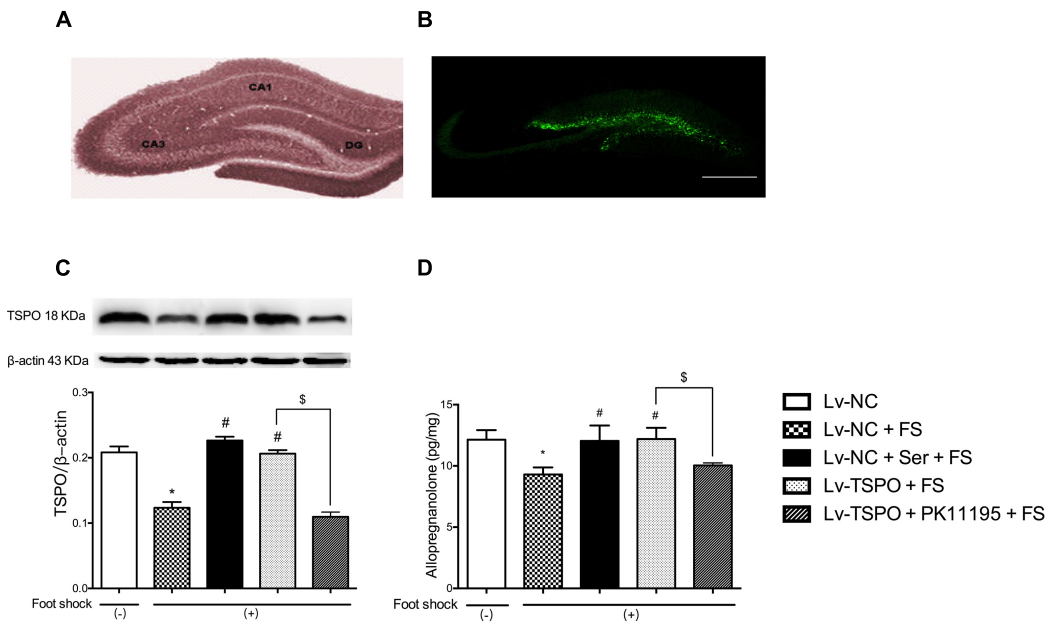

FIGURE 2 | Effects of lenti-GFP-TSPO on expression of TSPO and level of Allo in the DG of mice. **(A,B)** Fluorescence microscopy captured the microinjection sites shown as expression of GFP in the DG; Scale bars = 500 mm; $n = 3$. **(C)** Representative immunoblots of TSPO and the histogram represents semi-quantitative results of western blot analysis. The protein expression of TSPO were normalized by β-actin; $n = 3$; **(D)** The level of Allo of hippocampal tissues; $n = 3$. Data were presented as the means ± SEM. $^{*}P < 0.05$ compared with the Lv-NC+foot-shock (–) group; $^{\#}P < 0.05$ compared with the Lv-NC+FS group; $^{\$}P < 0.05$ compared with the Lv-TSPO+FS group.

reduced the Allo level in the hippocampus compared to shock-free control mice (P = 0.0140), which was clearly reversed by daily administration of Ser or Lv-TSPO injection (P = 0.0475 for Lv-NC+Ser+FS vs. Lv-NC+FS; P = 0.0375 for Lv-TSPO+FS vs. Lv-NC+FS). And the increase of Allo by administration of LV-TSPO was blocked by TSPO antagonist PK11195 (P = 0.0312; **Figure 2D**).

Effect of Hippocampal TSPO Overexpression on the Number of BrdU-Positive Cells in the Hippocampus in Mice After Electric Foot-Shocks

Given the view that neurogenesis could be reduced by PTSD, we then labeled BrdU-positive cells in the hippocampus DG to determine the effect of TSPO on neurogenesis. Mice were sacrificed 30 day after the beginning of BrdU labeling. Cells labeled with BrdU were counted per bilateral, entire hippocampal dentate gyri. BrdU-positive cells were predominantly localized in the subgranular layer (**Figure 3A**) and co-localized with NeuN-cells (**Figure 3B**). Statistical analysis revealed that the foot-shock procedure significantly decreased the number of BrdU (+) cells present in the DG when compared with foot-shocked (−) mice (P = 0.0269 for Lv-NC+FS vs. Lv-NC). And this effect could be reversed by the chronic administration of Ser or Lv-TSPO injection which exhibited significantly more BrdU (+) cells than Lv-NC treated animals (P = 0.0085 for Lv-NC+Ser+FS vs. Lv-NC+FS; P = 0.0334 for Lv-TSPO+FS vs. Lv-NC+FS; **Figure 3C**). These data indicated that the overexpression of TSPO alleviated the impaired hippocampal neurogenesis induced by foot-shocked procedure.

DISCUSSION

In this study, we showed that Lv-TSPO mediated overexpression of TSPO in the DG attenuated PTSD-like behaviors without significantly affecting locomotor activity. These results suggested that overexpression of TSPO in the DG may reverse the PTSD-like behaviors, which can be reversed by a TSPO antagonist PK11195. Furthermore, hippocampal TSPO overexpression increased the level of Allo and improved hippocampal neurogenesis.

In the present study, the lentivirus was selected for its stable and long-term gene overexpression as the vector (Schratt et al., 2006; Krassnig et al., 2015; Chen et al., 2016). It was reported that lentiviral vectors can receive a big fragment of exogenous target gene, express constantly in cells, and possess satisfactory safety (Escors and Breckpot, 2010; Sauer et al., 2014). Till now, no studies about the application of lentiviral vectors to PTSD therapy were reported worldwide. Our previous study suggested that the lentiviral vectors carrying TSPO gene were well-expressed in the DG of hippocampus (Wang et al., 2016), which fits the evidence of our present study. As the lentiviral vectors efficiently inspired hippocampal TSPO signaling, it was examined whether TSPO expression change can modify the PTSD-like responses of mice after foot-shock paradigms, a reliable animal model for PTSD. Interestingly, our studies showed that intra-hippocampal injection of the Lv-TSPO reversed the behavioral impairment including the anxiogenic-like effect in mice after foot-shock exposure, which is consistent with the effect of chronic administration of sertraline. Sertraline, as a selective serotonin reuptake inhibitor (SSRI), is a FDA-approved medication for PTSD. It has shown its certain anti-PTSD effect independent on the shock exposure in many rodent models (Miao et al., 2014; Zhang et al., 2015; Qiu et al., 2017; Zhang Z.S. et al., 2017). Our results suggested that the dose of intra-hippocampal injection of the Lv-TSPO was efficient to exert the anti-PTSD effect as sertraline. We also found that neither the foot-shock procedure nor the intra-hippocampal injection of the Lv-TSPO significantly impacted on the locomotor activity in mice, suggesting that the observed behavioral differences were independent on the basal locomotor activity changes.

In order to investigate the anti-PTSD-like effect of intra-hippocampal injection of the Lv-TSPO, it was tested whether blocking the TSPO pathway by PK11195 affected the behavioral effects in the foot-shock procedure. According to our previous studies, chronic administration of PK11195 alone would not change the foot-shock induced PTSD-like behavior or the level of Allo in serum or brain (Zhang et al., 2014b). However, in this study when PK11195 intervened the effect of intra-hippocampal injection of Lv-TSPO in foot-shock model, it was shown that the TSPO antagonist PK11195 administration reversed all the attenuated behavioral effects induced by DG Lv-TSPO. These results were in line with our previous studies which demonstrated that PK11195 completely blocked the anti-PTSD-like effects of TSPO ligand YL-IPA08 (Zhang et al., 2013). Overall, these findings indicated that the anti-PTSD effects of intra-hippocampal injection of the Lv-TSPO could be mediated by TSPO activation.

The expression level of TSPO protein were measured and further verified the overexpression of TSPO in DG in the Lv-TSPO+FS group. The decreased TSPO expression induced by foot shock in PTSD mice model and increased TSPO expression by sertraline agree with our previous studies (Qiu et al., 2013; Zhang et al., 2014b; Li et al., 2017; Zhang L.M. et al., 2017). Interestingly, we found that PK11195 blocked the effects of TPSO over-expression on TPSO protein levels in the hippocampus. We think it might be some compensatory mechanism, for that TSPO is technically not a classic receptor. On the other hand, PK11195 was reported to be able to induce changes in expression of immediate early genes and transcription factors in U118MG glioblastoma cells which were studied for TSPO functions for years. These changes also included gene products that are part of the canonical pathway serving to modulate general gene expression (Yasin et al., 2017). It might be other reason for the TSPO expression change and needs further investigation.

Allo is the most abundant neurosteroid in the central nervous system potently and selectively acting with $GABA_A$ the receptors and modulating of $GABA_A$ signaling action (Puia et al., 1990; Lambert et al., 1995, 2003). The decreased level of Allo in the central nervous system was reported to associate with the symptoms of PTSD (Vaiva et al., 2004; Rasmusson et al., 2006).

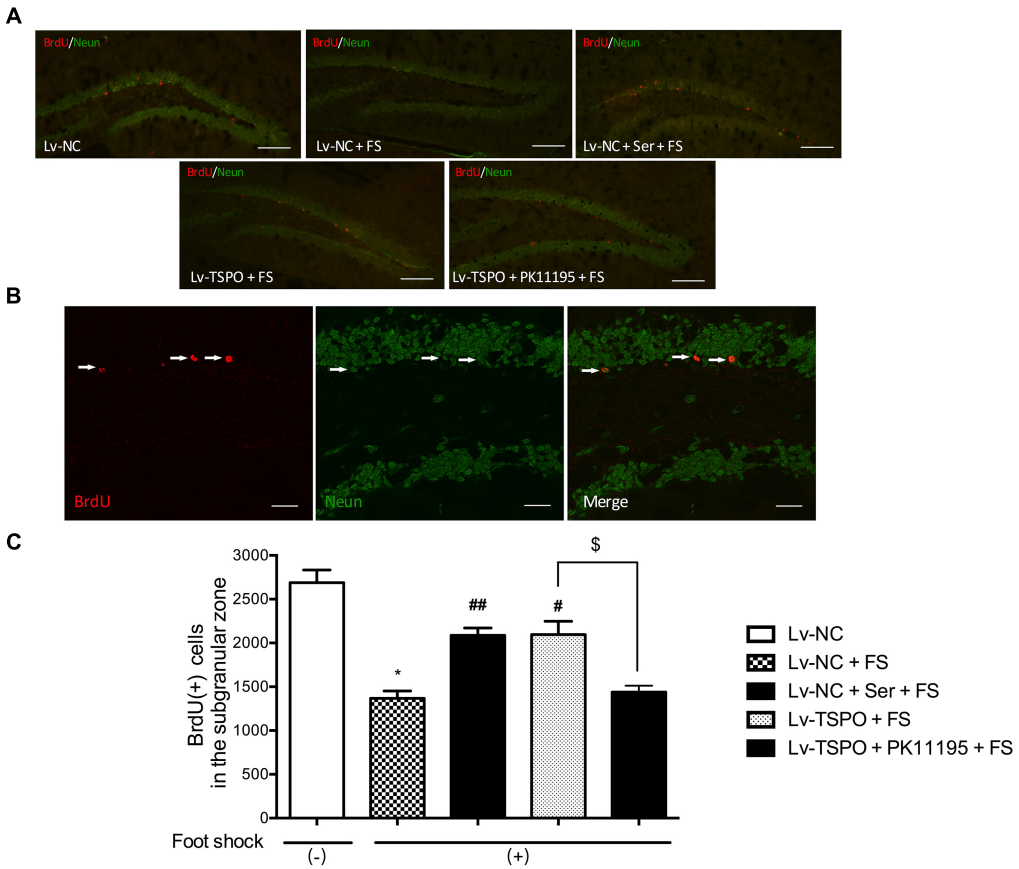

FIGURE 3 | Effect of TSPO overexpression in the DG on the number of BrdU-positive cells in the hippocampus in mice after electric foot-shocks. **(A)** A confocal laser-scanning microscope micrographs of BrdU-labeled cells (red) in the dentate gyrus of hippocampus. Scale bar = 200 μm. **(B)** NeuN (green) and BrdU (red) co-labeled cells in the dentate gyrus were photographed and quantified under confocal microscope. Scale bar = 50 μm. **(C)** Statistical analysis showed a significant decrease of the number of BrdU-positive cells in mice of foot-shock exposure (LV-NC + FS) compared with non-foot-shocked mice (Lv-NC), whereas it was prevented in the group of Lenti-GFP-TSPO injection (Lv-TSPO +FS). The improvement of Lv-TSPO was reversed by PK11195 (Lv-TSPO + PK11195 +FS). Data were presented as the means ± SEM (n = 3). $^{*}P < 0.05$ compared with the Lv-N+foot-shock (–) group; $^{\#}P < 0.05$ compared with the Lv-NC+FS group; $^{\$}P < 0.05$ compared with the Lv-TSPO+FS group. $^{\#\#}P < 0.01$ compared with the Lv-NC+FS group.

Numerous studies have demonstrated that Allo plays a pivotal role in the mediation of contextual fear memory cued by the trauma-related events and the incapacity of Allo biosynthesis may be one of the molecular mechanisms underlying the etiology of PTSD (Uzunova et al., 1998; Rasmusson et al., 2006; Pibiri et al., 2008; Pinna and Rasmusson, 2012). In this current study, we detected the level of Allo in mice to substantiate this hypothesis that the normalization of brain Allo levels may underlie the anti-PTSD-like effects of intra-hippocampal injection of the Lv-TSPO. Our results showed a remarkable decreased Allo level in the hippocampus in post-foot-shock mice, which was reversed by sertraline in hippocampus, and the result is in line with the previous knowledge that anti-PTSD-like activities of sertraline were closely associated with elevated biosynthesis of Allo (Pinna and Rasmusson, 2012; Xu et al., 2018). Likewise, the intra-hippocampal injection of the Lv-TSPO reversed the decreased Allo level in the hippocampus in post-foot-shock mice, further suggesting that the anti-PTSD-like effects of Lv-TSPO could be mediated by the subsequent synthesis of Allo in hippocampus DG. It is consistent with the results of previous studies on TSPO ligands, such as YL-IPA08 and AC-5216, which have been shown to display efficacy toward the treatment of psychiatric disorders by increasing neurosteroid biosynthesis in the brain, inducing anxiolytic and antidepressant activities in some rodent models and improving behavioral deficits in a mouse model of PTSD (Qiu et al., 2013; Zhang et al., 2014a; Wang et al., 2016; Li et al., 2017; Zhang L.M. et al., 2017). It is important to note that other neurosteroids were not examined in our present study, but their roles cannot be excluded and their potential contribution need further studies to identify. Our present study also found that sertraline could reverse the lowered Allo levels in post-foot-shock mice and further suggested that the anti-PTSD-like effects of sertraline were partly mediated by the subsequent synthesis of Allo.

A vast literature demonstrated the important role of adult-born neurons in buffering stress responses and in mediating anti-PTSD-like effects. Functional studies that have

described the impaired adult hippocampal neurogenesis in PTSD patients and different animal models and the hippocampal neurogenesis became one of the treatment targets of anti-PTSD interventions (Kheirbek et al., 2012; Peng et al., 2013; Besnard and Sahay, 2016). This study showed that similar as sertraline, intra-hippocampal injection of the Lv-TSPO in post-foot-shock mice induced a significant proliferation of progenitor cells as shown by BrdU immunohistochemistry. Interestingly, evidences reported that exogenous administration of Allo could prevent the occurrence of depression/anxiety-like behavior, as well as alleviate the damage of hippocampal neurogenesis (Evans et al., 2012) and promote cell survival (Brinton, 1994; Djebaili et al., 2005). Furthermore, recent studies have demonstrated the neuroprotective role of Allo against the hippocampal neurogenesis impairment in a transgenic mouse model of Alzheimer's disease (Wang et al., 2010; Singh et al., 2012). In addition, it was reported that besides GABAergic mechanisms, Allo could also enhance neurogenesis, which contribute to the regulation of depression and anxiety (Wang et al., 2005). These observations suggested that impaired hippocampal neurogenesis may participate the pathology of PTSD, and thus the hippocampal neurogenesis also provides a promising target for anti-PTSD treatment (Kempermann and Kronenberg, 2003).

In summary, the over-expression of TSPO in hippocampal DG exerted anti-PTSD effect in mice submitted to the foot-shock, which may be related to the up-regulation of Allo synthesis and subsequent stimulation of the adult hippocampal neurogenesis. It should also be stated that in this present study, we did not measure other neurosteroids or the new-born neurons in hippocampus DG, so we could not attribute the anti-PTSD effect of TSPO overexpression completely to increased Allo level. Nevertheless, our study advances our knowledge of the PTSD theory and provide a promising implication for the treatment of this mental disorder.

AUTHOR CONTRIBUTIONS

X-YZ helped to conceive the study, carried out the study execution and data analysis, and contributed to the manuscript draft. WW and QF contributed to the analysis of immunohistochemistry. L-MZ participated in the research design, the construction of recombinant lentiviruses, and the draft of manuscript. Y-ZZ, W-DM, and Y-FL contributed to the research design, data analysis, and manuscript revision.

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REVIEW
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The Role of G-proteins and G-protein Regulating Proteins in Depressive Disorders

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Progress toward new antidepressant therapies has been relatively slow over the past few decades, with the result that individuals suffering from depression often struggle to find an effective treatment – a process often requiring months. Furthermore, the neural factors that contribute to depression remain poorly understood, and there are many open questions regarding the mechanism of action of existing antidepressants. A better understanding of the molecular processes that underlie depression and contribute to antidepressant efficacy is therefore badly needed. In this review we highlight research investigating the role of G-proteins and the regulators of G-protein signaling (RGS) proteins, two protein families that are intimately involved in both the genesis of depressive states and the action of antidepressant drugs. Many antidepressants are known to indirectly affect the function of these proteins. Conversely, dysfunction of the G-protein and RGS systems can affect antidepressant efficacy. However, a great deal remains unknown about how these proteins interact with antidepressants. Findings pertinent to each individual G-protein and RGS protein are summarized from *in vitro*, *in vivo,* and clinical studies.

Keywords: G-protein, RGS, antidepressant, depression, GPCR

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INTRODUCTION

Major depressive disorder (MDD) is one of the most prevalent psychiatric disorders with over 16% of adults in the US experiencing a depressive event within their lifetime, and over half of these events leading to severe or very severe role impairment (Kessler et al., 2003; García-Velázquez et al., 2017). While a multitude of antidepressant drugs are now available, no one treatment is fully effective in all patients, with about one third failing to remit even after 4th line treatments (Insel and Wang, 2009). This high rate of treatment failure combined with the high prevalence of depressive disorders highlights the need not only for improved treatment options, but also for a better understanding of the molecular and cellular factors that determine whether a given treatment will succeed or fail.

In this review we highlight the role of G-proteins and their signaling partners, especially the Regulators of G-protein Signaling (RGS) proteins, in both the etiology and treatment of depression.

GPCRs IN DEPRESSION

The vast majority of drugs prescribed for depressive disorders either interact directly with G-protein coupled receptors (GPCRs), e.g., buspirone with the 5-HT1A receptor or

aripiprazole with a multitude of monoaminergic GPCRs, or indirectly regulate GPCR function by affecting endogenous neurotransmitter levels, e.g., selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine and monoamine oxidase inhibitors (MAOIs) such as selegiline (Kantor et al., 2015).

G-protein coupled receptors are 7-transmembrane domain proteins that form a multi-protein complex with members of an intracellular family of heterotrimeric G-proteins comprised of a Gα subunit and a βγ dimer (**Figure 1**). There are several members of the Gα family that couple to different cohorts of effectors in the cell. These include the so-called inhibitory Gα proteins ($G\alpha_{i/o}$ and $G\alpha_z$), which inhibit adenylyl cyclase to decrease cyclic adenosine monophosphate (cAMP), the adenylyl cyclase stimulatory $G\alpha_s$ and $G\alpha_{olf}$, $G\alpha_q$ which activates phospholipase C, and $G\alpha_{12/13}$ that couple to the Rho family of small GTPases. There are 5 β subunits and 11 γ subunits that can be engaged to make the associated βγ dimer in mammals. There is selectivity of receptors for particular types of Gα proteins, and also for activation or inhibition of downstream effectors in the cell. Following GPCR activation by endogenous neurotransmitters or exogenous agonists both the Gα subunit and the Gβγ complex functionally dissociate from the receptor and go on to stimulate or inhibit a range of intracellular effectors. The Gα activation process involves a loss of bound GDP (inactive form) in exchange for GTP (active form). Signaling is terminated by the hydrolysis of the bound GTP back to GDP by the intrinsic GTPase activity of the Gα subunit. However, for certain Gα proteins this enzymatic process is slow. To accelerate this process a regulator of G-protein signaling (RGS) protein binds to the active GTP-bound Gα and facilitates its inactivation, acting as a GAP (GTPase accelerating protein; **Figure 1**). This allows return to the resting, inactive state. The inactive GDP-bound Gα subunit can then recouple with both the Gβγ complex and receptor until the receptor is again activated and the cycle repeats. Although RGS proteins can substantially limit $G\alpha_{i/o}$ and $G\alpha_q$ signaling (see Oldham and Hamm, 2008 for review) their impact on $G\alpha_s$ mediated signaling is less pronounced. While an RGS for $G\alpha_s$ has been identified, it is not clear that this protein facilitates the GTPase activity of $G\alpha_s$ (Zheng et al., 2001; Ha et al., 2015).

In addition to mediating the actions of antidepressant drugs, many GPCRs have been associated with the development of depression. Aberrations in both α- and β-adrenergic receptor signaling have been found in depressed patients (Matussek et al., 1980; Ebstein et al., 1988) and the brains of suicide victims consistently have alterations in 5-HT1A receptor expression (and various nuclear receptors) resembling the alterations produced by chronic stress in animal models (López et al., 1998). In contrast, study of 5-HT2A and 5-HT2C receptor expression levels has produced inconsistent results (see Stockmeier, 2003 for review). Nonetheless, a 5-HT2C receptor polymorphism in the N-terminal extracellular domain has been associated with MDD in a large population study (Lerer et al., 2001). Preclinical models also implicate the 5-HT1B receptor both in the genesis of depressive states and in antidepressant action (Svenningsson et al., 2006). Polymorphisms in both the dopamine D3 and D4 receptors have been correlated with the development of MDD (Dikeos et al., 1999; López León et al., 2005), while D1 and D2 receptors have instead been linked to bipolar disorder (Massat et al., 2002; Dmitrzak-Weglarz et al., 2006). Interestingly the GABA-B receptor agonist baclofen produces a transient depressive state in some patients (Post et al., 1991), suggesting this receptor may play a role in MDD. A corticotropin-releasing hormone receptor 1 antagonist has also been found to have antidepressant activity (Zobel et al., 2000), in agreement with predictions from preclinical studies (Mansbach et al., 1997).

Downstream signaling bias is also affected by proteins in complex with the receptor. For example, the 5-HT1A receptor can form receptor/receptor complexes with the 5-HT7 receptor, the ghrelin receptor family member GPR39, or fibroblast growth factor receptor 1 (FGFR1), with each heterodimer complex producing a unique downstream signaling profile (Renner et al., 2012; Tena-Campos et al., 2015; Borroto-Escuela et al., 2017). The 5-HT1A/5-HT7 heterodimer complex promotes internalization of the 5-HT1A receptor (Renner et al., 2012), suggesting that the high degree of colocalization between these receptors in the dorsal raphe nucleus may contribute to 5-HT1A autoreceptor desensitization observed during SSRI treatment (Naumenko et al., 2014). These results imply that strategies which promote 5-HT1A/5-HT7 heterodimer formation could facilitate the antidepressant effects of SSRIs by attenuating 5-HT1A autoreceptor activity. In contrast, combined 5-HT1A receptor and FGFR1 agonist treatment fails to produce antidepressant-like behavior in rats with low hippocampal 5-HT1A/FGFR1 heterodimer expression (Borroto-Escuela et al., 2017), while these agonists produce robust antidepressant-like effects in rats with increased dimer formation. These heterodimer dependent behavioral effects are likely mediated by a reduced ability of the 5-HT1A/FRGR1 heterodimer to activate G-protein coupled inwardly rectifying potassium (GIRK) channels, compared to the free 5-HT1A receptor (Borroto-Escuela et al., 2017). These results suggest that 5-HT1A receptor stimulated GIRK activity in the hippocampus is in fact detrimental to antidepressant activity, and that agonists which preferentially couple to 5-HT1A/FGFR1 heterodimers would be superior to unbiased ligands. For a recent review on how GPCR oligomerization can affect receptor activity, see Szafran et al. (2013) and Farran (2017) for review of GPCR heterodimers in depression.

The above discussion suggests that insight into the roles of specific G-proteins and their cognate RGS proteins in antidepressant action may facilitate future antidepressant drug development. Furthermore, studying dysfunction of these systems in the depressed brain may provide insight into the etiology of depression.

G-PROTEIN SUBUNITS IN DEPRESSION AND ANTIDEPRESSANT ACTION

G-protein Expression Levels

In preclinical studies, central nervous system (CNS) G-protein expression levels do not appear to change consistently as a result of antidepressant drug treatment. $G\alpha_s$, $G\alpha_o$ and $G\alpha_i$ mRNA expression in the rat hippocampus remain constant

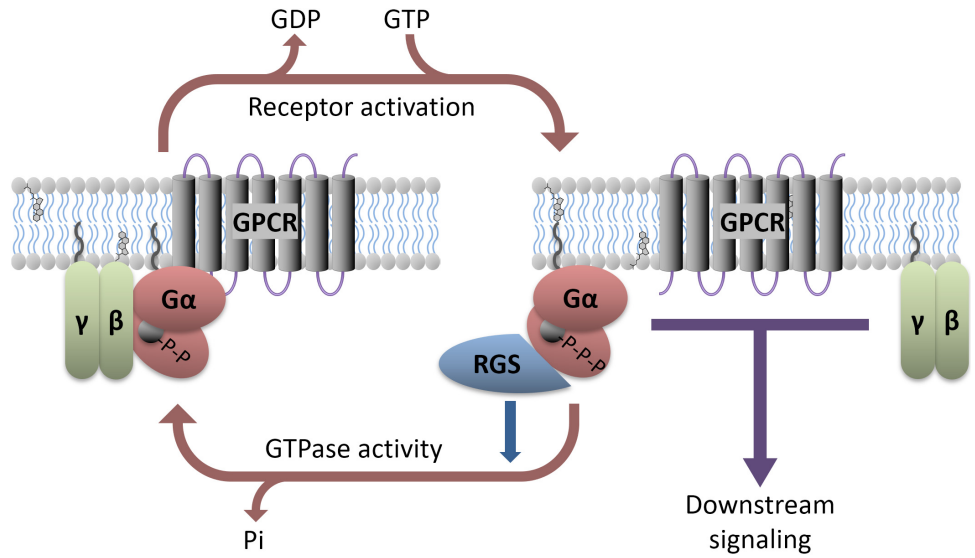

FIGURE 1 | Heterotrimeric G-protein signaling with RGS regulation. GPCR activation, either due to agonist binding or constitutive activity, causes downstream signaling through both the α and βγ subunits. Various antidepressants modulate this process directly (e.g., Buspirone) or indirectly (e.g., SSRIs). RGS proteins interact with active Gα and accelerate its GTPase activity, facilitating a return to the GDP-bound inactive state. Preclinical models suggest that direct manipulation of the RGS or G-proteins can affect antidepressant response.

following chronic treatment with the tricyclic antidepressant imipramine (Lasoń and Przewłocki, 1993). However, levels of G-protein mRNA and protein expression do not always have a strong correlation (Krumins and Gilman, 2006), and so these results do not necessarily reflect the amount of G-protein present. Chronic treatment with the dual serotonin norepinephrine reuptake inhibitor (SNRI) amitriptyline, the tricyclic antidepressant desipramine, the MAOI tranylcypromine or electroconvulsive shock did not affect protein levels of $G\alpha_s$, $G\alpha_o$, $G\alpha_i$ or Gβ in the rat cerebral cortex (Chen and Rasenick, 1995a; Emamghoreishi et al., 1996; Dwivedi and Pandey, 1997). In contrast, brief treatment with the MAOI antidepressant phenelzine increased $G\alpha_{i2}$ protein expression in the rat cortex and hippocampus without affecting $G\alpha_s$, $G\alpha_o$, $G\alpha_q$ or $G\alpha_{i1}$ expression in any brain region (Dwivedi and Pandey, 1997). However, this does not appear to be a conserved effect for all MAOI antidepressants on $G\alpha_{i2}$, as chronic treatment with tranylcypromine did not affect cortical $G\alpha_{i2}$ expression while chronic clorgiline instead produced a small decrease (Lesch et al., 1991; Emamghoreishi et al., 1996). Three-week treatment with various tricyclic antidepressants (imipramine, desipramine, or chlomipramine) produced slight increases of brain $G\alpha_o$ and decreases of $G\alpha_s$ and $G\alpha_i$, although the magnitude of these changes (∼10–30% from baseline) may not be great enough to produce functional consequences (Lesch et al., 1991). Note that it has been suggested that $G\alpha_s$ expression is over 30-fold higher than expression of downstream effectors (Ostrom et al., 2000), thus minor variations in the expression of $G\alpha_s$ are not expected to influence downstream signaling. Furthermore, tricyclics such as desipramine and amitriptyline had no effect on G-protein expression (Chen and Rasenick, 1995a; Emamghoreishi et al., 1996).

