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TNFα disrupts blood brain barrier integrity to maintain prolonged depressive-like behavior in mice

Yuyan Cheng[1,2], **Sachi Desse**[3], **Ana Martinez**[4], **Ryan J. Worthen**[1,2], **Richard S. Jope**[1,2,a,*], and **Eleonore Beurel**[1,2,*]

[1]Department of Psychiatry and Behavioral Sciences, Miller School of Medicine, University of Miami, Miami, FL 33136

[2]Department of Biochemistry and Molecular Biology, Miller School of Medicine, University of Miami, Miami, FL 33136

[3]Department of Biology, University of Miami, Coral Gables, FL 33146

[4]Centro de Investigaciones Biologicas-CSIC, 28040 Madrid, Spain

Abstract

Recovery from major depressive disorder is difficult, particularly in patients who are refractory to antidepressant treatments. To examine factors that regulate recovery, we developed a prolonged learned helplessness depression model in mice. After the induction of learned helplessness, mice were separated into groups that recovered or did not recover within 4 weeks. Comparisons were made between groups in hippocampal proteins, inflammatory cytokines, and blood brain barrier (BBB) permeability. Compared with mice that recovered and control mice, non-recovered mice displaying prolonged learned helplessness had greater hippocampal activation of glycogen synthase kinase-3 (GSK3), higher levels of tumor necrosis factor-α (TNFα), interleukin-17A, and interleukin-23, increased permeability of the blood brain barrier (BBB), and lower levels of the BBB tight junction proteins occludin, ZO1, and claudin-5. Treatment with the GSK3 inhibitor TDZD-8 reduced inflammatory cytokine levels, increased tight junction protein levels, and reversed impaired recovery from learned helplessness, demonstrating that prolonged learned helplessness is reversible and is maintained by abnormally active GSK3. In non-recovered mice with prolonged learned helpless, stimulation of sphingosine 1-phosphate receptors by Fingolimod or administration of the TNFα inhibitor etanercept repaired the BBB and reversed impaired recovery from prolonged learned helplessness. Thus, disrupted BBB integrity mediated in part by TNFα contributes to blocking recovery from prolonged learned helplessness depression-like behavior. Overall, this report describes a new model of prolonged depression-like behavior and demonstrates that stress-induced GSK3 activation contributes to disruption of BBB integrity

[a]Corresponding author: Richard S. Jope, Miller School of Medicine, University of Miami, 1011 NW 15th Street, Gautier Building room 416, Miami, Florida 33136 USA, phone: 305-243-0262, rjope@med.miami.edu.
[*]These authors contributed equally to this research.

mediated by inflammation, particularly TNFα, which contributes to impaired recovery from prolonged learned helplessness.

Keywords

blood brain barrier; depression recovery; GSK3; learned helplessness; neuroinflammation; TNFα

1. Introduction

Major depressive disorder, which afflicts nearly 17% of people in the United States, is defined by the presence of multiple debilitating symptoms and by their prolonged duration (Kessler et al., 2005). Stress is a common precipitating factor of depression, and stress is often used to induce rodent models of depression (Kessler, 1997; Pariante and Lightman, 2008). These rodent models are evaluated by depression-like behaviors because rodents are not thought to experience the equivalent of major depressive disorder (Gould and Gottesman, 2006; Nestler and Hyman, 2010).

One robust rodent depression model is learned helplessness, in which rodents are exposed to uncontrollable and inescapable mild foot shocks as the aversive stimuli (Chourbaji et al., 2005; Maier and Seligman, 2016; Vollmayr and Gass, 2013). Upon subsequent exposure to foot shocks with escape freely available, rodents that display learned helplessness fail to leave the foot shock chamber. Learned helplessness is considered a behavioral model of depression, particularly involving despair and a coping deficit in response to stress, because it is accompanied by changes typical of depression, including decreased brain regional levels of serotonin, norepinephrine, dopamine, and brain-derived neurotrophic factor (BDNF), decreased home cage activity, elevated corticosteroid levels, increased rapid eye movement during sleep, and displays of other depression-like behaviors, such as impaired social interactions, anhedonia, and increased immobility in the tail suspension and forced swim tests (Chourbaji et al., 2005; Henn and Vollmayr, 2005; Vollmayr and Gass, 2013). Furthermore, susceptibility to learned helplessness is diminished by treatment with antidepressants and other drugs, such as a high dose of lithium (Beurel et al., 2011; Vollmayr and Gass, 2013). Lithium is an inhibitor of glycogen synthase kinase-3 (GSK3), which refers to two isoforms that are predominantly controlled by inhibitory phosphorylation on serine21-GSK3α and serine9-GSK3β (Beurel et al., 2015). Substantial evidence indicates that abnormally active GSK3 promotes depression and animal models of depression, which may derive in part from the promotion of inflammation by GSK3 (Jope, 2011).

Numerous studies have reported treatments and mechanisms that regulate susceptibility to onset of depression-like behaviors in rodents (Gould and Gottesman, 2006; Nestler and Hyman, 2010), but less is known about treatments and mechanisms that control recovery from prolonged depression-like behaviors. We observed that some mice display prolonged learned helplessness, rather than recovering in the usual 2–4 week period. This led us to examine mechanisms that may contribute to impaired recovery. Inflammatory cytokines are elevated in depressed patients, including treatment refractory patients. One of the possible mechanisms whereby cytokines can maintain depressive symptoms is by affecting the blood

brain barrier (BBB). There is evidence of impaired integrity of the BBB in depressed patients (Lavoie et al., 2010; Najjar et al., 2013) and rodent models of depression (Esposito et al., 2001; Friedman et al., 1996; Santha et al., 2015; Sharma et al., 1995), and inflammatory cytokines, such as tumor necrosis factor-α (TNFα), can impair BBB integrity (Rochfort and Cummins, 2015). Endothelial cells connected by tight junctions (TJs) constitute an integral component of the BBB. TJs are comprised of occludin, claudin, ZO-1, ZO-2 and other junction adhesion molecules that maintain structural and functional integrity of the brain endothelium (Obermeier et al., 2013). GSK3 inhibition promotes TJ stability in brain endothelial cells by increasing the levels of occludin and claudin-5 (Ramirez et al., 2013), suggesting GSK3 inhibition may protect the BBB by stabilizing TJ proteins. Decreased levels of TJ proteins and BBB breakdown occur after trauma, stroke, multiple sclerosis, and Alzheimer's disease, conditions associated with increased activated GSK3, neuroinflammation and increased prevalence of depression.

Therefore, we examined links between recovery from prolonged learned helplessness and GSK3, inflammation and BBB integrity. The results show that BBB permeability is increased in mice that do not recover from prolonged learned helplessness, and that recovery and BBB integrity can be improved pharmacologically by treatment with a GSK3 inhibitor, Fingolimod, or anti-TNFα. These results indicate that stress-induced GSK3 activation promotes inflammation that contributes to disruption of BBB integrity, and this is associated with impaired recovery from prolonged learned helplessness.

