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Full-length Article

Toll-like receptor-4 regulates anxiety-like behavior and DARPP-32 phosphorylation

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ABSTRACT

Toll-like receptors (TLRs) play a crucial role in early innate immune responses to inflammatory agents and pathogens. In the brain, some members of the TLR family are expressed in glial cells and neurons. In particular, TLR4 has been involved in learning and memory processes, stress-induced adaptations, and pathogenesis of neurodegenerative disorders. However, the role of TLR4 in emotional behaviors and their underlying mechanisms are poorly understood. In this study, we investigated the role of TLR4 in emotional and social behavior by using different behavioral approaches, and assessed potential molecular alterations in important brain areas involved in emotional responses. TLR4 knockout (KO) mice displayed increased anxiety-like behavior and reduced social interaction compared to wild type control mice. This behavioral phenotype was associated with an altered expression of genes known to be involved in emotional behavior [e.g., brain-derived neurotrophic factor (BDNF) and metabotropic glutamate receptors (mGluRs)]. Interestingly, the mRNA expression of dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32) was strongly upregulated in emotion-related regions of the brain in TLR4 KO mice. In addition, the phosphorylation levels at Thr75 and Ser97 in DARPP-32 were increased in the frontal cortex of TLR4 KO male mice. These findings indicate that TLR4 signaling is involved in emotional regulation through modulation of DARPP-32, which is a signaling hub that plays a critical role in the integration of numerous neurotransmitter systems, including dopamine and glutamate.

1. Introduction

Toll-like receptors (TLRs) are transmembrane pattern recognition receptors that recognize damage-associated molecular patterns and pathogen-associated molecular patterns (Kaisho and Akira, 2001; Takeda et al., 2003). TLRs play a critical role in early innate immune responses to inflammatory agents and pathogens (Hennessy et al., 2010) and facilitate the activation of the adaptive immune response (Wong et al., 2010). Mammalian TLRs exhibit differential expression patterns in the brain and facilitate diverse functions within the developing and adult central nervous system (Okun et al., 2011). In the brain, some members of the TLR family are expressed in glial cells (astrocytes, microglia, and oligodendrocytes), neurons, and neural progenitor cells, suggesting that TLRs also participate in brain development and behavior and regulation of brain physiology. Indeed, recent research demonstrates that TLRs are critical in mediating neurogenesis during brain development, as well as hippocampal neurogenesis and plasticity during adulthood (Bercik and Collins, 2014; Okun et al., 2011; Rolls et al., 2007) (Trudler et al., 2010). However, little is known about the specific function of TLRs and their crosstalk with other brain signaling pathways.

Recent studies investigating the role of TLRs in the brain have shown that TLR type 4 receptors (TLR4) are involved in learning and memory (Okun et al., 2012), aging (Letiembre et al., 2007), and neurodegenerative disorders such as Parkinson's disease (Noelker et al., 2013) and Alzheimer's disease (Walter et al., 2007). An emotion-related role for TLR4, especially in mediating stress and depression, has also been described (Liu et al., 2014). Interestingly, the translocation of lipopolysaccharide (LPS)-containing gram-negative enterobacteria, which stimulate TLR4, has been potentially associated with the onset of a number of inflammatory and oxidative/nitrosamine stress pathways in major depressive disorder (Garate et al., 2011; Maes et al., 2008), in the regulation of the adrenal response to stress and inflammatory stimuli (Bornstein et al., 2006; Zacharowski et al., 2006), and in stress-induced neuroinflammation in the brain (Caso et al., 2008; Garate et al., 2013). In autism spectrum disorder, which is

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characterized by poor social adaptations and is often co-associated with anxiety (van Steensel et al., 2011), a marked increase in the levels of the pro-inflammatory cytokine IL-1B in response to the stimulation of TLR4 expressing monocytes by its specific ligand (LPS) has been reported (Enstrom et al., 2010). In addition, cytokine levels have been found to increase upon TLR4 activation in patients with schizophrenia and bipolar disorder (Garcia-Bueno et al., 2016; McKernan et al., 2011). These findings indicate that dysregulation of the TLR 4 signaling may contribute to the pathogenesis of neurodevelopmental and psychiatric disorders.

To date, few studies have investigated the role of TLR4 in anxiety using animal models of alcohol-induced (Montesinos et al., 2016) and diet-induced obesity (Strekalova et al., 2015). Moreover, the activation of TLR4 by LPS treatment in neonatal rats has been shown to produce long-lasting effects during adolescence, including reduced anxiety and enhanced patterns of locomotion and rearing in the open field and elevated plus maze (EPM) tests (Rico et al., 2010). However, the precise role of TLR4 modulation in anxiety-like behavioral responses is poorly understood (Okun et al., 2012; Li et al., 2016), particularly in the modulation of brain pathways such as metabotropic glutamate receptors (mGluRs) (Swanson et al., 2005) and synaptic plasticity markers such as brain-derived neurotrophic factor (BDNF) (Martinowich et al., 2007), which are closely associated with the regulation of emotions, including anxiety.

Here, we investigated the involvement of TLR4 in the regulation of anxiety-like behavior and social interaction in male and female mice. We also assessed the impact of TLR4 deficiency on the expression of several genes implicated in the processing of emotions and social stimuli within the cortical-limbic circuits, and evaluated potential alterations in the expression and activity of dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32), a signaling hub that integrates signals from multiple neurotransmitter systems involved in emotion and cognition.

2. Methods

2.1. Animals

All experiments were performed in 10–11-week-old wild type and TLR4 knockout (KO) mice (C57BL/6J genetic background) of both sexes. Homozygous TLR4 KO and wild type breeding pairs were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were bred and housed in our animal facility under specific-pathogen-free (SPF) conditions in standard plastic cages (Type III Makrolon®, Tecniplast, Buguggiate, Italy) under controlled temperature, humidity, and light (12:12 h light-dark cycle; the light cycle started at 7:00 AM). Food and water were available *ad libitum*. Wild type and TLR4 KO mice were obtained from multiple breeding pairs (n = 10). All protocols were approved by the Animal Research Ethics Committee of Stockholm North and complied with the European Communities Council Directive 2010/63/EU.

2.2. Behavioral studies

2.2.1. General behavioral procedure

All behavioral testing occurred between 9:00 AM and 4:00 PM. Before any behavioral procedure, mice were brought in their home cages to a room adjacent to the testing room and were allowed to habituate for at least 60 min before testing to minimize stress caused by environmental changes. Carry-over effects were avoided by using distinct animal groups for each behavioral task (10 animals per group in the open field test, 13–15 per group in the light and dark (LD) box and EPM test, and 8–9 per group in the social interaction test).

