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Toll-like receptor 2 (TLR2)-deficiency impairs male mouse recovery from a depression-like state

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Abstract

Major depression is a prevalent, debilitating disease, yet therapeutic interventions for depression are frequently inadequate. Many clinical and pre-clinical studies have demonstrated that depression is associated with aberrant activation of the inflammatory system, raising the possibility that reducing inflammation may provide antidepressant effects. Using the learned helplessness mouse model, we tested if susceptibility or recovery were affected by deficiency in either of two receptors that initiate inflammatory signaling, Toll-like receptor-4 (TLR4) and TLR2, using knockout male mice. TLR4^{-/-} mice displayed a strong resistance to learned helplessness, confirming that blocking inflammatory signaling through TLR4 provides robust protection against this depression-like behavior. Surprisingly, TLR2^{-/-} mice displayed increased susceptibility to learned helplessness, indicating that TLR2-mediated signaling counteracts susceptibility. TLR2-mediated signaling also promotes recovery, as TLR2^{-/-} mice demonstrated a severe impairment in recovery from learned helplessness. That TLR2 actually protects from learned helplessness was further verified by the finding that administration of the TLR2 agonist Pam3CSK4 reduced susceptibility to learned helplessness. Treatment with Pam3CSK4 also blocked chronic stress-induced impaired sociability and impaired learning in the novel object recognition paradigm, demonstrating that TLR2 stimulation can protect from multiple impairments caused by stress. In summary, these results demonstrate that TLR2-mediated signaling provides a counter-signal to oppose deleterious effects of stress that may be related to depression, and indicate that TLR2 and TLR4 act oppositely to balance mood-relevant responses to stress.

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Keywords

depression; inflammation; Pam3CSK4; Toll-like receptor-2

1. Introduction

Major depression is a prevalent, debilitating disease, yet therapeutic interventions for depression are frequently inadequate (Belmaker and Agam, 2008). The critical need for improved treatments is impeded by an insufficient understanding of the causes of depression, and the essential identification of new therapeutic targets. Both onset and duration are important criteria for major depression, including multiple defining symptoms that cause significant distress and impaired function, and persistence for at least 2 weeks. However, most animal model studies of depression address only onset, raising the possibility that limited preclinical studies on duration may be contributing to the difficulty in identifying new therapeutic targets.

Substantial evidence demonstrates that depression is associated with aberrant activation of the innate immune system's inflammatory response (Dantzer et al., 2008; Medina-Rodriguez et al., 2018; Miller et al., 2009; Rivest, 2009). Activation of the inflammatory system induces both inflammation and suppressive mechanisms that limit the severity and duration of the inflammation, without which immune-mediated host damage would occur. Although the role of inflammation in depression remains to be more definitively defined, the general dogma suggests that inflammation promotes depression. However, there is much evidence that, as in the periphery, inflammatory signaling in the CNS performs beneficial, as well as more often studied deleterious, functions (Okun et al., 2011; Schwartz and Shechter, 2010), supporting the concept that the inflammatory system is composed of many checks and balances that serve to limit damage to the host. Thus, perturbations in inflammatory signaling that occur in depression may involve detrimental inflammatory signaling and/or loss of inflammatory control due to insufficient immunosuppressive signaling. Furthermore, antidepressant drugs have been shown to increase certain cytokines (Kenis and Maes, 2002; Munzer et al., 2013; Warner-Schmidt et al., 2011), further indicating that the role of inflammation in depression is complex and not well-defined.

Understanding of the immune system has recently evolved from considering it solely as a defender from microbial invaders to the realization that its overall role is the maintenance and restoration of homeostasis. In particular, the immune system is now recognized as critical in sensing stresses and other insults besides traditional pathogens (Gay and Gangloff, 2007; Hanisch et al., 2008; Jin and Lee, 2008; Okun et al., 2011). Sensing mechanisms include Toll-like receptors (TLRs), transmembrane glycoproteins that are activated by both pathogen-associated molecular patterns (PAMPs) of microbes and insult-induced endogenous ligands called danger- or damage-associated molecular patterns (DAMPs) that can be induced by stress as well as by tissue damage (Akira, 2006). TLR activation by DAMPs occurs in many non-infectious CNS conditions, such as neurodegenerative diseases, stroke, spinal cord trauma or nerve damage, and likely depression (Cheng et al., 2016; Hanisch et al., 2008).