On the other hand, a series of post-mortem studies examining the involvement of G-proteins in depressive states contrast with the findings from pre-clinical studies discussed above. Post-mortem studies indicate that a downregulation of $G\alpha_o$ and $G\alpha_{i2}$ protein and mRNA co-occurs with an upregulation of $G\alpha_s$ protein levels and mRNA in the prefrontal cortex of adult suicide cases (Dwivedi et al., 2002). This fits with data from $G\alpha_{i2}$ knock-out mice which show that loss of $G\alpha_{i2}$ contributes to depressive behaviors (Talbot et al., 2010), suggesting that the observed alterations of $G\alpha_{i2}$ in these subjects may have contributed to their pathology. However, the work of Donati et al. (2008) shows only minor changes in $G\alpha_s$ expression and none in other Gα proteins. Thus, there is no consistent effect on G-protein expression in the brain following chronic antidepressant treatment, the effects that have been seen are not consistent between antidepressant drugs with similar pharmacology, and any changes observed are of relatively small magnitude.

Effects on $G\alpha_s$ Localization and Signaling

Despite the lack of any clear effect on G-protein expression levels, chronic but not acute antidepressant drug treatment (including amitriptyline, desipramine and iprindole) increases cAMP concentrations in a $G\alpha_s$ dependent manner in the rat brain, but not liver or kidney (Menkes et al., 1983; Ozawa and Rasenick, 1989; De Montis et al., 1990). In addition to antidepressant drug treatments, chronic electroconvulsive treatment increases coupling between $G\alpha_s$ and adenylyl cyclase (Ozawa and Rasenick, 1991). Consistent with this increased adenylyl cyclase activity, increased activity of cAMP dependent kinases (e.g., Protein kinase A) have also been observed in

the rat brain following chronic antidepressant treatment. These changes occurred with chronic but not acute treatment with desmethylimipramine, and were seen in the cerebral cortex but not hippocampus, striatum or cerebellum (Perez et al., 1989). This suggests a more general role for brain $G\alpha_s$/adenylyl cyclase coupling in antidepressant action downstream of their better characterized direct effects on transporters and GPCRs.

In order to understand how antidepressant drugs affect G-protein signaling, it is necessary to consider not only the expression levels of these proteins and their binding partners, but also their subcellular localization in microdomains. Within the plasma membrane bilayer there are lipid raft microdomains which contain an increased proportion of both cholesterol and sphingomyelin (Simons and Toomre, 2000) and the scaffolding protein Caveolin 1 (**Figure 2**). G-proteins are known to accumulate in these lipid raft domains, with $G\alpha_s$, $G\alpha_q$, and $G\alpha_{i/o}$ subunits all found at higher concentrations in these regions (Pesanová et al., 1999; Dunphy et al., 2001; Allen et al., 2005). These microdomains can affect G-protein mediated signaling, with either faciliatory or inhibitory effects on signaling depending on the G-protein. For example, localization to raft regions inhibits the ability of $G\alpha_s$ proteins to increase cAMP levels through adenylyl cyclase activation (Allen et al., 2009), while raft localization of $G\alpha_q$ greatly enhances signaling downstream of 5-HT2A receptor activation (Rybin et al., 2000; Bhatnagar et al., 2004; Allen et al., 2007 for review). In addition to an upregulation of $G\alpha_s$ protein expression, a shift in $G\alpha_s$ subcellular membrane localization also occurs in the brains of depressed humans. Thus, compared to non-psychiatric control subjects there was an approximately two-fold increase in the localization of $G\alpha_s$ to lipid raft domains in both cerebral cortex and cerebellum from suicide cases with documented depression (Donati et al., 2008). As these lipid raft domains are known to inhibit signaling downstream of $G\alpha_s$ (Rybin et al., 2000; Miura et al., 2001; Head et al., 2006; Allen et al., 2009), including coupling to adenylyl cyclase, this increased lipid raft localization likely indicates decreased $G\alpha_s$ signaling in the depressed brain. In fact, a $G\alpha_s$ dependent adenylyl cyclase dysfunction in the depressed brain is supported by impairment in the ability of forskolin to stimulate adenylyl cyclase activity in post-mortem tissue from individuals who completed suicide (Cowburn et al., 1994). This loss of adenylyl cyclase activity is associated with decreased expression and activity of the cAMP dependent kinase (PKA) in the frontal cortex, but not hippocampus of suicide completers (Pandey et al., 2005).

Preclinical and *in vitro* models suggest that antidepressants cause both increased association of $G\alpha_s$ with adenylyl cyclase (**Table 1** and **Figure 2**) and reduce the relative amount of $G\alpha_s$ localized in lipid rafts (Chen and Rasenick, 1995a,b; Toki et al., 1999; Donati and Rasenick, 2005; Allen et al., 2009; Zhang and Rasenick, 2010; Singh et al., 2018; Wray et al., 2018). Both of these effects of antidepressants would in theory directly counteract changes detected in post-mortem brain tissue from suicide completers, namely impaired adenylyl cyclase activity and increased accumulation of $G\alpha_s$ in lipid rafts (Cowburn et al., 1994; Donati et al., 2008). While these results predict that antidepressant treatment should correct these deficits observed in the depressed brain, this hypothesis has not yet been fully tested.

It is noteworthy, however, that recent PET imaging studies reveal decreased [^{11}C]-Rolipram (a phosphodiesterase-4 inhibitor) binding in all brain regions of depressed subjects (Fujita et al., 2017). Subsequent to successful antidepressant treatment [^{11}C]-Rolipram binding returns to baseline throughout the CNS (Fujita et al., 2017). This is consistent with the predictions from the work described above, as [^{11}C]-Rolipram binding is known to reflect levels of cAMP (Hoffmann et al., 1998), due to a feedback mechanism involving cAMP, PKA, and phosphodiesterase-4 (Houslay et al., 1998).

In cellular models, multiple G-protein subtypes accumulate in lipid rafts and antidepressant drug treatment reduces the amount of lipid raft associated $G\alpha_s$ without changing the abundance of other G-proteins in the rafts (Toki et al., 1999; Donati and Rasenick, 2005). This reduction in lipid raft $G\alpha_s$ content occurs without changes in the overall expression level of $G\alpha_s$ protein, or in the expression of other G-proteins including

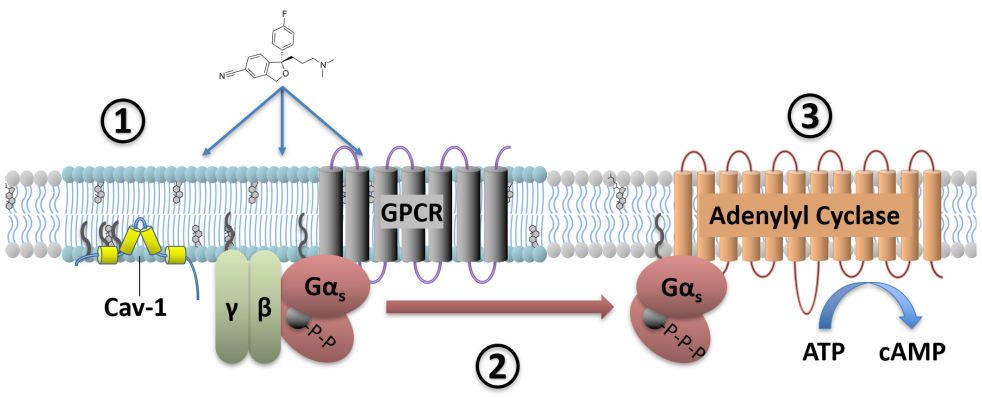

FIGURE 2 | Antidepressant effects on $G\alpha_s$ signaling. Antidepressant drugs (e.g., escitalopram, depicted) slowly accumulate in lipid raft domains **(1)**. These areas are defined in part by high cholesterol content, detergent insolubility, and expression of membrane proteins such as Caveolin-1. Antidepressant accumulation causes a translocation of $G\alpha_s$ from lipid raft to non-raft membrane **(2)**, where coupling to downstream effectors such as adenylyl cyclase increases. This facilitates downstream signaling, including increased cAMP production **(3)**.

TABLE 1 | Antidepressants effects on $G\alpha_s$ signaling.

	$G\alpha_s$ Translocation	$G\alpha_s$ Membrane mobility	cAMP	Reference
Antidepressants				
Amitriptyline	+		+	Menkes et al., 1983; Ozawa and Rasenick, 1989; Chen and Rasenick, 1995a,b; Toki et al., 1999
Bupropion		+		Czysz et al., 2015
Desipramine	+	+	+	Menkes et al., 1983; Ozawa and Rasenick, 1989; Chen and Rasenick, 1995a,b; Toki et al., 1999; Donati and Rasenick, 2005; Czysz et al., 2015
Electroconvulsive Therapy			+	Menkes et al., 1983; Ozawa and Rasenick, 1991; Chen and Rasenick, 1995a
Escitalopram	+	+	+	Zhang and Rasenick, 2010; Czysz et al., 2015; Donati et al., 2015; Erb et al., 2016; Singh et al., 2018
Fluoxetine	+	+		Toki et al., 1999; Donati and Rasenick, 2005; Czysz et al., 2015
Imipramine	+	+	+	Menkes et al., 1983; Czysz et al., 2015; Singh et al., 2018
Iprindole	+		+	Menkes et al., 1983; Ozawa and Rasenick, 1989; Toki et al., 1999
Phenelzine		+		Czysz et al., 2015
Sertraline		+		Czysz et al., 2015
Tianeptine		+		Czysz et al., 2015
Venlafaxine		+		Czysz et al., 2015
Putative antidepressants				
ABT 200			+	Chen and Rasenick, 1995a
Ketamine	+	+	+	Wray et al., 2018
Tubastatin-A	+	+		Singh et al., 2018
Non-antidepressants				
Amphetamine		–	–	Chen and Rasenick, 1995a
Chlorpromazine	–			Toki et al., 1999
Diazepam		–		Czysz et al., 2015
Haloperidol		–		Czysz et al., 2015
Lithium	–	–		Donati et al., 2015
LY368514	–			Donati et al., 2015
Olanzapine		–		Czysz et al., 2015
R-citalopram	–	–	–	Zhang and Rasenick, 2010; Czysz et al., 2015
Valproate	–	–		Donati et al., 2015

*Many antidepressant drugs are known to affect $G\alpha_s$ localization and signaling. These effects include a reduction of $G\alpha_s$ localized to lipid raft membrane regions, alongside increased $G\alpha_s$ found in non-raft membranes (**$G\alpha_s$ Translocation** column), reduced $G\alpha_s$ lateral membrane mobility following antidepressant treatment, quantified by Fluorescence Recovery After Photobleaching ($G\alpha_s$ membrane mobility column), and a faciliatory effect on cAMP accumulation (**cAMP** column). In general, these effects have been observed with antidepressant drugs, but not closely related analogues. For example, the antidepressant Escitalopram is known to produce these effects, while the inactive stereoisomer, R-citalopram, does not. A number of compounds with putative antidepressant qualities, including Tubastatin-A, ABT 200, and ketamine also show this $G\alpha_s$ mediated antidepressant biosignature, suggesting these readouts may be useful for detecting novel antidepressants. A '+' in each column indicates this drug is known to cause this effect, while a '–' indicates this drug does not. Blank entries indicate that data has not been published for this effect.*

$G\alpha_i$, $G\alpha_o$ or $G\beta$ (Chen and Rasenick, 1995b). The shift of $G\alpha_s$ from lipid raft regions to non-lipid raft regions coincides with increased coupling between $G\alpha_s$ and adenylyl cyclase as well as increased adenylyl cyclase activation and cAMP accumulation (**Table 1**; Chen and Rasenick, 1995a,b). Indeed, antidepressants including the tricyclic desipramine (Czysz et al., 2015), the SSRI escitalopram (Erb et al., 2016), and the NMDA antagonist ketamine (Wray et al., 2018) reduce the lateral membrane mobility of $G\alpha_s$ (**Table 1**). This likely reflects increased association with the large, relatively immobile adenylyl cyclase protein following $G\alpha_s$ translocation out of lipid raft domains (Czysz et al., 2015). These effects occur with tricyclic antidepressants, SSRIs and atypical antidepressants, suggesting a potential conserved antidepressant mechanism independent of the known sites of action.

Importantly, the transfer of $G\alpha_s$ out of lipid raft domains occurs following chronic treatment with the antidepressant (S)-stereoisomer of the SSRI citalopram, but not the

(R)-stereoisomer which lacks antidepressant effects (Zhang and Rasenick, 2010). This stereospecific effect of citalopram occurs in C6 cells lacking the serotonin transporter, suggesting that $G\alpha_s$ translocation out of lipid rafts occurs due to interaction of the antidepressant with some other protein target. Furthermore, antidepressant drugs with diverse mechanisms of action (including desipramine, reboxetine and fluoxetine) themselves accumulate in these lipid raft domains over time (Eisensamer et al., 2005). Initially these drugs distribute evenly throughout the plasma membrane, but gradually partition into lipid rafts over a 3-day treatment period (Erb et al., 2016). While together these data suggest that a specific binding site for antidepressants within lipid rafts may exist, a suitable candidate site has yet to be identified.

Therefore, in general antidepressant drugs liberate $G\alpha_s$ from the inhibitory effects of lipid raft localization (Head et al., 2006) allowing this subunit to signal more effectively through downstream effectors including adenylyl cyclase (**Table 1** and **Figure 2**). These effects are relatively specific to the brain as peripheral tissues do not show the same response, and even within the brain there is regional specificity as brain regions other than the cerebral cortex show reduced $G\alpha_s$ translocation, if any (Dwivedi et al., 2002). This provides a plausible mechanism for the long-recognized ability of antidepressant drugs to increase the coupling between $G\alpha_s$ and adenylyl cyclase (Menkes et al., 1983). This enhancement occurs only following extended antidepressant treatment, consistent with the hysteresis observed between the initiation of antidepressant treatment and the onset of therapeutic effects (Chen and Rasenick, 1995a). Notably, ketamine, which is reported to have a rapid therapeutic onset, shows the antidepressant biosignature described above after only 15 min of treatment (Wray et al., 2018).

Although $G\alpha_s$ coupling to adenylyl cyclase is unaffected by antidepressant treatment in peripheral tissues (e.g., liver and kidney; Menkes et al., 1983), a variety of blood cells undergo changes similar to those observed in the brain. Lymphocytes and platelets from depressed subjects have diminished cAMP production due to decreased $G\alpha_s$-adenylyl cyclase coupling (Hines et al., 2005) and this is resolved in subjects who respond to antidepressants (Pandey et al., 1985; Mooney et al., 2013). These data raise the possibility of using $G\alpha_s$ signaling changes in peripheral blood cells to test for depression and antidepressant response, a strategy that has already seen some success with other markers detected in patient blood samples (Rivera-Baltanas et al., 2014).

Together the above discussion suggests a dysregulation of G-protein signaling in the brains of depressed individuals. The dysregulation likely involves changes in overall G-protein expression, as well as translocation of $G\alpha_s$ into a more restrictive membrane microenvironment where signaling to downstream effectors is inhibited, with apparent consequences for cyclase dependent signaling pathways including protein kinase A. It remains unclear whether these changes contribute to suicidal behavior and depression, or are simply correlated with the expression of these states without a causative effect. In either case the existence of these changes may allow for new strategies to diagnose and potentially treat depressive disorders.

RGS PROTEINS IN DEPRESSION AND ANTIDEPRESSANT DRUG ACTION

As mentioned earlier RGS proteins are essential modulators of signaling downstream of the G-proteins discussed above. There are more than 20 members of the RGS protein family, designated by the presence of a 120 amino-acid RH domain and divided into subfamilies based on their structures outside of the RH domain (Hollinger and Hepler, 2002; Traynor and Neubig, 2005). This variety indicates that RGS proteins have some degree of specificity for which Gα subunits they can regulate. For example, RGS4 and RGS8 potently inhibit signaling downstream of $G\alpha_{i2}$ *in vitro* while RGS7 has no effect (Talbot et al., 2009). RGS-PX1 and RGS-PX2 regulate signaling downstream of $G\alpha_s$ (Zheng et al., 2001; Ha et al., 2015), while RGS2 preferentially modulates $G\alpha_q$ signaling (Heximer et al., 1997). RGS proteins also appear to have specificity in terms of which GPCRs they modulate, even when those GPCRs signal through the same type of G-proteins. The dopamine D2 receptor and 5-HT1A receptors are both $G\alpha_{i/o}$ coupled GPCRs, however, RGS4, RGS10 and RGSZ1 reduced 5-HT1A receptor-mediated signaling *in vitro* but did not affect D2-mediated signaling (Ghavami et al., 2003). It remains unclear whether RGS proteins achieve this specificity through direct interaction with certain GPCRs or by interactions with other intracellular binding partners.

Membrane anchoring can also affect how RGS proteins regulate G-protein action. For example, palmitoylation of RGS16 is necessary for regulation of $G\alpha_i$ and $G\alpha_q$ signaling (Hiol et al., 2003). RGS proteins in complex with a Gα subunit can also directly affect signaling to downstream effectors, independent of their GAP function. This level of regulation also depends on the specific RGS protein involved, for instance RGS2 in complex with $G\alpha_q$ can prevent $G\alpha_q$ from binding to the downstream effectors p63 RhoGEF and GRK2, while RGS4 in complex with $G\alpha_q$ has little effect on binding to these effectors (Shankaranarayanan et al., 2008).

Therefore, while different RGS proteins have classically been thought to have largely redundant actions, current evidence suggests that different family members might have considerable variation in G-protein preference, receptor selectivity, and scaffolding functions toward downstream effectors. This highlights the need for a better understanding of what role the RGS proteins play in neuropsychiatric disorders and treatments.

RGS INSENSITIVE G-PROTEINS

When an individual RGS protein is knocked out or genetically modified, other available RGS proteins can at least partially compensate. Similarly, when an individual G-protein is lost, a GPCR may be able to continue signaling through other available G-proteins. In order to overcome these difficulties a series of modified G-proteins were developed which are insensitive to the negative regulatory effects of RGS proteins. These mutated G proteins have a Gly in the "switch 3" region of the Gα protein that binds RGS proteins, replaced with a Ser (Tesmer et al., 1997).

This prevents the protein-protein interaction necessary for GAP activity.

The potential utility of RGS insensitive (RGSi) G-proteins was quickly recognized and a series of novel mammalian RGSi G-proteins were created (DiBello et al., 1998; Lan et al., 1998). These mutations do not affect the kinetics of GDP release, GTP hydrolysis, Gβγ binding, or interaction with the receptor, but produce up to 100-fold loss of affinity for RGS proteins (Day et al., 2004; Fu et al., 2004). These properties allowed investigators to probe the effects of removing all RGS control at a specific G-protein without affecting G-protein signaling otherwise, avoiding the difficulties posed by RGS protein redundancy.

Mice expressing an RGSi knock-in variant of $G\alpha_{i2}$ display a profound antidepressant-like phenotype across a number of behavioral tests including tail suspension, forced swim, elevated plus maze and novelty induced hypophagia (Talbot et al., 2010; Senese et al., 2018). These mice also have antidepressant-like signaling changes in the hippocampus and frontal cortex, including reduced glycogen synthase kinase 3 beta (GSK3β) activity (Talbot et al., 2010). GSK3β inhibition produces neurogenesis in the adult hippocampus, and this neurogenic effect may be an important component of antidepressant action (Malberg et al., 2000; Li et al., 2004; Tsai et al., 2008), although the significance of these antidepressant induced neurogenic effects has been disputed (Hanson et al., 2011).

Both the changes in hippocampal GSK3β and antidepressant-like behaviors observed in RGSi $G\alpha_{i2}$ knock-in mice are fully reversed by pretreatment with a 5-HT1A receptor antagonist (Talbot et al., 2010). Furthermore, direct application of this 5-HT1A receptor antagonist to the hippocampus of the RGSi $G\alpha_{i2}$ knock-in mice reversed their antidepressant-like behavior (Senese et al., 2018), suggesting that enhanced 5-HT1A receptor activity in the hippocampus is necessary for this behavior. Coupled with the fact that 5-HT1A receptor agonists produce more potent antidepressant-like effects in these animals, it appears that the loss of RGS control at $G\alpha_{i2}$ promotes 5-HT1A receptor signaling leading to robust antidepressant-like effects. Mice heterozygous for RGSi $G\alpha_o$ also show antidepressant-like effects in the tail suspension test, but the GPCR involved is not the 5-HT1A receptor and remains unidentified (Senese et al., 2018). The culprit in this case may be the delta opioid receptor (DOPR) since the potency of the delta agonist SNC80 to produce antidepressant-like effects is enhanced in these mice (Dripps et al., 2018).

Although activating postsynaptic 5-HT1A heteroreceptors is generally considered beneficial for antidepressant action, presynaptic 5-HT1A autoreceptor activation can limit antidepressant action and may contribute to the hysteresis observed between initiation of treatment and the onset of therapeutic effects (Hjorth and Sharp, 1993; Artigas et al., 1994; Le Poul et al., 1995; Matsuda et al., 1995). Interestingly, RGSi $G\alpha_{i2}$ knock-in mice display enhancements of responses known to depend on 5-HT1A heteroreceptor activity (e.g., antidepressant-like behaviors, hippocampal GSK3β inhibition), but not responses dependent on 5-HT1A autoreceptor activity (e.g., hypothermia; Hillegaart, 1991; Matsuda et al., 1995; Li et al., 2004; Talbot et al., 2010). 5-HT1A receptor agonists also produce exaggerated responses in hippocampal neurons from these mice, with more robust 5-HT1A receptor dependent suppression of neuronal excitability observed in pyramidal neurons (Senese et al., 2018). As the hippocampus does not contain 5-HT cell bodies (Ren et al., 2018), this provides direct evidence for increased 5-HT1A heteroreceptor activity in these animals.

The above data suggest that disrupting RGS control of $G\alpha_{i2}$ may represent a novel strategy to selectively enhance the antidepressant effects of 5-HT1A receptor activation without promoting the detrimental effects of autoreceptor activation. Unfortunately, specific RGS protein(s) responsible for the antidepressant-like phenotype in the RGSi-$G\alpha_{i2}$ mice are not currently known, but two candidates (RGS6 and RGS19) are discussed later in this review.

Although the above studies highlight roles for RGS proteins in modulating depressive-like behaviors in preclinical models, they do not identify which RGS protein or proteins are involved. However, a limited number of studies have been performed on specific RGS proteins as described below.

RGS2

Mice lacking RGS2 showed a baseline increase in anxious and depressive-like behaviors (Oliveira-dos-Santos et al., 2000; Lifschytz et al., 2012). These behavioral alterations occur alongside decreased presynaptic 5-HT1A receptor expression and function, suggesting that these receptors may play a role in the observed behavioral phenotype (Lifschytz et al., 2012; Muma, 2012 for review). In contrast, a genetic manipulation which specifically increases raphe 5-HT1A receptor expression in mice increases vulnerability to stress and decreases response to antidepressants (Richardson-Jones et al., 2010), suggesting that reductions in raphe 5-HT1A receptor availability following disruption of RGS2 may be a compensatory change rather than a causative factor of the pro-depressant behaviors. Nonetheless, these data demonstrate that RGS2 may have a protective effect against anxiety and depression, and that RGS2 disruption may have detrimental neuropsychiatric effects.

In line with these pre-clinical results, individuals expressing any of several single nucleotide polymorphisms (SNPs) in the RGS2 gene experience anxiety disorders and suicidal ideations at an increased rate (Leygraf et al., 2006; Smoller et al., 2008; Amstadter et al., 2009). In addition, various RGS2 genetic variants were found at an increased rate in suicide completers than controls (Cui et al., 2008). However, an increase in RGS2 immunoreactivity was also found in post-mortem tissue from both the prefrontal cortex and amygdala of these subjects (Cui et al., 2008), although this may represent a compensatory upregulation due to a loss of RGS2 functionality. Together these findings provide solid evidence that genetic alterations in the RGS2 gene can influence the development or expression of affective disorders in human populations, in line with findings from RGS2 knockout mice.

While it remains difficult to study antidepressant action *in vitro*, a number of reports have provided evidence on how

RGS2 can affect cellular processes in ways that might modify antidepressant action. For example, an increase in hippocampal synaptic plasticity typically occurs following antidepressant treatment, while depressive states cause decreased plasticity (Kozisek et al., 2008; Nissen et al., 2010). Loss of RGS2 produces a similar loss of synaptic plasticity in mouse hippocampal tissue potentially by altering $G\alpha_{i/o}$-mediated inhibition of hippocampal Ca^{2+} channels (Han et al., 2006). This suggests that a loss of neural plasticity due to genetic variation in RGS2 could have detrimental neuropsychiatric effects and might impair the function of antidepressant drugs.

RGS4

Studies in rodent models have consistently shown that 5-HT1A receptor agonists such as 8-OH-DPAT cause a reduction in extracellular serotonin levels in the brain by activating 5-HT1A autoreceptors in the dorsal raphe nucleus (Casanovas and Artigas, 1996; Adell and Artigas, 1998; Celada et al., 2001). It is thought that this 5-HT1A receptor-dependent reduction in central serotonin may delay the beneficial effects of SSRI antidepressants. Indeed, strategies to limit 5-HT1A autoreceptor activity during SSRI treatment, such as co-administration of 5-HT1A receptor antagonists or weak partial agonists, have shown promising results (Artigas et al., 1994; Pérez et al., 1997; Tome et al., 1997; Maes et al., 1999). Although RGS4 mRNA is not normally expressed in the dorsal raphe nucleus (Gold et al., 1997), RGS4 overexpression in this region attenuates the ability of 5-HT1A receptors to reduce central serotonin levels (Beyer et al., 2004). Based on these results, overexpression or stimulation of RGS4 in brain regions containing 5-HT1A autoreceptors should have beneficial effects on antidepressant drug action, similar to the results obtained by combining 5-HT1A antagonists with traditional antidepressants (Artigas et al., 1994).

Delta opioid receptor agonists produce antidepressant-like behavioral effects in rodent models including the tail suspension and forced swim tests (Broom et al., 2002; Naidu et al., 2007). RGS4 knockout mice show an enhanced antidepressant-like response to DOPR agonists in the forced swim test, but not tail suspension test, suggesting that these antidepressant-like behaviors may depend on distinct neural pathways or signaling intermediates downstream of DOPR activation (Stratinaki et al., 2013; Dripps et al., 2017). This effect on forced swim test behavior appears to depend on nucleus accumbens RGS4 expression, as specific RGS4 knockdown in this region produces similar effects as global RGS4 knockout (Stratinaki et al., 2013). RGS proteins capable of modulating DOPR-mediated tail suspension test behavior have not yet been identified.