2.2.2. Open field test

Mice were placed individually in the center of an open field box (48 cm × 48 cm; Acti-Mot detection system; TSE, Bad Homburg, Germany), and spontaneous motor activity was recorded for 90 mins. The computer program automatically recorded the following parameters: distance traveled (in meters), number of rears, and time spent in the center and periphery (in seconds) using an infrared photo-beam system.

2.2.3. Light and dark box test

The light and dark (LD) apparatus consisted of two equal-sized (24 cm × 24 cm) compartments, in which one compartment was transparent and lit with a light bulb on top and the other compartment was black and contained a black cover on top. A rectangular door in the middle wall connected the two compartments. In this test, each mouse was placed in the lighted compartment and was allowed to freely explore both arenas for 5 min. The following parameters were analyzed in the LD compartments: time spent, distance traveled, and number of rears. These parameters were automatically recorded by the Acti-mot detection system (TSE, Bad Homburg, Germany) using photo-beam cells.

2.2.4. Elevated plus maze test

The elevated plus maze (EPM) apparatus (Kinder Scientific) was made of black Plexiglas and consisted of two open arms (36 × 5 cm), two enclosed arms (36 × 5 cm), and a central area (5 × 5 cm) elevated 64 cm above the floor level. Two arms were open, and two arms were closed with 16-cm high walls made of the same material. Mice were individually placed in the center, always facing the same open arm, and were allowed to explore all four arms for 5 min. The following behaviors were recorded using infrared photo-beams: number of entries and time spent and distance traveled in the open arms, closed arms, and intersection (center).

2.2.5. Social interaction

The social interaction test was carried out with two mice of the same age, weight, and sex, which were allowed to freely interact in a new arena with the size of the home cage. On the day of the experiment, the test mice were housed individually in a new clean home cage for 2 h before testing (Type III Makrolon®, Tecniplast, Buguggiate, Italy) for habituation. During the habituation period, the mice had access to water and food, which was removed immediately before the start of the test. The behavioral task started with the introduction of a novel (unfamiliar) stimulus mouse into the home cage of the test animal. Social interactions were video recorded for 10 mins with a video camera (Samsung, Seoul, South Korea) placed in front of the cage (horizontal plane). The time the test mouse spent interacting with the stimulus mouse was manually scored. Active interaction was defined as sniffing, close following, and allogrooming.

2.3. Gene expression studies

2.3.1. Quantitative real-time polymerase chain reaction (qRT-PCR)

Naïve wild type and TLR4 KO mice of both sexes (n = 4–5 per group) were sacrificed via cervical dislocation, and the areas of the frontal cortex, striatum, and hippocampus were rapidly dissected on ice, frozen on dry ice, and stored at −80 °C until use. Total RNA was isolated using RNeasy® Mini Kits (QIAGEN AB, Sollentuna, Sweden) according to the manufacturer's instructions (including the optional DNase digestion step for 15 min at room temperature) and quantified via spectrophotometry using a NanoDrop® ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand cDNAs were synthesized from equal amounts of total RNA (1 μg/reaction) using the iScript cDNA synthesis kit (Bio-Rad, Sundbyberg, Sweden) according to the man-

ufacturer's instructions and stored at −20 °C until use. Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Sundbyberg, Sweden). Briefly, each PCR reaction contained 30 ng cDNA, 0.5 μM each of primer and nuclease-free water, and 5 μL iQ™ SYBR® Green Supermix (SYBR® Green I dye: Bio-Rad), 50 U/ml iTaq™ DNA polymerase (Bio-Rad, Sundbyberg, Sweden), Deoxynucleotide triphosphates (dNTPs), 6 mM $MgCl_2$, 100 mM KCl, 20 nM fluorescein including stabilizers, and 40 mM Tris–HCl (pH 8.4) in a 10-μl reaction. The housekeeping gene, TATA-binding protein (TBP) was used for normalization. All samples were performed in triplicate. The cycling program was set as follows: Step 1, 98 °C for 30 s; Step 2, 40 cycles of 98 °C for 5 s, followed by 50–60 °C for 15 s; Step 3, 90 cycles for 5 s each, beginning at 50 °C and increasing by 0.5 °C with each subsequent cycle. Subsequent to the amplification procedure, a melting curve analysis was performed (set point, 50 °C) in order to confirm amplification specificity. The specificity of the gene products was determined via melting curve analyses. The sequences and annealing temperatures of primers used are listed in Supplementary Table 1. The data analysis was based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The normalized ΔC_t for each gene of interest (GOI) was calculated by deducting the C_t of the housekeeping gene TBP (because the expression of this gene was not statistically different in the RNA samples) from the C_t of each GOI. Then, the double delta C_t ($\Delta\Delta C_t$) for each GOI was calculated by deducting the average ΔC_t of GOI in the male wild type control group from the ΔC_t of each GOI in the other groups. The fold-change of each GOI relative to the wild type group was calculated as $2^{-\Delta\Delta Ct}$. Therefore, the expression level of each evaluated gene was normalized to the levels of TBP and expressed relative to male wild type controls.

2.3.2. In situ hybridization

Following cervical dislocation, the brains of wild type and TLR4 KO mice of both sexes (n = 5 per group) were rapidly dissected, frozen on dry ice, and stored at −80 °C. Coronal sections (14 μm) containing frontal areas such as the medial prefrontal cortex and orbitofrontal cortex were prepared on a cryostat and stored at −80 °C until use. Fixation, prehybridization, and hybridization were performed as previously described (Diaz Heijtz et al., 2004). The BDNF and DARPP-32 probes were prepared as previously described (Diaz Heijtz et al., 2011). All comparisons between groups were made using sections hybridized together, under identical conditions, and exposed to β-Max autoradiographic film for the same period. Films were scanned with an Epson Perfection V700 Photo Scanner in a grayscale format at 1200 dpi and were saved as high-quality TIFF files. For each animal, the mRNA levels were determined by measuring the optical density values using the appropriate software (NIH Image J version 1.48, National Institutes of Health). A ^{14}C step standard (GE Healthcare, Uppsala, Sweden) was included to calibrate optical density readings and convert the measured values into nCi/g. The analyzed regions were the medial prefrontal cortex (2.7 mm to 2.5 mm anterior to bregma) and the orbital frontal cortex (2.7 mm to 2.5 mm anterior to bregma).