Signaling by TLRs can either reduce or exacerbate diseases depending on the conditions involved and the TLR subtypes activated (Hanisch et al., 2008; Kurt-Jones et al., 2004). TLR4 is activated by lipopolysaccharide (LPS) and has been implicated in the sickness behavior model of depression induced by a low dose of LPS (Dantzer et al., 2008). Activation of TLR2 in the CNS promotes pathology in models of autoimmune disease (Reynolds et al., 2010), Parkinson's disease (Kim et al., 2018), and ischemia (Lu et al., 2011; Tang et al., 2007; Ziegler et al., 2007), but protects against damage from spinal cord injury (Kigerl et al., 2007), prion disease (Carroll et al., 2018), and cognitive decline in mouse Alzheimer's disease models (Pourbadie et al., 2018; Richard et al., 2008). The expression of both TLR4 and TLR2 was found to be higher in prefrontal cortices from depressed suicide subjects than normal control subjects (Pandey et al., 2019) and TLR2 polymorphisms were found to be linked to bipolar disorder (Oliveira et al., 2015; Oliveira et al., 2014). TLR2 recognizes a number of pathogen-associated molecular patterns (PAMPs), including components of the outer membrane of Gram-positive bacteria, such as triacetylated lipoproteins and the synthetic ligand Pam3CSK4 (palmitoyl3-cysteinyl-seryl-[lysyl4]). Although they share many of the same signaling pathway components, the actions of TLR2 and TLR4 are not redundant and it has been recognized that they can have counterbalancing actions (Hua et al., 2009; Rolls et al., 2007). For example, TLR4 reduces neurogenesis whereas TLR2 promotes it (Rolls et al., 2007; Seong et al., 2018). Their actions in mediating the inflammatory response suggest that targeting TLRs rather than individual cytokines during depressive behavior may provide a therapeutic strategy.

In this study, we addressed the actions of TLR2 and TLR4 on both duration and susceptibility of mice to the learned helplessness model of depression-like behavior. The results show that TLR4 and TLR2 have opposing roles in learned helplessness, as TLR4 promotes learned helplessness, whereas TLR2 promotes recovery from learned helplessness. Furthermore, pharmacologically activating the TLR2 receptor reduced stress-induced impaired sociability and learning and memory.

2. Material and methods

2.1. Mice and behavioral assessments

C57BL/6 TLR4 global knockout (TLR4^{-/-}) mice and C57BL/6 TLR2 global knockout (TLR2^{-/-}) mice were initially provided by co-author Dr. Michalek, and C57BL/6 wild-type mice were obtained from Charles Rivers Laboratories. Mice were bred using the following paradigm: TLR^{+/-} × TLR^{+/-} to obtain 25% TLR^{-/-}, 25% wild-type, and 50% TLR^{+/-} mice, and male littermates were used at 8–12 weeks of age for experiments. Mice were housed in the University of Miami vivarium with light and temperature controlled rooms and were treated in accordance with NIH and the University of Miami Institutional Animal Care and Use Committee regulations. Behavioral experiments were carried out with littermates that were randomized to their treatment groups. Where indicated, mice were treated i.v. via a tail vein with the TLR2 agonists Pam3CSK4 (palmitoyl3-cysteinyl-seryl-[lysyl4]; 50 µg/mouse) or FSL-1 (a synthetic diacylated lipoprotein; 5 µg/mouse).