2. Materials and methods

2.1. Mice and drug administration

These studies used 8–12 week old male wild-type C57BL/6 mice from the National Cancer Institute and Charles River Laboratory. All mice were housed and treated in accordance with NIH and the University of Miami Institutional Animal Care and Use Committee regulations. Mice were treated TDZD-8 (5mg/kg; produced in the Martinez laboratory (Martinez et al., 2002)) which directly inhibits both isoforms of GSK3, the sphingosine 1-phosphate receptor (S1PR) agonist Fingolimod (FTY-720; 1mg/kg; Novartis) intraperitoneally (i.p.) three days before the last session of escapable foot shocks, or the TNFα antagonist etanercept (100μg/mouse, i.p.; Enbrel® Amgen) every other day for a week.

2.2. Learned helplessness

Learned helplessness was induced as described previously (Beurel et al., 2013). Mice were placed in one side of a shuttle box (Med Associates, St. Albans, VT) with the gate between chambers closed, and 180 inescapable foot shocks (IES) were delivered at an amplitude of 0.3 mA, a duration of 8 sec, and a randomized inter-shock interval of 5–45 sec. 24 hr after one session of inescapable foot shocks, mice were returned to the shuttle box and 30 escape trials were carried out with a 0.3 mA foot shock for a maximum duration of 24 sec with door of the chamber opening at the beginning of the foot shock administration to allow mice to escape. Latency to escape the shock was recorded using software from Med Associates, and trials in which the mouse did not escape within the 24 sec time limit were counted as escape failures. Mice with greater than 15 escape failures were defined as learned helpless. Mice

were retested with escapable foot shocks once a week to distinguish mice that failed to recover from learned helplessness (continually failing to escape), termed here prolonged learned helplessness, from mice that spontaneously recovered from learned helplessness. Where indicated, mice displaying prolonged learned helplessness were treated with drugs followed by texting learned helplessness.

2.3. Cytokine measurements

Cytokine levels were measured as previously described (Cheng et al., 2016). Hippocampi were homogenized in ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium vanadate, 50 mM sodium fluoride, and 100 nM okadaic acid. The lysates were centrifuged at 15,000 g for 10 min to remove insoluble debris, and protein concentrations in the supernatants were determined using the Bradford protein assay. Enzyme-linked immunosorbent assays (ELISA) were performed according to the manufacturer's instructions (eBioscience) using 100–150 μg protein. ProcartaPlex Multiplex Immunoassay for IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-9, IL-10, IL-12(p70), IL-13, IL-17A, IL-18, IL-23, IL-27, IL-28, IFN-γ, CXCL10, M-CSF, CCL7, CCL11, CXCL5 and TNFα were measured using 50 μg protein from hippocampal extracts according to the manufacturer's instructions (eBiosience) with the same control samples loaded on multiple plates to control for inter-plate variability. Cytokine concentrations were determined by the multiplex assay reader (Magpix; Luminex) and Bio-Plex manager software (Bio-Rad). We noted that the ELISA and multiplex assays reported different absolute levels of cytokines, so only one or the other was used for determining all of the values shown in each panel.

2.4. Western blotting

Brain regions were isolated 3 hr after the last escapable foot shock treatment, and western blotting was performed as described previously (Cheng et al., 2016). In brief, mouse hippocampus and prefrontal cortex were rapidly dissected in ice-cold phosphate-buffered saline, snap-frozen, and stored at −80° C before use. Brain regions were homogenized in ice-cold Triton-lysis buffer, and protein concentrations in the supernatants were determined using the Bradford protein assay. Proteins (10–20 μg) in brain region extracts were resolved with SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies to high mobility group box 1 protein (HMGB1; ab18256, Abcam), phospho-Ser9-GSK3β (#9322, Cell Signaling Technology), phospho-Ser21-GSK3α (#9316, Cell Signaling Technology), total GSK3α/β (05–412, Millipore), Rac1 (#ARC03, Cytoskeleton, Inc.), occludin (33–1500, Thermo Fisher Scientific), claudin-5 (35–2500, Thermo Fisher Scientific), ZO-1 (40–2200, Thermo Fisher Scientific), ZO-2 (71–1400, Thermo Fisher Scientific) and reblotted with β-actin (Sigma Aldrich) as the loading control. Chemiluminescent signals were acquired using an Amersham Imager 600 (GE Healthcare Life science), which determines the most high resolution digital image. The images were quantified using the IQTL software (GE Healthcare Life science).

2.5. Blood brain barrier permeability assay

Mice were given 200 μl 10% sodium fluorescein in PBS (i.p.). After 2 hr, cardiac blood was collected, and mice were transcardially perfused with 0.9% saline. Prefrontal cortex and hippocampus were isolated, weighed, homogenized in PBS, and the lysates were centrifuged at 14,000g for 5 min. 500 μL of the supernatant was mixed with 500 μL of 15% TCA and centrifuged at 1,000g for 10 min. 500 μL of the supernatant was mixed with 125 μl 5 N NaOH, and fluorescence was measured in100 μl of the mixture by SpectraMaxIII (excitation 485 nm, emission 530 nm). Serum was recovered from the blood by centrifuging at 1,000 g for 10 min. Serum was mixed with 15% TCA (1:10), neutralized with 5 N NaOH (4:1), diluted 1:500 in PBS, and fluorescence was measured in 100 μl of diluted serum. BBB permeability was calculated as the ratio of brain region to serum fluorescence intensity, and displayed as fold increase compared to the permeability in non-stressed control mice.

2.6. Statistical analysis

The data were analyzed with Student's t-test, Wilcoxon matched-pairs signed-ranks test, or one-way or two-way ANOVA with Bonferroni post-hoc test for multiple comparisons. Data represent means±s.e.m., and significance was set at $p<0.05$.

3. Results

3.1. Mice unable to recover from learned helplessness display increased active hippocampal GSK3

After inducing learned helplessness, mice were retested with escapable foot shocks once a week to determine the rate of recovery. Whereas ~80% of mice recovered from learned helplessness within 3–4 weeks (Figure 1A), after 4 and 5 weeks ~20% of mice remained learned helpless (hereafter referred to as non-recovered mice). The mice that remained learned helpless for 5 weeks had 29 ± 0.3, 29 ± 0.5, 28 ± 0.7, 28 ± 0.7 and 27 ± 0.9 escape failures in weeks 1, 2, 3, 4, and 5, respectively, demonstrating the consistent failure to escape of the mice that displayed prolonged learned helplessness. To test if weekly exposure to escapable foot shocks influenced the failure of a cohort of mice to recover, learned helplessness was induced and the mice were not tested again for 4 weeks. This paradigm resulted in 30% of mice remaining learned helpless (Figure S1A), closely matching the results with weekly testing. Further experiments used the weekly testing paradigm. We also noted that recovered mice have adapted to foot shock stress and are not equivalent to untreated control mice, as re-exposure to the learned helpless paradigm did not induce learned helplessness in any recovered mice, whereas typically ~70% of naive mice develop learned helplessness (Figure S1B).