2.3.3. Western blot analysis

Naïve wild type and TLR4 KO mice of both sexes (n = 5 per group) were sacrificed by cervical dislocation, and the frontal cortex was rapidly dissected on ice, frozen on dry ice, and stored at −80 °C until use. Tissue samples were homogenized in 700 μL of 1% SDS and 50 mM NaF and then boiled for 10 min as previously described (Kuroiwa et al., 2012). Aliquots (10 μL) of the homogenate were used for protein determination using the Quick StartTM Bradford Protein Assay kit (Bio-Rad, Sundbyberg, Sweden). Equal amounts of protein (10 μg) from each sample were loaded onto 12% polyacrylamide gels. Proteins were separated using SDS-PAGE and transferred to Immobilon® PVDF membranes (Millipore, Solna, Sweden). The membranes were immunoblotted using polyclonal antibodies against phosphorylated threonine 34 (pThr34-DARPP-32) (Cat No. 12,438, 1:1000), phosphorylated threonine 75 (pThr75-DARPP-32) (Cat No. 2301, 1:5000), phosphorylated serine 97 (pSer97-DARPP-32) (Cat No. 3401, 1:10,000, all from Cell Signaling Technology, Beverly, MA, USA), and total DARPP-32 (Cat No. 2306, 1:40,000; Cell Signaling Technology, Beverly, MA, USA). All the antibodies used have been described previously (Qian et al., 2015; Engmann et al., 2015). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Sundbyberg, Sweden) and Clarity™ Western ECL Substrate (Bio-Rad, Sundbyberg, Sweden). Protein bands were detected using the ChemiDoc™ XRS + System with Image Lab™ Software (Bio-Rad, Sundbyberg, Sweden), and the optical density of each blot was quantitated using NIH Image J version 1.29 (National Institutes of Health). Rabbit polyclonal antibody against heat shock protein 90 (Hsp90; Cat No. 4874, 1:10,000; Cell Signaling Technology, Beverly, MA, USA) served as a loading control. All blots were normalized to its corresponding loading control, Hsp90 (Cat No. 4874, 1:10,000; Cell Signaling Technology, Beverly, MA, USA).

2.4. Statistics

All statistical analyses were performed using STATVIEW version 5.1 software. Data from the open field, LD and EPM tests were analyzed using three-way repeated-measures analysis of variance (ANOVA). Genotype and Sex were considered the main factors in all the analyses. The repeated factor depended upon the outcome measure (see Supplementary Table 2). Statistically significant main effects or interactions were followed up with a two-way ANOVA for each level of the repeated variable, with Genotype and Sex as main factors. Data from the social interaction test and gene expression were analyzed with two-way ANOVA, with genotype and sex as main factors. All *post hoc* comparisons were made with the Bonferroni test in the presence of significant ANOVA effects. Western blot analysis was analyzed using two-tailed unpaired *t*-test. The threshold for statistical significance was set at $p \leq 0.05$. All data are presented as the means (±S.E.M.).

3. Results

The results from the ANOVA analyses for the open field, LD and EPM tests are presented in Supplementary Tables 2–5.

3.1. Mice lacking the TLR4 receptor displayed anxiety-like phenotype

Male and female TLR4 KO mice spent significantly less time in the center and more time in the periphery of the open field during the habituation period (15–90 min) compared to wild type mice (Genotype as main factor for time in center and for time in periphery: $F_{1,36} = 15.109$, $p = 0.0004$, and $F_{1,36} = 15.108$, $p = 0.0004$, respectively; Fig. 1A and B), whereas the time spent during the novelty period (0–15 min) did not change. Interestingly, female wild type mice spent significantly more time in the center and less time in the periphery of the open field during the habituation period compared to male wild type mice (Sex as main factor for time in center and for time in periphery: $F_{1,36} = 6.803$, $p = 0.0132$, and $F_{1,36} = 6.799$, $p = 0.0132$, respectively; Fig. 1A and B). There were no significant differences in the total distance traveled and number of rears in the open field in the 90-min period (Fig. S1).

The time spent in the light compartment of the LD box was significantly reduced in male and female TLR4 KO mice compared to

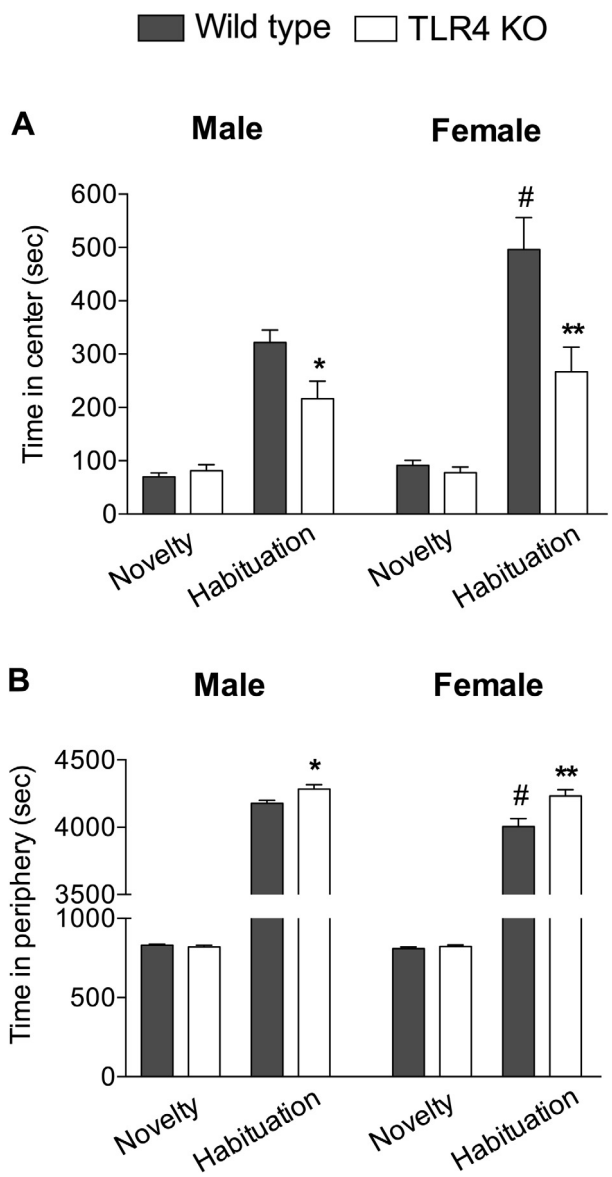


Fig. 1. Open field test in wild type and TLR4 KO mice of both sexes. Time spent (sec) in the center (A) and periphery (B) of the open field arena in the novelty phase (0–15 min) and habituation phase (15–90 min). All data (A–B) are presented as means (±S.E.M; n = 10 mice per group). Bonferroni's multiple comparison test: **p < 0.01; ***P < 0.001 compared with control wild type mice; #p < 0.05 female vs. male from the same genotype.