2.1.1 Learned helplessness—Learned helplessness was measured using a standard learned helplessness paradigm or a modified protocol that does not induce learned helplessness in most wild-type mice, as described previously (Polter et al., 2010). Mice were placed in one side of a Gemini Avoidance system shuttle box (San Diego Instruments, San Diego, CA, USA) with the gate between chambers closed. For standard learned helplessness, 180 inescapable foot shocks (IES) were delivered at an amplitude of 0.3 mA, a duration of 6–10 s per shock (averaging 8 s), and a randomized inter-shock interval of 5–45 s (Duman et al., 2007; Polter et al., 2010). For the modified inescapable shock protocol that allows detection of increased vulnerability, mice were given 180 foot shocks at an amplitude of 0.3 mA, a duration of 2–6 s shock (averaging 4 s), and a randomized inter-shock interval of 5–25 s (Polter et al., 2010). Twenty-four hours after 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 s 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 Gemini software, and trials in which the mouse did not escape within the 24 s time limit were counted as escape failures. Mice with greater than 15 escape failures were defined as learned helpless.

2.1.2 Social interactions—Diminished social interaction is associated with depression in humans and rodents and were measured as we previously described (Beurel et al., 2013; Mines et al., 2010). Mice were tested in social interactions using a sociability apparatus, which is a rectangular, transparent, Plexiglas box (24 cm × 19 cm, 19 cm high) divided into three equal sized chambers with doors. Chambers 1 and 3 have a wire cage; Chamber 2 in the middle is empty. Mice were habituated individually by being placed in Chamber 2 and were allowed to freely explore the entire apparatus for 25 min the day prior to testing, and, separately, stimulus mice were habituated for 20 min to the wire cage in Chamber 1. Testing consisted of 5 min rehabituation followed by 10 min access to all chambers with an unfamiliar stimulus mouse (age- and sex-matched) in the wire enclosure in Chamber 1. Each session was videotaped and videos were analyzed by pre-trained investigators blind to treatments and genotypes. Videos were quantitated for the number of nose contacts with the stimulus mouse.

2.1.3 Novel Object Recognition—Novel object recognition was measured as described previously (Pardo et al., 2016) by allowing each mouse individually to explore two identical objects for 5 min, and after a 5 min period in an opaque chamber, mice were allowed to explore an unused familiar object and a novel object for 5 min. Time spent exploring the objects (sniffing or touching the object with its nose, vibrissa, mouth or forepaws) was quantified.

2.1.4 Chronic stress—Chronic restraint stress is widely used to induce depression-like behaviors and has high face and predictive validity (Henn and Vollmayr, 2005). For chronic restraint stress, mice were restrained for 2 h/day for 2 weeks, as we previously described (Beurel et al., 2013), and behavioral tests were carried out beginning the day after the last day of chronic restraint stress. Mice were treated with Pam3CSK4 or saline 8–10 hr after the last session of chronic restraint stress and on the following days 1 hr prior to the behavioral

tests. The day after chronic restraint stress mice were tested for activity in a novel open field, and later habituated to the social interaction apparatus. The following day mice were tested for social interactions, and the following day novel object recognition was measured.

2.1.5 Open field activity—For the open field activity measurements (Schwartz and Shechter, 2010), mice were placed in a Plexiglas open field (Med Associates, St Albans, VT) outfitted with photo beam detectors under soft overhead lighting, and activity was monitored during 30 min using activity monitoring software (Med Associates). The number of beam breaks was calculated for each 5 min period.

2.1.6 Pain sensitivity—Mice were put on a hot plate at 42°C, and the time until they lick or lift a paw was recorded.

2.2. Quantitative real-time polymerase chain reaction

Brains were collected 3 hr after the escapable foot shocks in the fourth week of testing as previously described (Cheng et al., 2018). RNA from the hippocampus was extracted with TRIzol Reagent (Life Technologies) and cDNA was synthesized with ImProm-II™ Reverse Transcriptase and random primers (Promega). TLR2 and TLR4 expression were measured by SYBR green RT- qPCR or Taqman gene expression assay in a Jena Analytika instrument and the results were quantified by the 2⁻ Ct method. Primers used: TLR2: 5'- TTTCTACTTTACCCAGCTCGCTCA -3' and 5'- GGAAGTGTCTGGAGGTAGAGTTCCG -3'; TLR4 (Mm00445273_m1; Thermo Fisher Scientific); Values were normalized to B2M (Mm00437762_m1; Thermo Fisher Scientific).