Mice were separated into two cohorts, those that recovered or did not recover from learned helplessness after 5 weeks. The induction of learned helplessness causes activation of hippocampal GSK3 by decreased phosphorylation on the inhibitory serines in both isoforms (Cheng et al., 2016; Polter et al., 2010). We found that GSK3 activation persisted in mice that did not recover from learned helplessness. Compared with recovered mice, non-recovered mice had lower hippocampal levels of the inhibitory serine-phosphorylated GSK3α (Figure 1B; one-way ANOVA; $F(2,12)=5.641$; $p<0.05$) and serine-phosphorylated

GSK3β (Figure 1C; $F(2,12)=16.80$; $p<0.05$). Total levels of GSK3α and GSK3β did not differ between groups (Figure S1C and S1D), indicating changes in activation, not expression, occurred. Reduced levels of phospho-serine-GSK3α/β were not due to the recent exposure to escapable foot shocks because GSK3α/β serine-phosphorylation was not altered by a single session of escapable foot shocks in otherwise untreated mice, although there appeared to be a tendency for reduced phospho-Ser9-GSK3β, which would affect both recovered and non-recovered mice in the prolonged learned helplessness paradigm (Figure S2). Thus, GSK3 remains activated during prolonged learned helplessness, suggesting that maintenance of active GSK3 may contribute to impaired recovery from learned helplessness. To test this, non-recovered mice were treated with the selective GSK3 inhibitor TDZD-8 (5mg/kg; i.p.) for 3 days prior to the 4th week of testing escapable foot shocks. TDZD-8 treatment induced recovery from learned helplessness in 75% of the non-recovered mice, whereas all non-recovered mice treated with saline remained learned helpless (Figure 1D; Wilcoxon $W=24.00$; $n=8$ for TDZD-8 treated mice; $p<0.05$). These results demonstrate that maintenance of active GSK3 contributes to impaired recovery and that prolonged learned helplessness is reversible.

3.2. Mice unable to recover from learned helplessness display increased cytokines in the hippocampus

Learned helplessness induction is accompanied by increases in several cytokines in the hippocampus mediated in part by a signaling pathway involving increased hippocampal levels of the alarmin protein HMGB1, an agonist of Toll-like receptor-4 (TLR4), activation of TLR4, and activation of GSK3 (Cheng et al., 2016). Compared with recovered mice, non-recovered mice had higher hippocampal HMGB1 levels (Figure 2A; one-way ANOVA; $F(2,16)=6.369$; $p<0.05$), TNFα (Figure 2B; $F(2,15)=9.689$; $p<0.05$), IL-17A (Figure 2C; $F(2,15)=12.55$; $p<0.05$) and IL-23 (Figure 2D; $F(2,15)=14.80$; $p<0.05$), whereas hippocampal levels of 18 other cytokines did not differ between groups (Figure S3). In non-recovered mice that were treated with TDZD-8, compared with saline-treated non-recovered mice there were significantly lower hippocampal levels of TNFα (Figure 2E; Student's t-test; $t(9)=2.508$; $p<0.05$), IL-17A (Figure 2F; $t(8)=2.979$; $p<0.05$) and IL-23 (Figure 2G; $t(9)=3.239$; $p<0.05$). Thus, increased activation of hippocampal GSK3 and elevated hippocampal levels of three inflammatory cytokines are associated with impaired ability to recover from learned helplessness, and inhibition of GSK3 effectively decreases hippocampal cytokine levels and promotes recovery.

3.3. Mice unable to recover from learned helplessness display decreased hippocampal levels of Rac1 and tight junction proteins

To determine possible mechanisms by which cytokines may prevent recovery from learned helplessness, we examined levels of Rac1 because it is decreased in the nucleus accumbens in the chronic social defeat mouse model of depression (Golden et al., 2013), Rac1 is regulated by GSK3 (Rehani et al., 2009), and Rac1 regulates TJs. Hippocampal levels of Rac1 were lower in non-recovered mice than recovered mice (Figure 3A; one-way ANOVA; $F(2,10)=6.119$; $p<0.05$). Rac1, as well as GSK3 and inflammatory cytokines, can regulate TJ proteins that contribute to the BBB, leading us to test if there were differences between the groups of mice in BBB permeability and hippocampal levels of tight junction proteins.

BBB permeability assessed by the hippocampal accumulation of sodium fluorescein was significantly greater in non-recovered than recovered mice (Figure 3B; $F(2,16)=9.276$; $p<0.05$), indicating increased BBB permeability in the hippocampus. BBB permeability was also significantly higher in the prefrontal cortex of non-recovered mice compared to non-stressed control mice (Figure S4; $F(2,8)=6.602$; $p<0.05$), demonstrating that increased BBB permeability in prolonged learned helplessness is not limited to the hippocampus. Compared with recovered mice, non-recovered mice had lower hippocampal levels of the TJ proteins occludin (Figure 3C; $F(2,10)=5.934$, $p<0.05$), ZO-1 (Figure 3D; $(2,10)=7.437$; $p<0.05$) and claudin-5 (Figure 3E; $F(2,10)=5.549$; $p<0.05$), but not ZO-2 (Figure 3F; $F(2,10)=0.877$; $p>0.05$). We tested if the weekly exposure to escapable foot shocks could affect any of these measures, and found none altered by exposure of mice to only escapable foot shocks without previous inescapable foot shock treatment (Figure S2). TDZD-8 treatment of non-recovered mice, which induced recovery from learned helplessness, increased the hippocampal levels of Rac1 (Figure 3G; Student's t-test; $t(9)=3.086$; $p<0.05$), occludin (Figure 3H; $t(5)=2.845$; $p<0.05$), ZO-1 (Figure 3I; $t(9)=2.292$; $p<0.05$) and claudin-5 (Figure 3J; $t(5)=5.116$; $p<0.05$) compared with saline-treated mice that remained learned helpless. Interestingly, treatment with TDZD-8 of mice that had recovered from learned helplessness did not alter hippocampal levels of TJ proteins (Figure S5), indicating that inhibition of GSK3 normalizes the learned helplessness-associated deficits in TJ protein levels. These results demonstrate that non-recovery from learned helplessness is associated with impaired integrity of the BBB and decreased hippocampal levels of TJ proteins.

3.4. Acute learned helplessness increases permeability of the BBB

To determine if the differences between non-recovered and recovered mice occurred early or if they developed later, measurements were made after the initial induction of learned helplessness. Hippocampi were analyzed separately in mice that developed learned helplessness or did not (called non-depressed for brevity) (Figure 4A). We previously reported that GSK3 is activated by inducing learned helplessness (Cheng et al., 2016; Polter et al., 2010). Compared to non-depressed mice, learned helpless mice had significantly higher hippocampal levels of TNFα (Figure 4B; Student's t-test; $t(6)=2.979$; $p<0.05$) and IL-12 p70 (Figure 4C; $t(6)=3.241$; $p<0.05$), but not IL-17A, IL-23, or several other cytokines (Figure S6). Mice displaying learned helplessness had significantly greater hippocampal BBB permeability than non-depressed mice (Figure 4D; one-way ANOVA; $F(2,11)=8.273$; $p<0.05$), lower hippocampal levels of occludin (Figure 4E; $F(2,21)=5.501$; $p<0.05$) than non-depressed mice, and lower levels of ZO-1 (Figure 4F; $F(2,21)=7.594$; $p<0.05$) than untreated mice, whereas there were not significant difference in hippocampal levels of claudin-5 and Rac1 (Figure S7A and S7B). These results suggest that the changes identified in non-recovered mice reflect a continuum of alterations initially induced in the hippocampus during acute learned helplessness, including GSK3 activation, increased TNFα, increased BBB permeability, and decreased occludin, whereas increased IL-17A and IL-23, and decreased claudin-5 and ZO-1 developed during prolonged learned helplessness.