In addition to inhibiting the antidepressant-like effects of DOPR agonists, loss of RGS4 appears to inhibit the antidepressant-like effects of SSRIs, norepinephrine reuptake inhibitors and the *N*-methyl-D-aspartate (NMDA) receptor antagonist ketamine (Stratinaki et al., 2013). Acute treatment with either a DOPR agonist or ketamine decreases frontal cortex RGS4 expression yet only chronic treatment with a DOPR agonist increases RGS4 expression in the nucleus accumbens (Stratinaki et al., 2013). While this would suggest that nucleus accumbens and frontal cortex RGS4 have opposing effects on antidepressant action, this has yet to be conclusively demonstrated. Although RGS4 expression in the brain does not appear to differ between post-mortem tissue from depressed and healthy individuals, an upregulation of RGS4 has been observed in post-mortem nucleus accumbens tissue from depressed individuals undergoing antidepressant treatment compared to untreated depressed individuals (Stratinaki et al., 2013). Coupled with findings from rodent models showing increased antidepressant effectiveness in animals with overexpression of RGS4 in this brain region, it appears possible that nucleus accumbens RGS4 has a facilitatory effect on antidepressant treatment.

The RGS4 inhibitor CCG-203769 produces antidepressant-like effects on the tail suspension test in a mouse model (Senese et al., 2018). As RGS4 knockout mice do not have behavioral changes on the tail suspension test (Dripps et al., 2017), and loss of RGS4 inhibits the antidepressant-like effects of drugs with diverse mechanisms of action (Stratinaki et al., 2013), RGS4 inhibition alone cannot explain the antidepressant-like effect of CCG-203769. However, CCG-203769 also inhibits RGS19, and downregulation of RGS19 has been shown to increase 5-HT1A receptor signaling in hippocampal neurons (Wang et al., 2014). CCCG-203769's antidepressant-like effects are discussed in more detail in the section on RGS19.

RGS6

Mice lacking RGS6 display antidepressant-like and anxiolytic behaviors at baseline, including in the elevated plus maze and novelty induced hyponeophagia test (Stewart et al., 2014). This behavioral phenotype is fully reversible by 5-HT1A receptor antagonist pretreatment, and by direct activation of adenylyl cyclase with forskolin (Stewart et al., 2014). Loss of RGS6 did not affect mitogen-activated protein kinase (MAPK) or GSK3β signaling (Stewart et al., 2014), changes that have been detected in mice with a 5-HT1A receptor dependent antidepressant-like phenotype due to loss of RGS control at $G\alpha_{i2}$ (Talbot et al., 2010). Instead the RGS6 knockout mouse phenotype appears to depend on increased phospho-CREB in the hippocampus and cortex, areas with high 5-HT1A receptor expression (Stewart et al., 2014). The mechanistic differences across the two mouse models calls into question whether RGS6 is the primary mediator of the RGSi-$G\alpha_{i2}$ knock-in mouse behavioral phenotype, although it may be one of several RGS proteins involved. Nonetheless, the findings suggest that RGS6 may normally limit the actions of serotonergic antidepressants by reducing adenylyl cyclase inhibition downstream of 5-HT1A receptor activation, and that strategies to limit RGS6 activity may have beneficial effects for antidepressant treatment.

RGS8

Overexpression of RGS8 in mouse brain produces an antidepressant-like behavioral phenotype in the forced swim test

(Kobayashi et al., 2018). RGS8 is a potent negative modulator of melanin-concentrating hormone receptor 1 (MCHR1) signaling (Miyamoto-Matsubara et al., 2008). Inhibitors of MCHR1 have antidepressant-like effects, however, this action is lost in mice overexpressing RGS8, suggesting signaling downstream of MCHR1 is the target for RGS8. Whether loss of central RGS8 produces a pro-depressant phenotype has not yet been tested, although small molecule compounds with inhibitory action against RGS8 (Blazer et al., 2011; Storaska et al., 2013) could provide the means to examine these behaviors in future studies. While these RGS8 inhibitors are not highly potent or selective, a detailed analysis of the binding sites for these inhibitors (Shaw et al., 2018) will facilitate the discovery of more selective compounds.

RGS16

Although RGS16 has not been directly linked to depression, palmitoylation causes the accumulation of RGS16 in lipid raft domains, a subcellular membrane compartment known to generally promote signaling downstream of $G\alpha_{q.}$ (Rybin et al., 2000; Hiol et al., 2003). As discussed earlier, this localization places RGS16 at or near the putative, but not yet positively identified, binding site for antidepressant drugs within lipid rafts (Eisensamer et al., 2005; Erb et al., 2016). This localization may provide RGS16 with an increased ability to regulate antidepressant drug action, although this prediction has yet to be tested.

RGS17 (RGSZ2)

Levels of RGS17 mRNA (also known as RGSZ2) were shown to be markedly reduced in post-mortem brain tissue from individuals diagnosed with MDD (Shelton et al., 2011) in a large-scale RNA microarray analysis. Unfortunately, this study did not provide a potential mechanism by which MDD affects RGS17, nor evidence that these changes in RGS17 mRNA reflect changes in RGS17 activity.

RGS19 (GAIP)

RGS19, also known as G Alpha Interacting Protein (GAIP; De Vries et al., 1995) has been shown to regulate 5-HT1A receptor signaling in both C6 and SH-SY5Y cells (Wang et al., 2014). RGS19 knockdown facilitated 5-HT1A receptor agonist induced activation of MAPK and inhibition of adenylyl cyclase (Wang et al., 2014), while RGS4 knockdown did not significantly affect signaling. The effect of RGS19 knockdown was magnified when the cells were co-treated with fibroblast growth factor 2 (FGF2), a factor known to act synergistically with 5-HT1A receptor activity in the hippocampus to facilitate synaptic plasticity (Borroto-Escuela et al., 2012; Wang et al., 2014). The enhancement of 5-HT1A receptor agonist stimulated MAPK activity following RGS19 knockdown seen in cellular models was replicated in mouse primary hippocampal neurons, including the synergistic enhancement by co-treatment with FGF2 and a 5-HT1A receptor agonist (Wang et al., 2014). This suggests that reducing RGS19 activity may facilitate the action of serotonergic antidepressants due to disinhibition of hippocampal 5-HT1A receptor activity.

CCG-203769, an RGS inhibitor with 100-fold selectivity for RGS19 and RGS4 compared to other RGS proteins (Blazer et al., 2015), produces antidepressant-like effects in a mouse model (Senese et al., 2018). These effects occur after repeated intra-hippocampal administration in female, but not male mice (Senese et al., 2018), suggesting that behavioral responses to RGS inhibitors may be sexually dimorphic. This behavioral sex difference occurs despite similar hippocampal expression of RGS19 between male and female mice (Senese et al., 2018).

As discussed above, loss of RGS4 activity attenuates the effects of various antidepressants (Stratinaki et al., 2013), suggesting that the antidepressant-like behavioral effects observed following CCG-203769 administration are not likely to be mediated by its inhibitory effect on RGS4 (Blazer et al., 2015; Senese et al., 2018). In contrast, loss of RGS19 activity facilitates 5-HT1A receptor mediated MAPK activity (Wang et al., 2014), signaling changes that have been associated with antidepressant-like behaviors in animal models (Talbot et al., 2010). While not conclusive, these data suggest that the antidepressant-like effects of CCG-203769 in female mice are due to its inhibitory effects on RGS19 (Senese et al., 2018). This is the first report of antidepressant-like behaviors produced by a compound which targets RGS proteins directly, although the requirement for central delivery of CCG-203769, and its action as an irreversible inhibitor, suggests that novel RGS inhibitors with improved drug-like properties are needed before this class of compounds can be fully explored for potential clinical utility.

RGS20 (RGSZ1)

In post-mortem tissue from individuals with MDD, RGS20 (also known as RGSZ1) mRNA is significantly decreased in the anterior cingulate cortex (Tomita et al., 2013). This change was not observed in tissue from individuals with bipolar disorder (Tomita et al., 2013). It remains unclear whether this change in RGS20 mRNA is reflected by changes in protein expression or activity. Furthermore, as the majority of these individuals were undergoing neuropsychiatric treatment at time of death, it is impossible to determine whether RGS20 mRNA was reduced due to antidepressant treatment, or reflects an innate difference in the depressed brain.

Chronic estradiol treatments both desensitize hypothalamic 5-HT1A receptors and cause an increase in plasma membrane RGS20 expression (Carrasco et al., 2004; McAllister et al., 2014). Increased RGS20 is predicted to reduce signaling downstream of 5-HT1A receptor and therefore may contribute to the observed 5-HT1A receptor desensitization, although this interaction has not been conclusively demonstrated (Carrasco et al., 2004). 5-HT1A receptor desensitization in the dorsal raphe nucleus, not the hypothalamus, is generally considered a critical step in antidepressant action. However, RGS20 expression in the dorsal raphe has not been assessed following chronic antidepressant

treatment, so it remains possible that a similar process contributes to 5-HT1A receptor desensitization in this brain region.

CONCLUSION

G-proteins and their RGS protein modulators likely play important roles in the development of depressive states, and also influence the effectiveness of antidepressant therapies. Changes in activity of these proteins can have dramatic effects on these complex disorders, while even more subtle alterations, such as G-protein translocation between subcellular microdomains, can profoundly regulate antidepressant action. Although preclinical studies provide multiple hypotheses for how these proteins behave in depressed populations in the clinic, only a handful of these theories have been addressed in humans. Considering how alterations in G-proteins and/or RGS differentially affect responses to a variety of antidepressant treatments, it appears reasonable that a better understanding of these proteins could aid in the tailoring of personalized treatment strategies for depression. Screening for changes in G-protein signaling pathways could also provide new insight into susceptibility toward depressive disorders at an individual level. In addition, direct RGS-inhibiting compounds have been proposed as novel treatment options for a variety of indications, and selective small molecule RGS inhibitors have already been identified (Zhong and Neubig, 2001; Roman et al., 2006; Shaw et al., 2018). A more complete understanding of how these G-proteins and their partners interact with antidepressant therapies and the development of depressive states is both needed and welcome.

AUTHOR CONTRIBUTIONS

NS provided the original draft, and editing of subsequent drafts. MR and JT provided project funding, and manuscript review and editing.

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Non-linear Entropy Analysis in EEG to Predict Treatment Response to Repetitive Transcranial Magnetic Stimulation in Depression

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Background: Biomarkers that predict clinical outcomes in depression are essential for increasing the precision of treatments and clinical outcomes. The electroencephalogram (EEG) is a non-invasive neurophysiological test that has promise as a biomarker sensitive to treatment effects. The aim of our study was to investigate a novel non-linear index of resting state EEG activity as a predictor of clinical outcome, and compare its predictive capacity to traditional frequency-based indices.

Methods: EEG was recorded from 62 patients with treatment resistant depression (TRD) and 25 healthy comparison (HC) subjects. TRD patients were treated with excitatory repetitive transcranial magnetic stimulation (rTMS) to the dorsolateral prefrontal cortex (DLPFC) for 4 to 6 weeks. EEG signals were first decomposed using the empirical mode decomposition (EMD) method into band-limited intrinsic mode functions (IMFs). Subsequently, Permutation Entropy (PE) was computed from the obtained second IMF to yield an index named PEIMF2. Receiver Operator Characteristic (ROC) curve analysis and ANOVA test were used to evaluate the efficiency of this index (PEIMF2) and were compared to frequency-band based methods.

Results: Responders (RP) to rTMS exhibited an increase in the PEIMF2 index compared to non-responders (NR) at F3, FCz and FC3 sites ($p < 0.01$). The area under the curve (AUC) for ROC analysis was 0.8 for PEIMF2 index for the FC3 electrode. The PEIMF2 index was superior to ordinary frequency band measures.

Conclusion: Our data show that the PEIMF2 index, yields superior outcome prediction performance compared to traditional frequency band indices. Our findings warrant further investigation of EEG-based biomarkers in depression; specifically entropy indices applied in band-limited EEG components. Registration in ClinicalTrials.Gov; identifiers NCT02800226 and NCT01887782.

Keywords: EEG, rTMS, major depressive disorder, permutation entropy, empirical mode decomposition, biomarker

INTRODUCTION

Major depressive disorder (MDD) is a global public health concern (World Health Organization [WHO], 2016) as the disease is the leading cause of disability (Zarate et al., 2013) and affects approximately 5.4% of the population worldwide (Ferrari et al., 2013). Furthermore, it is estimated that 40% of those living with the disorder do not respond to first line treatments such as pharmacological or psychosocial treatments and have treatment resistant depression (TRD) (Berlim et al., 2008).

Repetitive transcranial magnetic stimulation (rTMS) is a safe and effective treatment for TRD with 50–55% response and 30–35% remission rates (Galletly et al., 2011; McDonald et al., 2011), and rTMS is considered a first-line treatment option for TRD (Milev et al., 2016). rTMS induces an electric field in the brain strong enough to depolarize neurons and trigger action potentials, and the treatment is delivered non-invasively by applying a coil in contact with the scalp. Neuroimaging studies have shown a degree of hypoactivity in the left dorsolateral cortex (L-DLPFC) in MDD (Hallett, 2007), and therefore rTMS protocols that increase cortical excitability have been applied to the L-DLPFC for the treatment of MDD.

The prescription of rTMS, similar to antidepressant medication prescription, is currently based on clinical assessment and a process of trial and error. Identification of effective biomarkers that can inform clinical decisions is lacking, and this absence may contribute to higher health-care costs (Silverstein et al., 2015). Developing reliable biomarkers may have profound implications for clinical practice as it would shift the prescription process to a more precise and personalized approach that would further improve clinical outcomes and efficiency during treatment initiation (Collins et al., 2011).

The search for biomarkers of response has expanded to molecular, neurophysiological and neuroimaging methods (Silverstein et al., 2015). The resting-state electroencephalogram (rsEEG) has merited particular interest due to its ease of use, cost-effectiveness and non-invasive nature which are optimal characteristics for its implementation in clinical settings (Baskaran et al., 2012; Shalbaf et al., 2015b; Wade and Iosifescu, 2016).

Several frequency-based rsEEG measures have been proposed as predictors of response in TRD in the context of rTMS. Examples include theta (4–7 Hz) activity in the subgenual zone of the anterior cingulate cortex (Narushima et al., 2010), anterior alpha (8–12 Hz) peak frequency (Arns et al., 2012), prefrontal cordance (combination of absolute and relative EEG power at different bands), (Bares et al., 2015; Erguzel et al., 2015) and Lempel-Ziv analysis on the alpha band (Arns et al., 2014). However, these frequency-based methods are susceptible to artifacts and are more suitable for the analysis of stationary signals. Furthermore, these frequency-based measures require a Fourier transform, and this transform precludes precise estimation of temporal patterns in EEG (Huang et al., 1998).

Abbreviations: DLPFC, dorsolateral prefrontal cortex; EMD, empirical mode decomposition; HDRS, hamilton depression rating scale; MDD, major depressive disorder; ROC, receiver operating characteristic; rTMS, repetitive transcranial magnetic stimulation; TRD, treatment-resistant depression.

Complexity and non-linear behavior are characteristics of typical brain functioning, (Elbert et al., 1994) and therefore the application of non-linear dynamics analyses to the EEG signal may prove to be a better measure of neural activity (Hosseini et al., 2010). Recently, a non-linear parameter called permutation entropy (PE) (Bandt and Pompe, 2002; Cao et al., 2004) has been developed to dissect the complexity of EEG signals by deciphering the local order structure of a dynamical time series (Cao et al., 2004). In addition, PE properly tracks the dynamics of brain activity (Shalbaf et al., 2013), is conceptually simple, computationally efficient, and robust against artifacts (Shalbaf et al., 2015a).

However, PE can be underestimated if the signals are superimposed with local or global trends. Research has suggested that properly removing the trends in biological signals with a decomposition approach may improve the performance of non-linear signal analysis (Lo et al., 2009; Tsai et al., 2012). Different variations of decompositions are suitable due to their ability to derive dynamical features from the signals with an enhanced resolution (Kevric and Subasi, 2017). One such method, empirical mode decomposition (EMD), was developed for analyzing non-stationary data (Huang et al., 1998). EMD can decompose a complicated signal without a basis function, such as sine or wavelet functions, into several intrinsic mode functions (IMFs) that are embedded in the original signal. The decomposition procedure is adaptive, data-driven and highly efficient (Kevric and Subasi, 2017). Therefore, entropy index applied in band-limited EEG component extracted with EMD method may optimally quantify non-linear neuronal oscillations.

The purpose of this study is to examine rsEEG features as predictors of treatment response in TRD patients receiving excitatory rTMS to the L-DLPFC. We hypothesized that rsEEG decomposition components will hold different energies for different patients and that these would differentiate responders (RP) from non-responders (NR). Furthermore, we hypothesized that non-linear methods would be better suited and more efficient predictors of rTMS treatment response compared to traditional linear frequency-band power metrics.

MATERIALS AND METHODS

Participants and Experimental Procedures

The neurophysiology dataset was part of two randomized, single-blinded trials in which patients with TRD were assigned to receive either intermittent theta burst stimulation (iTBS) or high frequency left (HFL) rTMS protocols to the left DLPFC (parameters discussed below). Patients received a 4–6 week course of rTMS.

Participants were part of two separate clinical trials with identical inclusion and exclusion criteria registered in ClinicalTrials.Gov, identifier NCT02800226 and NCT01887782 (Blumberger et al., 2018). All participants provided informed consent and both experimental protocols were approved by both the UBC Clinical Research Ethics Board as well as the Vancouver Coastal Health Research Institute.

Subjects were 62 TRD patients and 25 healthy comparison (HC) subjects. Demographic characteristics were collected at baseline (**Table 1**). All participants completed rsEEG at baseline, prior to receiving treatment. Six patients did not complete the full 4 weeks of rTMS treatment and were excluded from analysis. Data from an additional 5 patients were removed from analysis due to random noise after quality control analysis. Thus, a dataset with a total of 76 participants was used in this study, including 51 TRD patients (26 randomized to iTBS and 25 to HFL) and 25 HC. The inclusion and exclusion criteria of patients and HC are outlined in **Supplementary Material**.

Stimulation Technique and Parameters

Stimulation techniques have been previously described (Ge et al., 2017). Briefly, a MagPro X100 stimulator with a Cool-B70 fluid-cooled coil was used to deliver rTMS for all patients (Magventure, Farum, Denmark). Resting motor threshold was determined by visual inspection of right interpolicis brevis muscle contraction with the aid of the TMS Motor Threshold Assessment Tool (Dobek et al., 2016). All treatments were delivered at 120% resting motor threshold (Blumberger et al., 2018). Following randomization, TRD patients received either HFL stimulation or iTBS over the left DLPFC, using a Neuronavigation system (Visor 2.0, ANT Neuro, Enschede, Netherlands) and the target location specified by reverse coregistration from a stereotaxic coordinate on the standard Montreal neurological institute (MNI-152) template brain [x − 38, y + 44, z + 26] identified as optimal based on functional connectivity and clinical outcome (Dobek et al., 2016).

Clinical Measures

Primary clinical outcome was measured using the 17-item Hamilton Depression Rating Scale (HDRS). For each patient, HDRS scores were collected at baseline and at the end of the rTMS course. Interviewers were blinded to patient treatment allocation. Responders (RP) were defined as those having a 50% or greater reduction in HDRS scores between baseline and end of treatment. Out of the 51 patients included in the analysis, there were 31 responders and 20 non-responders to rTMS treatment.

Pre-treatment EEG Acquisition

rsEEG was collected using Brain Products EEG systems (Brain Products, Gilching, Germany) at two UBC sites part of the Canadian Biomarker Integration Network in Depression (Lam et al., 2016). The process of acquiring data with different systems has been carefully considered and addressed (Farzan et al., 2017a). Continuous rsEEG was recorded using 31 (site A) or 64 (site B) recording sites determined using the 10–20 system of electrode placement, an EasyCap electrode cap, and sintered Ag-AgCl electrodes. rsEEG data were recorded using a QuickAmp amplifier (Brain Products, Gilching, Germany; 1000 Hz A/D rate; 0.10 Hz high pass, 499 Hz low pass; common average reference; impedances $\leq$ 10 kΩ). rsEEG was obtain within 7 days of treatment initiation in all participants (mean 3.7 days).

Participants were given the same resting state instructions, "Please close your eyes for 3 min while we collect your brain activity at rest. Let your mind wander and try not to fall asleep." All rsEEGs were conducted in a sound-attenuated room with reduced lighting to limit distraction and noise. Two sets of bipolar electrodes were placed around the participant's eyes for collecting Electrooculogram (EOG) to track eye movements for artifact rejection.

EEG Preprocessing

Two levels of pre-processing steps were implemented in order to standardize the EEG data collected from both sites. These steps are done in MATLAB (The Mathworks, Inc., Natick, MA, United States) via the open-source EEGLAB toolbox (Delorme and Makeig, 2004).

The aim of the first pre-processing step was to minimize raw data heterogeneity across two sites and prepare the data for integration. First, data from Site B were reduced because site B had more recording electrodes, and thus some electrodes were removed from analyses to match the same number of electrodes as Site A. The electrode locations in both caps were identical as per manufacturer description (Brain Products, Gilching, Germany in both site A and site B). Second, data from Site B were re-referenced to common average reference such that data from the two sites possess the equivalent electrode reference. Then, length of recording for all participants modified to have the same length. Also, separate frequency analysis and statistical tests

TABLE 1 | Demographics and clinical characteristics of HC and TRD patients by responder and non-responder groups.

	TRD patients (*n* = 51)			
	Responders (*n* = 31)	**Non-responders (*n* = 20)**	**Healthy volunteers (*n* = 25)**	***p***
Sex (F/M)	19/12	12/8	18/7	ns[a]
Age (*SD*)	43.4 (11.6)	42.5 (14.1)	39.8 (13.1)	ns[b]
Years of education (*SD*)	15.2 (2.5)	15.0 (1.9)	16.0 (1.9)	ns[b]
Handedness (R/L/A)	26/5/0	15/3/2	21/2/2	ns[a]
HDRS (*SD*)	22.5 (4.3)	2249 (4.0)	–	ns[b]
Treatment (HFL/iTBS)	13/18	12/8	–	ns[a]

M, male; F, female; SD, standard deviation; L, left; R, right; A, ambidextrous; HDRS, 17-item Hamilton Depression Rating Scale. [a]Chi-square test. [b]Two sample t-test to compare healthy controls to patients, since in the original analysis, a one-way ANOVA was used to compare age and education across the 3 groups.

from the same two healthy volunteers were done to show data were equivalent in quality across the two sites.

The second pre-processing step was to implement EEG artifact removal, since these artifacts interfere with the identification of true neurophysiological signal. First, the sampling rate of all data was decreased from 1000 to 256 samples per second to reduce white noise. Second, data segments contaminated with large-amplitude or random noise sources that cannot be extracted through filtering were removed with GUI workflow. Third, the high and low band pass filters were set to at 0.5 and 55 Hz respectively, to remove low and high frequency noise. The notch filter was also set at 60 Hz to remove industrial noise. Finally, blind source separation techniques via independent component analysis (ICA) (Makeig et al., 1996) were used to extract eye movements and blinks, muscle activity, and cardiac signals in order to separate neural activity from these sources of noise.

Linear EEG Analyses

Time-frequency analyses using short time windowed Fast Fourier Transform (FFT) applied to resting EEG epochs were computed using MATLAB and EEGLAB software (Delorme and Makeig, 2004). Some conventional frequency band measures such as Delta (1–4 Hz), Theta (4–8 Hz), Alpha (8–12 Hz), Beta (12–24 Hz) and Gamma (30–50 Hz) relative powers were extracted from the rsEEG signals for both HC and TRD groups. 1024 points discrete FFT with a 100% Hanning window was computed over segments of 8 s with an overlap of 6 s and the average of all segments in a recording was considered the relative powers index for each participant.

Non-linear EEG Analyses

Non-linear features were extracted from the eyes-closed resting state EEG signals of both HC and TRD groups. Details of the algorithm will be described in following sections.

Empirical Mode Decomposition (EMD)

EMD is a method of signal analysis (Huang et al., 1998) that has recently been applied to biological signals (Shalbaf et al., 2012). Using EMD, any complex signal can be decomposed into a small number of intrinsic mode functions (IMFs) through a sifting process. The IMFs should fulfill two requirements:

(1) The IMF is symmetric with respect to the local mean.
(2) Each IMF has the same number of zero crossings and extrema, or they differ at most by one.

The detailed algorithm has been previously described and the number of IMFs was established to be six (Shalbaf et al., 2012). An EEG epoch of 8 s from one participant in FC3 electrode is plotted in **Figure 1A**. The EMD of this EEG epoch is composed of six IMFs which are shown in **Figure 1B**. These IMFs are almost orthogonal components.

Permutation Entropy (PE)

Permutation entropy (PE) is a new non-linear parameter that quantifies the amount of regularity in EEG data (Bandt and Pompe, 2002; Cao et al., 2004). This feature converts a given EEG series into a sequence of ordinal patterns, meaning that a non-stationary series can be transformed to an almost stationary ordinal series. The smallest and the largest values of PE are zero and one, with zero reflecting a highly regular time series and one reflecting equal probability of all permutations. The detailed algorithm and parameter sets have been previously published (Shalbaf et al., 2013).

Permutation Entropy Intrinsic Mode Functions (PEIMF2)

The EEG signals were first decomposed by applying the EMD method into symmetric and band-limited IMFs which are arranged from high to low frequency components. EMD decomposition was computed over segments of 8 s with an overlap of 6 s in order to consistently track the transient changes in the EEG recording. Then, PE was computed from each of the 8-s IMF2 segments. The average PE of all segments in a recording was considered the PEIMF2 index for each participant.

Statistical Analysis

Differences in neurophysiological variables between RP, NR, and HC groups were examined using 1-way analysis of variance (ANOVA). The normality of the data was investigated before performing analyses, and a p-value of 0.01 was set as the criteria for statistical significance for greater stringency than conventional levels.

A Receiver Operator Characteristic (ROC) curve was plotted as a two-dimensional depiction of the classifier's performance in predicting treatment outcomes using the proposed biomarkers. The two axes of this graph represent tradeoffs between errors (false positives) and successes (true positives) that a classifier makes between two classes. To corroborate the results of this analysis, the area under the ROC curve, abbreviated as AUC was calculated.

RESULTS

Demographic and Clinical Characteristics

Table 1 summarizes the demographic and clinical characteristics of all participants. The responders, non-responders, and healthy comparison groups had similar age, sex, years of education, and handedness.

There were no differences between RP and NR as well as between MDD and HC in age and sex. Also Baseline HDRS scores of responders were similar to that of non-responders.

Response Prediction Based on PEIMF2 Index

The calculated PEIMF2 indices for the RP, NR and HC groups are plotted onto scalp topographic maps in **Figure 2** with scales to the right of the maps. Greater PEIMF2 index are observed in RP and HC groups compared to NR, especially at left frontal sites.