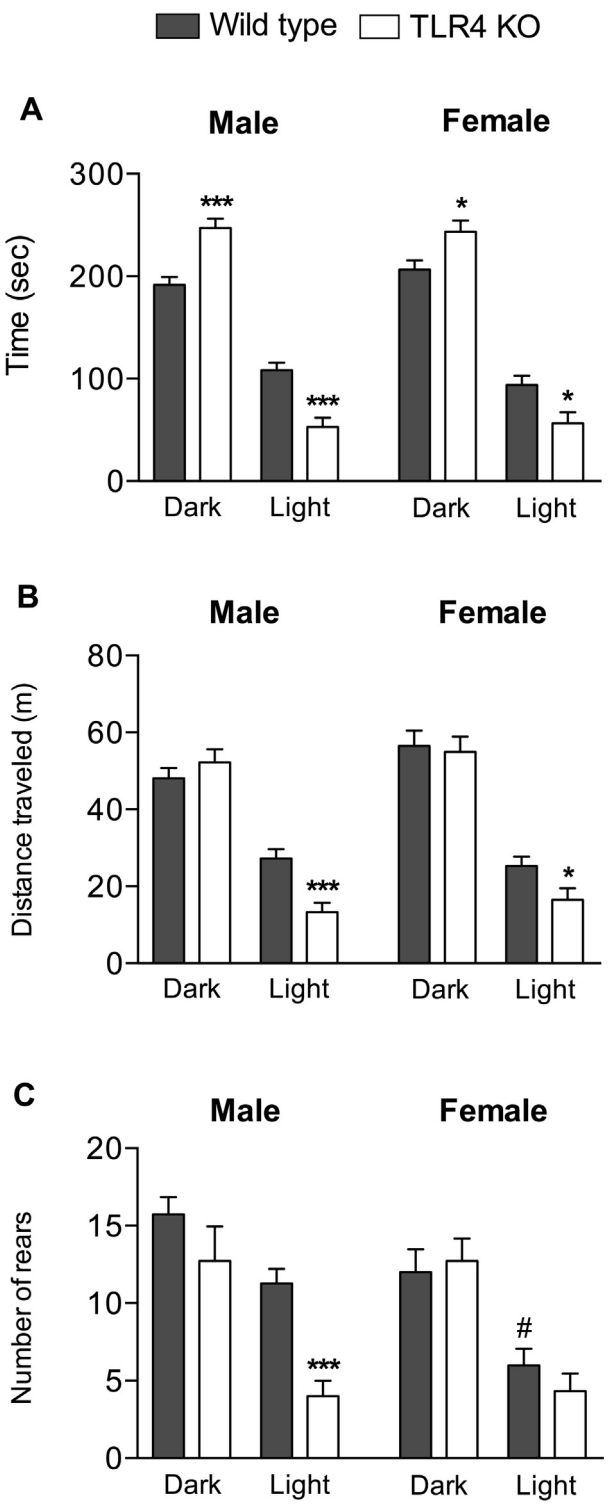


Fig. 2. Light and dark box test in wild type and TLR4 KO mice of both sexes. Time spent (sec) (A), distance traveled (m) (B), and number of rears (C) in dark and light compartments measured for 5 mins. All data (A–C) are presented as means (±S.E.M; n = 13–15 mice per group). Bonferroni's multiple comparison test: *p < 0.05; **p < 0.01; ***p < 0.001 compared with control wild type mice; #p < 0.05 female vs. male from the same genotype.

their respective wild type controls, while the time spent in the dark compartment was significantly increased (Genotype as main factor for time in light and for time in dark: $F_{1,54} = 25.626$, $p < 0.0001$, and $F_{1,54} = 25.517$, $p < 0.0001$, respectively; Fig. 2A). The distance traveled in the light compartment was significantly shorter in both male and female TLR4 KO mice compared to their respective wild type controls (Genotype as main factor: $F_{1,54} = 19.166$, $p < 0.0001$; Fig. 2B). In addition, the number of rears in the light compartment was significantly lower in male TLR4 KO mice compared to male wild type mice (Genotype as main factor: $F_{1,54} = 18.564$, $p < 0.0001$; Fig. 2C). In contrast, the number of rears in the LD test did not differ significantly in female TLR4 KO mice (Fig. 2C). There were also significant sex differences in the numbers of rears in the light compartment (Sex as main factor: $F_{1,54} = 5.662$, $p = 0.0209$; Fig. 2C), with female wild type mice displaying significantly lower number of rears compared to male wild type mice.

In the EPM test, male TLR4 KO mice spent significantly less time in the open arms and more time in the closed arms compared to male wild type mice (Arm × Genotype interaction for time: $F_{2,108} = 3.48$, $p = 0.0343$; Fig. 3A). In contrast, the time spent in these compartments did not differ significantly in female TLR4