3.5. Fingolimod and etanercept stabilize the BBB and promote recovery from learned helplessness

To test if increased BBB permeability was important in prolonging learned helplessness, we used Fingolimod, an agonist of sphingosine 1-phosphate (S1P) receptors that enhances endothelial barrier integrity (McVerry and Garcia, 2004; Prager et al., 2015). We first examined the effects of Fingolimod administration on acute learned helplessness. Fingolimod treatment blocked the increased hippocampal BBB permeability caused by acute induction of learned helplessness (Figure 5A; one-way ANOVA; $F(2,19)=7.174$; $p<0.05$). Repair of the BBB permeability by Fingolimod was accompanied by reduced susceptibility to the induction of acute learned helplessness, as only 37.5% of mice treated with Fingolimod developed learned helplessness compared with 75% of saline-treated mice (Figure 5B).

Fingolimod treatment also repaired the integrity of the BBB in mice with prolonged learned helplessness (Figure 5C; Student's t-test; $t(10)=3.352$; $p<0.05$), reduced hippocampal TNFα (Figure 5D; $t(10)=2.602$; $p<0.05$) and IL-23 (Figure 5E; $t(10)=3.302$; $p<0.05$) levels, but not IL-17A (Figure S8A; $t(9)=1.208$; $p>0.05$), and increased hippocampal levels of occludin (Figure 5F; one-way ANOVA; $F(2,14)=11.36$; $p<0.05$), and Rac1 (Figure 5G; $F(2,14)=14.02$; $p<0.05$), but not claudin-5 (Figure S8B; $F(2,14)=3.669$; $p>0.05$) or ZO-1 (Figure S8C; $F(2,8)=1.826$; $p>0.05$). Along with repairing the BBB, Fingolimod treatment of non-recovered mice with prolonged learned helplessness induced a rapid recovery in 62.5% of mice, whereas no mice treated with saline recovered (Figure 5H; Wilcoxon $W=21$; $n=8$ for Fingolimod treated mice; $p<0.05$). Thus, Fingolimod treatment bolstered BBB integrity and promoted recovery from prolonged learned helplessness.

The consistent association of elevated hippocampal TNFα levels with impaired BBB integrity and inability to recover from learned helplessness led us to test the effects of the TNFα inhibitor etanercept. Etanercept (100μg/mouse, i.p.) treatment induced recovery of 58% of mice with prolonged learned helplessness, whereas all mice treated with PBS remained learned helpless (Figure 5I; Wilcoxon $W=36$; $n=12$ for etanercept treated mice; $p<0.05$). The etanercept-induced recovery from learned helplessness was associated with reduced BBB permeability (Figure 5J; $F(2,16)=9.707$; $p<0.05$) and reduced hippocampal levels of TNFα (Figure 5K; $t(9)=3.16$; $p<0.05$) and IL-23 (Figure 5L; $t(9)=12.85$; $p<0.05$), but not IL-17A (Figure S8D; $t(9)=0.3572$; $p>0.05$), whereas tight junction proteins were unaltered (Figure S8E–H; Rac1: $F(2,11)=3.723$; occludin: $F(2,11)=0.5005$; claudin-5: $F(2,11)=1.864$; ZO-1: $F(2,11)=0.08858$).

4. Discussion

We describe here a mouse model for studying mechanisms that regulate recovery from a long-term depression-like behavior, which revealed that disrupted BBB integrity is associated with impaired recovery from prolonged learned helplessness. In this model, ~20% of mice maintained learned helplessness for at least 5 weeks, whereas ~80% spontaneously recovered within 1–4 weeks. This model differs from others that apply the inducing stressors repeatedly to maintain depression, such as chronic unpredictable mild stress (several weeks), chronic restraint stress (2–3 weeks), and repeated social defeat stress (6 days). In the

prolonged learned helplessness model, although mice are exposed weekly to escapable foot shocks, those are not sufficient to induce learned helplessness, and 30% of mice maintained learned helplessness for 4 weeks without intermittent escapable foot shocks. Identified characteristics that contribute to impaired recovery from learned helplessness include activated GSK3, increased BBB permeability, and increased TNFα in the hippocampus. Impaired recovery was not irreversible, as treatment with a GSK3 inhibitor, an S1P receptor agonist, or anti-TNFα, induced rapid recovery from prolonged learned helplessness in most mice.

4.1. Blood-brain barrier

The BBB permeability increased in the hippocampus of mice immediately after learned helplessness induction, and this was maintained in mice with prolonged learned helplessness, whereas the BBB permeability had normalized in mice that recovered from learned helplessness. This disrupted integrity of the BBB associated with learned helplessness agrees with previous reports of increased BBB permeability in rodent brain after immobilization stress or forced swimming stress (Esposito et al., 2001; Friedman et al., 1996; Santha et al., 2015; Sharma et al., 1995). Some evidence also indicates that there is increased BBB permeability in depressed patients (Lavoie et al., 2010; Najjar et al., 2013). A limitation of our results is that we focused on the hippocampus because of its association with depression (Campbell and MacQueen, 2004), but obviously mechanisms regulating depression and recovery also include other brain regions that should be examined in the future. Indeed, the key finding of impaired BBB integrity was also identified in the prefrontal cortex of mice with prolonged learned helplessness. The compromised integrity of the BBB associated with impaired recovery from learned helplessness led us to examine potential contributors and interventions.

4.2. Neuroinflammation in prolonged learned helplessness

Inflammation is induced by stress and is associated with depression (Miller and Raison, 2016). TNFα, IL-1β, and IL-6 are particularly increased in the blood and postmortem brains of depressed patients, and in animal models of depression, including learned helplessness (Cheng et al., 2016; Hughes et al., 2016; Miller and Raison, 2016). Mice with impaired recovery from learned helplessness had elevated hippocampal levels of TNFα, IL-17A and IL-23, but not IL-6, IL-1β, or other cytokines that were elevated by acute learned helplessness (Cheng et al., 2016), demonstrating a high selectivity in the elevation of these three cytokines and raising the possibility that they may contribute to impaired recovery. Increases in TNFα, IL-17A, and IL-23 may be caused by the prolonged increase in HMGB1, an agonist of TLR4 receptors, but it is not clear why only these three cytokines would be elevated by this mechanism. IL-17A is primarily produced by T helper 17 (Th17) cells, and IL-23 promotes the differentiation of Th17 cells and IL-17A release (McKenzie et al., 2006). Increases in these two cytokines in non-recovered mice are interesting in light of previous reports that Th17 cells are increased in brain regions of learned helplessness mice (Beurel et al., 2013) and in the blood of depressed patients (Chen et al., 2011). TNFα, but not IL-17A or IL-23, was increased after acute induction of learned helplessness, suggesting that elevated TNFα is maintained for a long period in mice that do not recover, and that TNFα may contribute to failure to recover from prolonged learned helplessness. This might