One-way ANOVAs were calculated to investigate whether there were significant differences between RP and NR groups on PEIMF2 index for each electrode site (**Table 2**). The ANOVA

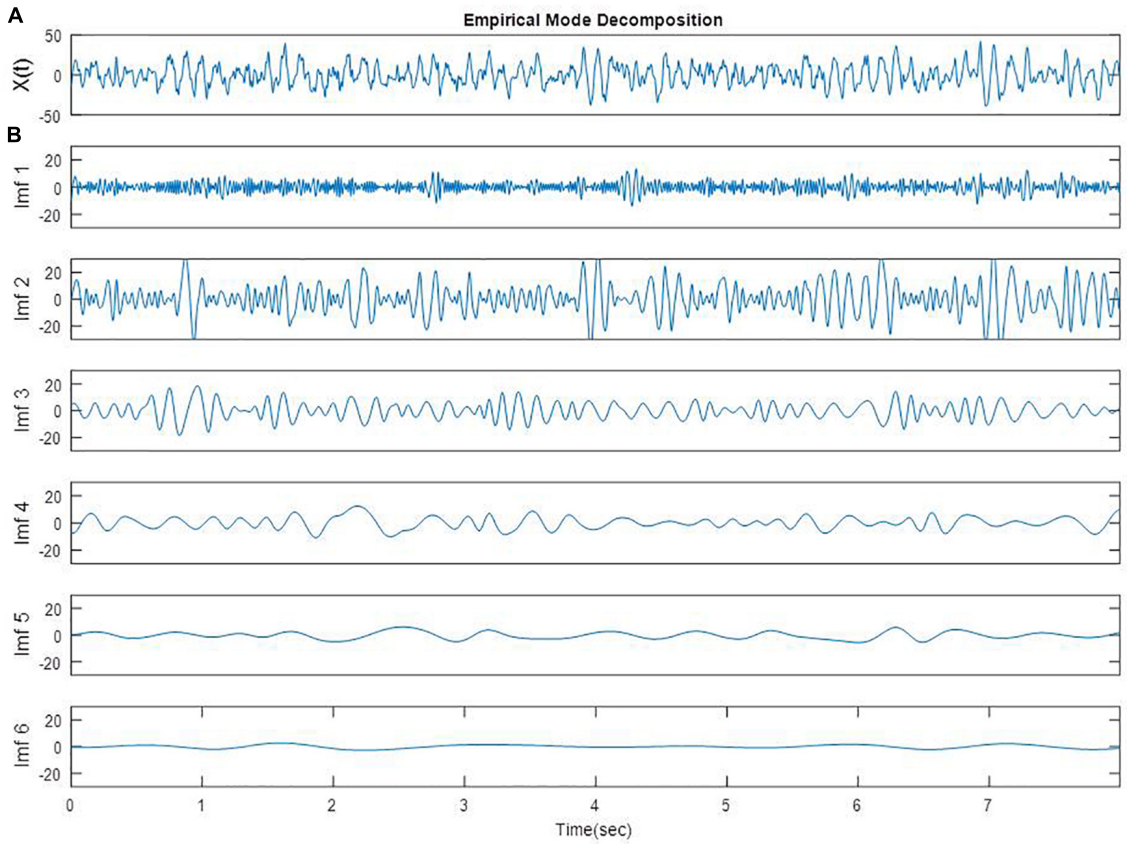

FIGURE 1 | A segment of EEG signal from one participant in FC3 site **(A)** [X(t)] and EMD of the same segment (**B**, Imf 1 to Imf 6).

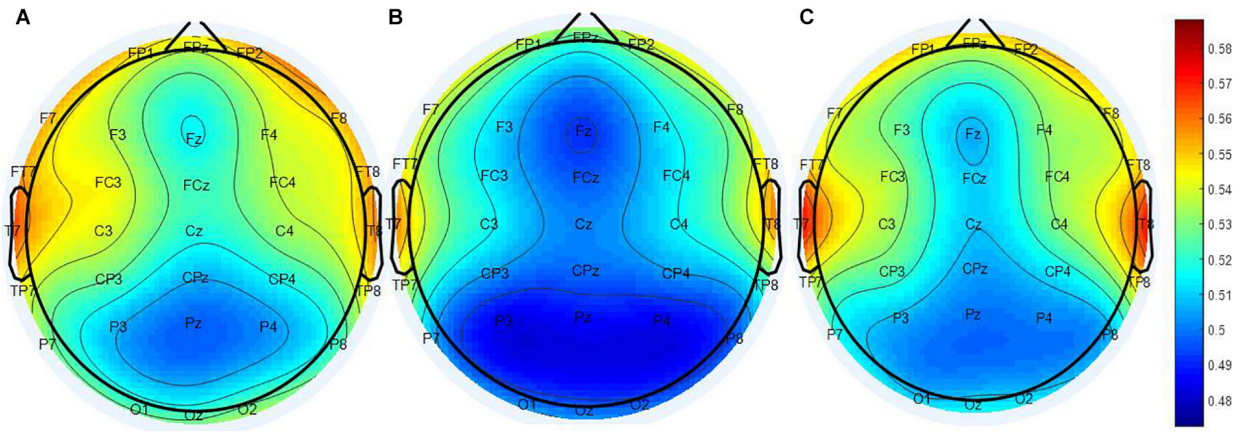

FIGURE 2 | Scalp topographical maps of PEIMF2 index (resting state, eyes closed). From left to right: topographies of the **(A)** RP, **(B)** the NR, and **(C)** HC. PEIMF2 index in RP and HC groups is higher than NR group especially at left frontal electrodes.

showed that there were significant differences in the PEIMF2 index between the two groups in FC4 ($p = 0.009$), FCz ($p = 0.001$), F3 ($p = 0.002$), F4 ($p = 0.003$), Fz ($p = 0.003$), CP3 ($p = 0.0049$), P3 ($p = 0.005$) and FC3 ($p < 0.001$) electrodes. Also, as shown in **Figure 3**, the largest differences between RP and NR were observed at FC3 [RP = 0.543 (0.033), NR = 0.521 (0.028), HC = 0.533 (0.027) (mean (standard deviation))], FCz (RP = 0.511 (0.021), NR = 0.496 (0.029), HC = 0.508 (0.024)), and F3 (RP = 0.534 (0.037), NR = 0.501 (0.022), HC = 0.525 (0.028)) electrode sites. These results suggest that the PEIMF2 index may be able to differentiate between RP and NR groups, particularly at the frontal regions. There were no significant

differences between RP and HC participants at these three electrode sites (p-value > 0.01), but there was a marginally significant difference between NR and HC (p-value $<< 0.01$), where aHC exhibited a higher PEIMF2 index as compared to NR. Results were unchanged when taking in consideration treatment group as a covariate (i.e., HFL vs. iTBS stimulation).

In this study, ROC curve analyses were used to explore the optimum component of EMD to undergo PE calculation in order to best differentiate between RP and NR patients. PE was computed for IMF1 to IMF4 to extract indices called PEIMF1 to PEIMF4 respectively, for FC3 electrode and multiple surrounding electrodes since this area best differentiated RP and NR. The AUC value for PEIMF2 index is 0.8, compared with 0.71 for PEIMF1, 0.76 for PEIMF3, and 0.74 for PEIMF4 in FC3 electrode and similar result gained from other electrodes. The AUC value of the ROC analysis classifying RP and NR was the greatest for PEIMF2 during resting state EEG, suggesting that PE calculated on the second IMF yields the best results for prediction of treatment response with moderate accuracy.

Response Prediction Based on Frequency Band Measures

Some conventional frequency band measures such as Delta, Theta, Alpha, Beta and Gamma relative powers were extracted from EEG signal of all electrodes. The efficiency of these indices was evaluated via AUC values on the best electrode for the classification of RP or NR. The AUC values of Delta at Oz, Theta at O1, Alpha at Oz, Beta at CPz and Gamma at O2 are 0.67, 0.64, 0.63, 0.60, and 0.68 respectively (**Figure 4**). The result show that there was a considerable difference between predictive value of PEIMF2 index (AUC = 0.8) and traditional frequency band measures on best electrode site.

DISCUSSION

The current study evaluated predictors of treatment response to rTMS administered to the left DLPFC in patients with TRD based on rsEEG signal. Our data shows that a non-linear entropy index, PEIMF2, yields superior outcome prediction performance compared to traditional frequency band indices. Our data indicate that TRD patients who responded to rTMS had higher entropy compared to NR, with the most prominent differences appearing in prefrontal areas (FCz, F3, and FC3 electrodes). Our findings extend previous the investigation of EEG-based biomarkers in depression, and position entropy indices applied in band-limited EEG components extracted with EMD method as a potential predictor for clinical use.

Entropy is becoming a valuable tool for the analysis of EEG activity and has received much attention in recent years in the study of brain disorders (Mizuno et al., 2010). Our data consistently show that NR patients have significantly lower entropy values in the prefrontal areas, and particularly the DLPFC region, compared to RP and HC subjects (**Figure 3**). Entropy indicates the complexity in a system, (Erguzel et al., 2015) and is also associated with the amount of "information" the signal carries. In the nervous system, higher levels of entropy have been consistently associated with healthy states where the nervous system is able to respond and adapt to dynamic changes. Conversely, lower entropy values (more regular, less information) are associated with pathological states and loss of the prime ability of the nervous system to respond to changes (Kevric and Subasi, 2017). A plausible hypothesis to explain the absence of difference between RP and HC would be that RP still have a system that is capable of such changes and this would make these patients amenable to respond to rTMS. Lower entropy levels in NR participants may indicate a reduction in typical intra-cortical information flow, a more regular, less complex EEG in the left frontal region and fewer chances for the system to change in response to rTMS. Therefore, lower levels of entropy (i.e., more regularity) may reflect a less preserved brain function that is not amenable to the effect of rTMS. This raises the question as to whether these patients would be amenable to a different type of stimulation (e.g., inhibitory rTMS), a different anatomical target (e.g., dorsomedial prefrontal cortex), or a higher dose of excitatory rTMS (e.g., more pulses per session or per day, accelerated protocols). Furthermore, our findings are convergent with those recently reported by Jaworska et al. (2018) who found that increased diffuse multi-scale entropy was predictive of treatment response to antidepressants and Farzan et al. (2017b) who showed that non-linear complexity measures was superior to power in explaining the therapeutic efficacy of seizure therapy.

The electrode sites that significantly distinguish RP from NR are located in left frontal (F3 and FC3), right frontal (F4, FC4), left parietal (CP3, P3) and central (FCz, Fz) sites (**Table 2**). A plausible explanation of our results is that the antidepressant effects of rTMS to the DLPFC are not restricted only to the local effect on the L-DLPFC, but rather that target circuits that underlie complex brain functions (e.g., affect regulation) including the frontal gyrus, anterior cingulate cortex, amygdala, and insula (Sheline et al., 2010; Smart et al., 2015). The current result converge with previous findings of brain areas related to MDD (Silverstein et al., 2015; Milev et al., 2016; Ge et al., 2017) and further extends it by adding new evidence that an entropy index indicating the complexity in a network could potentially serve as a predictor of clinical response to rTMS.

There are conflicting reports regarding the predictive capacity of frequency band metrics in the EEG such as alpha and theta activity. Some studies showed there was no correlation in alpha activity and treatment response (Price et al., 2008; Widge et al., 2013) whereas another showed that there was a negative correlation between the two (Micoulaud-Franchi et al., 2012). Moreover, one group reported that increased slow theta activity in the subgenual zone of the anterior cingulate cortex was correlated with positive response to rTMS (Hallett, 2007) while another group reported that theta rhythm increase in the frontal cortex is associated with non-response to rTMS treatment (Silverstein et al., 2015). Our own data would be convergent with the idea of a moderate predictive ability of frequency band (linear) metrics (**Figure 4**). One possible reason for the inferior predictive ability of linear metrics may be related to the non-linear nature of neural processes, as threshold and saturation phenomena control the dynamical behavior of individual neurons (Narushima et al., 2010). In contrast, our

TABLE 2 | Results of the ANOVA investigating differences in PEIMF2 index between RP and NR groups at all electrode sites ($\alpha = 0.01$).

Electrode site	*P-value*	Electrode site	*P-value*	Electrode site	*P-value*
FPz	0.107	FT7	0.108	TP8	0.024
FP2	0.123	FT8	0.481	Pz	0.275
FP1	0.120	Cz	0.022	**P3**	0.005
Fz	0.003	C3	0.01	P4	0.040
F3	0.002	C4	0.038	P7	0.014
F4	0.003	T7	0.524	P8	0.014
F7	0.053	T8	0.889	Oz	0.043
F8	0.054	CPz	0.251	O1	0.076
FCz	0.001	**CP3**	0.004	O2	0.012
FC3*	<0.001	CP4	0.084		
FC4	0.009	TP7	0.566		

FC3 asterisk denotes the most significant site for differentiating between RP and NR. Bold terms denote sites that significantly differentiate RP from NR.

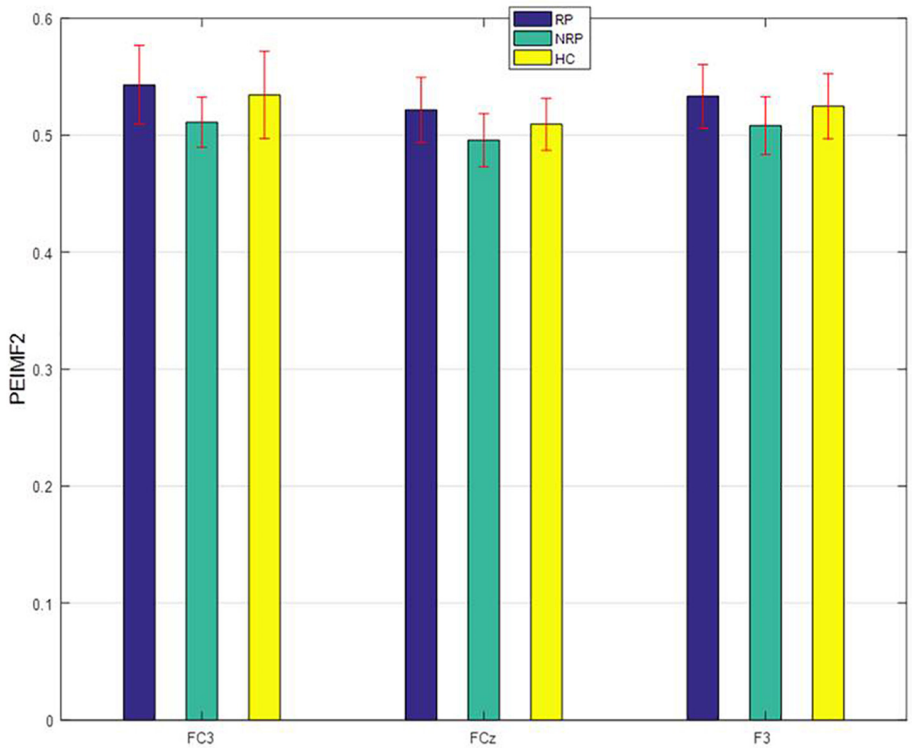

FIGURE 3 | PEIMF2 index as a function of electrode sites for RP and NR with comparison to HC participant groups. Error bars represent ± 1 standard error. RP differ than NR to rTMS treatment especially at FC3.

data supports the utility of non-linear metrics in predicting treatment outcome. Our measure quantified higher order non-linear complexity, which is not obtained using traditional EEG spectral-band analyses such as alpha or theta band power.

PEIMF2 measure is based on non-linear dynamics and has been found to indirectly index neuroplasticity (Hayley et al., 2005). PEIMF2 may represent the excitatory and inhibitory balance of the related networks in MDD which would also be associated with neuroplasticity. The scalp topography for the PEIMF2 values show that the most prominent differences between RP and NR groups are observed in the left frontal electrodes (**Figure 3**), which is consistent with previous findings of brain areas related to MDD. Considering this information, we would speculate that a plausible mechanism mediating the response to rTMS may be neuroplastic changes on relevant circuits involved in affect regulation and other symptomatic domains.

EMD adaptively and locally decomposes non-stationary EEG signals into a sum of IMFs that represent amplitude- and frequency- modulated components specific to the energy levels of

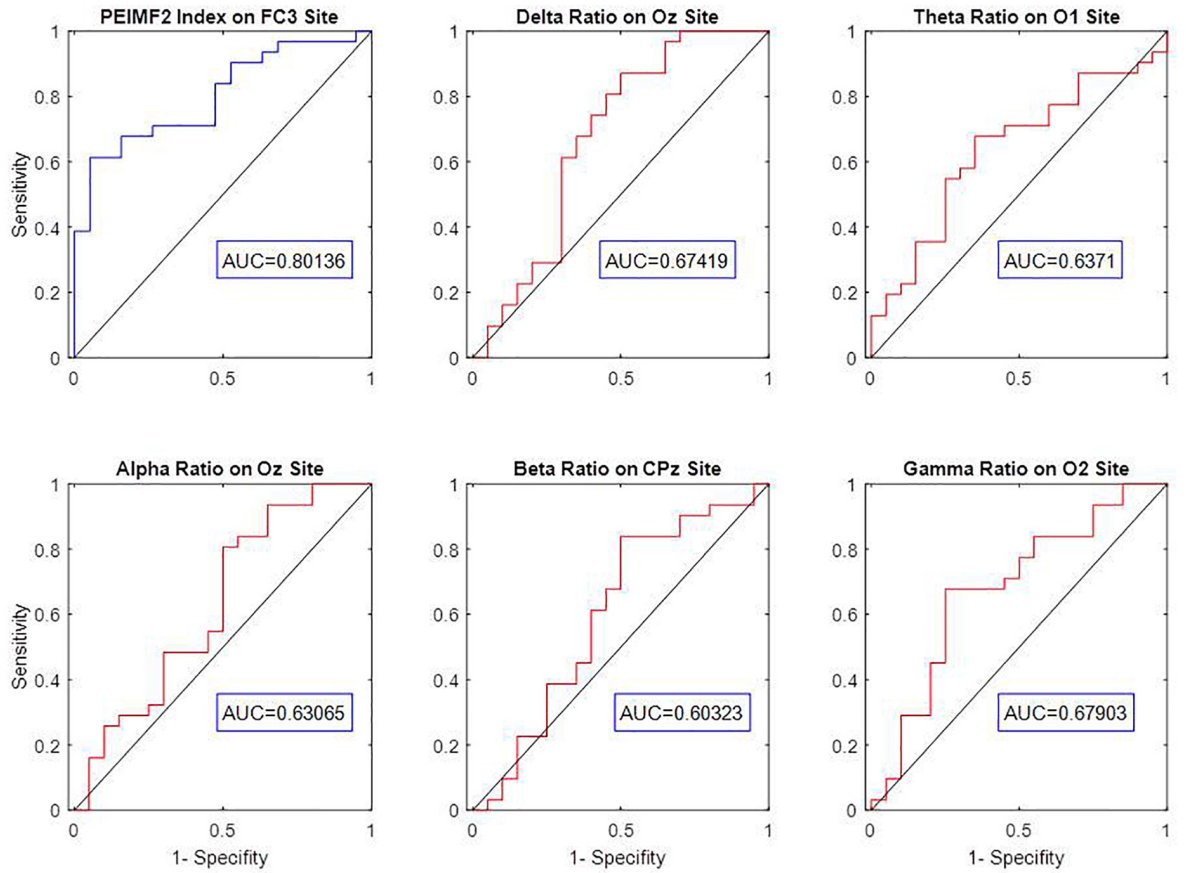

FIGURE 4 | ROC curve analysis and AUC value of Delta, Theta, Alpha, Beta, Gamma relative powers to discriminate between RP an NR on best electrodes. The low AUC value of frequency band measures indicates weak prediction accuracy of these linear approaches.

individual patients (Shalbaf et al., 2012). EMD has shown better properties over other methods of EEG decomposition such as the short-time Fourier transform (STFT), independent component analysis (ICA) (Makeig et al., 1996) and wavelet transform (WT) (Zikov et al., 2006). For instance, the STFT excludes EEG features with a short duration or narrow frequency band. ICA is hampered by the intrinsically non-stationary nature and the non-linear couplings involved in neural signal generation. Moreover, WT forces the decomposition of the signal into a pre-defined set of basic functions, therefore temporal patterns of EEG signals cannot be obtained precisely. Conversely, EMD is a decomposition technique that is completely data-driven and thus utilizes empirical knowledge of oscillations intrinsic to the given time series (Baskaran et al., 2012). Furthermore, unlike wavelet analysis, EMD does not depend on a fixed set of basic functions; instead it searches for IMF embedded within the data. Therefore, these pitfalls of the other methods, or the strength of the data-drive EMD method may make it a better biomarker of treatment response.

Some limitations of our work should be considered in order to better interpret our results. First, the decomposition procedure of EMD requires the arbitrary choice of the stopping criteria for the sifting and the spline-fitting scheme (Sweeney-Reed and Nasuto, 2007). The former could lead to uniform or deviated IMFs, and the latter may result in problems overshooting or distorting the beginning and ending of signals. Second, we believe that a multi-modal approach of response prediction that holistically integrates a variety of sources of data including clinical, neuroimaging, and neurophysiological measures may be most reliable because many clinical factors may affect the central nervous system including baseline physiological and neurological differences, thus decreasing the predictive power of related EEG measures. Finally, we acknowledge this work provides a preliminary proof-of-principle evidence and the real consistency will only be determined with future larger trials or replication of the two methods of analysis.

To conclude, this study addresses a new method to decompose the neuronal oscillations with EMD to obtain a series of IMFs. The results show that the permutation entropy measure applied to the second IMF yields the optimal result in predicting treatment response. This seems to indicate that second IMF oscillation plays an important role in discriminating between RP and NR in the context of rTMS treatment. The method described herein certainly merits further research in larger samples to replicate and improve its predictive power. Prospective studies applying our predictor to decide treatment interventions may be

used in the future, perhaps facilitating the precise prescription of rTMS as a first-line treatment when several favorable neurophysiological predictive factors are present, thus avoiding unsuccessful pharmacotherapy trials and expediting recovery.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations and approvals of UBC Clinical Research Ethics Board as well as the Vancouver Coastal Health Research Institute with written informed consent from all subjects in accordance with the Declaration of Helsinki. Trials were registered in ClinicalTrials.Gov, identifier NCT02800226 and NCT01887782.

AUTHOR CONTRIBUTIONS

RS, CB, and FV-R conceived and designed the study. DB, ZD, and RL provided input on the study design. RS, CB, and FV-R developed the plan for statistical analyses. RS analyzed the data. All authors contributed to the interpretation of data. RS and FV-R, drafted the manuscript. All authors made revisions to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01188/full#supplementary-material

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Liraglutide Activates mTORC1 Signaling and AMPA Receptors in Rat Hippocampal Neurons Under Toxic Conditions

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The aim of the present study was to determine whether treatment with liraglutide, a glucagon-like peptide 1 (GLP-1) receptor agonist, would alter mammalian target of rapamycin complex 1 (mTORC1) signaling and/or α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor activity under dexamethasone-induced toxic conditions. Western blot analyses were performed to assess changes in mTORC1-mediated proteins, brain-derived neurotrophic factor (BDNF), and various synaptic proteins (PSD-95, synapsin I, and GluA1) in rat hippocampal cultures under toxic conditions induced by dexamethasone, which causes hippocampal cell death. Hippocampal dendritic outgrowth and spine formation were measured using immunostaining procedures. Dexamethasone significantly decreased the phosphorylation levels of mTORC1 as well as its downstream proteins. However, treatment with liraglutide prevented these reductions and significantly increased BDNF expression. The increase in BDNF expression was completely blocked by rapamycin and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX). Liraglutide also recovered dexamethasone-induced decreases in the total length of hippocampal dendrites and reductions in spine density in a concentration-dependent manner. However, the positive effects of liraglutide on neural plasticity were abolished by the blockade of mTORC1 signaling and AMPA receptors. Furthermore, liraglutide significantly increased the expression levels of PSD-95, synapsin I, and GluA1, whereas rapamycin and NBQX blocked these effects. The present study demonstrated that liraglutide activated mTORC1 signaling and AMPA receptor activity as well as increased dendritic outgrowth, spine density, and synaptic proteins under toxic conditions in rat primary hippocampal neurons. These findings suggest that GLP-1 receptor (GLP-1R) activation by liraglutide may affect neuroplasticity through mTORC1 and AMPA receptors.

Keywords: mammalian target of rapamycin complex 1 signaling, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor, glucagon-like peptide 1 receptor, liraglutide, neuroplasticity, toxic condition

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INTRODUCTION

Depressive disorder is a very common psychiatric illness that typically has a chronic course (Andrade et al., 2003). The lifetime prevalence of depressive disorder is approximately 17% in the United States (Andrade et al., 2003; Kessler et al., 2003). In addition to experiencing a depressed mood, patients with depressive disorder may exhibit compromised social functioning, decreased quality of life, and an elevated risk of medical illnesses (Brown et al., 2009; Dhar and Barton, 2016; Kupferberg et al., 2016). Thus, the burden of illness associated with depressive disorder necessitates genuinely novel treatment approaches (Millan et al., 2015). More than 50 years after the monoamine hypothesis of depression has been proposed, several monoamine-based antidepressants have been developed. It is now widely used as a pharmacological treatment for depression (Hillhouse and Porter, 2015). But, many difficulties are still associated with pharmacotherapies for depressive disorder (Lee et al., 2010; Popa-Velea et al., 2015). Thus, there is a need for innovative treatment strategies that utilize newly identified molecular mechanisms.

Novel agents that target molecular effector systems relevant to neuroplasticity and neurogenesis represent a promising research opportunity for psychiatry (Massart et al., 2012; Vialou et al., 2013; Pilar-Cuellar et al., 2014). In terms of neuroplastic mechanisms, much attention has been focused on plastic changes in neural connectivity that operate via the mammalian target of rapamycin complex 1 (mTORC1) pathway (Li et al., 2010; Duman et al., 2012). For example, Li et al. (2010) observed that the *N*-methyl-D-aspartate (NMDA) receptor antagonist ketamine decreases immobility time in the forced swimming test (FST), activates mTORC1 signaling, and increases synaptic protein levels (PSD-95, synapsin I, and GluA1) in the prefrontal cortex of mice. Furthermore, Maeng et al. (2008) demonstrated that the activation of mTORC1 signaling by ketamine and the associated antidepressant behavioral responses require the activation of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors. The activations of AMPA and neurotrophic receptors play important roles in the synthesis of synaptic proteins that support synaptic plasticity (Ignacio et al., 2016).

Glucagon-like peptide 1 (GLP-1) is an endogenous incretin hormone composed of 30 amino acids that exerts differential effects based on its synthesis site and release (Holst et al., 2011). When GLP-1 is released in response to fat and carbohydrates by small intestinal L-cells, the activation of the GLP-1 receptor (GLP-1R) stimulates glucose-dependent insulin secretion, the synthesis of insulin, β-cell proliferation, and the inhibition of β-cell apoptosis (Drucker, 2001; Zhou et al., 2015). In contrast, when GLP-1 is released in the brain, it functions as a neuropeptide (Holst et al., 2011). GLP-1Rs are widely expressed throughout the brain in rodents and humans in areas such as the caudate putamen, cerebral cortex, and hippocampus (Perry et al., 2002; Perry and Greig, 2004; Li M. et al., 2015). Accumulating evidence indicates that GLP-1 acts as a neuronal growth factor and it has been shown that GLP-1 enhances neurite outgrowth and protects against oxidative injury in PC12 and SK-N-SH human neuroblastoma cells (Li M. et al., 2015).

Liraglutide, which is a GLP-1R agonist, is approved by the U.S. Food and Drug Administration as an adjunct to diet and exercise and is aimed at improving weight loss and glycemic control in adults with type 2 diabetes (Gross, 2013). However, in addition to its anti-diabetic treatment effects, recent studies have shown that liraglutide may have neuroprotective effects and can modulate neuroplasticity as well as cognitive function. Liraglutide exerts neurotrophin-like activity at least in part via the MEK-ERK signaling pathway (Li M. et al., 2015) and its neuroprotective effects appear to operate via reductions in excessive levels of reactive oxygen species (ROS; Zhu et al., 2016). Liraglutide also decreases the hyperphosphorylation of tau and neurofilament proteins and improves learning and memory ability in mice (Xiong et al., 2013). Although several studies have suggested possible molecular mechanisms underlying the effects of liraglutide (O'Neill, 2013), no studies have investigated the neuroplastic effects of liraglutide and its association with mTORC1 signaling or AMPA receptors. Thus, the present study aimed to determine whether there is an association of the neuroplastic effects of liraglutide with the activations of mTORC1 signaling and AMPA receptors. For this study, we used a synthetic glucocorticoid dexamethasone as neurotoxic model for screening the neuroplastic effects of liraglutide. Excessive glucocorticoid levels in depression lead to hippocampal atrophy and disturbances of neuroplasticity (McEwen et al., 1992). Dexamethasone in neuronal cells was reported to reduce cell viability, cell proliferation, and neurite outgrowth (Obradovic et al., 2006; Leskiewicz et al., 2013). Thus, the effects of liraglutide on mTORC1 signaling, AMPA receptor activity, synaptic protein expression, neurite outgrowth, and synaptic density were investigated using rat primary hippocampal neurons under dexamethasone-induced toxic conditions.