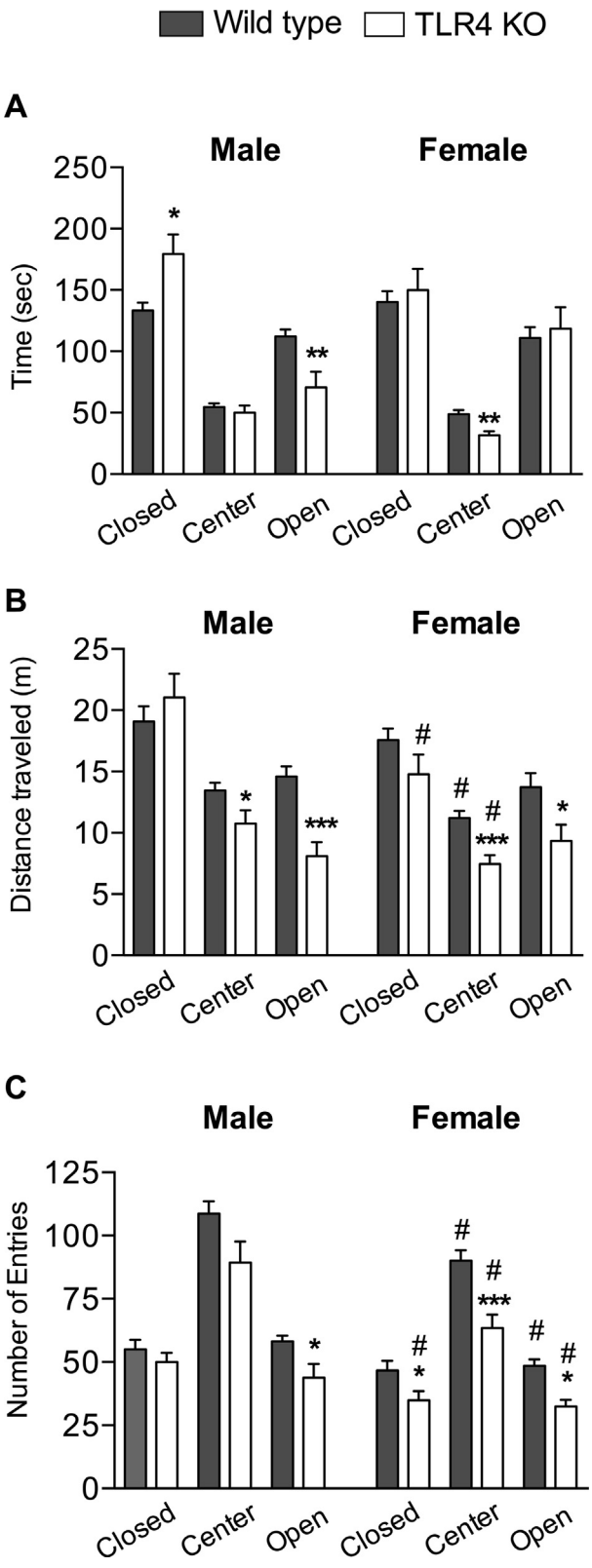


Fig. 3. Elevated plus maze test in wild type and TLR4 KO mice of both sexes. Time spent (sec) (A), distance traveled (m) (B), and number of entries (C) into open arms, closed arms, and center during a 5-min test session. All data (A–C) are presented as means (±S.E.M; n = 13–15 per group). Bonferroni's multiple comparison test: *p < 0.05; **p < 0.01; ***p < 0.001 compared with control wild type mice; #p < 0.05 female vs. male from the same genotype.

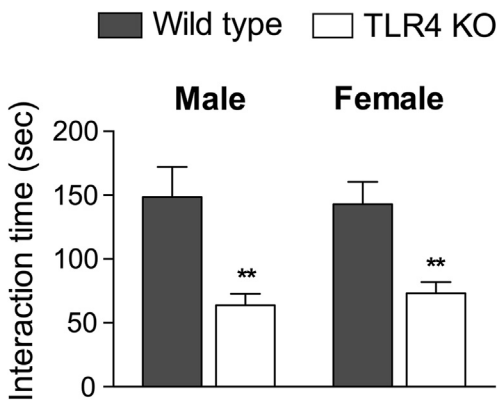


Fig. 4. Interaction time (sec) of wild type and TLR4 KO male mice and female mice in the social interaction test. All data are presented as means (±S.E.M; n = 8–9 per group). Bonferroni's multiple comparison test: **p < 0.01 compared with control wild type mice.

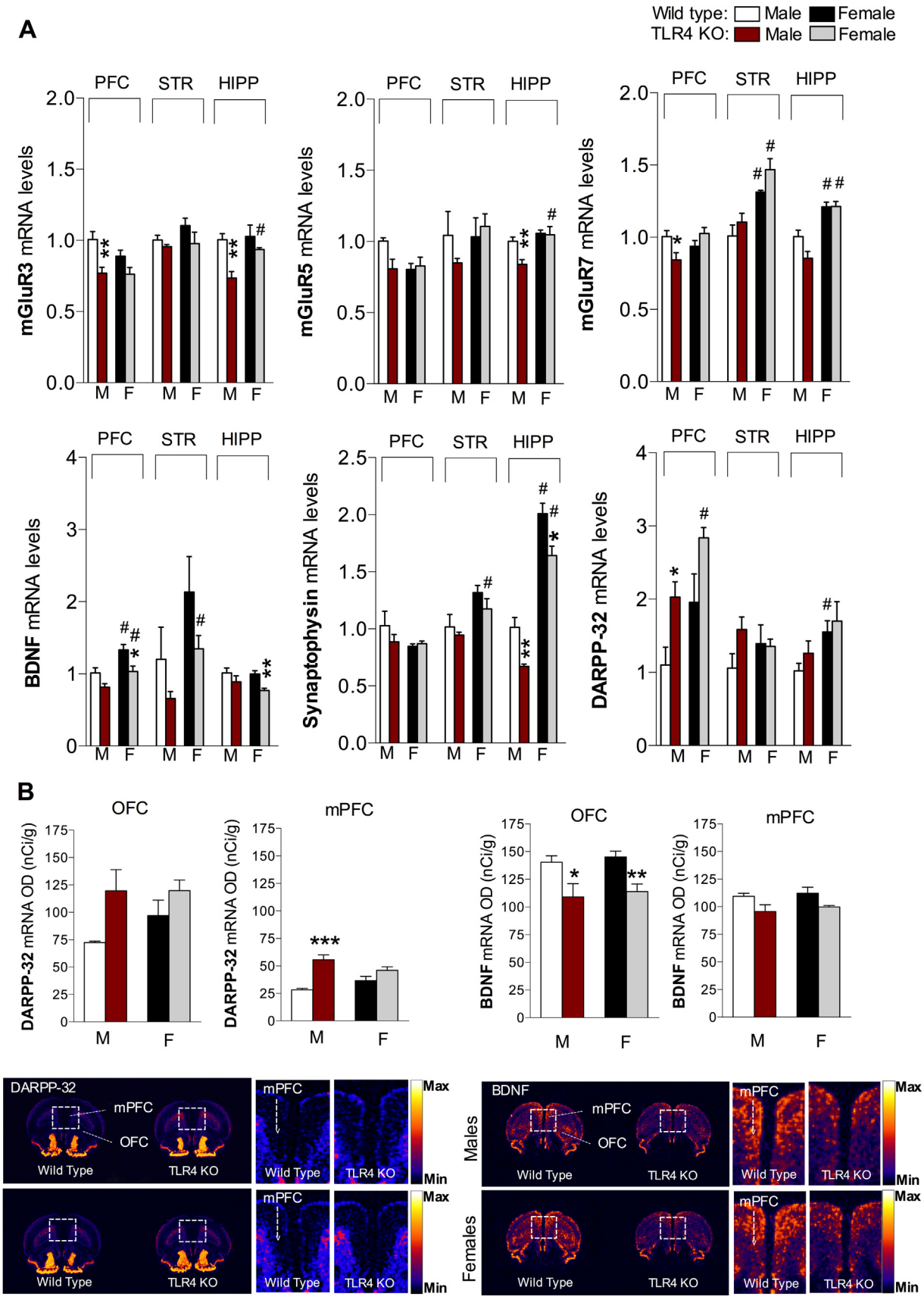
KO mice (Fig. 3A). However, female TLR4 KO mice spent significantly less time in the center compared to female wild type mice (Fig. 3A). The distance traveled in the center and in the open arm was decreased significantly in male and female TLR4 KO mice compared to their respective control mice (Arm × Genotype interaction: $F_{2,108} = 5.54$, $p = 0.0051$; Fig. 3B). Moreover, the number of entries in the open arm was significantly lower in male and female TLR4 KO mice compared with their respective wild type controls. In addition, female TLR4 KO showed significant less entries in the center compared to female wild type mice (Arm × Genotype interaction for number of entries: $F_{2,108} = 24.16$, $p < 0.0001$; Fig. 3C). Of note, there were significant sex differences in the distanced traveled and the number of entries (Sex as main factor: $F_{1,54}$=7.34, $p = 0.0090$, and $F_{1,54} = 4.95$, $p = 0.0302$, respectively; Fig. 3B and C).

