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Anti-depressive-like behaviors of APN KO mice involve Trkb/BDNF signaling related neuroinflammatory changes

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Major depression disorder is a severe mental illness often linked with metabolic disorders. Adiponectin is an adipocyte-secreted circulatory hormone with antidiabetic and glucose/lipid modulation capacities. Studies have demonstrated the pathophysiological roles of adiponectin involved in various neurological disorders, including depression. However, the underlying mechanisms are poorly understood. Here we showed that adiponectin deprivation enhanced antidepressive-like behaviors in the LPS-induced model of depression. APN KO mice displayed increased cytokines (both pro and anti-inflammatory), accompanied by an impaired expression of adiponectin receptors (mRNA/protein level) and decreasing IBA-1 level in the cortex and primary microglia of LPS treated APN KO mice. Further, LPS-treatment significantly reduced p-NFκB expression in the microglia of APN KO mice. However, the Bay11-7082 treatment recovered p-NFκB expression in the cortex of APN KO mice in the presence of LPS. Interestingly, the antidepressant potentials of APN KO mice were abolished by TrkB antagonist K252a, IKK inhibitor Bay11-7082, and AdipoRon suggesting crosstalk between TrkB/BDNF signaling and NFκB in depression. Furthermore, the effects of Bay11-7082 were abolished by a TrkB/BDNF activator (7,8-DHF), indicating a critical role of TrkB/BDNF signaling. Taken together, these findings showed that dysregulated neuroinflammatory status and BDNF signaling might underlie the antidepressive-like behaviors of APN KO mice. NFκB elicited BDNF changes may be accountable for the pathogenesis of LPS induced depression, where APN might present an alternative therapeutic target for depressive disorders.

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INTRODUCTION

Major depressive disorder (MDD) is a complex, inhomogeneous, debilitating, and highly prevalent mental illness associated with significant morbidity and mortality [1, 2]. The symptomology of MDD spans a broad spectrum, including lack of energy, low self-esteem, sadness, loss of interest and pleasure, and social withdrawal [3, 4]. Current treatments for depressive disorders mainly rely on monoaminergic system regulation; however, about 30% of depressive patients are partially or entirely resistant to this treatment [5–7], demanding an urgent need for further investigation.

Multiple interacting factors, including genetic, social, physiological, and biological processes, may contribute to the pathogenesis of depression [1, 4, 8]. Recent studies reported neurotransmitter or metabolic system dysregulation is involved in the onset of depression [9, 10]. However, as one of the critical indicators of treatment-resistant depression, the link and causal relationship of the "binomial stress inflammation" link to depression have not been elucidated. An increasing level of cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), have been demonstrated in the peripheral blood and CSF of depression patients [11]. Similarly, previous studies, including ours, have shown that lipopolysaccharides (LPS) administration induced depression exhibited impaired chemokines and cytokines levels [12, 13]. Impaired cytokine levels could affect the metabolism of norepinephrine, 5-HT, dopamine, and neuroendocrine function [14–16], leading to altered glial cell activities, BDNF dysregulation, and impaired neurogenesis [11, 12, 15, 17], demonstrating a possible causal relationship between inflammation and depression. Additionally, accompanied redox signaling modulates inflammasome activation and cell migration and adherence [18, 19]. Inflammatory responses recruit redox system and pro-oxidant signals, which play a pathological role in depression progression [20–22]. Mitochondria-generated ROS (reactive oxygen species) contributes to innate immunity activation, intending a correlation among inflammasome activation, ER stress, and mitochondrial process impairment under stress conditions [23–25]. Altered NLRP3 and complex I levels have been reported in the MDD patients' frontal cortex after post-mortem [26–28], suggesting a causative relationship among ROS, inflammation, and neurological disorders.

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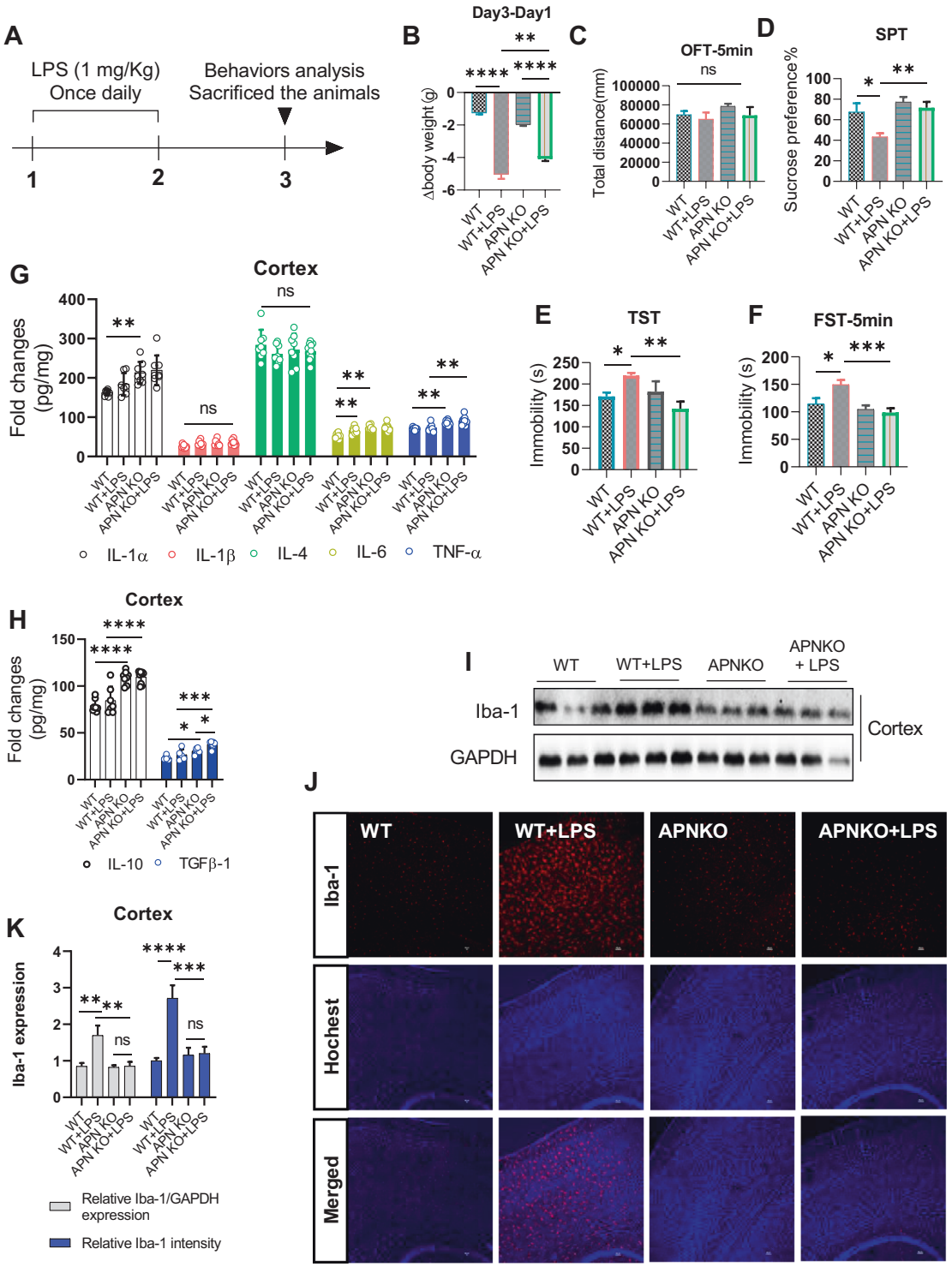