2.3 Statistical analyses

The data were analyzed with Student's t-test, Mann-Whitney U test, or one-way or two-way ANOVA with Bonferroni post-hoc test for multiple comparisons. Data represent means ± SEM, and significance was set at $p < 0.05$. The statistical analyses were performed using GraphPad Prism 6 software.

3. Results

We tested if TLR4^{-/-} mice and TLR2^{-/-} mice had different responses than wild-type mice in stress-induced learned helplessness. As expected, most wild-type mice (80%) developed learned helplessness (Fig 1A). As we recently reported (Cheng et al., 2016), TLR4^{-/-} mice were largely resistant to the induction of learned helplessness, with only 33% displaying learned helplessness (Fig 1A; One-way ANOVA, $F(2,28)=4.073$, Bonferroni post-hoc test $p<0.05$), demonstrating that blocking this major pathway leading to cytokine production is sufficient to largely ameliorate susceptibility to learned helplessness. Surprisingly, TLR2^{-/-} mice responded similarly to wild-type mice, with 90% exhibiting learned helplessness (Fig 1A). This was not the result of decreased sensitivity to pain because in new cohorts of mice exposed to only escapable foot shocks (no previous inescapable foot shocks), both wild-type and TLR2^{-/-} mice escaped (Suppl Fig 1A), and in another new cohort of mice there were no differences between groups in the hot plate pain sensitivity test (Suppl Fig 1B).

The slight increase in the percentage of TLR2^{-/-} mice displaying learned helplessness compared with wild-type mice suggested that TLR2 deficiency may actually promote susceptibility to learned helplessness. To test this, the duration of the foot shocks during the learned helplessness protocol was reduced, which eliminates the “ceiling effect” in which most wild-type mice develop learned helplessness. Instead, in the modified paradigm most wild-type mice do not develop learned helplessness, allowing studies of conditions that increase susceptibility to learned helplessness (Polter et al., 2010). Using the modified learned helplessness protocol, only 30% of wild-type mice displayed learned helplessness, but 80% of TLR2^{-/-} mice displayed learned helplessness (Fig 1B; Mann-Whitney U=24, $p<0.05$). This demonstrates that TLR2 deficiency increased susceptibility to learned helplessness. Thus, TLR4 promotes, but TLR2 opposes, the induction of the learned helplessness model of depressive behavior.

Most rodent measures of depressive-like behaviors involve acute assessments, after either acute or chronic stress, which fail to model the prolonged duration of depression in patients. Therefore, we tested the duration of learned helplessness and tested if deficiency in TLR2 affected recovery. Recovery from learned helplessness was measured with weekly tests of responses to escapable foot shocks without additional exposure to inescapable foot shocks after the initial induction of learned helplessness. After 0, 1, 2 and 3 weeks (Fig 2A, B), 70–80%, 50%, 40% and 20%, respectively, of wild-type mice displayed learned helplessness, confirming our previous report that ~80% of wild-type mice recover within about 3 weeks (Cheng et al., 2018). Remarkably, TLR2^{-/-} mice exhibited dramatically impaired recovery from learned helplessness. After 3 weeks, 70% of TLR2^{-/-} mice still displayed learned helplessness compared to 20% of wild-type mice (Fig 2A; $F(2,28)=4.015$, Bonferroni post-hoc test $p<0.05$). After recovery from learned helplessness, no mouse reverted to the learned helpless condition, and no initially resilient mouse (non-learned helpless after the initial learned helplessness protocol) later displayed increased failures to escape from escapable foot shocks (Suppl Fig. 2). Impaired recovery of TLR2^{-/-} mice was replicated in another cohort of mice, and this study demonstrated the impaired recovery was maintained through 9 weeks after the induction of learned helplessness (Fig 2B; Mann-Whitney, U=4, $p<0.05$). These results reveal that TLR2 deficiency promotes the maintenance of a long-term depression-like behavior, indicating that signaling through TLR2 is important for recovery from learned helplessness.