result from TNFα-induced impairments in the BBB integrity, as BBB permeability was increased in TNFα-treated mice (Rosenberg et al., 1995), and anti-TNFα treatment blocked increased BBB permeability induced by sepsis or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Tsao et al., 2001; Zhao et al., 2007). These results are consistent with a previous report that deletion of either TNFR1 or TNFR2 is antidepressant in mice (Simen et al., 2006). Furthermore, etanercept treatment rescued BBB integrity and recovery from prolonged learned helplessness. This is particularly important considering that several clinical trials have found effective antidepressant effects of anti-TNFα drugs in a portion of patients (Kappelmann et al., 2016; Raison et al., 2013) and chronic etanercept treatment was antidepressant in rats in the forced swim test (Bayramgurler et al., 2013; Krugel et al., 2013). Thus, one mechanism by which learned helplessness can be prolonged appears to be via elevated levels of TNFα, which contribute to disruption of the integrity of the BBB.

4.3. Tight junction proteins

We tested if decreased tight junction proteins might contribute to increased BBB permeability during prolonged learned helplessness because other stresses both reduce tight junction proteins and increase BBB permeability (Jiao et al., 2011; Lochhead et al., 2010). There was a tendency for acute learned helplessness to lower hippocampal tight junction proteins, particularly occludin, but the decreases were modest compared to the increase in BBB permeability. More robust reductions of occludin, ZO-1, and claudin-5 were evident in the hippocampus of mice with prolonged learned helplessness, suggesting that reductions in tight junction proteins develop over time during prolonged learned helplessness, possibly contributing to increased BBB permeability and to impaired recovery. These decreases might be linked to the elevations in TNFα and IL-17A in non-recovered mice because treatment with either TNFα or IL-17A reportedly decrease tight junction protein levels and increase BBB permeability (Kebir et al., 2007). Decreased Rac1 also may contribute to changes in tight junction proteins and BBB permeability because both are reduced by deficient Rac1 (Garcia et al., 2001; Obermeier et al., 2013), and GSK3 activity decreases Rac1 activity (Rehani et al., 2009), which might exacerbate the stress-induced lowering of Rac1. Furthermore, Rac1 levels are low in postmortem nucleus accumbens from patients with major depressive disorder and in mice that are susceptible, but not resilient, to chronic social defeat stress (Golden et al., 2013). Thus, low Rac1 levels and elevated TNFα and IL-17A may contribute to impaired tight junctions and disrupted BBB to promote prolonged depression-like behavior.

4.4. Treatment of prolonged learned helplessness

Hippocampal GSK3 was activated in mice with impaired recovery from prolonged learned helplessness, and TDZD-8 treatment promoted recovery in 75% of previously non-recovered mice, demonstrating that active GSK3 contributes to maintaining prolonged learned helplessness. Accompanying recovery, TDZD-8 treatment lowered hippocampal levels of TNFα, IL-17A, and IL-23, consistent with much evidence that GSK3 inhibition reduces the production of inflammatory cytokines (Martin et al., 2005) and raising the possibility that GSK3 may impair recovery from learned helplessness by promoting cytokine production. TDZD-8 treatment also increased hippocampal levels of Rac1 and the tight junction proteins occludin, claudin-5, and ZO-1 in mice with prolonged learned helplessness. This is

consistent with previous evidence that GSK3 inhibitors increased the levels of occludin and claudin-5 in brain endothelial cells, which was attributed to increased stability, not transcriptional regulation, and increased barrier tightness, and expression of mutated constitutively active GSK3 reduced barrier integrity (Ramirez et al., 2013). Also, GSK3 inhibitor-treated brain endothelial cells had increased association of ZO-1 and β-catenin (Ramirez et al., 2013), a target of GSK3 (Beurel et al., 2015), and β-catenin signaling maintains BBB integrity (Liebner et al., 2008; Tran et al., 2016). Thus, abnormally active GSK3 in prolonged learned helplessness contributes to both maintenance of elevated inflammatory cytokines and decreased tight junction protein levels, and therefore appears to be a target for reversing prolonged depression-like behavior.

Fingolimod treatment bolstered BBB integrity, and this was associated with rapid recovery in 63% of prolonged learned helpless mice. Fingolimod is a lipophilic agonist of S1P receptors that crosses the BBB and it is approved for treating multiple sclerosis (Miron et al., 2008). Fingolimod-induced increased BBB integrity is consistent with substantial previous evidence. Activation of the S1P1 receptor activates Rac1, modifies tight junctions, and enhances endothelial barrier integrity (Garcia et al., 2001; Gonzalez et al., 2006; Nishihara et al., 2015; Prager et al., 2015; Singleton et al., 2005), demonstrating that Fingolimod protects the BBB. Fingolimod also reduced BBB permeability in human brain endothelial cells (Brinkmann et al., 2004; Lee et al., 2006; Spampinato et al., 2015). Moreover, a recent study reported that Fingolimod is antidepressant in the forced swim test after chronic stress (di Nuzzo et al., 2015). Although Fingolimod treatment of non-recovered mice normalized BBB permeability, this may not result from changes in tight junction proteins, as Fingolimod treatment only increased the level of hippocampal occludin, suggesting that Fingolimod increases BBB integrity by other mechanisms. Fingolimod could repair the BBB by its reduction of hippocampal TNFα and IL-23, which were elevated in the mice unable to recover from learned helplessness. This supports previous evidence of anti-inflammatory effects of Fingolimod, which downregulated TNFα and IL-1β in the CNS of animal models of multiple sclerosis or epilepsy (Foster et al., 2009; Gao et al., 2012). Thus, Fingolimod treatment increased the integrity of the BBB and promoted recovery from prolonged learned helplessness.

4.5. Conclusions

In summary, this study provides a new model of prolonged depression-like behavior that can be used to identify mechanisms that distinguish differences between mice that are not able to recover from learned helplessness from those that recover spontaneously. Impaired inhibitory control of GSK3, elevated TNFα and IL-17A, and impaired BBB integrity were identified as key components contributing to blocking the recovery from prolonged learned helplessness. We speculate that impaired capacity to recover from stress-induced GSK3 activation causes its well-documented promotion of the production of cytokines that in turn impair BBB integrity to hinder recovery from learned helplessness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- We describe a prolonged (>4 weeks) learned helplessness (LH) depression model in mice
- Prolonged LH was associated with elevated hippocampal TNFα, IL-17A and IL-23 levels
- Blood-brain barrier (BBB) disruption was evident in mice with prolonged LH
- Prolonged LH and elevated cytokines were rapidly reversed by Fingolimod or TNFα inhibitor treatment
- TNFα-mediated disruption of the BBB may contribute to impaired recovery from LH