Fig. 1 LPS-treatment did not enhance depressive-like behaviors and Iba-1, while modified cytokines level in APN KO mice. A Experimental approach, **B** Relative body weight changes, **C** We didn't detect any significant difference among the animals during Open field test analysis, $n = 8–10$, The significant difference was recorded among the experimental groups during the Sucrose preference test (**D**), Tail suspension test (**E**), and Forced swimming test (**F**) $n = 8–10$. **G–H** LPS-treatment did not alter IL-1 α, IL-1β, IL-4, while it significantly enhanced IL-6, TNF-α, TGFβ−1, IL−10 in the APN KO mice cortex. LPS-administration did not alter IBA-1 expression in the cortex **I**, **J**, and **K** of APN KO mice, measured by immunofluorescence and immunoblotting (bar graphs showing relative IBA-1 intensities) $n = 6$. Mean ± SEM, ANOVA, Tukey's test. $p > 0.05$. *$p < 0.05$, **$p < 0.01$), ***$p < 0.001$, ****$p < 0.0001$.

Adiponectin (APN) is an adipocyte-secreted circulatory hormone that plays a significant role in glucose and lipid metabolism [29, 30] and emerging as a promising molecular target to treat metabolic disorders. Interestingly, accumulated pieces of evidence suggest the association of APN to mental disorders, including depression, as impaired APN level has been detected in MDD patients [31–34]. Studies have shown that APN regulates fear memory extension via its receptors AdipoR1 and AdipoR2

expressed throughout the brain. Further investigation shows selective deletion of AdipoR1 in 5-HT neurons induced depression-related behaviors in mice, presumably resulting from 5-HT deficits that can be reversed fluoxetine but not desipramine [35]. Moreover, APN signaling contributes to hippocampal neurogenesis, synaptic functions, and energy expenditure [30, 32–34, 36–38]. However, the exact role and contribution of APN to the pathophysiology of treatment-resistant depression remain controversial and rudimentary. In the present study, we used Adiponectin knockout mice treated with LPS to evaluate and investigate adiponectin's mechanistic interplay in depressive-like behaviors. The detailed molecular mechanisms underlying depression, including BDNF, neuroinflammation, redox systems, and depression-related molecular signaling changes, were examined.

MATERIAL AND METHODS

Animals

The APN KO mice (12–14 weeks) with a C57BL/6 background were obtained from the Jackson Laboratory. Wild type (WT) C57BL/6 J was purchased from Guangdong medical laboratory animal center, China. All the experimental animals used here were 12–14 weeks old. The experimental animals were housed at Laboratory Animal Research Center, Peking University Shenzhen Graduate School, under a 12 h light/12 h dark cycle at 18–22 °C. They had free access to diet and tap water throughout the study. The experimental procedures were set in such a way as to minimize mice suffering. All the experimental procedures were performed according to the protocols approved by the *Institutional Animal Care and Use Committee of Peking University Shenzhen Graduate School*.

Drug administration and schedule

The present investigation is comprised of the following experiments.

1. The experimental mice (total $n = 40$: and 10/group) (APN KO and wild type, WT) were treated with LPS (1 mg/kg, i.p) for 2 days (Fig. 1A), and groups were: WT, WT + LPS, APN KO, and APN KO + LPS. 24 h after the final LPS administration, behavior analysis (SPT, TST, OFT, and FST) were performed as previously reported [39, 40]. Finally, the animals were sacrificed, and the serum and tissue were collected for further analysis.
2. In the second experiment mice (total $n = 50$: and 10/group) were treated with, K252a (25 ug/kg, ip, dilute in 0.1% DMSO), or AdipoRon (50 mg/kg, ig, dilute in 0.5%CMCC), or Bay11-7082 (5 mg/kg, i.p, dilute in ddH_2O), followed by LPS administration, and groups were: WT + LPS, APN KO + LPS, APN KO + LPS + K252a, APN KO + LPS + Bay11-7082, and APN KO + LPS + AR. The drug treatment schedule has been shown in Fig. 3A. 24 h after the final LPS administration, mice behavior analysis was performed and sacrificed, followed by brain tissues collection.
3. In the 3rd experiment, mice ($n = 30$: and 10/group) were treated with 7,8-dihydroxyflavone (5 mg/kg, i.p, diluted in 0.1% DMSO), along with Bay11-7082, followed by LPS. The groups were: APN KO + LPS, APN KO + LPS + Bay11-7082, and APN KO + LPS + Bay11-7082 + 7,8-DHF. The drug treatment schedule has been shown in Fig. 4A. 24 h after the final LPS administration, behavior analyses were performed, sacrificed the mice and collected the brain tissues.
4. In the next experiment, AdipoR2 KO mice ($n = 32$: and 8/group) were treated with LPS (1 mg/kg) for 2 days. Groups were: WT, WT + LPS, AdipoR2 KO, AdipoR2 KO + LPS. 24 h after the final LPS administration, behavior analysis (OFT, TST, and SPT) were performed and analyzed. The drug treatment schedule is shown in Fig. 5G.

Finally, the animals were sacrificed, and the serum and tissue were collected for further analysis.