3.2. Social behavior was reduced in TLR4 KO mice

In the social interaction test, both male and female TLR4 KO mice interacted for a significantly shorter time with the stimulus mouse compared with wild type mice (Genotype as main factor: $F_{1,29} = 24.79$, $p < 0.0001$; Fig. 4). No significant sex differences were found (Sex as main factor: $F_{1,29} = 0.015$, $p > 0.1$; Genotype x Sex interaction: $F_{1,29} = 0.244$, $p > 0.1$).

3.3. Altered gene expression in different brain regions was associated with emotions in TLR4 KO mice

The expression levels of metabotropic glutamate receptor 3 (mGluR3) were significantly decreased in the prefrontal cortex and hippocampus of male TLR4 KO mice compared to male wild type mice (Genotype as main factor: $F_{1,16} = 15.25$, p = 0.0013; and $F_{1,16} = 12.90$, p = 0.0024, respectively; Fig. 5A, top left panel). Similarly, mGluR5 mRNA levels were decreased in the hippocampus of male TLR4 KO mice compared with their wild type controls (Genotype as main factor: $F_{1,16} = 5.41$; p = 0.0335; Fig. 5A, top center panel). In the prefrontal cortex, mGluR7 mRNA levels were significantly decreased in male TLR4 KO mice (Genotype × Sex interaction: $F_{1,16} = 8.344$, p = 0.0107; Fig. 5A, top right panel). Interestingly, there were significant sex differences in mGluR3 mRNA levels in the hippocampus and in mGluR7 mRNA levels in the striatum and hippocampus (Sex as main factor: $F_{1,16} = 5.03$, p = 0.0395; $F_{1,12} = 28.23$, p = 0.0002; and $F_{1,16} = 48.01$, p = 0.0001;


A
Wild type: Male Female
TLR4 KO: Male Female
PFC STR HIPP
mGluR3 mRNA levels
mGluR5 mRNA levels
mGluR7 mRNA levels
BDNF mRNA levels
Synaptophysin mRNA levels
DARPP-32 mRNA levels
M F
B
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mPFC
DARPP-32 mRNA OD (nCi/g)
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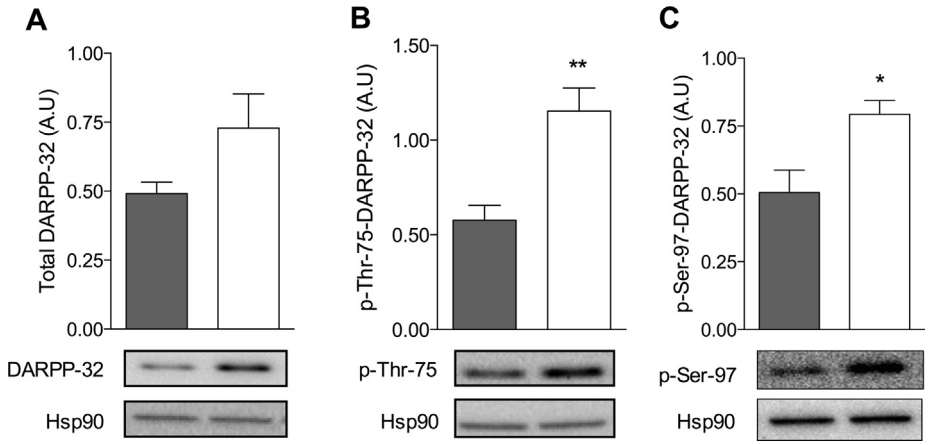

Fig. 6. Protein expression levels of total DARPP-32 (A), p-Thr-75-DARPP-32 (B), and p-Ser-97-DARPP-32 (C) in male TLR4 KO mice in the prefrontal cortex. All data (A-C) are presented as means (±S.E.M; n = 5 per group). Student *t*-test: *p < 0.05; **p < 0.01 compared with control wild type mice.

Fig. 5A, top right panel). In wild type and KO mice, the mRNA expression levels of mGluR7 were significantly higher in female mice than in male mice ($p < 0.05$; Fig. 5A, top right panel).

The total mRNA levels of BDNF were significantly decreased in the prefrontal cortex and hippocampus of female TLR4 KO mice but not of male TLR4 KO mice compared with their wild type controls (Genotype as main factor: $F_{1,16} = 12.80$, $p = 0.0025$; and $F_{1,16} = 7.877$, $p = 0.0127$, respectively; Fig. 5A, bottom left panel). In the prefrontal cortex and striatum, there were significant sex differences in the expression levels of BDNF (Sex as main factor: $F_{1,16} = 14.94$, $p = 0.0014$; and $F_{1,12} = 5.45$, $p = 0.0374$, respectively; Fig. 5A, bottom right panel). In both genotypes, the mRNA expression levels of BDNF in the prefrontal cortex of female mice were significantly higher than those in the prefrontal cortex of male mice. However, in the striatum, this increase was only evident in TLR4 KO mice. It is worth mentioning that the mRNA levels of BDNF were significantly lower in the striatum than in the prefrontal cortex and hippocampus, as reflected by the higher C_t values (29 vs. 22–25 in the other regions).