MATERIALS AND METHODS

Drugs and Reagents

Liraglutide and dexamethasone were purchased from GL Biochem Ltd. (Shanghai, China) and Sigma (St. Louis, MO, United States), respectively. The reagents used for the primary hippocampal cultures were purchased from Invitrogen (Carlsbad, CA, United States) and included dimethyl sulfoxide (DMSO), neurobasal medium, fetal bovine serum (FBS), horse serum (HS), B27 supplement, L-glutamine, penicillin-streptomycin, and trypsin. The antibodies used for the Western blot analyses were purchased from the following sources: anti-BDNF (sc-546), anti-synapsin I (sc-8295), anti-rabbit (sc-2004), and anti-goat (sc-2020) IgG-horseradish peroxide-conjugates from Santa Cruz Biotechnology (Santa Cruz, CA, United States); monoclonal anti-α-tubulin (T9026) and anti-mouse IgG peroxidase conjugates (A4416) from Sigma; anti-phospho-mTORC1 (Ser2448, #2971), anti-mTORC1 (#2972), anti-phospho-4E-BP-1 (Thr37/46, #2855), anti-4E-BP-1 (#9452), anti-phospho-p70S6K (Thr389, #9205), and anti-p70S6K (#9202) from Cell Signaling Technology (Beverly, MA, United States); anti-PSD-95 (AB9634) from Millipore (Temecula, CA, United States); and anti-GluA1 (ab109450) from Abcam (Cambridge, United Kingdom).

The antibodies used for immunostaining were purchased from the following sources: anti-microtubule-associated protein 2 (MAP2; MAB3418) from Millipore; Alexa Fluor® 568 goat anti-mouse IgG (A11031) and Hoechst 33258 (H21491) from Invitrogen; and Alexa Fluor® 488 Phalloidin from Molecular Probes (Eugene, OR, United States). Specific kinase inhibitors were purchased from the following sources: PI3K inhibitor LY294002 from Cell Signaling Technology; MEK inhibitor PD98059 and mTORC1 inhibitor rapamycin from Calbiochem (San Diego, CA, United States); and the AMPA receptor inhibitor 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f] quinoxaline-7-sulfonamide (NBQX) from Tocris Bioscience (Ballwin, MO, United States).

Primary Hippocampal Cell Cultures

Pregnant Sprague-Dawley rats were obtained from Orient Bio (Seongnam, South Korea) and all experimental procedures were approved by the Inje Medical College Committee for Animal Experimentation and the Institutional Animal Laboratory Review Board (Approval no. 2015-027).

Hippocampal cell cultures were prepared from the hippocampi of fetuses at embryonic day 17 in a manner similar to that reported by Kaech and Banker (2006). Briefly, the hippocampi were carefully removed and dispersed in neurobasal medium containing 0.03% trypsin for digestion at 37°C (5% CO2) for 20 min. Next, the cells were suspended in a medium including 1% FBS, 1% HS, 2% serum-free growth medium B27, 0.25% L-glutamine, and 50 U/ml penicillin-streptomycin; this was the control condition. For the Western blot analyses, the cells were plated on 6-well dishes coated with poly-L-lysine at a density of 2×10^5 cells per dish. For the neurite and spine assays, the cells were plated on 12-well dishes at a density of 2×10^4 or 5×10^3 cells per dish. They were grown under the control condition for either 7 days (for the neurite and spine assays) or 10 days (for Western blots). After incubation for 7 or 10 days, the cells were treated with liraglutide in the presence or absence of 500 μM dexamethasone for either 4 days (for the Western blots) or 5 days (for the neurite and spine density assays) before being harvested for further analysis. In a preliminary experiment performed by our research group, exposure to 500 μM dexamethasone caused a significant reduction in hippocampal cell viability (approximately 22% decrease; **Supplementary Figure 1**).

Drug Treatment

Liraglutide (1 μM) was completely dissolved in distilled water and then diluted to various concentrations also using distilled water prior to use. Dexamethasone (50 mM) was dissolved in DMSO (Amresco, OH, United States) and then diluted to a final concentration of 500 μM using 1% DMSO prior to use. For the Western blot analyses, the cells were cultured for 4 days with liraglutide (10 or 100 nM) and, for the neurite and spine density assays, the cells were cultured for 5 days with liraglutide (100 nM) in the presence or absence of dexamethasone. The range of liraglutide concentration used in the present study was based on studies showing that liraglutide exerts neuroprotective effects in various cell cultures (Miao et al., 2013; Sharma et al., 2014; Li Y. et al., 2015).

Western Blot Analysis

The Western blot experiments were performed as previously described (Seo et al., 2014). Briefly, the hippocampal cells were homogenized in ice-cold lysis buffer (20 mM Tris–HCl, 137 mM NaCl, 10% glycerol, 1% Nonidet^{TM} P-40, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, and 2 mM ethylenediaminetetraacetic acid [EDTA]) and one complete protease inhibitor tablet (Roche; Laval, QC, Canada). The lysates were centrifuged (1000 × g, 15 min, 4°C) and the samples were stored at −80°C until use. Immunoblotting was performed with one of the primary antibodies (anti-phospho-mTORC1, anti-mTORC1, anti-phospho-4E-BP-1, anti-4E-BP-1, anti-phospho-p70S6K, anti-p70S6K, anti-BDNF, anti-PSD-95, anti-synapsin I, or anti-GluA1 [1:1000] and anti-α-tubulin [1:2000]) in Tris-buffered saline with Tween-20 (TBS-T) at 4°C overnight and then the membranes were washed three times in TBS-T for 10 min. The membranes were then incubated for 1 hr in TBS-T containing a horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit IgG for anti-phospho-mTORC1, anti-mTORC1, anti-phospho-4E-BP-1, anti-4E-BP-1, anti-phospho-p70S6K, anti-BDNF, anti-p70S6K, anti-phospho-eIF4B, anti-eIF4B, anti-phospho-S6, anti-S6, anti-BDNF, anti-PSD-95, or anti-GluA1 [1:2000]; donkey-anti-goat IgG for anti-synapsin I [1:2000]; or anti-mouse IgG for anti-α-tubulin [1:10,000]) at room temperature. The Western blot analyses were repeated two times per group for each of the two independent cultures.

Neurite Assay

Dendrites were visualized via immunostaining using a MAP-2 antibody, which is a dendritic marker, as previously described (Park et al., 2016). To analyze total dendritic length, five fields were randomly selected from each group and two independent cultures were performed. All neurons in a given field were counted, including both basal and apical dendrites, and dendritic length was determined to be the distance between the edge of the cell body and the tip of the growth cone. Total dendritic length was obtained by summing the lengths of all dendrites from a single neuron and then averaging this measure in each group using MetaMorph (Molecular Devices, Downingtown, PA, United States), an automated image-analysis program (Klimaschewski et al., 2002). At least 400 cells were analyzed in 10 fields by a researcher blind to the groups.

Spine Density Assay

Spines were stained with phalloidin as previously described (Park et al., 2016). To analyze spine density, spines, and filopodia were differentiated by shape and length such that spines were defined as less than 3 μm long with a rounder or mushroom shape while filopodia were defined as between 3 and 10 μm long with a narrower shape. Ten neurons were randomly selected from each group and two independent cultures were performed. Using 12–20 neurons per group, two dendritic segments per neuron

(50 μm) were analyzed (23–40 dendritic segments per group) by a researcher blind to the groups. To represent average spine density in a 10-μm dendrite, the spine density of a 50-μm dendritic segment was divided by 5.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software (ver. 7.03; GraphPad Software, La Jolla, CA, United States). Changes in the levels of mTORC1-mediated proteins were analyzed by one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple comparisons. Two-way ANOVA was used to assess the main effect of liraglutide or the inhibitor and the interaction between liraglutide and the inhibitor; when warranted, Tukey's multiple comparisons were carried out. *P*-values <0.05 were considered to indicate statistical significance.

RESULTS

Effects of Liraglutide on mTORC1, 4E-BP-1, and p70SK Levels in Rat Hippocampal Cells Under the Dexamethasone-Induced Toxic Condition

Dexamethasone treatment significantly reduced the phosphorylation levels of mTORC1, 4E-BP-1, and p70S6K (phospho-Ser^{2448}-mTORC1 levels: 40% of control, $p < 0.001$; phospho-$Thr^{37/46}$-4E-BP-1 levels: 30% of control, $p < 0.001$; phospho-Thr^{389}-p70S6K levels: 28% of control, $p < 0.001$, **Figures 1A–C**). In the dexamethasone condition, liraglutide (100 nM) significantly prevented reductions in the phosphorylation levels of mTORC1, 4E-BP-1, and p70S6K (phospho-Ser^{2448}-mTORC1 levels: ANOVA, $F_{[3,12]} = 42.310$, $p < 0.001$; *post hoc*, $p = 0.001$, **Figure 1A**; phospho-$Thr^{37/46}$-4E-BP-1 levels: ANOVA, $F_{[3,12]} = 49.800$, $p < 0.001$; *post hoc*, $p = 0.001$, **Figure 1B**; phospho-Thr^{389}-p70SK levels: ANOVA, $F_{[3,12]} = 26.630$, $p < 0.001$; *post hoc*, $p < 0.001$, **Figure 1C**). In addition, liraglutide prevented the dexamethasone-induced decrease in the phosphorylation levels of eIF4B and S6 (**Supplementary Figures 2A,B**).

Activations of mTORC1 Signaling and AMPA Receptors Were Necessary for the Effects of Liraglutide on BDNF Expression in Rat Hippocampal Cells Under Dexamethasone-Induced Toxic Conditions

The present study further examined whether the activations of mTORC1 signaling and AMPA receptors would contribute to the regulation of BDNF expression induced by liraglutide following the administration of dexamethasone. Two-way ANOVA revealed significant effects of liraglutide ($F_{[1,12]} = 10.130$, $p = 0.008$), rapamycin ($F_{[1,12]} = 11.080$, $p = 0.006$), and the liraglutide × rapamycin interaction ($F_{[1,12]} = 8.279$, $p = 0.010$) as well as liraglutide ($F_{[1,12]} = 13.100$, $p = 0.004$), NBQX ($F_{[1,12]} = 7.774$, $p = 0.020$), and the liraglutide × NBQX interaction ($F_{[1,12]} = 10.140$, $p = 0.008$) for BDNF expression. *Post hoc* analyses revealed that liraglutide significantly increased BDNF expression ($p = 0.001$) and that this increase was blocked by rapamycin ($p = 0.002$) and NBQX ($p < 0.010$; **Figure 2**).

Effects of Liraglutide on Dendritic Outgrowth and Spine Formation in Rat Hippocampal Cells Under Dexamethasone-Induced Toxic Conditions

Hippocampal cells were incubated with liraglutide (10 or 100 nM) under either control or dexamethasone (500 μM) condition for 5 days. They were then photographed and assessed to quantify total dendritic length (**Figure 3A**) and spine density (**Figure 3B**) following treatment with liraglutide. Liraglutide did not affect total dendritic length or spine density in hippocampal cells under the control condition (**Supplementary Table 1**). In the absence of dexamethasone, control cells exhibited modest dendritic differentiation and an average dendritic length of approximately 50 μm (**Figure 3A**). On the other hand, dexamethasone-treated cells exhibited a decreased total dendritic length compared to control cells (dexamethasone-treated cells vs. control cells: 40 μm vs. 50 μm, $p = 0.020$, **Figure 3A**) but this reduction was prevented by liraglutide in a concentration-dependent manner (ANOVA; $F_{[3,1596]} = 12.860$, $p < 0.001$; 10 nM = 53 μm, $p < 0.001$; 100 nM = 61 μm, $p < 0.001$, **Figure 3A**).

The changes in spine density were similar to those observed for total dendritic length. Spine density was significantly reduced in dexamethasone-treated cells compared to that in control cells (dexamethasone-treated cells vs. control cells: 0.6 vs. 1.2, $p < 0.001$, **Figure 3B**), but liraglutide significantly prevented the reduction in dendritic spine density in a concentration-dependent manner under the dexamethasone condition (ANOVA; $F_{[3,156]} = 17.000$, $p < 0.001$; 10 nM = 0.9, $p = 0.030$; 100 nM = 1.4, $p < 0.001$, **Figure 3B**).

Effects of mTORC1 and AMPA Receptor Inhibitors on Liraglutide-Induced Increases in Dendritic Outgrowth and Spine Formation

To investigate the roles that mTORC1 signaling and AMPA receptors play in the enhancement of liraglutide-induced dendritic outgrowth and spine formation, hippocampal cells were pretreated with rapamycin or NBQX under the dexamethasone condition. Two-way ANOVA revealed significant differences for rapamycin ($F_{[1,1596]} = 23.380$, $p < 0.001$), liraglutide ($F_{[1,1596]} = 183.800$, $p < 0.001$), and the rapamycin × liraglutide interaction ($F_{[1,1596]} = 57.790$, $p < 0.001$) as well as NBQX ($F_{[1,1596]} = 50.610$, $p < 0.001$), liraglutide ($F_{[1,1596]} = 192.300$, $p < 0.001$), and the NBQX × liraglutide interaction ($F_{[1,1596]} = 86.210$, $p < 0.001$) for total dendritic length. More specifically, rapamycin and NBQX inhibited the enhancement of total dendritic length induced by liraglutide

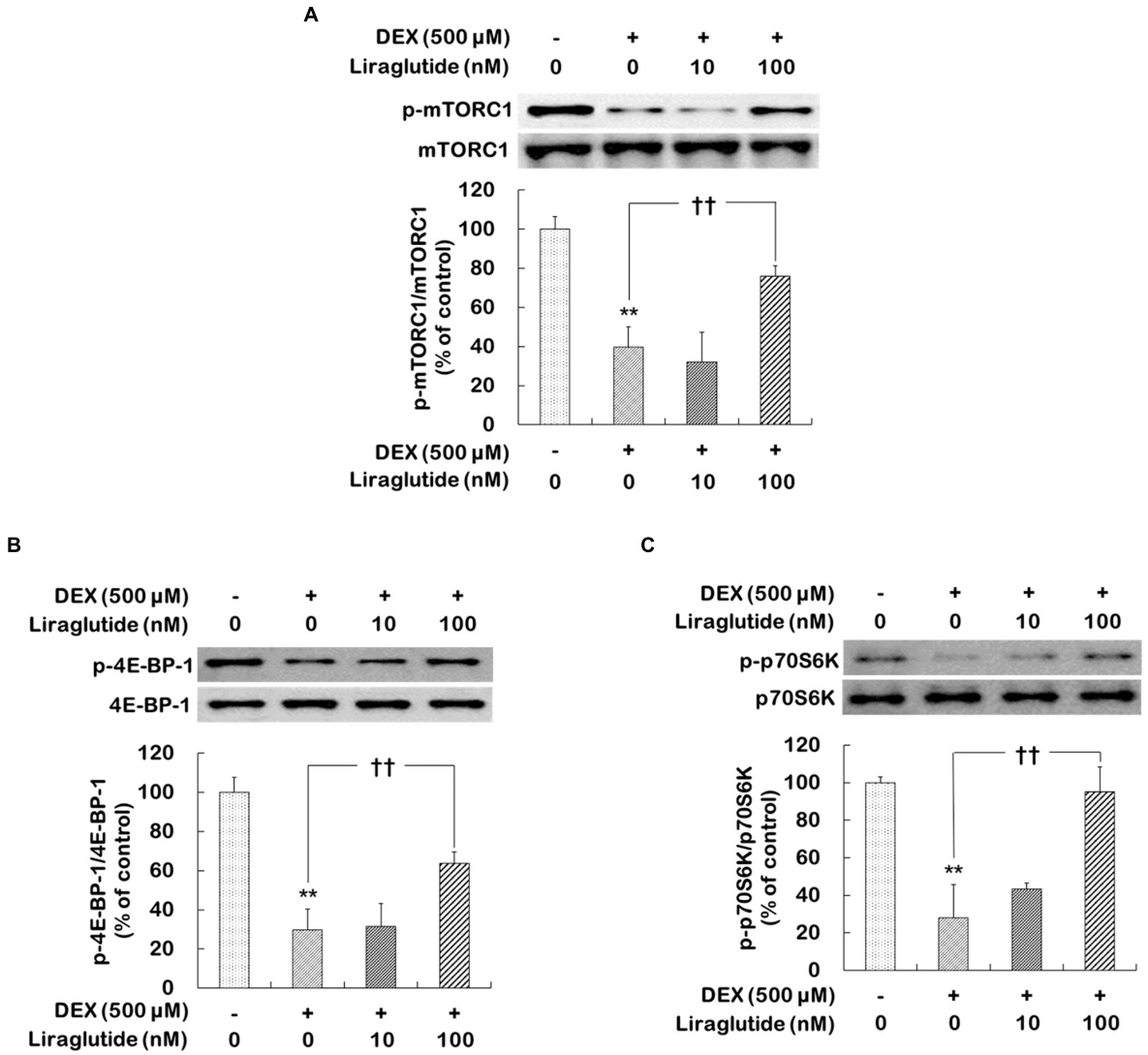

FIGURE 1 | Effects of liraglutide on mTORC1, 4E-BP-1, and p70SK phosphorylation levels in hippocampal cells treated with dexamethasone. Cells were treated with liraglutide (10 or 100 nM) or distilled water (control) for 4 days either with (+; dexamethasone condition) or without (–; control condition) dexamethasone (DEX; 500 μM). In two different wells per group of each of two independent cultures (total 4 wells), cell lysates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses with each of the primary antibodies. The Western blot analyses revealed the levels of phospho-Ser^{2448}-mTORC1 **(A)**, phospho-Thr$^{37/46}$-4E-BP-1 **(B)**, and phospho-Thr^{389}-p70S6K **(C)**. Representative images and quantitative analyses normalized to the total levels for each protein are shown; values (n = 4) are shown as the mean ± standard error of the mean (SEM) expressed as a percentage of the control cell values. $^{**}p < 0.01$ vs. control cells (-DEX, no liraglutide), $^{††}p < 0.01$ vs. dexamethasone-treated cells (+DEX, no liraglutide).

(liraglutide vs. rapamycin + liraglutide: 64 μm vs. 55 μm, $p < 0.001$; liraglutide vs. NBQX + liraglutide: 64 μm vs. 53 μm, $p < 0.001$; **Figure 4A**).

Statistical analyses of spine density revealed significant effects of rapamycin ($F_{[1,101]} = 29.840$, $p < 0.001$), liraglutide ($F_{[1,101]} = 78.260$, $p < 0.001$), and the rapamycin × liraglutide interaction ($F_{[1,101]} = 26.680$, $p < 0.001$) as well as NBQX ($F_{[1,103]} = 33.470$, $p < 0.001$), liraglutide ($F_{[1,103]} = 34.370$, $p < 0.001$), and the NBQX × liraglutide interaction ($F_{[1,103]} = 75.040$, $p < 0.001$). *Post hoc* analyses showed that rapamycin and NBQX inhibited the liraglutide-induced enhancement of spine density (liraglutide vs. rapamycin + liraglutide: 3.0 vs. 1.6, $p < 0.001$; liraglutide vs. NBQX + liraglutide: 3.0 vs. 1.1, $p < 0.001$, **Figure 4B**).

Activations of mTORC1 Signaling and AMPA Receptors Necessary for the Effects of Liraglutide on Synaptic Protein Expression in Rat Hippocampal Cells Under the Dexamethasone-Induced Toxic Condition

Whether liraglutide would similarly enhance the expression of various synaptic proteins, including PSD-95, synapsin I,

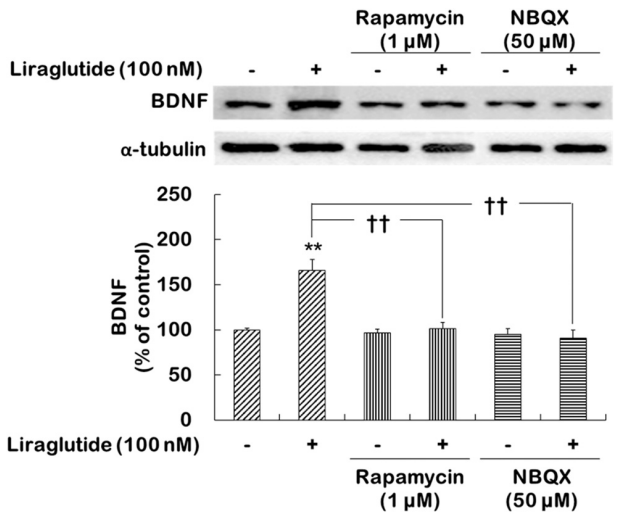

FIGURE 2 | Effects of rapamycin and NBQX on the regulation of liraglutide-induced BDNF expression in hippocampal cells treated with dexamethasone. Cells were exposed to rapamycin (1 μM) or NBQX (50 μM) for 30 min prior to the addition of distilled water (control) or liraglutide (100 nM) for 4 days with dexamethasone (500 μM). In two different wells per group of each of two independent cultures (total 4 wells), cell lysates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses for each of the primary antibodies. The Western blot analyses revealed the levels of BDNF. A representative image and quantitative analysis normalized to the α-tubulin band are shown. Values (n = 4) are mean ± standard error of the mean (SEM) expressed as a percentage of the control cell values. $^{**}p < 0.01$ vs. control cells (no liraglutide and no inhibitors), $^{††}p < 0.01$ vs. liraglutide-only-treated cells.

and GluA1, through the activation of mTORC1 signaling and AMPA receptors was investigated in hippocampal cells under the dexamethasone condition.

Two-way ANOVA revealed significant effects of liraglutide ($F_{[1,12]}$ = 26.750, $p < 0.001$), rapamycin ($F_{[1,12]}$ = 10.370, p = 0.007), and their interaction ($F_{[1,12]}$ = 9.630, p = 0.009) as well as main effects of liraglutide ($F_{[1,12]}$ = 17.330, p = 0.001), NBQX ($F_{[1,12]}$ = 5.381, p = 0.040), and their interaction ($F_{[1,12]}$ = 5.186, p = 0.040) for PSD-95 expression; significant effects of liraglutide ($F_{[1,12]}$ = 16.750, p = 0.008), rapamycin ($F_{[1,12]}$ = 10.290, p = 0.001), and their interaction ($F_{[1,12]}$ = 15.810, p = 0.002) as well as significant effects of liraglutide ($F_{[1,12]}$ = 5.710, p = 0.030), NBQX ($F_{[1,12]}$ = 15.630, p = 0.002), and their interaction ($F_{[1,12]}$ = 5.349, p = 0.040) for synapsin I expression; and significant effects of liraglutide ($F_{[1,12]}$ = 35.800, $p < 0.001$), rapamycin ($F_{[1,12]}$ = 35.800, $p < 0.001$), and their interaction ($F_{[1,12]}$ = 22.310, $p < 0.001$), as well as significant effects of liraglutide ($F_{[1,12]}$ = 26.350, $p < 0.001$), NBQX ($F_{[1,12]}$ = 31.440, $p < 0.001$), and their interaction ($F_{[1,12]}$ = 26.350, $p < 0.001$) for GluA1 expression. Liraglutide significantly increased the expression of PSD-95 (156% of control, p = 0.020; **Figure 5A**), synapsin I (174% of control, p = 0.001; **Figure 5B**), and GluA1 (160% of control, $p < 0.001$; **Figure 5C**). However, the positive effects of liraglutide were blocked by rapamycin (p = 0.002 for PSD-95, **Figure 5A**; $p < 0.001$ for synapsin I, **Figure 5B**; and $p < 0.001$ for GluA1, **Figure 5C**) and NBQX (p = 0.001 for PSD-95, **Figure 5A**; p = 0.020 for synapsin I, **Figure 5B**; and $p < 0.001$ for GluA1, **Figure 5C**).

DISCUSSION

The present study demonstrated that liraglutide influenced mTORC1 signaling and AMPA receptor activities and was associated with changes in the expression of synaptic proteins, neurite outgrowth, and synaptic density in rat primary hippocampal neurons treated with toxic levels of dexamethasone.

Liraglutide is a long-acting human GLP-1 analog that has a 97% amino acid sequence identity with human GLP-1 (Miao et al., 2013). As a result, liraglutide is a potent antidiabetic agent that is protective against pancreatic β-cell apoptosis *in vitro* and increases pancreatic β-cell mass *in vivo* (Rolin et al., 2002; Bregenholt et al., 2005). In addition to these reported effects, liraglutide may also be neuroprotective and alter neuroplasticity in the brain (Holst et al., 2011; Hunter and Holscher, 2012; Xiong et al., 2013; Zhu et al., 2016). In preclinical studies, GLP-1 and its longer-lasting analogs decrease apoptosis, protect neurons from oxidative stress and inflammation, induce neurite outgrowth, protect neural plasticity, and enhance memory formation in the brains of animals in mouse models of Alzheimer's disease, Parkinson's disease, and other neurodegenerative diseases (Holscher, 2010, 2012; Xiong et al., 2013; Liu et al., 2015). In a study of GLP-1R knockout mice, GLP-1R-deficient mice exhibited a phenotype characterized by learning deficits that were restored by hippocampal Glp1r transfer (During et al., 2003). Additionally, GLP-1R affects neurite outgrowth and neuroplasticity. In a separate study, Li M. et al. (2015) found that liraglutide induces neurite outgrowth in C57BL/6 mouse primary cortical neurons and that this effect is blocked by the MEK-ERK inhibitor U0126. Hunter and Holscher (2012) reported that peripherally administered liraglutide and lixisenatide cross the blood–brain barrier in C57BL/6 mice where they enhance cyclic adenosine monophosphate (cAMP) levels and increase neurogenesis. Taken together, these results provide the basis for speculations that liraglutide may have neuroplastic effects.

However, the underlying mechanisms by which GLP-1 and GLP-1 analogs induce neuroplastic effects via mTORC1 signaling remained unclear. Most studies have examined the effects of GLP-1 or GLP-1 analogs on mTORC1 signaling and its protective effects using pancreatic β-cells. Miao et al. (2013) found that liraglutide increases INS-1 cells and activates mTOR as well as its downstream effectors, including 70-kDa ribosomal protein S6 kinase and eIF4E-binding proteins. These authors also observed that liraglutide prevents pancreatic β-cell glucolipotoxicity via mTOR and that the actions of liraglutide are attenuated by AICAR, which is an AMPK activator, and rapamycin, which is an mTOR inhibitor (Miao et al., 2013).