K252a: inhibitor of TrkB, Bay11-7982: inhibitor of NFkB and IKKβ; 7,8-DHF: is the BDNF agonist.

Behavioral assays

Open field test (OFT), Tail suspension test (TST), Forced swimming test (FST), and Sucrose preference test (SPT) were performed according to the previously described protocols [41, 42]. Details of the assays are in the supplementary data.

Primary microglia culture and treatment

Microglia primary culturing was performed according to the previously developed protocol [43]. In brief, brain tissues from postnatal (day 1-3) APN KO mice and WT mice were isolated, cut into tiny pieces, and followed by digestion with trypsin for 15 min. Dissociated tissues mixed with glia were then plated into T-25 culture flasks containing DMEM/F12 with 10% fetal bovine serum, GlutaMAX (Invitrogen), and 1% penicillin/streptomycin. After culture in a 5% CO2/37°C incubator for 14 days, the flasks were shaken at 220 rpm for 4 h at 37 °C to harvest the primary microglia. After that, the microglia were plated in 6-well plates at a density of 5×10^5 cells per well for further analysis.

Nitric oxides and H_2O_2 measurement

The NO and H_2O_2 were analyzed by a commercially available kit (Beyotime Institute of Biotechnology, China, CAT# S0021M, and CAT# S0038, respectively) [44, 45]. Details are in supplementary data.

ELISA

Frozen hippocampal and cortical tissue was lysed with RIPA buffer and homogenized on ice. Supernatants were collected after centrifugation and stored at freezing temperature for further analysis. According to the manufacturer's protocols, ELISA kits (ABclonal, and malbio) were used to quantify cytokine expression. Briefly, after washing the wells of the 96-well plate, 100 µL standard/sample was added and incubated for 2 h at 37 °C. Next, the plates were cleaned, and each well was added with a biotin-conjugated antibody (1:30), followed by incubation for 1 h at 37 °C and Streptavidin-HRP for 30 min at 37 °C. Finally, the reaction was stopped, and the optical density was measured accordingly.

Quantitative real-time PCR analysis

Briefly, total RNA was extracted from cortex tissues with Trizol reagent (Invitrogen), and cDNA was synthesized using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (Transgen Biotech). qRT-PCR was performed using TransScript Tip Green qPCR SuperMix (Transgen Biotech) according to the manufacturer's instructions, with each sample run at least in duplicate. Relative transcript abundance was normalized to β-actin.

Mitochondrial copy number

For relative mitochondrial copy number (mtCN) estimation, qPCR-based DNeasy Blood and Tissue Kit (Qiagen Ltd, UK) was used. Primer sequences for the mitochondrial segment were as follows: (F) GCCAGCCTGACCCATAGCCATAAT and (R) GCCGGCTGCGTATTCTACGTTA. Primer sequences for the single-copy nuclear control were as follows: (F) TTGAGACTGTGATTGGCAATGCCT and (R) CCAGAAATGCTGGGCGCTCACT. The mtCN was calculated relative to nuclear DNA using the following equations: 1. ΔC_T = mitochondrial C_T – nuclear C_T; 2. Relative mitochondrial DNA content = $2 \times 2^{-\Delta CT}$.

ATP assay

ATP assays were performed according to the instructions provided with ATP analysis ki (Cat # S0026, Beyotime technology, China). Briefly, the hippocampus of mice was split with ATP lysate, boiled at 100 °C for two minutes, centrifugated at 4 °C at 12000 rpm for 5 min, and collected the supernatant. Next, 50 uL of the sample was added into 100 uL of the reagents working solution and finally measured by the luminometer.

Immunofluorescence

Immunofluorescence staining was performed according to previously reported protocols [46]. Details are in supplementary data.

Short hairpin (sh)RNA expression constructs and treatment

shRNA plasmid coding was purchased from Haixing Biosciences (88 keling Road, Huqiu District, Suzhou, Jiangsu, China). The shRNA targeting mouse Adipor1 had the sequence 5′-GACGATGCTGAGACCAAATATCTCGAGATATTTGGTCTCAGCATCGTC-3′. The shRNA targeting mouse Adipor2 had the sequence 5′- CTTATGGCTAGCCTCTATATCCTCGAGGATATAGAGGCTAGCCATAAG-3′. The scrambled RNA sequence, used as a control, had the sequence 5′-