Considering the modulatory effects of TLR2 and TLR4 on learned helplessness described above, we tested if changes in TLR2 or TLR4 expression were evident in wild-type mice displaying prolonged learned helplessness. Wild-type mice were subjected to 4 weeks of testing for retention of learned helplessness and hippocampal mRNA levels of TLR2 and TLR4 were assessed because the hippocampus has previously been found to be important in both learned helplessness and depression (Chen et al., 2006). Groups of wild-type mice analyzed included those that were exposed to the learned helplessness protocol but never developed learned helplessness (non-LH), mice that remained learned helpless for four weeks (LH), and mice that never received foot shocks (NS). The TLR2 hippocampal mRNA level was significantly lower in mice that displayed prolonged learned helplessness compared with NS or non-LH mice (Fig 2C; one-way ANOVA, $F(2,18)=3.891$, Bonferroni post-hoc test $p<0.05$), corroborating the idea that deficient TLR2 actions contribute to

impaired recovery from learned helplessness and extending a previous report that TLR2 expression was reduced in the spleens of mice subjected to restraint stress (Hu et al., 2013). Oppositely, hippocampal TLR4 mRNA levels were significantly higher in mice that displayed prolonged learned helplessness compared with non-LH or NS mice (Fig 2D; one-way ANOVA $F(2,18)=5.878$, Bonferroni post-hoc test $p<0.05$), supporting a role for TLR4 in promoting learned helplessness.

Indications that TLR2-mediated signaling is important for modulating learned helplessness susceptibility and duration raised the possibility that activating TLR2 may reduce susceptibility to learned helplessness. TLR2 heterodimerized with TLR1 can be activated by triacetylated lipoproteins, such as the synthetic ligand Pam3CSK4 (palmitoyl3-cysteinyl-seryl-[lysyl4]), and TLR2 dimerized with TLR6 can be activated by diacetylated lipoproteins, such as FSL-1 (a synthetic diacylated lipoprotein) (Akira, 2006; Buwitt-Beckmann et al., 2006). Therefore, each of these TLR2 agonists was used to test if activation of the TLR2 receptor reduced susceptibility to learned helplessness. The TLR2 agonist or saline were injected i.v. via a tail vein 8–10 hr after inescapable foot shocks, a time of treatment that we previously reported was effective with other drugs (Beurel et al., 2011), and mice were tested with escapable foot shocks the next day. This protocol that was previously shown to be effective for detecting the ameliorative effects of other drugs in the learned helplessness model (Beurel et al., 2011; Li et al., 2010; Maeng et al., 2008). Stimulation of TLR2 with Pam3CSK4 or FSL-1 effectively reduced learned helplessness (Fig 3A; Student's t-test, $t=2,026$, $t=2,832$, $p<0.05$, $p<0.01$), as only 33% and 25%, respectively, of the treated wild-type mice exhibited learned helplessness compared with 71% of saline-treated mice. This demonstrates for the first time a strong and rapid ameliorative effect of TLR2 stimulation. As a control, Pam3CSK4 treatment did not have an effect on learned helplessness in TLR2^{-/-} mice (Fig 3B), verifying that the ameliorative effect of Pam3CSK4 in the learned helplessness test resulted from activation of TLR2, whereas FSL-1 had a modest effect in TLR2^{-/-} mice, suggesting a partial off-target effect of FSL-1. Altogether, these results demonstrate that selective stimulation of TLR2 provides resistance to the learned helplessness model of depression.