The present study investigated rat primary hippocampal neurons and found that liraglutide promoted mTORC1 signaling activities under dexamethasone-induced toxic conditions. Liraglutide increased the phosphorylation of mTORC1 downstream effectors (p70S6K and 4E-BP-1) as well as the

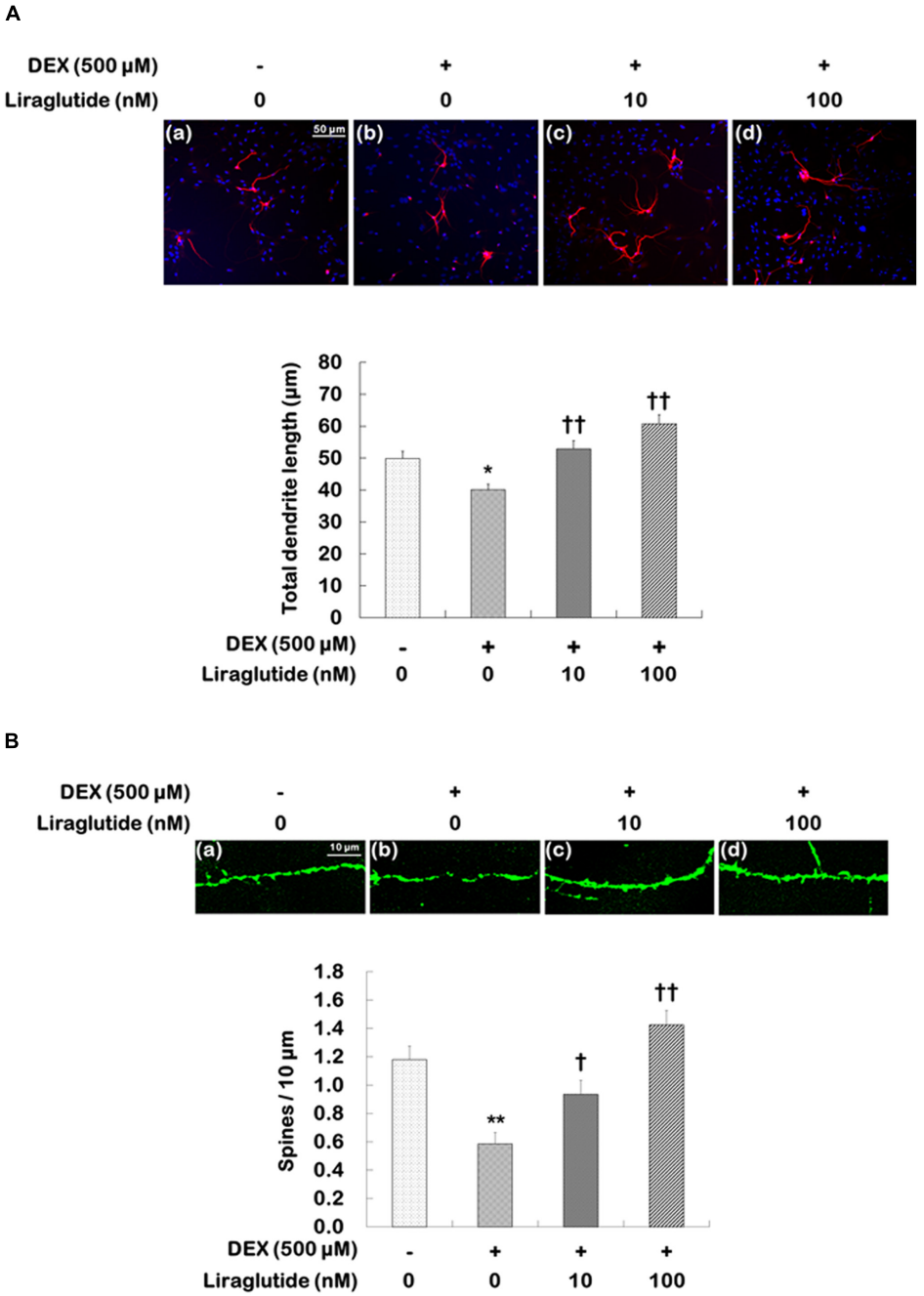

FIGURE 3 | Effects of liraglutide on total dendritic length and spine density in hippocampal cells treated with dexamethasone. Cells were treated with liraglutide (10 or 100 nM) or distilled water (control) for 5 days either with (+; dexamethasone condition) or without (–; control condition) dexamethasone (DEX; 500 μM). Two independent cultures were performed and the cells were photographed and scored according to the methods described above. In total, 400 cells of group were analyzed for total dendritic length **(A)**. In total, 40 dendritic segments per group were analyzed for spine density **(B)**. All data (n = 400 for dendritic length, n = 40 for spine density) are expressed as mean ± standard error of the mean (SEM). $^{*}p < 0.05$ vs. control cells (-DEX, no liraglutide), $^{**}p < 0.01$ vs. control cells, $^{\dagger}p < 0.05$ vs. dexamethasone-treated cells (+DEX, no liraglutide), $^{\dagger\dagger}p < 0.01$ vs. dexamethasone-treated cells.

levels of BDNF and various synaptic proteins (PSD-95, Synapsin I, and GluA1) and enhanced synaptic density and neurite outgrowth. In addition to its effects on mTORC1 signaling activation, recent studies have shown that ketamine rapidly increases glutamate release and stimulates AMPA receptors, mTORC1 signaling, and synaptogenesis through the increased release of BDNF and enhanced AKT activation (Duman et al., 2012). Subsequently, released BDNF stimulates the syntheses of synaptic proteins, including PSD-95, synapsin I, and GluA1, in the prefrontal cortex of mice and contributes to the rapid

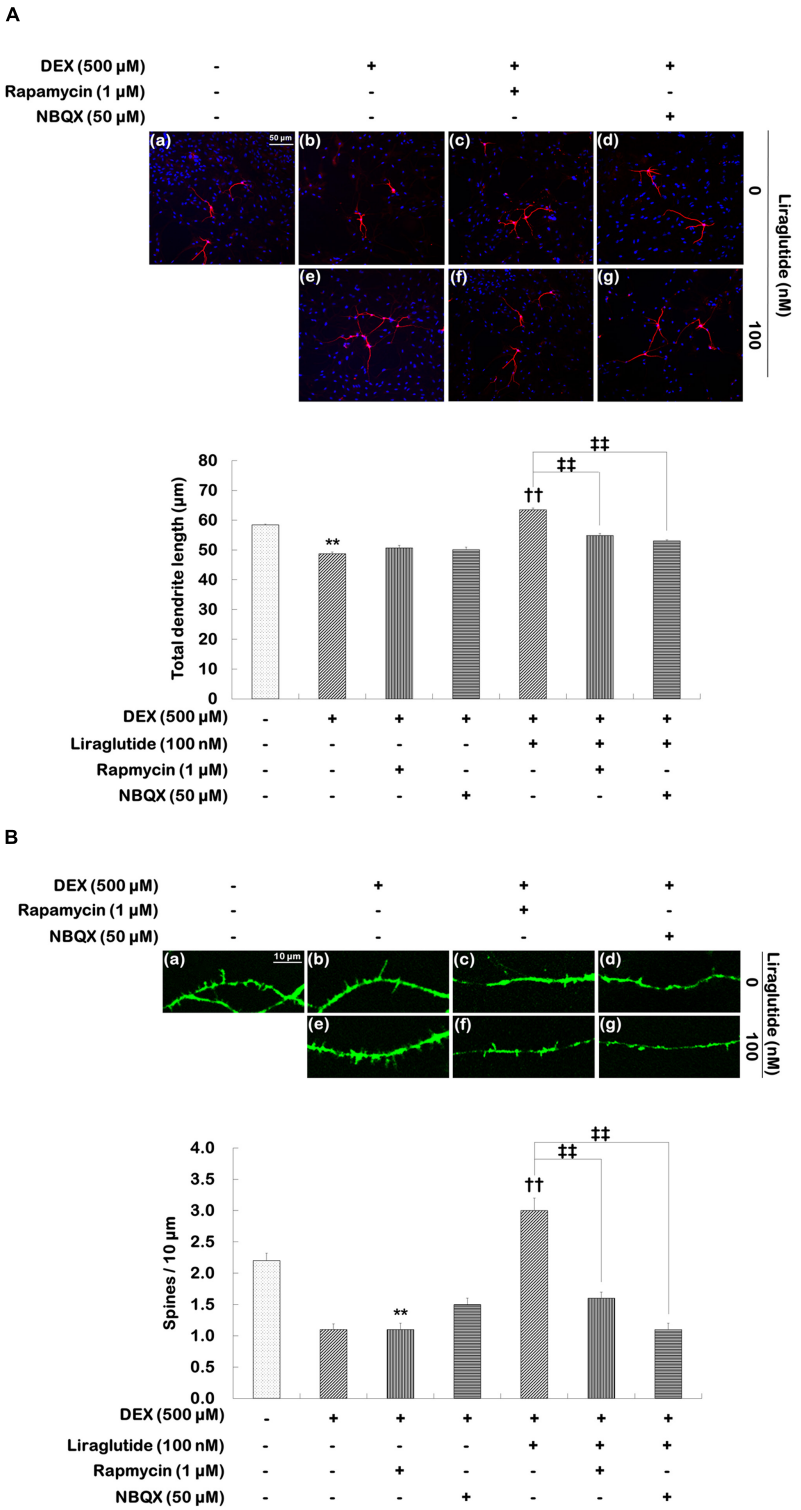

FIGURE 4 | Effects of rapamycin and NBQX on liraglutide-induced increases in total dendritic length and spine density in hippocampal cells treated with dexamethasone. Cells were exposed to rapamycin (1 μM) and NBQX (50 μM) for 30 min prior to the addition of distilled water (control) or liraglutide (100 nM) for 5 days either with (+; dexamethasone condition) or without (–; control condition) dexamethasone (DEX; 500 μM). Two independent cultures were performed and cells were photographed and scored according to the methods described above. In total, 400 cells per group were analyzed for total dendritic length **(A)**. In total, 23–31 dendritic segments per group were analyzed for spine density **(B)**. All data (n = 400 for dendritic length, n = 23–31 for spine density) are expressed as mean ± standard error of the mean (SEM). $^{**}p < 0.01$ vs. control cells (-DEX, no liraglutide and no inhibitors), $^{\dagger\dagger}p < 0.01$ vs. dexamethasone-treated cells (+DEX, no liraglutide and no inhibitors), $^{\ddagger\ddagger}p < 0.01$ vs. dexamethasone and liraglutide-treated cells (+DEX, liraglutide only).

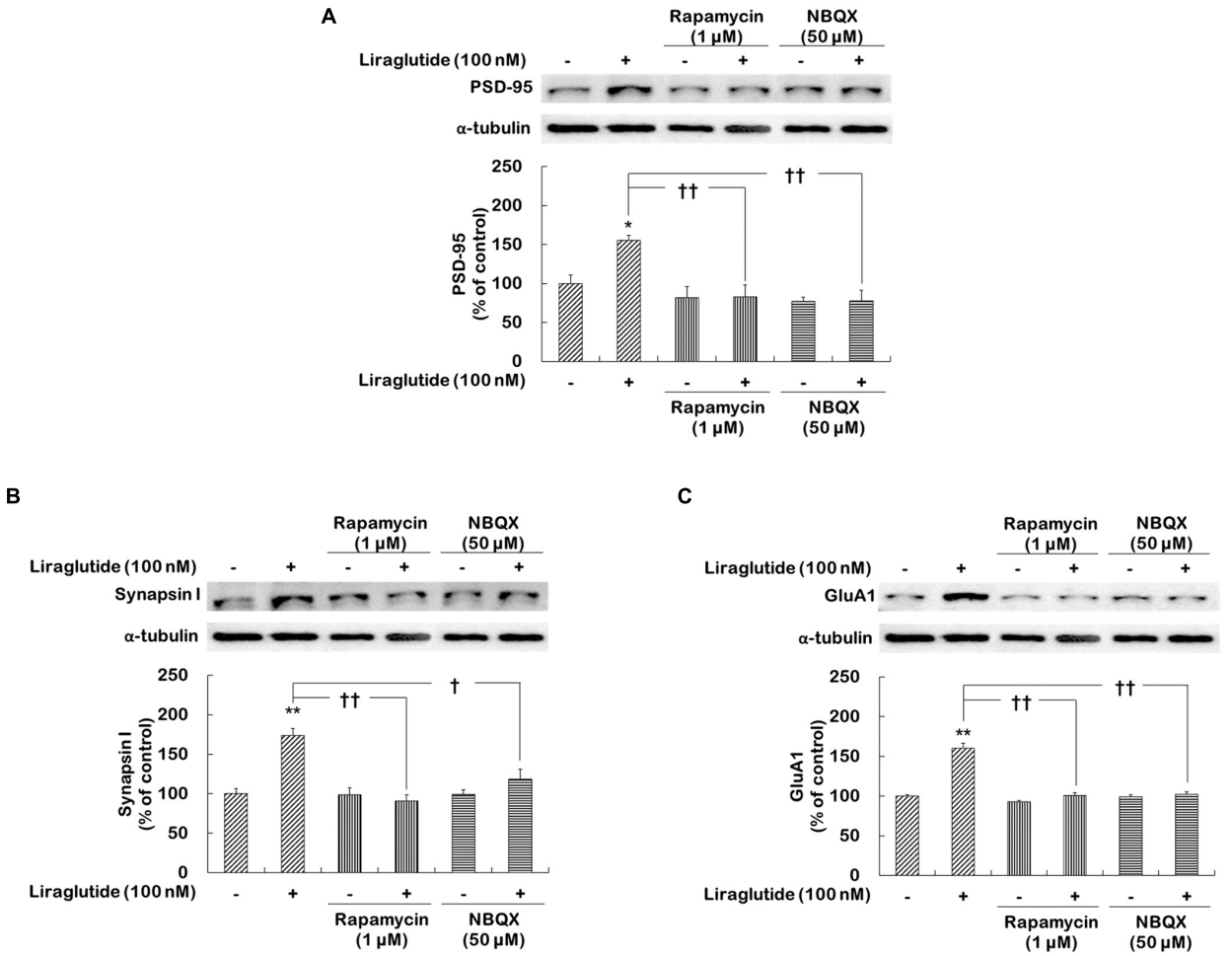

FIGURE 5 | Effects of rapamycin and NBQX on the regulation of liraglutide-induced synaptic protein expression in hippocampal cells treated with dexamethasone. Cells were exposed to rapamycin (1 μM) or NBQX (50 μM) for 30 min prior to the addition of distilled water (control) or liraglutide (100 nM) for 4 days with dexamethasone (500 μM). In two different wells per group of each of two independent cultures (total 4 wells), cell lysates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses for each of the primary antibodies. The Western blot and revealed the levels of PSD-95 **(A)**, synapsin I **(B)**, and GluA1 **(C)**. Representative images and quantitative analyses normalized to the α-tubulin band are shown. Values ($n = 4$) are mean ± standard error of the mean (SEM) expressed as a percentage of the control cell values. $^{*}p < 0.05$ vs. control cells (no liraglutide and no inhibitors), $^{**}p < 0.01$ vs. control cells, $^{\dagger}p < 0.05$ vs. liraglutide-only-treated cells, $^{\dagger\dagger}p < 0.01$ vs. liraglutide-only-treated cells.

antidepressant effects of ketamine (Duman et al., 2012; Dwyer and Duman, 2013; Abdallah et al., 2015). It has been proposed that PSD-95 plays an essential role in the maintenance and regulation of synaptic AMPA receptor function and AMPA receptor-dependent synaptic plasticity (Han and Kim, 2008). Increased levels of PSD-95 may be due to the increased number and size of dendritic spines and/or increases in the total number of synapses (Xu, 2011). Synapsin I is a modulator of neuronal development such that higher levels of synapsin I accelerate the development of synapses (Valtorta et al., 2011). GluA1 is a subunit of the AMPA receptor and is found in synapses where it enhances synaptic transmission (Santos et al., 2009). In the present study, liraglutide increased the expression of BDNF, PSD-95, synapsin I, and GluA1, whereas rapamycin and NBQX blocked the expression of each of these. In other studies, To date, no reports of studies investigating the activation of AMPA receptors on hippocampal neurons by liraglutide have been published. The present study is the first to report the effects of liraglutide on the activation of AMPA receptors in rat primary hippocampal neurons.

Chronic stress may cause depressive behavior by decreasing brain volume and reducing synaptic connections (Duman and Aghajanian, 2012). The activation of mTORC1 induces dendritic outgrowth but the induction of dendritic outgrowth is blocked by rapamycin (Park et al., 2014; Seo et al., 2016). Similarly, the present study demonstrated that liraglutide reversed decreases in neurite outgrowth in the dexamethasone-induced toxic condition and that the effects of liraglutide were blocked by rapamycin administration. Additionally, the increase in neurite outgrowth induced by liraglutide was blocked when NBQX and rapamycin were administered. Monnerie and Le Roux (2006) reported that kainate-induced dendritic outgrowth in mouse cortical

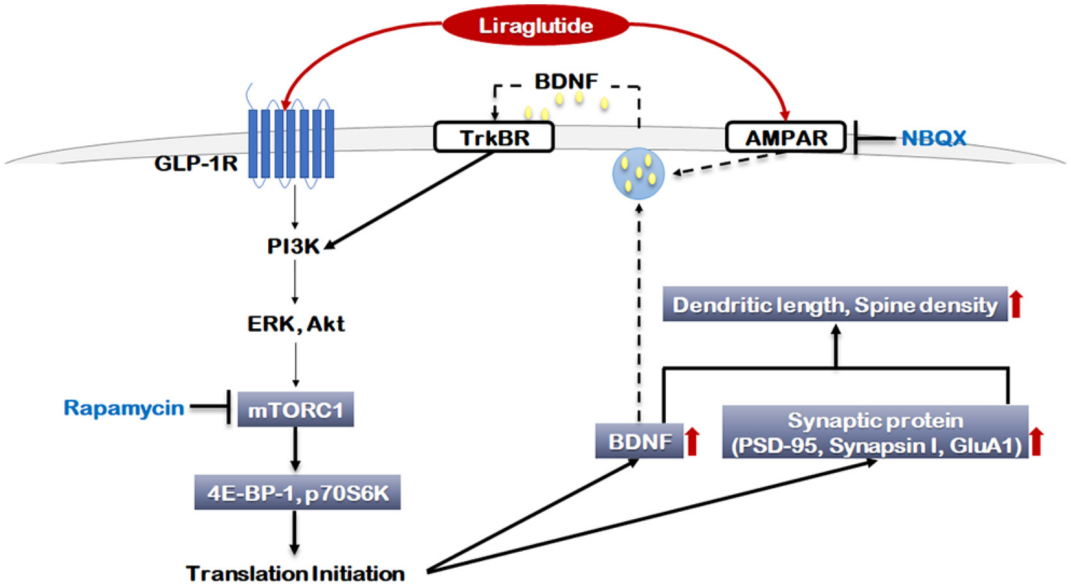

FIGURE 6 | Signaling pathways regulated by liraglutide. The molecular pathways shown in gray boxes illustrate our novel observations. Akt: Protein kinase B (PKB), AMPAR: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor, BDNF: brain-derived neurotrophic factor, ERK: extracellular signal-regulated kinase, GLP-1R: glucagon-like peptide 1 receptor, GluA1: AMPA receptor subunit GluR1, mTORC1: mammalian target of rapamycin complex 1, NBQX: 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide, p70S6K: P70S6 kinase, PI3K: Phosphoinositide 3-kinase, PSD-95: Post Synaptic Density 95 protein, TrKBR: tropomyosin receptor kinase B receptor, 4E-BP-1: eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1.

neurons is blocked by NBQX; therefore, it can be postulated that the activations of mTORC1 signaling and AMPA receptors by liraglutide may promote neurite outgrowth. The effects of liraglutide on synaptic density were also observed in the present study. Liraglutide prevented reductions in synaptic density in the dexamethasone-induced toxic condition, but this effect was blocked by rapamycin and NBQX. These results suggest that liraglutide-induced changes in neurite outgrowth and synaptic density may occur via the activations of mTORC1 signaling and AMPA receptors.

In the present study, there are several limitations to this study that should be noted. First, it remains unclear whether the concentration of liraglutide used in the present study can be used in *in vivo* environments, particularly in humans. Therefore, it will be necessary to confirm the effects of liraglutide on mTORC1 signaling and AMPA receptors in further experiments using animal models. Second, AMPA receptor activity was assessed using the administration of NBQX. To measure the activity of the AMPA receptor more accurately, measurements of the AMPA-excitatory synaptic current should be obtained following the administration of liraglutide (Shinohara, 2012). Third, only 100 nM rapamycin and 10 nM NBQX doses were used in the study and future studies with additional concentrations of these drugs will be necessary.

The present study was the first to investigate how liraglutide affects neuroplasticity via mTORC1 signaling and AMPA receptor activation. In this study, liraglutide activated mTORC1 signaling and AMPA receptors, increased the expression of BDNF and selected synaptic proteins, and enhanced neurite outgrowth and synaptic density under toxic conditions (**Figure 6**). Additionally, these liraglutide-induced changes were blocked by the mTORC1 inhibitor rapamycin and the AMPA receptor antagonist NBQX. These results indicate that liraglutide enacts neuroplastic regulation via mTORC1 signaling and AMPA receptor activation. Thus, it is possible that the activation of mTORC1 signaling and AMPA receptors may be a potential target for the modification of neural plasticity and the development of novel antidepressant drugs.

AUTHOR CONTRIBUTIONS

JL, SP, and RSM designed the study. MS and AC performed the experiments of this study. MS and SP wrote the protocol. SP and MS undertook the statistical analysis. RSM, RBM, YL, and J-HL contributed the methods and analysis tools. JL and SP wrote the first draft of the manuscript. All authors contributed to the approval of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00756/full#supplementary-material

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Changes in Membrane Protein Clustering in Peripheral Lymphocytes in an Animal Model of Depression Parallel Those Observed in Naïve Depression Patients: Implications for the Development of Novel Biomarkers of Depression

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Naïve depression patients show alterations in serotonin transporter (SERT) and serotonin 2A (5HT2A) receptor clustering in peripheral lymphocytes, and these alterations have been proposed as a biomarker of therapeutic efficacy in major depression. Repeated corticosterone (CORT) induces a consistent depression-like phenotype and has been widely used as an animal model to study neurobiological alterations underlying the depressive symptoms. In this experiment, we used the CORT paradigm to evaluate whether depression-like behavior is associated with similar changes in the pattern of SERT and 5HT2A membrane protein clustering as those observed in depression patients. We also analyzed the clustering of other proteins expressed in lipid rafts in lymphocytes. Rats received daily CORT or vehicle injections for 21 consecutive days. Afterward they underwent the forced swim test to evaluate depression-like behavior, and isolated lymphocytes were analyzed by immunocytochemistry coupled to image-analysis to study clustering parameters of the SERT, 5HT2A receptor, dopamine transporter (DAT), Beta2 adrenergic receptor (β2AR), NMDA 2B receptor (NR2B), Pannexin 1 (Pnx1), and prion cellular protein (PrPc). Our results showed that CORT increases the size of protein clusters for all proteins with the exception of β 2AR, which is decreased. CORT also increased the number of clusters for Pnx1 and PrPc only. Overall, these results indicate that alterations in SERT and 5HT2A protein clustering in naïve depression patients are paralleled by changes seen in an animal model of depression. The CORT paradigm may be a useful screen for examining additional proteins in lymphocytes as a preliminary step prior to their analysis as biomarkers of depression in human blood samples.

Keywords: depression, corticosterone, lipid rafts, biomarkers, lymphocytes

INTRODUCTION

Chronic stress and associated inflammatory events have been repeatedly shown to play a major role in the pathophysiology of depression (recently reviewed by Leonard, 2018). In fact several meta-analyses have revealed a consistent increased expression of pro-inflammatory cytokines – particularly IL-6, TNF-α, and CRP- in clinical depression (see Felger and Lotrich, 2013; Strawbridge et al., 2015; Haapakoski et al., 2016), and alterations in peripheral proteins related to inflammatory events have also been evaluated as possible biomarkers of depression (recently reviewed by Gururajan et al., 2016; Gadad et al., 2018). Also of importance in the context of the psychoneuroimmunology of stress/depression is the finding that high levels of stress cause a reduction in T and B lymphocyte proliferation, a reduction in immunoglobulin production, and an increase in neutrophils and macrophage activation and spreading (Dhabhar, 2008; Divyashree et al., 2016). Finally, we have recently reported that changes in the clustering of serotonin transporter (SERT) and serotonin 2A receptor (5HT2A) membrane proteins in lymphocytes in naïve depression patients give indications about the degree to which these patients will respond to antidepressant medication. These latter results have led us to propose that the pattern of clustering of these proteins could be a novel biomarker of therapeutic efficacy in major depression (Rivera-Baltanas et al., 2012, 2014, 2015).

Unsurprisingly, rodents subjected to different forms of chronic stress have consistently shown depression-like behavior and have been widely used to analyze the neurobiological events underlying the onset of a depressive phenotype (reviewed by Sterner and Kalynchuk, 2010). One well-characterized model makes use of repeated injections of corticosterone (CORT), which results in a robust and reliable increase in depression-like behavior as ascertained by different behavioral paradigms (Kalynchuk et al., 2004; Gregus et al., 2005; Marks et al., 2009), and a concomitant decrease in both adult hippocampal neurogenesis and expression of the extracellular matrix protein reelin in the proliferative subgranular zone of the dentate gyrus (Lussier et al., 2009, 2011, 2013). There appears to be a strong link between reelin and neurogenesis in the context of depression-like behavior. In addition, reelin is also involved in dendritogenesis, spinogenesis, and regulation of LTP, which are other forms of plasticity altered in depression, thereby indicating the interest in studying both central in peripheral actions of reelin within the context of depression, and fostering the interest in the study of the possible antidepressant-like characteristics of reelin peptides (Caruncho et al., 2016).

Following our studies in patient with depression, we now hypothesize that CORT will induce alterations in SERT and 5HT2A clustering along the cell membrane of peripheral lymphocytes that parallel those observed in human patients (Rivera-Baltanas et al., 2012, 2014). If so, and as these two proteins tend to cluster in specific microdomains such as lipid rafts (Magnani et al., 2004; Allen et al., 2007) and as interaction of antidepressants with lipid rafts is thought to be an important functional link for antidepressant efficacy (Donati and Rasenick, 2005; Zhang and Rasenick, 2010; Czysz and Rasenick, 2013; Czysz et al., 2015; Donati et al., 2015; Erb et al., 2016), we may further hypothesize that clustering of other membrane proteins that tend to accumulate in lipid rafts may be significantly changed in lymphocytes upon repeated CORT treatment, and that some parameters of alterations in membrane protein clustering may correlate with depression-like behavior as evaluated by the forced swim test (FST). As such, we also included in this experiment the analysis of membrane protein clustering for the dopamine transporter (DAT), the beta 2 adrenergic receptor (β2AR), the NMDA receptor 2B subunit (NR2B), pannexin 1 (Pnx1), and the prion cellular protein (PrPc). This experimental design will bring about our focus on the most translational component of our research, as will focus first in demonstrating if one can find in the CORT model of depression changes in the clustering pattern of SERT and 5HT2A that are similar to those found in naïve depression patients, and if so use this approach to analyze the patterns of clustering of other proteins that can thereby be screened for further analysis in depression patients (using a bedside to bench to bedside approach).

MATERIALS AND METHODS

Animals

We used a total of 53 adult male Long Evans rats in this experiment (purchased from Charles River Laboratories, Montreal, Quebec). Rats weighed 200–250 g at the time of arrival (rats were reared under conventional conditions by the breeder) and were single-housed with food and water provided *ad libitum*. The colony was kept under controlled conditions of light and temperature (12 h:12 h light-dark cycle, 21 ± 1°C). All the experimental procedures were conducted under protocol #20140038, approved by the Animal Research Ethics Board of the University of Saskatchewan.

About half of the animals were injected with vehicle and the other half with CORT as explained below, and thereafter they were evaluated for depression-like behavior in the FST. A set of 20 rats (10 vehicle, 10 CORT) was used to examine SERT clustering to compare with our previous analyses of SERT in patients with depression (Rivera-Baltanas et al., 2012, 2015); a set of 17 rats (9 vehicle, 8 CORT) was used to examine 5HT2A and β2AR clustering, to compare with previous studies on 5HT2A in depression patients (Rivera-Baltanas et al., 2014), and to analyze an additional G-protein coupled receptor; and a finals set of 16 rats (8 vehicle, 8 CORT) was used to examine novel membrane proteins (DAT, NR2B, Pnx 1, and PrPc) that could then be screened for further studies of membrane protein clustering in depression patients.

Corticosterone Treatment

Rats were handled briefly once per day for 7 days prior to the CORT or vehicle injections. Following this acclimatization period, rats were weight-matched into two groups that received either 21 days of CORT injections (CORT group) or 21 days of vehicle injections (vehicle group). All injections were administered subcutaneously once per day (between 9:00 and 2:00 pm). CORT (Steraloids) was suspended in 0.9% (w/v)

physiological saline with 2% (v/v) Tween-80 and given at a dose of 40 mg/kg in a volume of 1 ml/kg. Previous work in our laboratory and others has demonstrated that the dose of 40 mg/kg of CORT reliably increases depression-like behavior in rats (Kalynchuk et al., 2004; Marks et al., 2009; Fenton et al., 2015; Kott et al., 2016; Workman et al., 2016).

Forced Swim Test

The FST was conducted the day after the final CORT or vehicle injection. We used a modified version of the traditional Porsolt test (Porsolt et al., 1977). This version of the test is conducted over 1 day, which is as effective as 2-day version for assessing depression-like behavior in rats previously subjected to a period of chronic stress (Marks et al., 2009).

The FST was carried out in a different room from the one used for the CORT injections. Rats were placed into a rectangular Plexiglas swim tank (25 cm long × 25 cm wide × 60 cm high) filled with water to a depth of 30 cm. The temperature of the water was kept at 27°C (±2°C). Rats remained in the tank for 10 min and the session was videotaped for offline analyses. We scored both active and inactive components of behavior: swimming, struggling, and immobile (Marks et al., 2009).