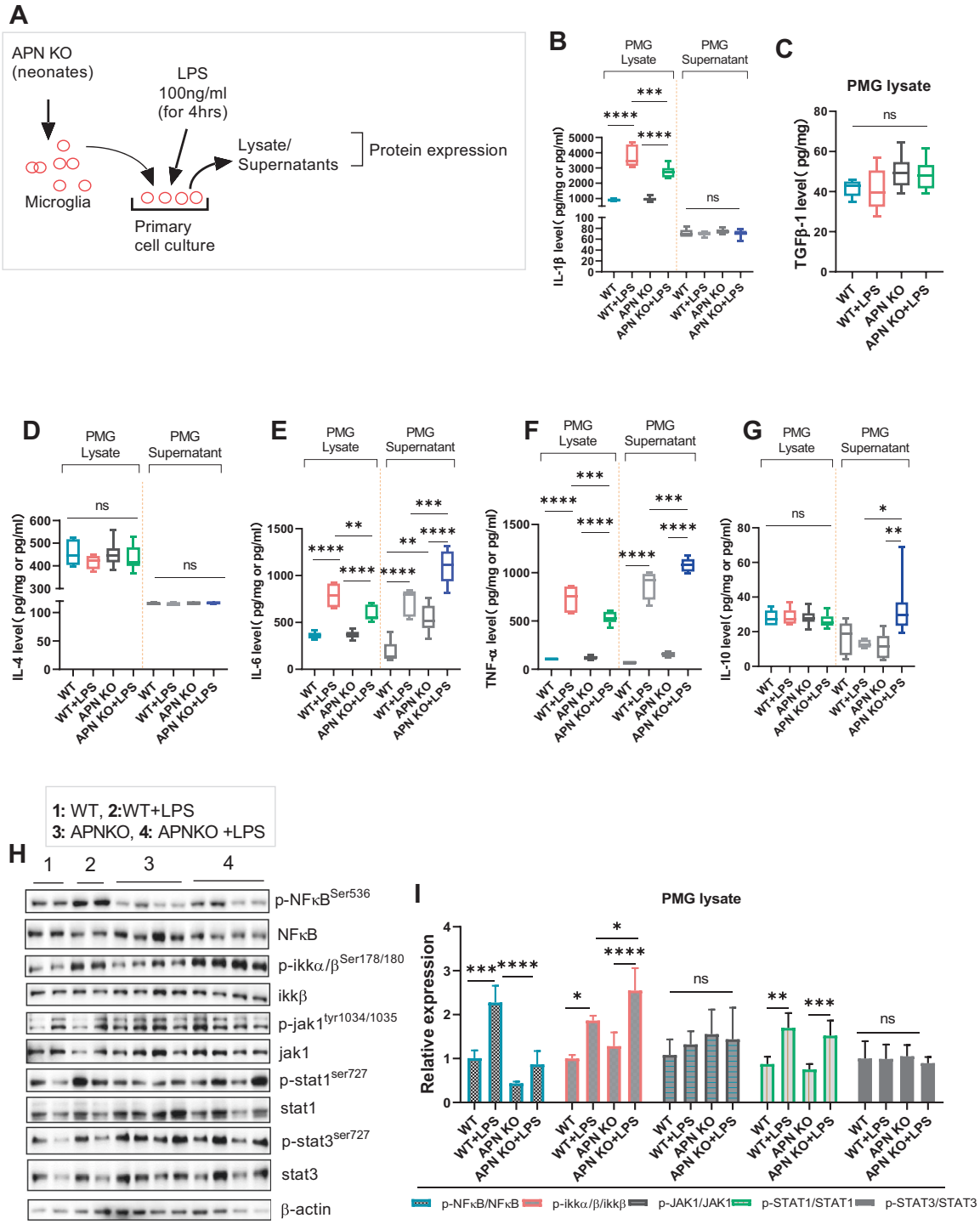


Fig. 2 LPS-treatment to PMG of APN KO mice. A Represents approach, **B** Representative bar graphs of cytokines, including IL-1β, **C** TGFβ-1, **D** IL-4, **E** IL-6, **F** TNF-α, **G** IL-10, in the PMG (lysates/supernatant) of APN KO mice. ELISA, $n = 8$–10, ANOVA, mean ± SEM, ANOVA, Tukey's test. **H**–**I** Representative immunoblots and bar graphs of p-(NFκB), p-ikkα/β, p-STAT1, and p-STAT3 expression ($n = 6$). Mean ± SEM, ANOVA, Tukey's test. $p > 0.05$. *$p < 0.05$, **$p < 0.01$), ***$p < 0.001$, ****$p < 0.0001$.

CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG-3. The plasmid (4 µg/well) containing shRNA was transfected to BV2 cells in a 6-well plate. After 44 h, the BV2 cells were treated with LPS (100 ng/ml). Finally, after 4 h of LPS treatment, cells were collected and proceeded for further analysis.

Western blot

Immunoblotting was performed according to the developed protocol in the lab [39]. The detail is in the supplementary data.

Statistical analysis

Western blot bands and morphological data were analyzed using ImageJ and image lab software (Image J 1.30) and analyzed by GraphPad Prism 8 software. Sample size selection was made as described previously [47, 48]. All the experiments were repeated three times independently. No randomization/blinding method was used. Data were presented as mean ± SEM. One-way ANOVA followed by posthoc Tukey Multiple Comparison tests were performed to compare different groups. $P < 0.05$ was regarded as significant. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$, and ****$p < 0.00001$.

RESULTS

APN KO mice displayed antidepressant-like behaviors and dysregulated neuroinflammatory responses with LPS administration

LPS is a well-known inflammatory agent and has been widely used to induced depressive-like behaviors in mice [42, 49]. Herein, mice (WT) treated with LPS showed significantly reduced body weight and sucrose preference (Fig. 1B, D) while exhibited increased immobility time in FST and TST tasks (Fig. 1E, F). However, LPS administration did not enhance depressive-like behavior of immobility in the APN KO mice (Fig.1E, F). The antidepressant-like behaviors of the APK KO mice were further validated by SPT analysis (Fig. 1D).

The interplay between neuroinflammation and depression has been well established and widely evidenced previously [41]. Herein, after LPS administration, impaired cytokine levels were detected in the APN KO mice cortex. As demonstrated in Fig. 1G, H, IL-1α, IL-6, TNF-α, TGFβ−1 were upregulated, with no significant changes of IL-1β and IL-4 (Fig. 1G, H). Interestingly, LPS-treatment also elevated anti-inflammatory cytokines, including IL-10 and TGFβ−1. We evaluated mRNA levels to validate these changes furtherly (Fig. S1A). IL-6 and TGFβ−1 mRNAs were upregulated, but IL-10 was decreased in the APN KO mice compared to the wild type. LPS treatment significantly increased TNF-α, TGFβ−1, and IL- 1β but decreased IL-10 mRNA levels in wild-type mice. However, mRNA levels of IL-6 were reduced in APN KO mice after LPS-administration. Next, IBA-1 and GFAP expression were then examined to validate LPS-induced neuroinflammation after LPS-treatment. Notably, LPS-treatment significantly enhanced GFAP expression in the cortex (Fig. S1B, C) and the hippocampus (Fig. S2) of the APN KO mice. Contrarily, LPS-administration did not alter IBA-1 expression in the cortex of APN KO mice compared to the marked increased in wild-type mice (Fig. 1I, J and K; Fig, S2). These results indicated dysregulated neuroinflammatory processes in APN KO mice.

To further evaluate the neuroinflammatory deficits, primary microglial cells (PMG) were isolated and cultured (Fig. 2A). After 4 h of LPS-treatment, cytokine levels were measured in the cell (PMG) lysate and supernatant. Elevated levels of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, were detected in the cell lysate of both APN KO and WT microglia after LPS-treatment (Fig. 2B, E and F). Similar to the in vivo finding, a notable increase of IL-10 (Supernatant) was detected in LPS-treated APN KO compared to WT (Fig. 2G). No significant changes of IL-1β (Supernatant), IL-4, IL-10 (Lysate), and TGFβ−1 in the PGM were detected after LPS-treatment (Fig. 2B, C, D, and G). Unexpectedly, we did not detect TGFβ−1 in the supernatant of PGM.