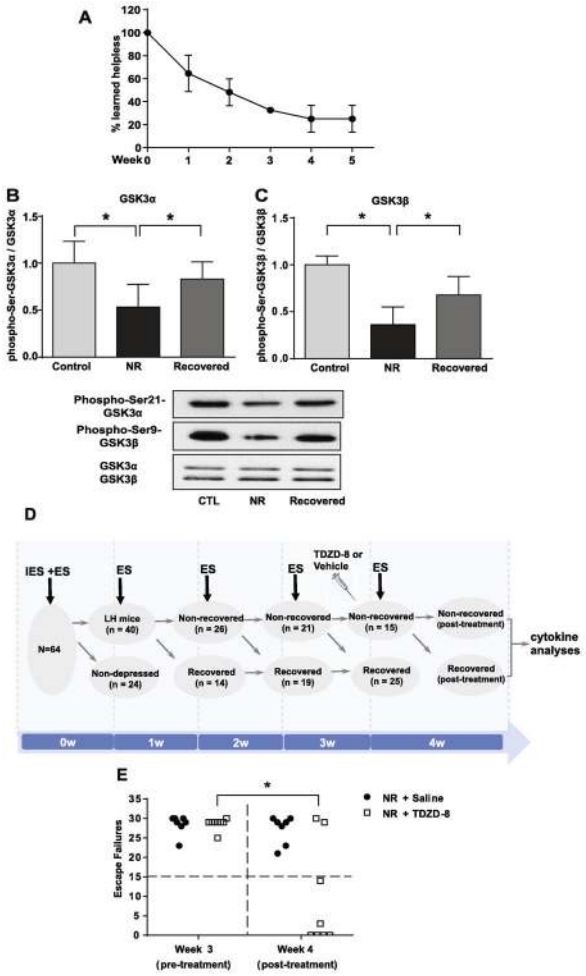

Figure 1. Mice unable to recover from learned helplessness display activated GSK3 in the hippocampus

(A) Mice were subjected to the learned helplessness paradigm at week 0, and recovery from learned helplessness was measured in weekly sessions of escapable foot shocks. Data are shown as the percent learned helpless mice (failing to escape >15 out of 30 trials) each week (n = 77 mice, in 5 different cohorts), excluding mice (n=35) that did not develop learned helplessness initially. Mice that were non-recovered (NR) from learned helplessness were separated from those that recovered (R) at week 5. Hippocampi in non-stressed control (CTL) mice, non-recovered mice, or recovered mice were collected 3 hr after the last escapable foot shocks, followed by measurement of hippocampal levels of serine-phosphorylated **(B)** GSK3α, **(C)** GSK3β and total GSK3α/β (GSK3) by western blot (n = 4–6 mice in each group). Graphs represent means±SEM; *p<0.05 compared to values in untreated control mice or recovered mice (one-way ANOVA with Bonferroni post-hoc test for multiple comparisons). Hippocampal serine-phosphorylated GSK3α/β in recovered mice did not significantly differ from control mice, e.g., for phospho-ser9-GSK3β t=2.67; p>0.05. **(D)** Experimental timeline of prolonged learned helplessness and recovery after treatment with the GSK3 inhibitor TDZD-8. For this experiment 64 mice were subjected to the learned helpless protocol at week 0 (IES = inescapable foot shocks, ES = escapable foot shocks) and

mice that initially displayed learned helplessness (LH) were given three weekly sessions of escapable foot shocks. After 3 weeks and 4 days, non-recovered LH mice were treated with TDZD-8 (5 mg/kg, i.p.) or saline daily for three days prior to testing escapable foot shocks at week 4. The numbers of mice in each category are shown. **(E)** Data show escape failures in non-recovered mice before (week 3) and after (week 4) treatment with TDZD-8 (n=8 mice) or saline (n = 7 mice). Each symbol represents escape failures for an individual mouse. *p<0.05 comparing escape failures in mice before (week 3) and after (week 4) treatment with TDZD-8 (Wilcoxon matched pair test).

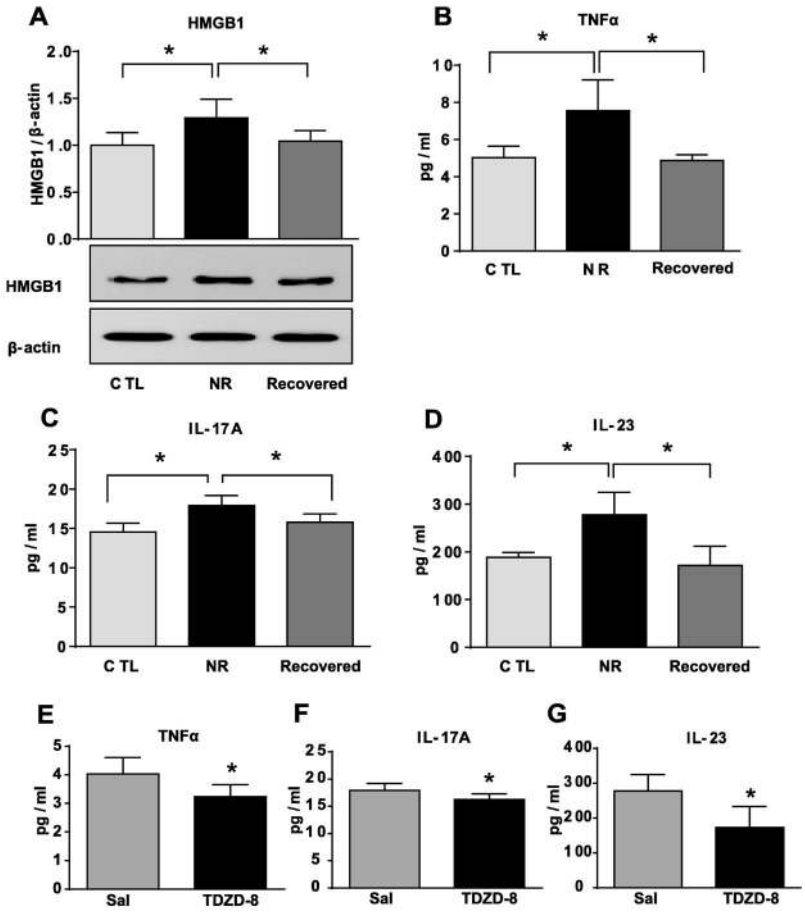

Figure 2. Mice unable to recover from learned helplessness display increased cytokine levels in the hippocampus

Mice were subjected to learned helplessness paradigm at week 0, and were retested with escapable foot shocks weekly for five weeks. Mice that were non-recovered (NR) from learned helplessness were separated from those recovered at week 5. Hippocampi in non-stressed control (CTL) mice, NR mice, or recovered mice were collected 3 hr after the last escapable foot shocks. Hippocampal levels of **(A)** HMGB1 were measured by western blot, and hippocampal levels of **(B)** TNFα, **(C)** IL-17A, and **(D)** IL-23 were measured by multiplex (n = 4–6 mice in each group). *p<0.05 compared to values in untreated control mice or recovered mice (one-way ANOVA with Bonferroni post-hoc test for multiple comparisons). Another set of mice was subjected to learned helplessness at week 0, and after three weekly sessions of escapable foot shocks, non-recovered (NR) mice were treated with TDZD-8 (5 mg/kg, i.p.) or saline for three days prior to testing escapable foot shocks at week 4. Hippocampi were collected at 3 hr after the last session of escapable foot shocks at week 4, followed by ELISA measurements of **(E)** TNFα, **(F)** IL-17A and **(G)** IL-23 in the mice that recovered from learned helplessness after TDZD-8 treatment and the mice that remained depressed after saline treatment (n = 6–7 mice in each group). Graphs represent means±SEM; *p<0.05 compared to values in saline-treated mice (Student's t-test).