Extraction of Lymphocytes and Immunocytochemistry

Rats were deeply anesthetized with isoflurane. Thereafter, six ml of blood were collected by heart puncture with ACD anticoagulant (85 mM trisodium citrate, 65 mM citric acid, 111 mM anhydrous glucose) at a ratio 1:7 (v/v). Blood was diluted 1:1 with phosphate buffer saline (PBS) and centrifuged in a Percoll gradient for 40 min at 800 × *g*. The lymphocyte band was then collected and cells were re-suspended in PBS solution and centrifuged at 1000 × *g* for 10 min. This step was repeated twice. Lymphocyte fixation involved incubation with 1% paraformaldehyde in 0.1 M phosphate buffer (PB) for 5 min (Rivera-Baltanas et al., 2010; Romay-Tallon et al., 2017).

Immunolabeling of specific membrane proteins was used to enable to assessment of membrane protein clustering. Prior to immunolabeling, we performed a preincubation step to avoid unspecific staining by incubating with a blocking solution [3% rat IgG (Sigma) and 1% bovine serum albumin (BSA)] in PBS for 10 min at room temperature. This was followed by overnight incubation at 4°C with a series of rabbit-polyclonal primary antibodies diluted in blocking solution: anti-SERT (1:250, Cat# AB10514P, Millipore), anti-5HT2A (1:150, Cat# RA24288, Neuromics); anti-β2AR (1:150, Cat# SAB1306036, Sigma); anti-PrPc (1:200, Cat# ab52604, Abcam); anti-NR2B (1:200, Cat# SAB4300711, Sigma); anti-DAT (1:250, Cat#D6944, Sigma); anti-Pannexin1 (PNX-1, 1:250, Cat#ab124131, Abcam).

The binding of the primary antibodies to the antigen was then revealed by incubation with a secondary antibody, goat anti rabbit Alexa Fluor 568 (1:200, Molecular Probes) diluted in 1% BSA in PBS for 1 h at room temperature. Several washes in PBS were performed after finishing the incubation with the secondary antibody, and thereafter samples were collected onto microscope slides and cover-slipped with Citifluor (Electron Microscope Science). Samples were stored at −20°C until analysis.

Omission of primary antibodies resulted in lack of immunostaining in all cases.

Imaging and Statistical Analysis

A total of 50 lymphocytes per animal were analyzed with a Nikon E800 microscope using computer software (MicroBrightfield) with a MicroFire digital camera (Optronics). The number and size of protein clusters were analyzed with ImageJ 1.48 software (NIH), using previously published methods (Romay-Tallon et al., 2017).

All data were analyzed with SPSS (Statistical Package for the Social Science, v16.0, Chicago). We used independent sample *t*-tests to evaluate differences between the vehicle and CORT groups in behavior and protein clustering. The data are expressed as the mean value ± SEM. Differences were considered statistically significant at $p < 0.05$.

RESULTS

CORT-Treated Rats Show Depression-Like Behavior in the FST

Figure 1 shows the effect of CORT on FST behavior. As expected, rats injected with CORT spent less time active than their vehicle counterparts. *T*-test analyses revealed a significant increase in the time the CORT rats spent immobile relative to the vehicle rats [$t(51)$: −6.140; $p = 0.0000001$], and a decrease in the amount of time spent swimming [$t(51)$: 4.108; $p = 0.0001$], and struggling [$t(51)$: 2.591; $p = 0.012$].

Serotonin Transporter

Figure 2A shows representative images of lymphocytes from vehicle and CORT rats, stained for SERT. The imaging system creates an outline representation of clusters (**Figure 2A**, right column) allowing for the quantification of the number and size of SERT clusters. This quantification is shown in **Figure 2B**. *T*-test analyses revealed no differences between the groups in the number of clusters [$t(18)$: 0.343, $p = 0.736$]. However, there was an increase of 13% in clusters size in CORT rats relative to vehicle-treated rats [$t(18)$: −2.458, $p = 0.030$]. **Figure 2C** further illustrates the distribution of SERT clusters based on their number and size, with numbers in the graph indicating individual animals. This analysis reveals that a number of CORT rats showed a low number and high size of SERT clusters in lymphocytes (indicated with a circle in the left panel of **Figure 2C**), interestingly these rats showed a higher level of immobility in the FST which parallels observations in SERT clustering in naïve depression patients (see section "Discussion" and Rivera-Baltanas et al., 2012).

Dopamine Transporter

As we demonstrated that changes in SERT clustering in lymphocytes from CORT treated animals parallel those observed

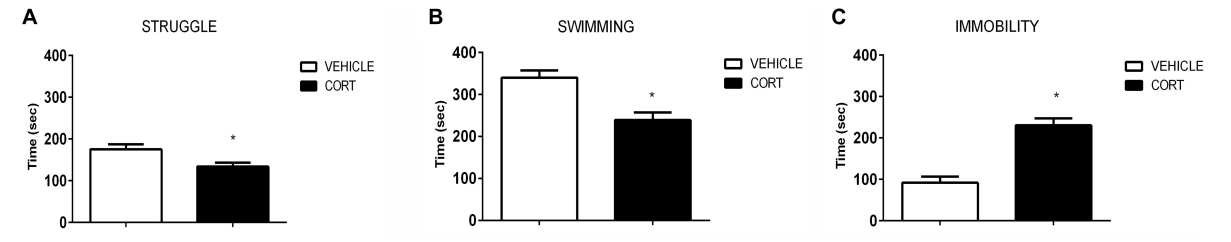

FIGURE 1 | Results of the forced swim test demonstrate that repeated CORT induces depressive-like behavior, as evidenced by analysis of the struggle **(A)**, swimming **(B)**, and immobility time **(C)**. Significant differences, p value < 0.05, are indicated by an asterisk (*).

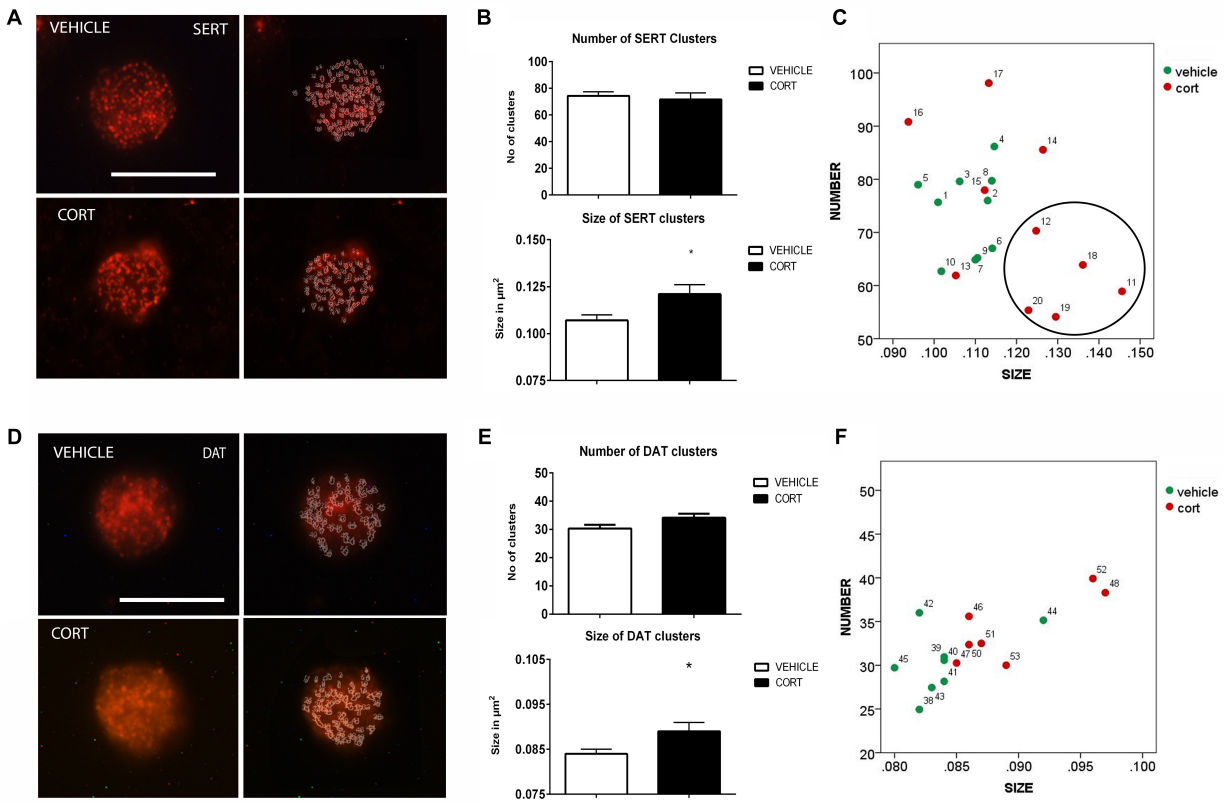

FIGURE 2 | Analysis of SERT and DAT MPC in lymphocytes. SERT immunolabeling is observed as patches of immunostaining in the plasma membrane of lymphocytes that are amenable to quantification by image analysis. Panel **(A)** shows SERT immunostaining in a representative lymphocyte from vehicle or CORT treated rats, and how these clusters are evaluated by using image-J. Calibration bar: 10 μm. Panel **(B)** shows that repeated CORT increases SERT clusters size but does not change the number of clusters per lymphocyte. Panel **(C)** left portraits the representation of the average number and size of SERT clusters in individual animals (identified by numbers). Note that a subset of CORT treated animals is identified by having a low number and high size of SERT clusters (circle). DAT immunolabeling is observed as patches of immunostaining in the plasma membrane of lymphocytes that are amenable to quantification by image analysis (Panel **D** Calibration bar: 10 μm). Panel **(E)** shows that repeated CORT increases DAT clusters size but does not change the number of clusters per lymphocyte. Panel **(F)** portraits a scatter plot of how CORT alters DAT MPC parameters. Significant differences, p value < 0.05, are indicated by an asterisk (*).

in naïve depression patients, we proceeded to analyze other neurotransmitter transporter that is also related to depression, but whose clustering has not been studied yet in depression patients. **Figure 2D** shows the effect of CORT on DAT-positive protein clusters on the cell membrane of lymphocytes. We found a significant (7%) increase in the size of DAT clusters in the CORT rats relative to the vehicle rats [$t(13)$: −2.505, p: 0.026], but no significant differences in the number of DAT clusters [$t(13)$: −1.908, p: 0.079]. This is shown in **Figure 2E**. **Figure 2F** shows the relationship between clusters numbers and size.

Serotonin Receptor 2A

Figure 3A illustrates the immunolabeling of 5HT2A clusters in representative lymphocytes from the vehicle and CORT rats (left panels), as well as the cluster outlines revealed

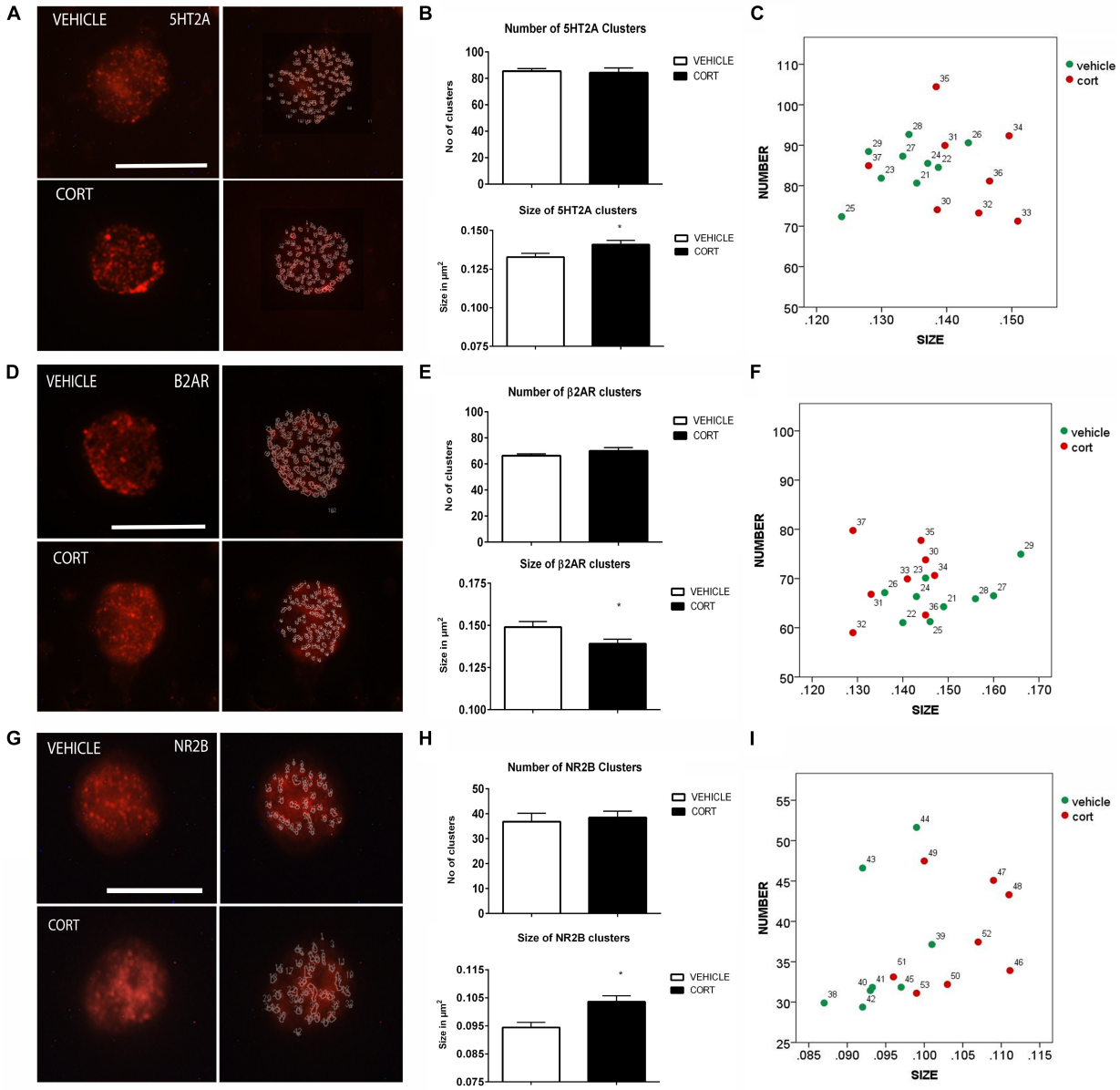

FIGURE 3 | Analysis of 5HT2A, β2AR, and NR2B receptors MPC in lymphocytes. 5HT2A immunolabeling is observed as patches of immunostaining in the plasma membrane of lymphocytes that are amenable to quantification by image analysis (Panel **A** Calibration bar: 10 μm). Panel **(B)** shows that repeated CORT increases 5HT2A clusters size but does not change the number of clusters per lymphocyte, while Panel **(C)** left portraits the alterations induced by CORT in number Vs size of 5HT2A clusters. Panel **(D)** illustrates the identification of β2AR clusters in peripheral lymphocytes (Calibration bar: 10 μm). Panel **(E)** shows the effect of repeated CORT in decreasing the size of β2AR clusters without altering their number. Panel **(F)** portraits the representation of the average number and size of 5HT2A clusters in individual animals (identified by numbers). Note that while for most protein clusters CORT induces an increase in clusters size in the case of β2AR the size of the clusters is decreased. Panel **(G)** presents representative micrographs of NR2B labeling in lymphocytes from vehicle or CORT-treated animals (Calibration bar: 10 μm). Panel **(H)** shows the effect of repeated CORT in increasing the size of NR2B clusters without altering their number. Panel **(I)** portraits the representation of the average number and size of NR2B clusters in individual animals. Significant differences, p value < 0.05, are indicated by an asterisk (*).

through the imaging system (right panels). Quantitative analysis showed that 5HT2A cluster size is increased 7% in the CORT rats [$t(15)$: -2.568, $p = 0.021$] but there were no groups differences in the number of clusters [$t(15)$: 0.218, p: 0.830] (**Figure 3B**). **Figure 3C** shows the distribution of 5HT2A clusters according to number and size in both groups. Similar changes have been previously observed in naïve depression patients (Rivera-Baltanas et al., 2014).

Beta 2 Adrenergic Receptor

As changes in 5HT2A clustering in lymphocytes from CORT treated animals parallel those observed in naïve

depression patients (see above), we proceeded to analyze other neurotransmitter receptors such as β2AR and NR2B, that are also related to depression, but whose clustering has not been studied yet in depression patients. **Figure 3D** shows the effect of CORT on β2AR protein clusters. Although there were no significant group differences in the number of clusters [$t(15)$: −1.294, p = 0.215], there was a significant decrease (7%) in the size of the β2AR clusters in the CORT rats relative to the vehicle rats [$t(15)$: 2.300, p = 0.036] (**Figure 3E**). **Figure 3F** shows the distribution of the clusters according with their number and size.

NMDA Receptor 2B Subunit

Figure 3G shows the effect of CORT on NR2B-immunopositive protein clusters. There were no group differences in the number of NR2B clusters [$t(14)$: −0.463, p: 0.650] but there was a significant increase in cluster size (i.e., 11%) in the CORT rats relative to the vehicle rats [$t(14)$: −3.936, p = 0.001] (**Figure 3H**). **Figure 3I** represents the distribution of NR2B clusters based on number and size.

Pannexin 1

On top of the analysis of the patterns of clustering of some neurotransmitter transporters and receptors we also proceeded to the analysis of other proteins that have been shown related to depression such as Pnx1 and PrPc, in order to ascertain the interest of further studying the patterns of clustering of these proteins in naïve depression patients. **Figure 4A** illustrates the effect of CORT on Pnx 1 immunopositive clusters along the cell membrane of lymphocytes. Quantification of Pnx 1 revealed a 26% increase in cluster number [$t(14)$: −3.624, p: 0.003], and a 7% increase in cluster size [$t(14)$: −2.592, p: 0.021] in the CORT rats relative to the vehicle rats (**Figure 4B**). The distribution of clusters based on the number and size is represented in **Figure 4C**.

Prion Cellular Protein

Figure 4D shows the effect of CORT on PrPc protein clusters. Quantitative analyses of these clusters revealed an increase of 21 and 15% in the number and size of clusters in the CORT rats, respectively [Number: $t(13)$: −3.852, p: 0.002; size: $t(13)$: −4.756, p = 0.0003], see **Figure 4E**. **Figure 4F** shows the distribution of PrPc positive clusters.

DISCUSSION

To our knowledge, this is the first time that a panel of membrane protein clusters has been analyzed in peripheral mononuclear

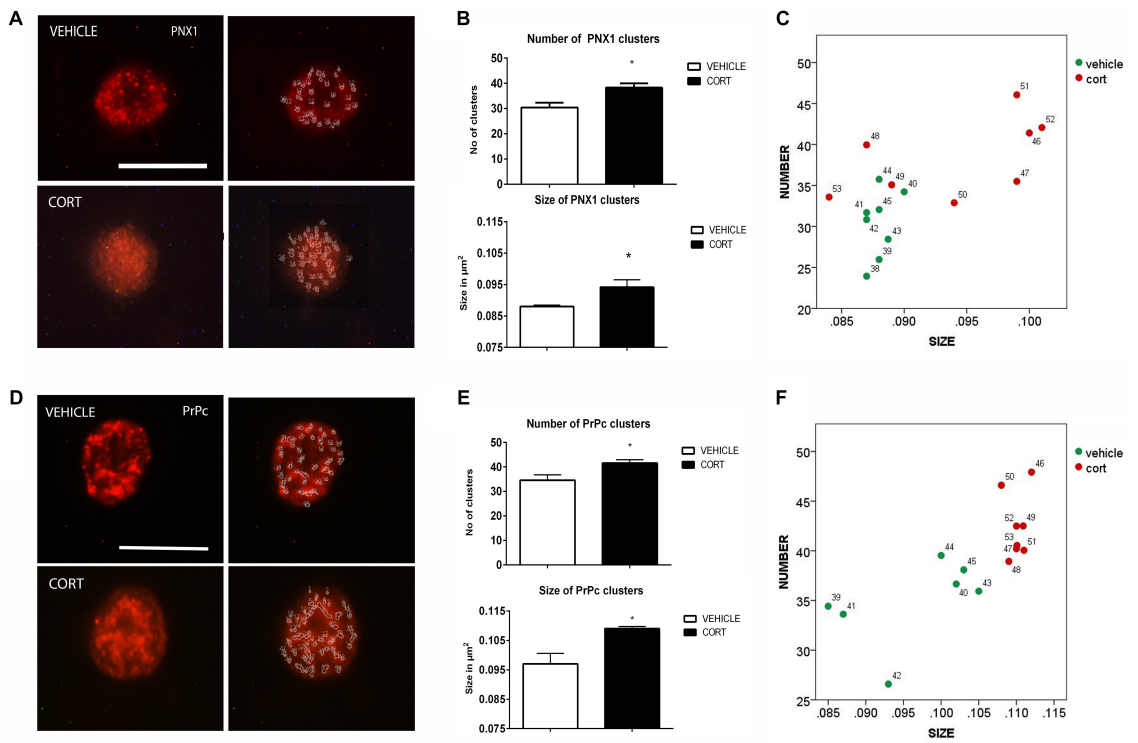

FIGURE 4 | Analysis of Pnx1 and PrPc MPC in lymphocytes. Pnx1 immunolabeling is observed as patches of immunostaining in the plasma membrane of lymphocytes that are amenable to quantification by image analysis (Panel **A** Calibration bar: 10 μm). Panel **(B)** shows that repeated CORT increases both Pnx1 clusters number and size. Panel **(C)** illustrates changes in individual animals in relation to number of clusters Vs size. PrPc labeling micrographs are shown in Panel **(D)** (Calibration bar: 10 μm). Panel **(E)** illustrates the effect of repeated CORT in increasing both the number and size of PrPc clusters. Panel **(F)** portraits the representation of the average number and size of PrPc clusters in individual animals (identified by numbers). Significant differences, p value < 0.05, are indicated by an asterisk (*).

blood cells and analyzed in relation to depression-like behavior in an animal model for depression. Ultimately, we conducted this analysis with the idea that positive results could suggest the use of animal models such as the CORT paradigm to screen biomarkers of depression to be further studied in samples from depression patients, which are not always easy to obtain. The main findings of this experiment indicate in the first place that repeated CORT induces alterations in the pattern of SERT and 5HT2A clustering along the cell membrane of peripheral lymphocytes that parallel those observed in naïve depression patients (Rivera-Baltanas et al., 2012, 2014), and second it establishes how other membrane proteins related to depression changes their pattern of clustering in the CORT model thereby indicating that this model can be used as a screening tool to select specific proteins whose pattern of clustering would be of interest to further study in samples from depression patients.

The behavioral results obtained here are consistent with previous literature showing that repeated CORT administration reliably increases depression-like behavior (reviewed in Sterner and Kalynchuk, 2010). We found that CORT-treated rats showed increased immobility and decreased struggling and swimming in the FST, which is a pattern of behavior that generally indicates a depressive phenotype (Cryan et al., 2005). This result was expected based on previous findings showing that the 40 mg/kg dose increases immobility behavior in both male and female rats (Kalynchuk et al., 2004) and that the effects of CORT on immobility can be reversed by administration of imipramine – a tricyclic antidepressant (Fenton et al., 2015). Importantly, the depressogenic effects of CORT are not restricted to the FST. Rodents subjected to repeated CORT also show impaired cognition, decreased interest in sexual behavior, decreased sucrose preference, decreased home cage activity, and increased immobility in a tail suspension test (Gorzalka and Hanson, 1998; Dwivedi et al., 2015; Sturm et al., 2015; Huston et al., 2016; Lui et al., 2017; Brymer et al., 2018), without altering nonspecific motor behavior (Marks et al., 2009). However, one should point out that the FST is more a tool to screen antidepressant-like activity that not ascertain depressive-like behavior (Nestler and Hyman, 2010; Molendijk and de Kloet, 2015; de Kloet and Molendijk, 2016). This issue is further discussed below in a section titled "Limitations."

The primary purpose of this experiment was to assess the utility of the CORT paradigm as a preliminary screen for changes in membrane protein clustering that might be good candidates for additional assessment in tissue samples from human patients. The evaluation of membrane protein clusters in lymphocytes have specific technical conditions that should be considered in terms of replicability of results. The time, concentration, and nature of the fixative, and the temperature and incubation time of lymphocytes with the primary antibody are crucial factors to be considered because they will affect the reorganization of the lipid rafts and also the quality and resolution of the staining. In this experiment we used the same protocol already presented in previous publications from our group (Rivera-Baltanas et al., 2010, 2012, 2014, 2015; Romay-Tallon et al., 2017). We analyzed the clustering of several neurotransmitter markers (transporters and receptors) as well as pannexin 1 and PrPc, on the cell membrane of peripheral blood lymphocytes. Immunolabeling of these proteins is observed on the membrane as microscopic dots (clusters) that are amenable quantification using images analysis programs (Romay-Tallon et al., 2017). Expression of these proteins has been repeatedly identified in lymphocytes populations (SERT: Marazziti et al., 1998; Gordon and Barnes, 2003; Barkan et al., 2004; Yang et al., 2007; Chen et al., 2015; Herr et al., 2017. 5HT2A: Padin et al., 2006; Yang et al., 2006. DAT: Marazziti et al., 2010; Buttarelli et al., 2011. b2AR: Fan and Wang, 2009; Sanders, 2012. NR2B: Miglio et al., 2005; Biermann et al., 2007. PNX1: Woehrle et al., 2010. PrPc: Isaacs et al., 2006).

The role of neurotransmitter transporters and receptors in the immune system seems to be mainly regulatory, although this is still unclear (Robson et al., 2017). For instance, monoamines regulate T lymphocyte activation and secretion of chemokines by neutrophils (Leon-Ponte et al., 2007; Maes et al., 2012); similarly, glutamate is modulating T lymphocyte proliferation (Miglio et al., 2005). Pnx1 regulates T-cell activation at the immune synapse (Woehrle et al., 2010), whereas PrPc also modulates T-cell activation and its cross-linking leads to rearrangement of components of lipid rafts translating into an increased phosphorylation of signaling proteins (Isaacs et al., 2006). Many studies have pointed out alterations in components in the immune system in major depression, and this has recently become a hot research topic particularly in relation to multiple evidences implicating an important role of inflammation in depression (see Miller and Raison, 2016), and the roles of serotonin in inflammation and immunity (recently reviewed by Wu et al., 2018). This, together with the demonstrated antidepressant effects of some anti-inflammatory drugs such as etanercept (Kappelmann et al., 2016; Brymer et al., 2018) brings about a novel interest in focussing on different components of the immune system as putative targets of novel antidepressant drugs.

Our analysis indicates that CORT brings about alterations in the pattern of membrane protein clustering, resulting in an increase in cluster size for all the proteins analyzed with the exception of β2AR, which shows a decrease in cluster size. In addition, cluster numbers tend to be unaltered by CORT treatment but they are increased in the case of Pnx1 and PrPc. As all these proteins tend to accumulate within the membrane domains of lipid rafts (see Magnani et al., 2004; Taylor and Hooper, 2006; Besshoh et al., 2007; Fallahi-Sichani and Linderman, 2009; Swanwick et al., 2009; Botto et al., 2014), the results could be interpreted as indicating that CORT treatment could alter protein clustering within lipid rafts, which may have important physiological/pathological consequences. Interestingly, most CORT induced alterations in membrane protein clustering tend to show a positive correlation with immobility in the FST, i.e., with a depressive phenotype. A series of elegant reports by Rasenick's lab point out that the action of antidepressant drugs in modulating Gα protein redistribution in rafts and non-rafts domains, and suggest that this action may be of importance for the onset of antidepressant activity, in fact they propose that it is this modulation of Gα protein distribution in raft domains by antidepressant drugs

what brings about alterations in G-protein functionality that underlie these drugs antidepressant effects more than these effects being mediated by the direct binding to specific neurotransmitter transporters (Allen et al., 2005, 2007, 2009; Donati and Rasenick, 2005; Sugama et al., 2007; Dave et al., 2009; Zhang and Rasenick, 2010; Czysz and Rasenick, 2013; Czysz et al., 2015; Erb et al., 2016). Considering this, it would thereby be of interest to evaluate whether antidepressant treatment is also able to reverse the alterations in membrane protein clustering in lipid rafts induced by CORT and if this correlates with a normalization of the depressive phenotype. In fact, preliminary data from our laboratory suggests that this is what is happening (Romay-Tallon, personal communication). It would also be of interest to analyze if similar alterations in the patterns of membrane protein clustering are also found in the CNS, while our preliminary studies also indicate that this is the case at least for SERT (Romay-Tallon, personal communication) additional experiments need to be performed to validate that this is so.