We further tested if pharmacological stimulation of TLR2 ameliorated behavioral impairments caused by another type of stress, chronic restraint stress, which has been widely used as a model of chronic stress to induce behavioral impairments associated with depression and anxiety (Chiba et al., 2012). After 2 weeks of daily restraint, mice were treated with Pam3CSK4 or saline and compared to control groups of mice that were subjected to the same behavioral tests and treatments without being exposed to chronic restraint stress. Impaired social interactions is commonly associated with depression (Krishnan and Nestler, 2008). In the three chambered social interaction test, non-stressed mice treated with Pam3CSK4 did not differ in their social interactions compared to mice treated with saline (Fig 3C). As expected, mice exposed to chronic restraint stress had 65% reduced number of nose contacts with the test mouse (Fig 3C; one-way ANOVA, $F(3,44)=16.08$; $p<0.001$). This impairment in chronic restraint-stressed mice in social interaction was reversed by TLR2 activation with Pam3CSK4 (Fig 3C). Pam3CSK4 administration to either control mice or mice subjected to chronic restraint stress did not alter locomotor activity in the open field (Fig 3D).

Impaired learning and memory can be caused by chronic stress and is often associated with depression (Burriss et al., 2008; Sun and Alkon, 2004). Mice were exposed to chronic restraint stress and subjected to the novel object recognition test. On the last day of chronic restraint stress and 1 h prior to the novel object recognition test, mice were treated with the TLR2 agonist Pam3CSK4 (50 µg/mouse) or saline. In parallel, control groups of mice were treated with Pam3CSK4 or saline and tested for novel object recognition without being exposed to chronic restraint stress. Of the time spent exploring objects, non-stressed control mice, with or without treatment with Pam3CSK4, spent 60% of the time exploring the novel object and 40% of the time exploring the familiar object (Fig 3E). Exposure to chronic restraint stress completely impaired novel object recognition, as these mice spent equal times exploring the two objects. Treatment with Pam3CSK4 completely reversed the stress-induced impairment in novel object recognition (Fig 3E; one-way ANOVA, $F(7,82)=26.95$, $p<0.001$). The total exploration time remained unchanged after chronic restraint stress or Pam3CSK4 treatment (Fig 3F). Thus, TLR2 stimulation can restore impaired memory in stressed mice.

4. Discussion

Since inflammation is linked to depression (Dantzer et al., 2008; Medina-Rodriguez et al., 2018; Miller et al., 2009; Rivest, 2009), and TLR4 and TLR2 are major contributors to inflammation, we tested the hypotheses that molecular deletion of TLR4 and TLR2 would reduce the susceptibility of mice to depression-like behavior in the learned helplessness test. Surprisingly, TLR4 and TLR2 were found to have very different roles in modulating learned helplessness, with TLR4 promoting susceptibility and TLR2 providing a counter-signal to reduce susceptibility and facilitate recovery. These results suggest that signaling through TLR2 and TLR4 provide mechanisms to balance mood-relevant responses to stress.

We recently reported the antidepressant-like effect of TLR4 deletion (Cheng et al., 2016) and present confirmatory findings here to provide a direct comparison to the surprising opposite role of TLR2. Attenuation by TLR4 depletion of depression-like responses to stress extends previous reports that administration of a low-dose of the specific TLR4 agonist lipopolysaccharide (LPS) induces a sickness behavior model of depression-like behavior (Frenois et al., 2007; Kullmann et al., 2013) and fits well with accumulating evidence that inflammation contributes to depression (Dantzer et al., 2008; Medina-Rodriguez et al., 2018; Miller and Raison, 2016).

In contrast to TLR4, signaling through TLR2 apparently promotes both resilience to stress and recovery from stress-induced impairments, since TLR2 deficiency increased susceptibility to learned helplessness, and even more remarkably impaired recovery from learned helplessness. The latter indicates that TLR2-mediated signaling provides an antidepressant-like signal to contribute to the termination of depression-like conditions. This supports previous evidence that certain components of inflammatory signaling can support recovery from depression, e.g., the Greengard group reported that administration of antidepressants to mice increased levels of certain cytokines and anti-inflammatory drugs impaired behavioral antidepressant responses (Warner-Schmidt et al., 2011). However, the experiments with TLR2^{-/-} mice have the limitation that TLR2 was absent throughout

development, a condition that might affect the establishment of circuits that are involved in modulating the measured behaviors.