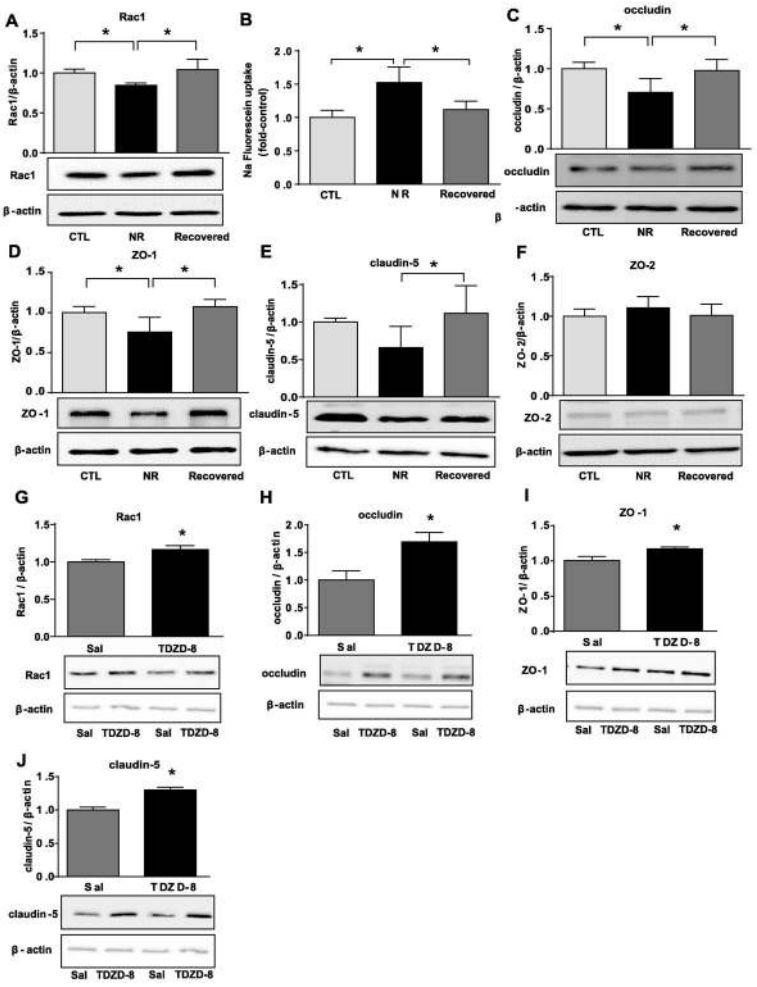

Figure 3. Mice unable to recover from learned helplessness display decreased hippocampal levels of Rac1 and tight junction proteins

Mice were subjected to learned helplessness paradigm at week 0, and were retested with escapable foot shocks weekly for five weeks. Mice that were non-recovered (NR) from learned helplessness were separated from those recovered at week 5. **(A)** Hippocampi in non-stressed control (CTL) mice, NR mice, or recovered mice were collected 3 hr after the last escapable foot shocks and hippocampal levels of Rac1 were measured by western blot. **(B)** BBB permeability in the hippocampus was measured by the sodium fluorescein assay (n = 5–7 mice in each group). Hippocampi in non-stressed control (CTL) mice, NR mice, or recovered mice were collected 3 hr after the last escapable foot shocks and hippocampal levels of **(C)** occludin**, (D)** ZO-1, **(E)** claudin-5 and **(F)** ZO-2 were measured by western blot (n = 4–6 mice in each group). *p<0.05 compared to values in untreated control mice or recovered mice (one-way ANOVA with Bonferroni post-hoc test for multiple comparisons). Another set of mice was subjected to learned helplessness at week 0, and after three weekly sessions of escapable foot shocks, non-recovered (NR) mice were treated with TDZD-8 (5 mg/kg, i.p.) or saline for three days prior to testing escapable foot shocks at week 4. Hippocampi were collected at 3 hr after the last session of escapable foot shocks at week 4, followed by measurement of **(G)** Rac1, **(H)** occludin**, (I)** ZO-1, **(J)** and claudin-5 by western

blot (n = 6–7 mice in each group). Graphs represent means±SEM; *p<0.05 compared to values in saline-treated mice (Student's t-test).

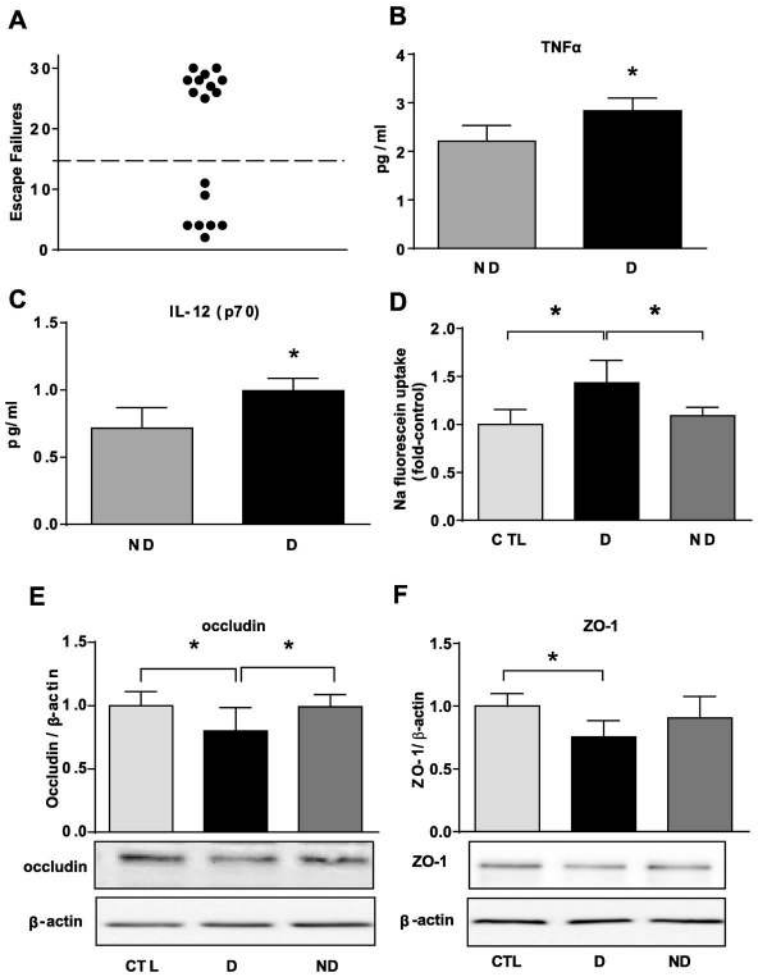

Figure 4. Acute learned helplessness increases permeability of the BBB

(A) Mice were subjected to the learned helplessness paradigm, and escape failures from escapable foot shocks were recorded. Each symbol represents escape failures for an individual mouse. Hippocampi were collected 3 hr after the escapable foot shocks from learned helpless (D; depressed) and non-learned helpless (ND; non-depressed) mice. Hippocampal levels of **(B)** TNFα and **(C)** IL-12 (p70) were measured by multiplex (n = 3–5 in each group). *p<0.05 compared to values in ND mice (*p<0.05; Student's t-test). **(C)** BBB permeability was determined by measuring fluorescein uptake into the hippocampus (n = 6–8 in each group). Tight junction proteins **(E)** occludin and **(F)** ZO-1 were measured by western blot (n = 6–8 in each group). *p<0.05 compared to values in untreated control mice or non-depressed mice (one-way ANOVA with Bonferroni post-hoc test for multiple comparisons). Graphs represent means±SEM;