Neuroinflammatory signaling and oxidative stress dysregulated in APN KO

Inflammation-relevant signaling pathway molecules were examined to assess LPS-induced neuroinflammatory disorders in APN KO mice. Unexpectedly, LPS-treatment did not alter the phosphorylation of NFκB, Jak1, STAT3 in PMG lysates of APN KO mice compared to the tremendous upregulation of p-NFκB and p-STAT1 in wild-type PMG (Fig. 2H, I). Interestingly, p-IKKα/β and p-STAT1 expression were markedly increased in LPS-treated PMG of APN KO mice (Fig. 2H, I). Overall, these findings suggested that APN deprivation elicitated neuroinflammation via their associated signalings with increases in both pro and anti-inflammatory cytokines.

Next, we sought to determine whether these in vitro neuroinflammatory signaling changes are consistent with in vivo results. We did not find changes in the expression of TLR9, p-NFκB, p53, BCl2, cleaved caspase 3, p38, ERK, p-JAK2, JAK2, p-Stat1, and p-Akt in LPS-treated both wild-type and APN KO mice (Fig. S3). However, APN deprivation enhanced p-Ikkα/β, JAK1, Stat1, Stat3, and p-AMPK expressions, which were significantly decreased (except Stat1 and p-AMPK) after LPS-administration (Fig. S3). Similarly, LPS-treatment significantly reduced p-ERK (in the APN KO mice), p-JNK (both in wild-type and APN KO), JNK (in wild-type), p-Stat3 (both in wild-type and APN KO), and p-mTOR (in wild type) (Fig. S3). These in vivo results further demonstrated disorganized neuroinflammatory signalings in APN KO mice.

Inflammation is associated with redox system dysregulation [50]. Thus to further illuminate the mechanisms of this phenomenon in APN KO condition, free radicals like (Reactive oxygen species (ROS): H_2O_2, NO (nitric oxide), HO-1, Nrf1, 2, and SOD2 levels were measured in the serum and cortex of the mice (Fig. S4). No significant changes in H_2O_2 and NO levels were detected in the serum and cortex from both WT and APN KO mice after LPS-administration (Fig. S4). However, APN KO mice exhibited a significantly accelerated free radical generation in the cortex. Next, redox signaling molecule changes showed that higher increasing NRF1, NRF2, HO1, and SOD2 levels existed in the serum and cortex of the APN KO mice after LPS-administration (Fig. S4), indicating the disorganized redox signaling in APN KO mice upon LPS- administration.

APN KO mice exhibited altered mitochondrial content and function

Mitochondria are the central origin of free radicals and critical contributors to several cellular processes, including ATP production and cell survival [25, 51]. The interplay between APN and mitochondrial has also been well documented [52]. Herein, we examined the mitochondrial and its function-associated molecules in the APN KO with and without LPS-stimulation. Our results showed increased mitochondrial DNA content in the APN KO mice cortex, which was further elevated LPS-administration (Fig. S5A). Similarly, LPS-administration significantly increased ATP level in the APN KO mice cortex than wild-type mice (Fig. S5B). Contrarily, a decline in ATP level was found in PMG of APN KO mice after LPS-treatment (Fig. S5C), demonstrating that ATP changes in the cortex might not directly result from microglia. Next, mitochondrial-associated protein expression in the APN KO condition after LPS-treatment was measured. Complex I and III expressions in APN KO mice's cortex were significantly higher than wild-type mice. However, after LPS-administration, complex III dramatically reduced, but not complex I (Fig. S5E). A similar reduction of complex II expression was also detected in APN KO mice after LPS-administration (Fig. S5E).

Furthermore, LPS-treatment significantly increased complex II and III expressions in the PMG of the APN KO mice compared to the wild-type mice (Fig. S5F). We found an enhanced level of DRP-1 and OPA1 in the PMG of APN KO mice, which were significantly reduced after LPS-treatment (Fig. S5G). These data indicated that mitochondrial dynamic and content abnormalities might underlie its functional disorders.

APN deprivation modified synaptic protein expression

Alterations in the synaptic protein are the hallmark of depression [53, 54]. Our previous results showed a dramatic loss of dendrites structure in LPS induced depression, which may partially result from homeostatic protein deficits and can be reversed by antidepressants [49, 55]. Herein, synaptophysin, SNAP25, and synapsin-1 were significantly upregulated in APN deprive state (Fig. S6A); however, remain unchanged upon LPS-administration (Fig. S6A). Furthermore, BNDF and its receptor TrkB were measured as crucial regulators of dendritic protein dynamics in the pathophysiology of depression. Interestingly, TrkB and BDNF were significantly increased in the APN KO mice compared to the wild-type and were not altered after LPS-administration (Fig. S6B). These findings were further validated by mRNA analysis (Fig. S6C).

To assess and differentiate whether BDNF/TrkB or neuroinflammatory related changes are responsible for the antidepressive-like behaviors, the APN KO mice were treated with K252a, Bay11-7082, and AR the presence of LPS (Fig. 3A). We did not find unspecific locomotor activity changes after drugs treatment (Fig. 3B). However, K252a, Bay11-7082, and AR treatments significantly reversed antidepressive-like effects in LPS-treated APN KO mice, as demonstrated by increased immobility time in FST and reduced sucrose intake (Fig. 3C, D,