That TLR2-mediated signaling may provide antidepressant-like effects was verified by the results of experiments involving the administration of Pam3CSK4, a specific TLR2 ligand (Buwitt-Beckmann et al., 2006). Pam3CSK4 administration provided protection from learned helplessness and also prevented chronic restraint stress-induced impairments in sociability and novel object recognition. The latter finding fits well with a recent report that TLR2 deletion promotes cognitive impairment in the Morris water maze test and increased immobile time in the tail suspension test in a mouse model of Alzheimer disease (Zhou et al., 2019). Clearly these are only three components of the many deleterious effects of stress and the many manifestations of depression, but they demonstrate proof-of-concept that stimulation of TLR2 receptors can ameliorate several impairments caused by stress. Further studies will be informative for clarifying the extent to which other deleterious effects of stress can be ameliorated by stimulating TLR2 receptors.

A limitation of this study is that it was based on measuring mouse behaviors (Nestler and Hyman, 2010). It is generally considered that mice do not exhibit major depressive disorder as that which afflicts humans. Therefore, such studies rely on examining behaviors that seem to model various components of major depressive disorder, which underlies changes associated with susceptibility and onset of depression using mouse behavioral models (Cryan and Mombereau, 2004; Gould and Gottesman, 2006; Henn and Vollmayr, 2005). Several models have been developed for studying depression-like behavior and antidepressant-like actions in rodents. Extensive reviews of these developments and their limitations have been published previously (Chen et al., 2010; Cryan and Mombereau, 2004; Nestler and Hyman, 2010). The learned helplessness model of depression is one of the most widely used because it provides a robust and reproducible response (Chourbaji et al., 2005; Henn and Vollmayr, 2005; Nestler et al., 2002; Overmier and Seligman, 1967). Importantly, it also has the advantages of flexibility, allowing detection of increased or decreased susceptibility to learned helplessness, and allowing long-term assessment of the duration of this model of depression. However, a variety of experimental variations in the learned helplessness protocol have been used and it remains to be established the causes of the failure to escape an escapable aversive situation, which may include some aspect of learning (Chourbaji et al., 2005; Henn and Vollmayr, 2005; Maier and Seligman, 2016; Nestler et al., 2002; Overmier and Seligman, 1967). Although this study focused on the hippocampus because it is important in mood regulation, cognition, and inflammation, the processes investigated here, a limitation of this study is that obviously other brain regions also participate in these processes that should be examined in the future (Campbell and Macqueen, 2004; Castanheira et al., 2019; Duman et al., 2019).

5. Conclusions

Altogether, it is now evident that TLR-mediated signaling in the CNS cannot be simplified as only causing deleterious inflammation, but involves complex signaling of checks and balances that maintain healthy functions, but also can contribute to detrimental outcomes of diseases. The present results show that that TLR4 and TLR2 are not redundant in their

regulation of learned helplessness, consistent with other studies showing non-redundant roles of TLR2 and TLR4 in immune responses (Hua et al., 2009; Rolls et al., 2007). Perhaps most interestingly, this study raises the possibility that stimulation of TLR2 may provide a new approach for limiting or recovering from the deleterious effects of stress that include depression-like states in mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TLR2^{-/-} mice are susceptible to learned helplessness, whereas TLR4^{-/-} mice are resistant

A, Wild-type (WT), TLR2^{-/-}, and TLR4^{-/-} mice were subjected to the learned helplessness paradigm and the number of failures to escape was recorded. Mice are considered learned helpless if they fail more than 15 out of 30 trials. Each point represents the number of failures to escape for an individual mouse. Bars represent means $\pm$ SEM. Percent of learned helpless mice is represented on the right. One-way ANOVA, $F(2,28)=4.073$, Bonferroni post-hoc test $*p<0.05$, $n=9-10$ mice/group. B, Wild-type and TLR2^{-/-} mice were subjected to the modified learned helplessness paradigm and the number of failures to escape was recorded. Each point represents the number of failures to escape for a single mouse. Bars represent means $\pm$ SEM. Percent of learned helpless mice is represented on the right $*p<0.05$, Mann-Whitney $U=24$, compared to wild-type mice, $n=10$ mice/group.