Previous studies from our laboratory have shown alterations in the size but not in the number of clusters that express SERT in patients with depression (Rivera-Baltanas et al., 2012, 2015). The analysis of SERT clustering in the repeated CORT model of depression reveals parallel alterations to those described in naïve depression patients (Rivera-Baltanas et al., 2012). More importantly, we have shown that alterations in SERT clusters size correlate with depressive-like behavior upon CORT treatment indicating that alterations in SERT clustering are related to the depressive phenotype. In a similar way, we have also shown that the therapeutic outcome of antidepressant treatment in depression can also be predicted by alterations in SERT clustering in lymphocytes in naïve patients and proposed that a SERT clustering assay could be used as a biomarker of therapeutic efficacy in major depression (Rivera-Baltanas et al., 2012, 2015). This brings about the interest to analyze in depression patients the patterns of MPC for the other proteins analyzed in this study, and strongly suggests that the repeated CORT model of depression could be used to screen MPC of multiple proteins previous to their analysis in depression patients. In fact, approaches using both animal models and samples from depression patients are becoming common place for the study and characterization of specific biomarkers (see as example Carrillo-Roa et al., 2017).

These series of hypotheses-based studies (bench to bedside to bench) initiated with the demonstration of altered patterns of SERT clustering in animals with low levels of reelin (Rivera-Baltanas et al., 2010) and the demonstration that those animals are more susceptible to some behavioral alterations induced by CORT (Lussier et al., 2011), followed by the demonstration of altered patterns of SERT and 5HT2A clustering in naïve depression patients and its proposal as putative biomarkers of therapeutic efficacy for major depression (Rivera-Baltanas et al., 2012, 2014, 2015). These experiments have been now followed with the demonstration of similar alterations in the CORT model of depression and thereby with the possible use of this model to screen for additional proteins to be further assayed in depression patients as putative biomarkers of depression. The use of this approach is perfectly compatible with the use of other "omics" approaches that are been widely assayed (i.e., genomics, proteomics, metabolomics), and it may well be that an algorithm encompassing several methodologies will be more effective for the developing of novel biomarkers with a clear clinical appeal, as for example it is currently been applied in cancer biomarkers research (see as review, Goossens et al., 2015).

LIMITATIONS

The present report primarily focusses on analyzing if changes in the pattern of membrane clustering of SERT and 5HT2A are also observed in the CORT model of depression, and thereafter in the analysis of the patters on clustering of several additional proteins with the purpose of using this test for their screening and further analysis in depression patients. While this has been so, one should be aware of some limitations of the present study, some of those pertaining to technological aspects on identification and quantification of protein clusters have been discussed above, but there are remain other limitations that should be considered that mostly pertain to the validity of the CORT model of depression and the use of specific behavioral tests.

The CORT model of depression has been widely used and validated in multiple behavioral tests (see as a review Sterner and Kalynchuk, 2010), but as it happens with most animal models it has some shortcomings and thereby data obtained by studying this model should always be related to the real thing, in this case what happens with alterations in membrane protein clustering in lymphocytes in depression patients. In the present report we indicate that changes in the pattern of clustering in SERT and 5HT2A parallel those observed in naïve depression patients and thereby we furthered the study by analyzing changes in the patterns of clustering of other membrane proteins. Although we are proposing to use this approach to screen for proteins to be thereafter studies in blood samples from depression patients, we cannot at the moment ascertain that the alterations found for these proteins in the CORT model would also be observed in depression patients. In addition, it would also be of interest to provide data from other animal models of depression that would further justify the use of animal models for screening of patterns of membrane protein clustering to be later translated to analyses in samples from depression patients.

A second shortcoming related to the use of the FST to establish depressive-like behavior. As mentioned at the beginning of the discussion, the FST is better used for screening of antidepressant-like activity than for demonstration of depression-like behavior (Nestler and Hyman, 2010; Molendijk and de Kloet, 2015; de Kloet and Molendijk, 2016). For the present experiments the FST was used only to demonstrate an effect of CORT similar to that we have shown multiple times (reviewed in Caruncho et al., 2016), but the CORT model also results in other emotional and cognitive alterations that are part of a depressive-like phenotype (see Kalynchuk et al., 2004; Gregus et al., 2005; Marks et al., 2009; Brymer et al., 2018).

Although out of the scope of the present report we understand that it would be of interest to ascertain the effects of antidepressants in membrane protein clustering and how these effects correlate with a reversal of different behavioral components in several models of depression, we are currently carrying out several studies to further on the knowledge on this topic.

CONCLUSION

We have observed that repeated CORT alters the clustering of several proteins that tend to accumulate within lipid rafts, and that these alterations correlate with depression-like behavior. Changes in membrane protein clustering parallel those observed in naïve depression patients, indicating a possible use of the repeated CORT model to screen clustering parameters in multiple proteins as a preliminary step to their analysis as biomarkers of therapeutic efficacy in human blood samples from major depression patients.

AUTHOR CONTRIBUTIONS

RR-T, JO, LK, and HC designed the experiments. RR-T, EK, KB, JA, and TR-B, carried out the experiments. RR-T and TR evaluated the statistics. All authors reviewed and discussed the complete set of data. RR-T wrote the original manuscript. LK and HC finalized the manuscript.

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Emerging Therapeutic Role of PPAR–α in Cognition and Emotions

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Neurosteroids and the endocannabinoid system are increasing in relevance as themes in the studies of many disorders and diseases (Berardi et al., 2016; Basavarajappa et al., 2017; Rasmusson et al., 2017). Correspondingly, psychiatric disorders, like post-traumatic stress disorder (PTSD), correlate with changes in endogenous neurosteroid and endocannabinoid availability, which may be related to the cause of its comorbidity with general cognitive decline (Qureshi et al., 2011; Schuitevoerder et al., 2013), neuroinflammation (Jeon and Kim, 2016; Mendoza et al., 2016), and neurodegenerative disorders (Cummings, 1992; Chi et al., 2014). These neuropathologies also reduce the quality of life and increase the socioeconomic burden.

Furthermore, increasing evidence shows an association of chronic maladaptive brain changes with neuroinflammation in PTSD (Jones and Thomsen, 2013). It is marked by the upregulation of pro-inflammatory cytokines such as IL−1β, IL−6, IL−10, and TNF–α in the CNS (Minami et al., 1991; Cunningham et al., 1992; Mogi et al., 1994, 1996). Particularly, IL−1β plays a critical role in the activation of the HPA axis (Shintani et al., 1995), and in the hippocampal formation where it regulates stress-enhanced fear learning (Jones et al., 2015). As such, the association between neurosteroids and neuroinflammation is unsurprising (Purdy et al., 1991; Webster et al., 2015; Villa et al., 2016). Indeed, neuroactive steroids, allopregnanolone and deoxycorticosterone have also been shown to increase during acute stress to levels that activate the $GABA_A$ receptor, and thereby influence the behavioral responses (Purdy et al., 1991). The chronic stress response has also been found to coincide with decreased cognitive function, especially learning and memory deficits (McEwen and Sapolsky, 1995), in episodic memory (Payne et al., 2006), and spatial learning and memory (Conrad, 2010). Chronic stress also negatively alters sleep patterns, social behavior, mood (Opp et al., 1988; Pinna et al., 2003, 2008; Hall et al., 2015; Olini et al., 2017 reviewed in Locci and Pinna, 2017), as well as decreases neurosteroids (Pinna et al., 2006, 2009; Bortolato et al., 2011), which affect synaptic plasticity (Serra et al., 2008; Fester and Rune, 2015) and neurogenesis (Wang, 2014).

The high prevalence of PTSD in the US makes it a high priority research topic. Approximately 7–12% of US adults are affected by mood and anxiety related disorders (Anxiety Depression Association of America, 2010-2016), while 4% of US adults (Harvard Medical School, 2007) and 20–30% of US veterans are affected by PTSD specifically (US Department of Veterans Affairs, 2015a). There are currently no reliable mechanisms or biomarkers to predict the onset or progression of PTSD, nor are there treatments that can consistently reduce the symptoms of PTSD. Currently, the only approved pharmacotherapies for PTSD are the selective serotonin reuptake inhibitors (SSRIs), however, these treatments have low response rates and only treat a small subset of patients (Hertzberg et al., 2000). The neurosteroid system is emerging as novel neuronal substrates in the pathogenesis of PTSD and its regulation may facilitate recovery (Yu et al., 2011; Zanettini et al., 2011; Litvin et al., 2013; Locci and Pinna, 2017; Pineles et al., 2018).

The goal of this opinion article is to examine the relationship between the endogenous fatty acid amides, including palmitoylethanolamide (PEA) and the biosynthesis of neurosteroids, particularly allopregnanolone, and their role in emotional and cognitive dysfunction in PTSD. Specifically, we focus on the function of the peroxisome proliferator–activated receptor (PPAR)–α, a target for PEA, which is best known for its role in reducing inflammation by decreasing cytokines,

pro-inflammatory enzymes and oxidative stress. For this, PPAR–α agonists act as neuroprotectants in various neurological disorders like Alzheimer's disease, Parkinson's disease, multiple sclerosis, and cerebral ischemia (Zolezzi et al., 2017). However, recent literature in the field suggest that PPAR–α has emerged as a new target that is useful as a novel approach to treat mood disorders by engaging neurosteroid biosynthesis.

THE ENDOCANNABINOID SYSTEM AND THE ROLE OF PPARS IN COGNITION AND EMOTIONS

The endocannabinoid system was curiously discovered in the 1990s because of the psychotropic effects that resulted from the use of *cannabis sativa* in medicinal and recreational settings (Di Marzo et al., 2004). The goal of early research was to elucidate the active agents, but, with time, research turned from the study of the psychotropic effects of the endocannabinoid system to the study of its medicinal properties. Eventually, treatment-oriented research revealed that the endocannabinoid system plays an important physiological role in homeostasis, pathogenesis and recovery in healthy and ill brain states (De Petrocellis et al., 2004), and is heavily involved in the regulation of emotions, cognition and stress (Viveros et al., 2005; Zanettini et al., 2011). The typical target of endocannabinoids in the CNS is the G-protein coupled, type-1, cannabinoid receptor (CB−1). Its role in pathogenesis and recovery is well investigated (Manzanares et al., 2004). However, much more recently, PPARs have emerged as new targets for cannabinoids and fatty acid amides for the regulation of pathophysiological functions, including inflammation, oxidative stress, alcohol addiction, and behavioral deficits (Le Foll et al., 2013; Mandrekar-Colucci et al., 2013; Rolland et al., 2013; Locci and Pinna, 2017; Rivera-Meza et al., 2017).

The PPAR family is a ligand-dependent, nuclear hormone receptor, transcription factor family of three isotypes: PPAR–α, PPAR–β/δ, and PPAR–γ (Fidaleo et al., 2014). Of the family, PPAR–β/δ is the least understood, yet it is known to have a role in the development of the CNS and cell survival (Berger and Moller, 2002; Abbott, 2009). PPAR–α and PPAR–γ have similar neurophysiological functions that include regulation of the redox response, neuroinflammation, neurogenesis, cellular differentiation, as well as secondary functions in the regulation of cognition, anxiety, and emotional behavior (Bordet et al., 2006; Bright et al., 2008; Panlilio et al., 2012; Fidaleo et al., 2014). PPAR–α and PPAR–γ are localized in brain regions that are selectively involved in the regulation of emotions and the stress response (Moreno et al., 2004). PPAR–α is most highly expressed in the basal ganglia, amygdala, prefrontal cortex and thalamic nuclei of healthy adults, with lower levels in the hippocampal formation (Warden et al., 2016). PPAR–γ is also highly expressed in the basal ganglia and amygdala, with lower levels in the hippocampal formation, and the thalamic nuclei (Moreno et al., 2004). The significance in the relationship between these regions and emotions has been extensively studied (Shin et al., 2006; Shin and Liberzon, 2010). Together, the basal ganglia, prefrontal cortex, amygdala, thalamus and hippocampus are all key components of the neuronal circuit for fear and anxiety (Shin and Liberzon, 2010), while the basal ganglia, prefrontal cortex and thalamus are critical to mediation of emotional drive and the planning of goal-directed behaviors—which are either exaggerated or depressed during a threat (Haber and Calzavara, 2009). The amygdala is crucial to learning threat-stimuli relationships and the expression of cue-specific fear (Davis, 1992). Its activity is heightened in PTSD, social phobias and related disorders (Shin and Liberzon, 2010). This hyperresponsivity of the amygdala likely dampens the responsivity of the prefrontal cortex, which manifests as hyporesponsivity in PTSD patients (Garcia et al., 1999; Shin and Liberzon, 2010). Additionally, the hippocampus which plays a fundamental role in memory acquisition, consolidation and retrieval, is likely influenced by the amygdala, especially in relation to threatening contexts (McGaugh, 2004).

PPAR–α activation has been shown as a natural response to stress, having the ability to mediate and modulate the stress response (Hillard, 2018). In healthy adults, PEA, an endogenous PPAR–α agonist, significantly increase after clinical stress tests, corresponding with increased cortisol levels (Dlugos et al., 2012). PEA levels increase when healthy individuals experience pain or a depressed mood transiently (Darmani et al., 2005). However, the levels of PEA in PTSD are low (Wilker et al., 2016), suggesting a significant role in emotion regulation. As such, endogenous and synthetic PPAR–α ligands have predictably and successfully stabilized emotions in preclinical models (Locci et al., 2017).

Enhanced fear memory, depressive-like behavior, and aggressive behavior are common characteristics of chronically stressed animals in murine models of PTSD that resemble human symptomology. PPAR–α activation has been assessed regarding its effect on this behavior. PPAR–α agonism rescued rodent behavior in response to stress induced fear. When PPAR–α was activated by exogenous PEA in socially isolated mice, a mouse model of PTSD, fear memory acquisition was reduced, and impaired fear extinction was rescued (Locci and Pinna, 2017; Locci et al., 2017). Similarly, PEA induced a dose-dependent anti-depressant effect (Yu et al., 2011), and reduced aggressive behavior that was blocked by pretreatment with antagonists (Locci et al., 2017). The relationship between PPAR activation and emotional regulation is further supported by its activity in neuroinflammation (O'Leary, 1990; Racke and Drew, 2008; Rolland et al., 2013; Esmaeili et al., 2015; Jeon and Kim, 2016), but even more so, by the localization of PPAR–α in brain areas that regulate mood and emotions.

In an analogous manner, the downregulation of PPAR–γ has been reported to exaggerate basal anxiety, enhance stress sensitivity and produce substantially different stress-induced neuronal activity in the amygdala and hippocampus (Domi et al., 2016). PPAR–γ antagonist, GW9662, produced an anxiogenic-like response, while PPAR–γ agonists did not affect basal anxiety-like behavior (Rosa et al., 2008). Similarly, treatment of rats with the PPAR–γ agonist, rosiglitazone, reduced the systemic response to acute stress, and reduced the heart rate in response to an

acute restraint stress (Ryan et al., 2012). In this study, treated rats also showed a blunted hormonal response (corticosterone levels). However, in contrast to the above, young, unstressed rats treated with rosiglitazone showed an improved response in the hippocampal-dependent fear conditioning task in comparison to control rats (Gemma et al., 2004). This may point to an analogous role for PPAR–γ activation in the treatment of anxiety and/or depression.

ROLE OF ALLOPREGNANOLONE IN COGNITION AND EMOTIONS

3α,5α-tetrahydroprogesterone, also known as allopregnanolone, is a neurosteroid that can be synthesized *de novo* from cholesterol, or from its precursors, pregnenolone and progesterone (Pinna et al., 2006; Schüle et al., 2014). In the CNS, allopregnanolone can function to rapidly alter neuronal excitability by acting as a potent and positive allosteric modulator at post- and extra-synaptic $GABA_A$ receptors, which are highly abundant in glutamatergic neurons (Pinna et al., 2000). These neurons participate in the circuit of fear, and are therefore involved in emotion and anxiety regulation (Möhler, 2012). As such, an imbalance of GABAergic neurotransmission, or endogenous neuromodulators results in abnormal regulation of emotion and abnormal stress responses (Möhler, 2012; Locci and Pinna, 2017). This inhibitory deficit is a known hallmark in anxiety and emotional disorders. Given that allopregnanolone directly binds this receptor, a reduction of allopregnanolone levels correlate to reduced $GABA_A$ receptor activity and dysfunctional behavior (Pinna et al., 2008, 2009).

Intriguingly, the allopregnanolone level in the blood and CSF are reduced in patients of MDD, impulsive aggression, premenstrual dysphoric disorder, PTSD and other disorders of mood and emotions (Rasmusson et al., 2006, 2016; Schüle et al., 2014; Šrámková et al., 2017; Pineles et al., 2018; Rasmusson

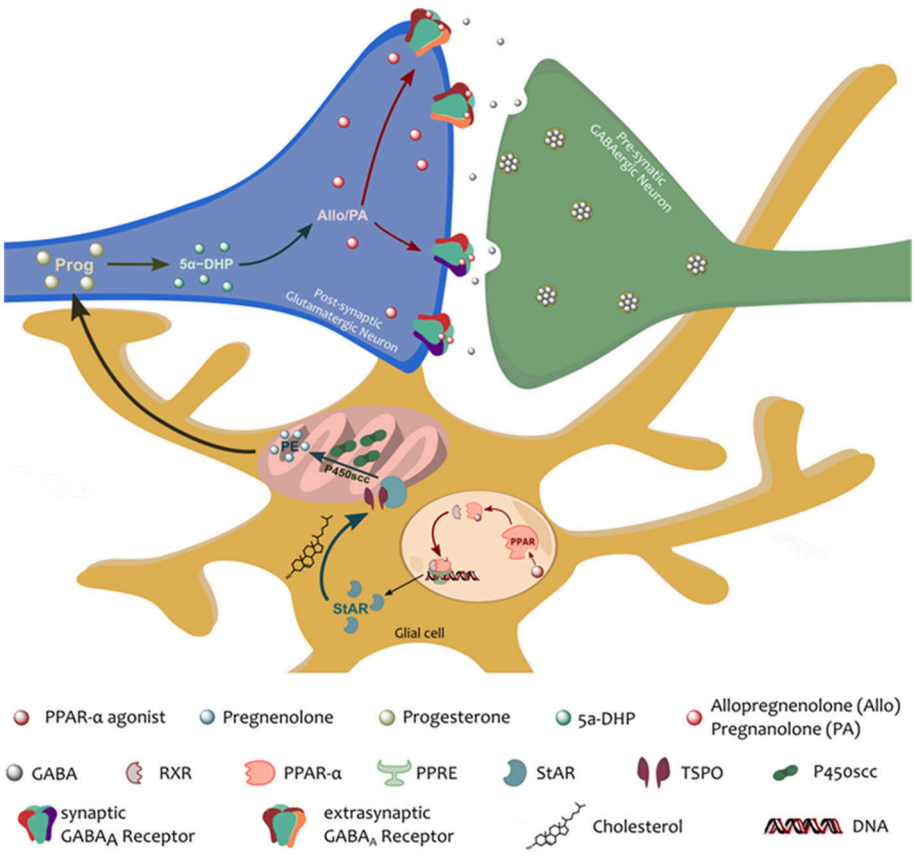

FIGURE 1 | Schematic representation of the proposed *PPAR–α-allopregnanolone biomarker axis*. PPAR–α, following its activation by an endogenous (e.g., PEA) or a synthetic agonist, heterodimerizes with a PPAR–α-specific retinoid X receptor (RXR). The PPAR-RXR dimer then binds the PPAR response element (PPRE) in specific promoter regions that up- or down-regulate gene expression. PPAR–α activation would therefore normalize the stress-induced downregulation of neurosteroidogenic proteins, StAR and p450scc. StAR, is crucial to the translocation of cholesterol into the inner mitochondrial membrane. There, cholesterol is metabolized by the action of the P450scc into pregnenolone (the precursors of all neurosteroids). Pregnenolone can be further converted to progesterone and 5α-dihydroprogesterone (5α-DHP) by the action of 5α-reductase type I. 5α-DHP can then be converted by 3α-hydroxysteroid dehydrogenase into allopregnanolone (Allo) and its equipotent isomer, pregnanolone (PA), which allows for potent, positive, allosteric potentiation of the $GABA_A$ receptors located in the post-synaptic membrane of pyramidal neurons of the frontal cortex and hippocampus, and pyramidal-like neurons of the basolateral amygdala (Agís-Balboa et al., 2006, 2007; Pinna et al., 2008).

and Pineles, 2018). Another interesting phenomenon is the observation that females are twice as likely to experience PTSD as males; 10% of women who experience trauma develop PTSD, compared to only 4% of men (US Department of Veteran Affairs, 2015b). The gender difference in PTSD patients further indicates that neurosteroids may play a large role in the progression and recovery of these disorders, as the difference in neurosteroid concentration contribute to the biological distinction of the sexes (Mendoza et al., 2016). As a specific example, the allopregnanolone level in the CSF of female PTSD patients were 40% lower than in controls, and the allopregnanolone/dehydroepiandrosterone (DHEA) ratio negatively correlates with PTSD re-experiencing (Rasmusson et al., 2006). To this end, studies are being pursued to verify lower levels of allopregnanolone during pregnancy as a predictor of postpartum depression (PPD) (Osborne et al., 2016; Kanes S. et al., 2017).

Early studies have shown that allopregnanolone levels in the brain increase to levels that can activate the GABA receptors, during acute stressful events (Purdy et al., 1991). Subsequently, it has been further hypothesized that the enhancement of GABAergic transmission decreases HPA activity and contributes to the behavioral stress response (Cullinan et al., 2008). Protracted stress, on the other hand, downregulates allopregnanolone biosynthesis (Pinna et al., 2003; Matsumoto et al., 2005, 2007). Indeed, preclinical studies demonstrate that socially isolated mice, known to exhibit enhanced contextual fear responses and impaired fear extinction, also exhibit time-related decreases in allopregnanolone levels in neurons of the medial prefrontal cortex, hippocampus and basolateral amygdala (Agís-Balboa et al., 2006, 2007; Pibiri et al., 2008). The decrease of allopregnanolone was the result of reduced levels of 5α-reductase type I mRNA and protein following social isolation (Dong et al., 2001; Matsumoto et al., 2005, 2007). Hence, these findings suggest that allopregnanolone, its precursors, and analogs of allopregnanolone are suitable treatments for emotional regulation (Pinna and Rasmusson, 2014; Locci et al., 2017). For example, exogenous allopregnanolone attenuated the contextual fear response in a dose-dependent manner. In a similar murine social isolation model of PTSD, researchers showed that allopregnanolone treatment normalized HPA responsiveness and interrupted depressive- and anxiety-like behavior, which are hallmarks of clinical PTSD (Evans et al., 2012). Allopregnanolone analogs, BR351 and BR297, effectively decreased aggression in socially isolated mice, with a lower non-response rate than SSRI-treated mice (Locci et al., 2017). Given preclinical successes, allopregnanolone, its precursors and its analogs are currently being sort after and tested as treatments in psychiatric and related disorders. Recently, allopregnanolone (brexanolone) was evaluated in phase 3 clinical trials for its efficacy against PPD, and successfully achieved primary endpoint (Kanes S. J. et al., 2017). For the phase 2 clinical trial, women were given an intravenous infusion of allopregnanolone, and outcomes were measured using HAM–D (Kanes S. et al., 2017). Of 21 enrolled patients, 70% of treated vs. only 9% of placebo-receiving patients exhibited remission of depressive symptoms. Researchers hypothesize that the action of this drug includes the potentiation of $GABA_A$ receptors (Kose and Cetin, 2017).

THE BRIDGE BETWEEN PPAR–α STIMULATION AND ALLOPREGNANOLONE BIOSYNTHESIS

The summaries above suggest that the role of allopregnanolone in the progression and recovery of psychiatric disorders is similar to the emerging role of PPAR–α. Importantly, these similarities are not limited to their function in emotion regulation. Comparable actions of PPAR–α and allopregnanolone have also been observed across cognition (Cuzzocrea et al., 2013; Fidaleo et al., 2014; Greene-Schloesser et al., 2014), neurogenesis (Ramanan et al., 2009; Fidaleo et al., 2014), neuroinflammation (Daynes and Jones, 2002), neurodegeneration (Naylor et al., 2010; Esmaeili et al., 2015), and substance use disorder (Le Foll et al., 2013; Blednov et al., 2015; Rivera-Meza et al., 2017). Raso et al. suggest that the PPAR–α and allopregnanolone are different substrates of the same mechanism, whereby PEA-induced activation of PPAR–α regulates the biogenesis of allopregnanolone in astrocytes (Raso et al., 2011). To this end, when astrocytes were treated with PEA *in vitro*, an increased expression of enzymes that are crucial to allopregnanolone biosynthesis [steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme (P450scc)] were reported along with increased cytoplasmic concentrations of allopregnanolone. This interdependent relationship between PPAR–α and allopregnanolone has also been alluded to in studies of pain perception. In studies of acute and persistent pain, researchers showed that the usual anti-nociceptive activity of PEA was reduced when activity of enzyme 5α-reductase and P450scc were blocked (Sasso et al., 2012). PEA restored enzyme expression and increased allopregnanolone level in the spinal cord. Further support for this relationship was shown when PEA was used as neuroprotector and regulator of the pentobarbital-evoked hypnotic effect (Sasso et al., 2010). In this case, PEA increased the expression of relevant enzymes and allopregnanolone concentrations in the spinal cord.

These findings suggest that allopregnanolone functions downstream of PPAR–α to mediate its therapeutic effects (**Figure 1**), thus, we further hypothesize that part of the mechanism of action of PPAR–α includes an upregulation of the biosynthesis of neurosteroids (Raso et al., 2011), by upregulating the expression of crucial neurosteroidogenic enzymes. A recent study by Locci and Pinna (2017) further demonstrated the allopregnanolone-dependent effect of PPAR–α-activation. In this study, a single dose of a PPAR–α agonist, PEA or GW7647, normalized the levels of allopregnanolone in socially isolated mice, improved depressive-like and anxiolytic-like behavior, and facilitated impaired extinction of fear memory. The therapeutic-like effects of the PPAR–α agonists were however obstructed by genetic ablation of PPAR–α, antagonism of PPAR–α, and inhibition of neurosteroidogenic enzymes. This and previous studies further support a possible *PPAR–α-allopregnanolone*

biomarker axis in PTSD, and a new therapeutic target for emotional disorders (discussed in Locci et al., 2018).

CONCLUSION

Collectively, these observations provide a relevant case for the design of novel molecules. It suggests that activating PPAR–α may induce a downstream increase of neurosteroid biosynthesis, and that allopregnanolone, pregnanolone, and their analogs can be synthesized to mimic neurosteroid actions at $GABA_A$ receptors. These can therefore provide important and novel steroid-based therapeutics for behavioral deficits in PTSD and other mood disorders. With overlapping symptoms spread across psychiatric disorders like PTSD, MDD and anxiety spectrum disorder, established methodical biomarkers will aid rapid differentiation, identification, prevention, and treatment of PTSD. Given the new relationship pointed out in this opinion article, the biochemical profile of PTSD may include a *PPAR–allopregnanolone biochemical axis* such that subpopulations of PTSD patients may display reduced allopregnanolone levels that can be increased by PPAR–α activation, only in allopregnanolone-deficient patients. Other components of the profile can also include changes in $GABA_A$ receptor subunit expression (Locci and Pinna, 2017), decreased levels of endogenous fatty acid amides such as PEA and OEA (Hillard, 2018), or downregulated expression of PPAR–α. The mechanism by which stress induces changes in these neurochemical targets may be a potential *biomarker axis* relevant to diagnosis and as a novel approach to treat emotional and cognitive impairment in PTSD.

AUTHOR CONTRIBUTIONS

KN wrote an initial draft of this opinion article. KN produced the graphics. GP revised the final version of the manuscript.

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