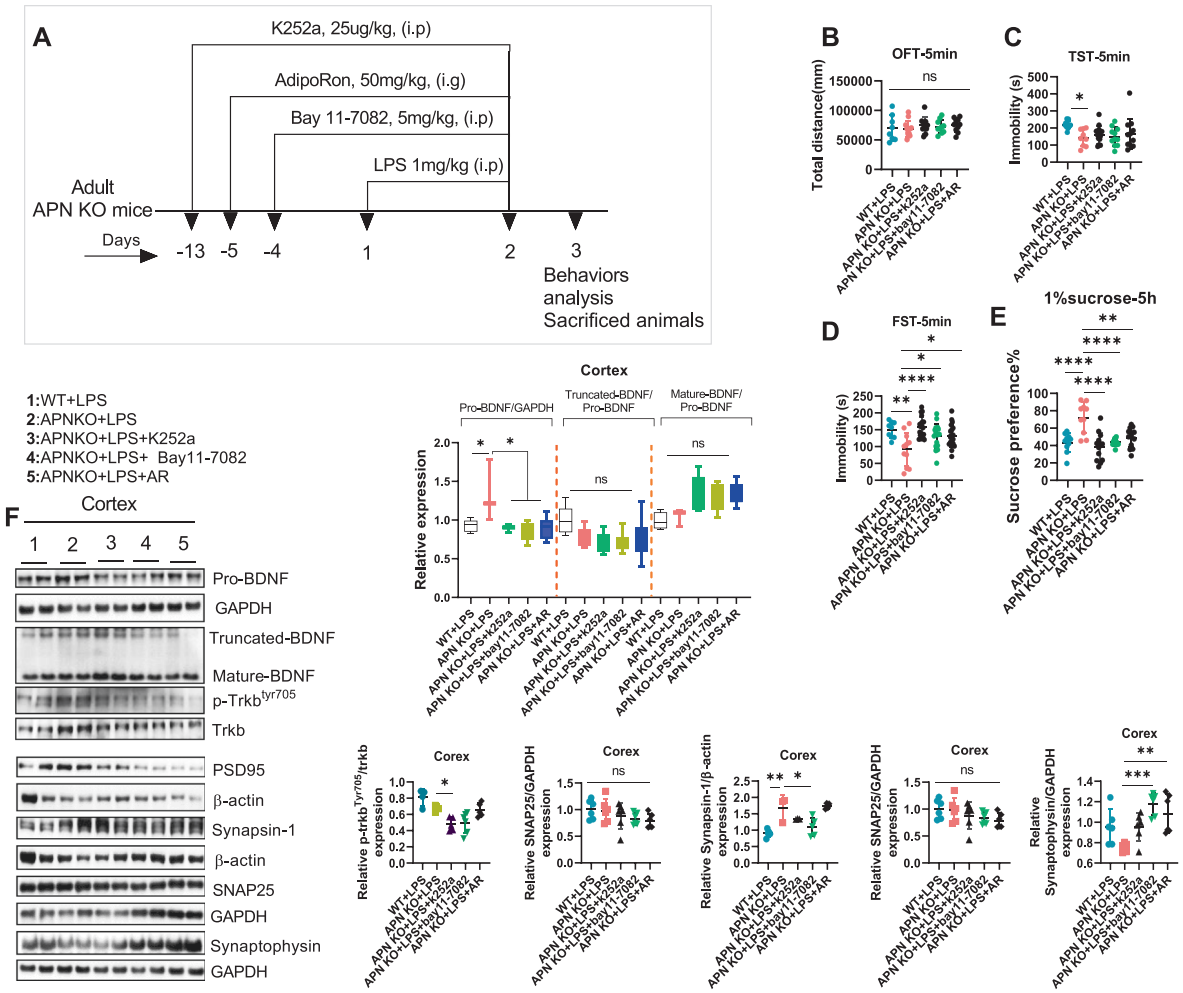


Fig. 3 LPS-treated APN KO mice lost anti-depressive-like behaviors and modified BNDF/TrkB and synaptic gene expression upon K252a, Bay11-7082, and AR treatment. A Shows study approach and drug treatment schedule, **B** Open field test, **C** Forced swimming test, **D** Tail suspension test, **E** Sucrose preference test. ($n = 9$–10). **F** Representative immune blots of BDNF (all form) and p-TrkB/TrkB, the Bar graph shows relative intensities of BDNF/TrkB expression, Synaptic protein, including PSD95, Synapsin-1, SNAP25, Synaptophysin level, detected by immunoblots, and the Bar graph shows the expression level of these proteins. $n = 6$, Mean ± SEM, ANOVA, Tukey's test. $p > 0.05$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

and E). To validate the underlying BDNF changes after treatments, BDNF (all forms) and TrkB expression in the cortex of mice were measured (Fig. 3F). Interestingly, inhibitors (K252a, Bay11-7082, and AR) significantly down-regulated pro-BDNF and p-Trkb expression, whereas the truncated and matured BDNF expression was not significantly altered (Fig. 3F). Next, we sought to determine whether the above treatments affected the synaptic molecules. Notably, PSD95 and synapsin-1 were downregulated considerably after treatment, which was enhanced in APN KO mice after LPS-administration (Fig. 3F). These findings suggest that APN KO mice could enhance Trkb/BDNF signaling activities, which could be accountable for why antidepressive behaviors of APN KO mice.

Redox and inflammatory signaling disorders may not account for the antidepressive like behavior in APN KO mice

Next, we measured cytokine levels and redox signaling changes after drugs treatment. K252a and Bay11-7082 did not change the pro-inflammatory cytokines, including IL-1β, IL-4, IL-6, and TNF-α expression (Fig. S7A). However, AR-treatment significantly enhanced both pro-inflammatory cytokines of IL-4, IL-6, and anti-inflammatory cytokine IL-10, TGF-α levels, compared to the LPS-treated APN KO mice (Fig. S7A). We further examined the inhibitor effects on JAK1 and STAT signaling (Fig. S7B). Surprisingly, the Bay11-7082 treatment enhanced JAK1 phosphorylation in LPS-treated APN KO mice. Similarly, p-AMPKα was significantly decreased by K252a administration in LPS-treated APN KO mice. Contrarily, the elevated expression of p-eif2α was considerably reduced in the LPS-treated APN KO mice after inhibitors treatment (except for K252a) (Fig. S7C).

Further insight into the free radicals showed that K252a treatment significantly enhanced H_2O_2 and NO levels in the mice's serum but not in the cortex (Fig. S8A). AR-treatment decreased H_2O_2 level, while Bay11-7082 treatment decreased NO level in the cortex (Fig. S8A). Similarly, AR-treatment significantly increased SOD2, HO-1, Nrf1, and Nrf2 levels in the serum of experimental mice but not in cortex tissues (Fig. S8B, C). Furthermore, no significant changes were found in the expression of SOD2, HO-1, Nrf1, and Nrf2 (Fig. S8B, C).