Figure 2. TLR2^{-/-} mice exhibit a prolonged duration of learned helplessness

A, Wild-type (WT), TLR2^{-/-}, and TLR4^{-/-} mice were subjected to the learned helplessness paradigm and the percent of learned helpless mice was calculated after 1, 2 and 3 weeks. n=10 mice/group, One-way ANOVA, +1 week: F(2,28)=4.738, +2 weeks: F(2,28)=3.434, +3 weeks: F(2,28)=4.015, Bonferroni post-hoc test *p<0.05. B, Different cohorts of wild-type and TLR2^{-/-} mice were subjected to the learned helplessness paradigm and the percent of learned helpless mice was calculated 24 hr after inescapable foot shocks on week 0, and 1, 2, 3, 4, 5 and 9 weeks later. n=8–15 mice in 8 different cohorts. Mann-Whitney, U=4, *p<0.05. C, TLR2 mRNA levels with respect to B2M were measured in the hippocampus of non-shocked (NS) wild-type mice, non-learned helplessness mice (non-LH) or mice presenting prolonged learned helplessness (LH). Each point represents an individual mouse. Bars represent means ± SEM. one-way ANOVA, F(2,18)=3.891, Bonferroni post-hoc test *p<0.05, n=4–11 mice/group. D, TLR4 mRNA levels with respect to B2M were calculated in the hippocampus of non-shocked (NS) wild-type mice, non-learned helplessness mice (non-LH) or mice presenting prolonged learned helplessness (LH). Each point represents an

individual mouse. Bars represent means $\pm$ SEM. One-way ANOVA, $F(2,18)=5.878$, Bonferroni post-hoc test $*p<0.05$, $**p<0.01$, $n=4-11$ mice/group.

Figure 3. Administration of the TLR2 agonist Pam3CSK4 reduces depression-like behaviors and improves stress-impaired learning and memory

A, Wild-type mice were subjected to inescapable foot shocks and the TLR2 agonists Pam3CSK4 (50 µg/mouse) or FSL-1 (5 µg/mouse) or saline (VEH) were administered i.v. ~8–10 h after inescapable foot shocks, and mice were tested with escapable foot shocks 24 h later. The number of failures to escape were recorded and the percentage of learned helpless mice was calculated. Each point represents the number of failures to escape for an individual mouse. Bars represent means ± SEM. Student's t-test, $t=2,026$, $t=2,832$, $*p<0.05$, $**p<0.01$, compared to saline-treated mice, $n=12$ – 28 mice/group. B, TLR2^{-/-} mice were subjected to

inescapable foot shocks and the TLR2 agonists Pam3CSK4, FSL-1, or saline (VEH) were administered i.v. ~8–10 h after inescapable foot shocks, and mice were tested with escapable foot shocks 24 h later. The number of failures to escape were recorded and the percentage of learned helpless mice was calculated. Each point represents the number of failures to escape for an individual mouse. Bars represent means $\pm$ SEM. n=5–8 mice/group. C-F, Wild-type mice were subjected to chronic restraint stress (CRS) for two weeks, treated with Pam3CSK4, and tested (C) in the three chambered sociability test, (D) the open field test, and (E,F) the novel object recognition test. The number of nose contacts (C), the beam breaks (D), the percentage of time exploring a familiar versus a novel object (E) and the total exploration time of these objects (F) were quantified. In parallel, control (CT) groups of mice was subjected to the same behavioral tests with Pam3CSK4 or saline treatments without being exposed to CRS. Each point represents an individual mouse. Bars represent means $\pm$ SEM. One-way ANOVA, $F(3,44)=16.08$ (C), $F(7,82)=26.95$ (E), Bonferroni post-hoc test *** $p<0.001$, n=8–15 mice/group.