The expression levels of synaptophysin were significantly decreased in the hippocampus of male and female TLR4 KO mice compared to their respective wild type controls (Genotype as main factor: $F_{1,16} = 22.37$, $p = 0.0002$; Fig. 5A, bottom center panel). In addition, there were significant sex differences in the mRNA levels of synaptophysin in the striatum and hippocampus (Sex as the main factor: $F_{1,12} = 11.86$, $p = 0.0049$; and $F_{1,16} = 172.81$, $p < 0.000\,1$, respectively; Fig. 5A, bottom center panel). In both genotypes, the mRNA levels of synaptophysin were significantly higher in the hippocampus of female mice compared to male mice (Fig. 5A, bottom center panel). However, this sex effect was less pronounced in the striatum.

The mRNA levels of DARPP-32 were robustly increased in the prefrontal cortex of male TLR4 KO mice compared to male wild type controls (Genotype as main factor: $F_{1,15} = 10.59$, $p = 0.0050$; Fig. 5A, bottom right panel). The same trend was observed in female TLR4 KO mice, but it was not significant ($p = 0.0613$). Moreover, there were significant sex differences in the expression of DARPP-32 in the prefrontal cortex and hippocampus (Sex as main factor: $F_{1,16} = 9.34$, $p = 0.0075$; and $F_{1,16} = 7.05$, $p = 0.0173$, respectively; Fig. 5A, bottom right panel), and the levels were higher in female mice than in male mice.

Changes in the expression levels of DARPP-32 and BDNF in specific frontal cortical regions (orbital and medial prefrontal cortex) of TLR4 KO mice were further confirmed via *in situ* hybridization. DARPP-32 mRNA levels were significantly increased in the medial prefrontal cortex of male TLR4 KO mice but not of female TLR4 KO mice compared to their respective wild type controls (Genotype as main factor: $F_{1,16} = 26.68$; $p < 0.0001$; Fig. 5B, top left panel). The same trend was observed in the orbital frontal cortex of male TLR4 KO mice, without reaching significance. In contrast, the mRNA levels of BDNF were significantly decreased in the orbital frontal cortex of male and female TLR4 KO mice compared to the respective wild type controls (Genotype as main factor: $F_{1,16} = 15.77$; $p = 0.0011$; Fig. 5B, top left panel).

3.4. TLR4 modulates the protein expression and phosphorylation state of DARPP-32

The total protein levels of DARPP-32 were non-significantly higher in the frontal cortex of male TLR4 KO mice compared to male wild type controls (Student *t*-test: $t = 1.824$, $df = 8$, $p = 0.1056$; Fig. 6A). The analysis of different phosphorylated sites in DARPP-32 revealed a significant increase in the phosphorylation levels at Thr-75 and Ser-97 (Student *t*-test: $t = 3.99$, $df = 8$, $p = 0.0040$; $t = 2.972$, $df = 8$, $p = 0.0178$, respectively; Fig. 6B–C). However, the phosphorylation levels at Thr-34 (which are very low under normal conditions) were not statistically different between TLR4 KO and wild type mice (data not shown).

4. Discussion

Here we demonstrated that mice lacking TLR4 had a robust anxiety-like phenotype and impaired social behavior. These behavioral responses indicate that TLR4 plays an important role in the regulation of anxiety. In line with this behavioral phenotype, the expression of several genes (e.g., BDNF and metabotropic glutamate receptors) fundamental for the functional regulation of emotional responses was altered in TLR4 KO mice. Furthermore, our

Fig. 5. Gene expression profile evaluated via qRT-PCR of metabotropic glutamate receptors, brain-derived neurotrophic factor (BDNF), synaptophysin, and dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32) related to anxiety in TLR4 KO and wild type male mice (M) and female mice (F) in the prefrontal cortex (PFC), striatum (STR), and hippocampus (HIPP) (A). All data are presented as means (±S.E.M; n = 4–5 per group). Bonferroni's multiple comparison test: *p < 0.05, **p < 0.01 compared with control wild type mice; #p < 0.05 female vs. male from the same genotype (A). Gene expression evaluated via *in situ* hybridization of DARPP-32 and BDNF in TLR4 KO and wild type male mice (M) and female mice (F) in the medial prefrontal cortex (mPFC) and orbitofrontal cortex (OFC) (B). All data are presented as means (±S.E.M; n = 4–5 per group). Bonferroni's multiple comparison test: *p < 0.05, **p < 0.01, ***p < 0.001 compared with control wild type mice (B). The lower panels indicate the regional localization and expression of the corresponding genes in the depicted areas. Colored bars represent the intensity of expression (minimal optical density–maximal optical density).

results revealed that TLR4 regulated the expression and activity of DARPP-32, which is essential for the integration of several neurotransmitter pathways involved in cellular and transcriptional responses.

Previous studies have suggested that TLR4 plays a role in emotional responses. However, the anxiety phenotype and associated underlying mechanisms have not been fully elucidated. In our study, no differences between TLR4 KO and wild type mice were observed in the first 15 min (novelty period) of the open field test. However, the overall exploration time in our experimental setup was 90 min and, during the habituation phase (15–90 min), TLR4 KO mice of both sexes spent less time in the center, indicating a higher discomfort in the open and illuminated area. Rodents that are exposed to an open field naturally avoid the central "stressful" area and spend more time in the periphery. Accordingly, TLR4 KO mice had increased anxiety levels in the open field during the habituation phase. Importantly, these results were not due to motor deficits in TLR4 KO mice because the overall distance traveled was similar for both genotypes. Okun and colleagues found similar results in the time spent by wild type mice in the central area of the open field after the central administration of a TLR4 antagonist (Okun et al., 2012), suggesting that the emotional alterations observed in adult mice lacking TLR4 are not solely due to developmental effects. In line with our findings, Li and colleagues demonstrated that mice lacking TLR4 had reduced exploratory behavior in the novelty-seeking test and social interaction test in large open spaces (Li et al., 2016). It is of interest that no differences were found between the genotypes when mice were tested in a less stressful apparatus (smaller in area but with a similar wall color of the mouse's cage) (Li et al., 2016). These observations indicate that the environmental conditions play a critical role in behavior. Therefore, TLR4 related behavioral responses may be sensitive to specific stressful stimuli because mice may have distinct responses depending on the intensity of the stressor and experimental conditions.