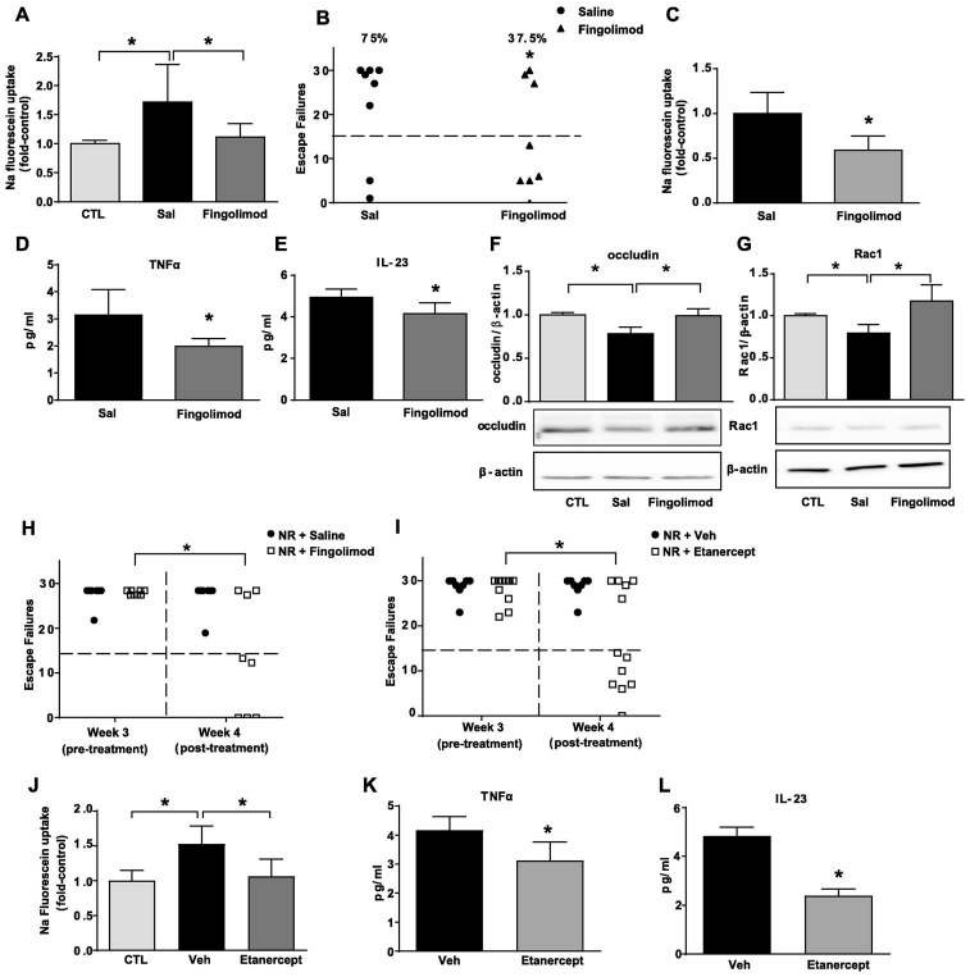

Figure 5. Effects of Fingolimod on the susceptibility and the recovery from learned helplessness

(A,B) Mice were treated with Fingolimod (1 mg/kg, i.p.) two times, 1 hr prior to inescapable foot shocks and 1 hr prior to escapable foot shocks in the learned helplessness paradigm. **(A)** BBB permeability was determined by measuring sodium fluorescein uptake into the hippocampus. *p<0.05 compared to values in untreated control mice or Fingolimod-treated mice (one-way ANOVA with Bonferroni post-hoc test for multiple comparisons). **(B)** Escape failures in mice treated with Fingolimod or saline. Each symbol represents escape failures for a single mouse. *p<0.05 compared to values in saline-treated mice; Student's t-test. **(C–H)** Following three weeks of escapable foot shocks after the induction of learned helplessness, mice were treated with Fingolimod (1 mg/kg, i.p.) or saline (Sal) for three days prior to the last session of escapable foot shocks at week 4. **(C)** BBB permeability (*p<0.05 compared to saline-treated mice; Student's t-test). **(D–G)** Hippocampi were collected from mice that recovered from learned helplessness after Fingolimod treatment and those that remained depressed after saline treatment 3 hr after the last session of escapable foot shocks at week 4, followed by measurements of the hippocampal levels of **(D)** TNFα **and (E)** IL-23 by ELISA (n = 6–8 in each group; *p<0.05 compared to values in saline-treated mice; Student's t-test), and of **(F)** occludin and **(G)** Rac1 by western blots (*p<0.05 compared to values in untreated control mice or Fingolimod-treated mice; one-way ANOVA with

Bonferroni post-hoc test for multiple comparisons). Values are means±SEM. **(H)** Escape failures in non-recovered mice before (week 3) and after (week 4) treatment with Fingolimod or saline. Each symbol represents escape failures for an individual mouse. n = 7–8 mice in each group; *p<0.05 comparing escape failures in mice before and after treatment with Fingolimod (Wilcoxon matched pair test). **(I–L)** Another group of mice that remained learned helplessness for three weeks were treated with etanercept (100 μg / mouse, i.p.) or PBS every other day for a week prior to the last session of escapable foot shocks at week 4. (I) Escape failures in non-recovered mice before (week 3) and after (week 4) treatment with etanercept or PBS. n = 8–12 mice in each group; each symbol represents escape failures for an individual mouse. *p<0.05 comparing escape failures in mice before and after treatment with etanercept (Wilcoxon matched pair test). Hippocampi were collected 3 hr after the last session of escapable foot shocks at week 4. (J) BBB permeability in the hippocampus was measured by the fluorescein assay in mice that remained depressed after etanercept or PBS treatment, mice that recovered after etanercept treatment, and non-stressed control mice. n = 5–7 mice in each group; *p<0.05 one-way ANOVA with Bonferroni post-hoc test for multiple comparisons. Hippocampal levels of (K) TNFα and (L) IL-23 were measured by ELISA (n= 5–6 mice in each group; *p < 0.05 compared to values in saline-treated mice; Student's t-test).