Next, to validate further the above redox system-related changes, we measured ATP levels and mitochondria-related protein expression (Fig. S9). The inhibitor treatments did not alter ATP levels (Fig. S9A); however, Bay11-7082 and AR treatment significantly reduced the elevated level of complex-I in the LPS-treated APN KO mice cortex (Fig. S9B). Furthermore, we did not detect any significant alterations in the expression of complex-II, TFAM, and other mitochondrial proteins (OPA1, Drp-1, and MFN)

after inhibitors administration (Fig. S9C). Overall, these results implicated that changes in redox signaling and iklammatory cytokines may not account for the antidepressive like behavior in APN KO mice

7,8-DHF attenuated Bay11-7082 induced effects on LPS-treated APN KO mice

Besides its role in neuroinflammation and redox signaling, previous results showed NFκB could regulate BDNF expression, which reciprocally activates NFκB via IκB phosphorylation [56]. This interaction was demonstrated to be involved in neurogenesis and synaptic transmission, which underly the antidepressant actions. To further dissect the effects of BDNF and NFκB, APN KO mice were treated with 7, 8-DHF in the presence of LPS (Fig. 4A). Bay11-7082 induced depressive-like behaviors were rescued by 7,8-DHF treatment, as demonstrated by decreased immobility time and increased sucrose preference of APN KO mice (Fig. 4B, C). Further, 7,8-DHF treatment did not induce any significant changes in the p-NFκB expression (Fig. 4E), suggesting NFκB may be independent of the antidepressant effects of TrkB/BDNF signaling. These findings suggested a possible causal association between BDNF/TrkB and antidepressive-like changes in APN KO mice.

APN receptors underlined the depressive-like behavior in APN KO mice

To test whether the anti-inflammatory and depressive-like behaviors of APN KO mice were linked to APN receptor modulation, we initially examined AdipoR1 and AdipoR2 expression. APN deprived mice displayed increased mRNAs, but not proteins of both AdipoR1 and AdipoR2. Interestingly, LPS-treatment enhanced both mRNA and protein levels of AdipoR2 (but not AdipoR1) in WT mice but reduced AdipoR1 protein levels in the cortex of APN KO mice, indicating dysregulated adiponectin receptor systems in APN KO mice on LPS stress (Fig. 5A, B, and C). We further evaluated adiponectin receptor changes were in PMG cells. Like APN KO mice cortex tissue, APN deprivation did not alter both receptor expressions compared to WT controls. However, LPS treatment decreased AdipoR1 while increasing AdipoR2 in the APN KO PMG cells compared to the LPS-treated WT (Fig. 5C). Furthermore, AR-treatment significantly increased AdipoR1 expression in the LPS-treated ANP KO mice cortex, while Bay11-7082 treatment decreased AdipoR2 in the presence of LPS, which could be recovered considerably by AR-treatment (Fig. 5D).

Next, to investigate the functional roles of adiponectin receptors, AdipoR1 and AdipoR2 (alone or both) were knocked down (KD) in BV2 cells with shRNA (Fig. 5E). After confirming the receptors' KD effects, BV2 cells were treated with LPS for 4 h, followed by BDNF and cytokines measurement. As shown in figure 11F, co-knockdown of AdipoR1/R2 increased BDNF level contrary to the solo (Fig. 5F). Besides, IL-1α and TNF-α (except in AdipoR1) were increased alone and in co-AdipoR1/R2 KD BV cells (Fig. S10A, C). IL-1β and IL-6 remain unchanged with and without LPS-treatment (Fig. S10B, D). however, IL-10 was increased in AdipoR2 KD cells (Fig. S10F). Additionally, similar to IL-4, LPS treatment decreased TGFβ−1 in AdipoR1 KD cells (Fig. S10E, F). These results imply that both adiponectin receptors may be involved in the dysregulated inflammatory responses (Fig. S10E, F, and G).

To further validate the roles of adiponectin receptors, AdipoR2 KO mice were treated with LPS followed by behavior analysis (Fig. 5G). AdipoR2 deprived mice displayed depressive-like behaviors, as demonstrated by decreased total distance traveled in OFT and increased immobility time. Surprisingly, LPS-treatment further enhanced immobility time during TST of AdipoR2 KO mice. However, we did not find any significant changes during the SPT analysis. Taken together, these results indicated that adiponectin receptors and related downstream signalings were partially involved in the inflammatory responses of LPS but showed a distinct phenotype with APN KO mice.

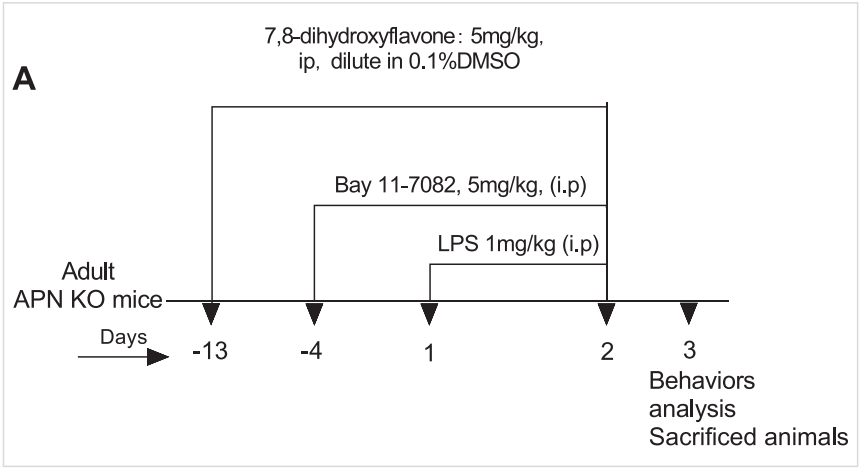
DISCUSSION

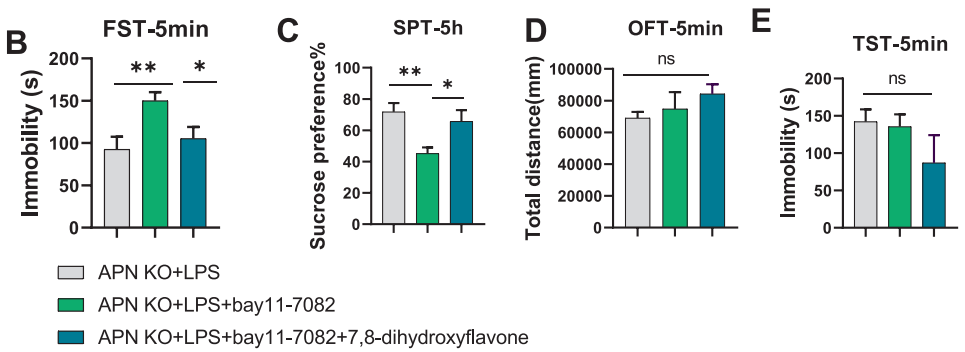
In the present study, we demonstrated the antidepressive-like effect of APN KO mice after LPS-treatment. Several key observation was made here; First, ANP KO mice showed antidepressive-like behaviors accompanied by neuroinflammatory impairment upon LPS-administration. Contrary to cytokines level, LPS-treatment did not enhance IBA-1 expression in the APN KO mice. Further, these changes were accompanied by redox system dysregulation, which was further validated via the primary microglia (PMG) culture of APN KO mice. Secondly, neuroinflammatory and redox signaling dysfunction may result from altered mitochondrial protein expression and function but may not be accountable for the antidepressant phenotypes of APN KO mice. Thirdly, altered synaptic related protein expression and signaling may result from the enhanced level of TrkB and BDNF expression, independent of NFκB changes that may underlie the antidepressive-like effect of APN KO mice. Finally, LPS-treatment impaired AdipoR1/R2 expression in the APN KO mice cortex (and PMG cells) while rescued (AdipoR1, but not AdipoR2) by AR administration. However, AdipoR1/R2 solo or co-knockdown BV2 cells displayed intricate and impaired levels of BDNF and cytokines. It proposed that APN KO mice's antidepressant-like behaviors may not depend on its receptors, as apparent from the LPS-treated AdipoR2 KO mice behaviors analysis.