The suggested anxiety phenotype was further investigated by evaluating TLR4 KO mice in two well-validated anxiety tests: the light and dark box test and the EPM test. Lighted, open and/or elevated spaces are used as strong stressful stimuli. In our experimental conditions, both male and female TLR4 KO mice spent less time in the stressful open areas and had less rearing activity than their counterparts, confirming an enhanced anxiety phenotype. Of note, this phenotype was more pronounced in male TLR4 KO mice than in female TLR4 KO mice. However, in the aforementioned study by Okun and colleagues, no differences were observed in the behavioral responses of male TLR4 KO mice in the EPM test (Okun et al., 2012). Since high anxiety levels have been associated with impaired social interaction (Iverach and Rapee, 2014), the social response of TLR4 KO mice upon exposure to a new mouse in a new home cage was evaluated. The results indicated that male and female mice lacking TLR4 interacted for significantly less time with an unfamiliar stimulus mouse than their respective controls. These findings are consistent with those of a recent study by Li and colleagues, wherein social interactions were reduced in male TLR4 KO mice (Li et al., 2016). Overall, these behavioral observations demonstrate that TLR4 plays a key role in modulating emotional and social behavioral responses associated with anxiety.

Next, we evaluated whether TLR4 affected genes known to be involved in emotional behavior, which could explain the anxiety phenotype. Several studies revealed that mGluRs might be used as new targets for treatment of anxiety disorders. In particular, various mGluR subtypes, including group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR7, and mGluR8), modulate excitability in critical brain structures involved in anxiety states (Swanson et al., 2005). In this study, the gene expression of mGluR3 and mGluR7 in the frontal cortex and the expression of mGluR3 and mGluR5 in the hippocampus were decreased in male TLR4 KO mice compared to wild type mice, supporting the observed anxiety-like phenotype of male TLR4 KO mice. Similarly, BDNF is known to regulate anxiety, neuroplasticity, and memory (Domingos da Silveira da Luz et al., 2013; Lu et al., 2014; Martinowich et al., 2007). Moreover, glutamate receptors are suggested to contribute to the maintenance of neurons by stimulating the production and release of trophic factors, including BDNF, thereby conferring neuroprotection (Di Liberto et al., 2010; Bessho et al., 1993; Jean et al., 2008). However, BDNF and mGluRs may be differentially regulated under certain conditions (e.g., stress) (Jia et al., 2015). Here, the expression levels of BDNF did not change significantly in male TLR4 KO mice. In turn, the BDNF expression in several brain regions involved in the processing of emotional stimuli, including the frontal cortex and hippocampus, was decreased in female TLR4 KO mice. These findings, at least those observed in the female mice, are in line with the hypothesis that anxiety disorders and depression are associated with decreased levels of BDNF (Berry et al., 2012; Lonsdorf et al., 2010). Notably, the baseline expression levels of various mGluRs and BDNF were increased in female mice, and these sex differences could be an important factor influencing the distinct changes observed in male and female TLR4 KO mice. Furthermore, in the hippocampus (a brain region primarily involved in memory and emotion), the synaptic plasticity marker synaptophysin was decreased in both male and female TLR4 KO mice. These findings demonstrate for the first time that TLR4 interacts with gene pathways closely associated with the regulation of anxiety responses.

In the present study, we further investigated the effect of TLR4 on DARPP-32, a well-known target for many neurotransmitters that regulate emotion and cognition, including glutamate, dopamine, serotonin, and GABA (Svenningsson et al., 2004). The mRNA levels of DARPP-32 were significantly increased in the prefrontal cortex of male TLR4 KO mice. DARPP-32 has received much attention in the past decade because of its function as a master switch in the cell to coordinate the degree of phosphorylation in different molecular targets in the cell membrane and cytoplasm (Fernandez et al., 2006; Reis et al., 2007; Yger and Girault, 2011). Considering that the most robust effect occurred in the prefrontal cortex of male TLR4 KO mice, the protein levels of DARPP-32 and its phosphorylation state were evaluated in this region. It is known that the phosphorylation of DARPP-32 at Thr34 may cause the inhibition of protein phosphatase 1 (PP-1), which amplifies the physiological effects of protein kinases on important targets, including transcription factors, receptors, voltage-gated ion channels, and protein kinases (for a review, see Svenningsson et al., 2004). This amplifying property of DARPP-32 is critical for dopaminergic signaling and other neurotransmitters. Interestingly, the level of phosphorylation of DARPP-32 at Thr34 was not changed, but the level of phosphorylation at Thr75 was significantly increased in male TLR4 KO mice. The phosphorylation of DARPP-32 at Thr75 inhibits PKA activity, which prevents the phosphorylation of Thr34 and promotes the activity of PP-1 (Svenningsson et al., 2004). Accordingly, our findings suggest that the lack of TLR4 would presumably diminish the action of transcription factors, receptors, voltage-gated ion channels and/or protein kinases. Furthermore, these changes were accompanied by the increased phosphorylation of DARPP-32 at Ser97 in male TLR4 KO mice, suggesting that the nuclear export of DARPP-32 to the cytoplasm is enhanced. In line with our findings, other studies reported that the levels of DARPP-32 and phospho-DARPP-32 were changed in mice with anxiety- and depression-like behaviors (Jin et al., 2015). In addition, DARPP-32 KO mice showed a reduced anxiety-like phenotype (Ehrman et al., 2006).

In conclusion, our findings demonstrate the relevant role of TLR4 in the regulation of anxiety-like behavior in rodents. We suggest that alterations in the expression and activity of DARPP-32

could be an important mechanism mediating the anxiogenic phenotype in TLR4 KO mice, as shown in the behavioral tests. One possibility is that TLR4 directly modulates DARPP-32 and subsequently modulates mGluRs and neurotrophic factors, and thus triggers emotional behavioral changes in this model. These results shed light on new potential therapeutic targets. However, additional studies using more advanced transgenic mouse models are necessary to clarify the functional modulation and interaction between the downstream TLR4 signaling targets and DARPP-32 signaling pathways. Furthermore, the role of gonadal hormones in TLR4 KO mice needs to be investigated as this may help explain the sex-dependent changes observed in the present study.

Conflict of interest

The authors declare that there are no conflicts of interest associated with this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbi.2017.11.022.

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