Adiponectin plays a significant role in various brain disorders [37, 57, 58]. Recent studies reported impaired adiponectin levels in MDD patients [59–61]. A unique and critical finding from this study is that APN deprivation enhanced TrkB/BDNF signaling, explaining its role in the LPS-induced model of depression. In contrast, previous studies have shown a decline of circulatory adiponectin in the social defeat model of depression [62]. Thus, it is reasonable to speculate that APN under pathological stress may contribute to the induction of depressive symptoms, which has a distinct difference from APN KO conditions. Indeed, we have also found protein homeostatic deficits in APN KO mice (Unpublished data). Herein, mice behaviors (FST, TST, and SPT) analysis revealed the antidepressant-like effect of APN KO mice upon LPS-administration. Interestingly, the impact of APN deprivation waned after blocking TrkB signaling (via K252a) and could be recovered again by TrkB activator (7,8-DHF), indicating the critical role of TrkB/BDNF signaling in the antidepressive behaviors of APN KO mice.

Further studies, including our, demonstrated that LPS-treatment could significantly induce depressive-like symptoms via neuroinflammation and related cytokines expression [41, 42, 49]. Interestingly, previous multiple meta-analyses revealed increased pro-inflammatory cytokines like IL-6, TNF, and C-reactive protein (CRP) in MDD patients [13, 63]. Moreover, this increase is closely associated with atypical symptoms of depression and suicidal cases [64, 65], supporting the previous idea that neuroinflammation is associated with treatment-resistant depression [66]. APN deprived mice displayed a complex cytokine expression pattern we detected upregulated both pro and anti-inflammatory cytokines upon LPS administration. Furthermore, LPS-treated mice showed increased GFAP and decreased IBA-1 expression, proposing that APN deprivation may contribute as an antidepressive via microglia regulation. More interestingly, dysregulated inflammatory status of APN KO mice coincides with other reports that elevated levels of anti-inflammatory cytokines in MDD patients, including transforming growth factor (TGF)-b and IL-10 [13, 63, 67]. Whether the pro-inflammatory and anti-inflammatory disorders contribute to the same extent and mechanism remained to be resolved. More specifically, the potential impact and details of neuroimmune changes that might contribute to treatment-resistant depression need further to be specified.

Along with dysregulated inflammatory cytokine expression, NFκB/Ikkα/β/STAT3 signaling also showed a similar complex





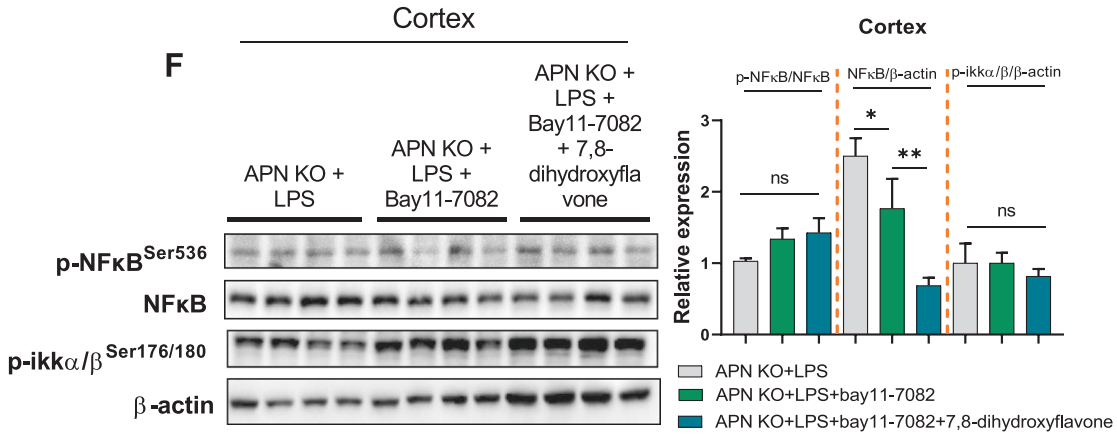


Fig. 4 7,8-DHF reversed Bay11-7082 modified changes in the LPS-treated APN KO mice. A Shows study approach and drug treatment schedule, **B** Forced swimming test, **C** Sucrose preference test, **D** Open field test, **E** Tail suspension test, ($n = 9$–10). **F** Representative immune blots and bar graphs showing expression of NFκB and IKKα/β in the cortex tissues of experimental mice ($n = 6$). Mean ± SEM, ANOVA, Tukey's test. $p > 0.05$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

pattern in LPS-treated primary microglia of APN KO mice. Furthermore, as demonstrated by increases NRF/SOD2 signaling, enhanced redox responsiveness in APN KO mice to LPS treatment indicated the neuroinflammation preventive effect of APN KO mice, which have been shown to relieve depression [41, 68–70]. NFκB plays a crucial role in producing pro-inflammatory cytokines upon LPS stimulation [71]. Surprisingly, we found a significant decline of p- NFκB expression in the LPS-treated PMG of APN KO mice, while the p-ikkα/β level was significantly elevated. Inhibition of ikkα/β and NFκB signaling pathway ameliorated their antidepressant behaviors (increasing immobility and decreasing sucrose preference) of APN KO mice,

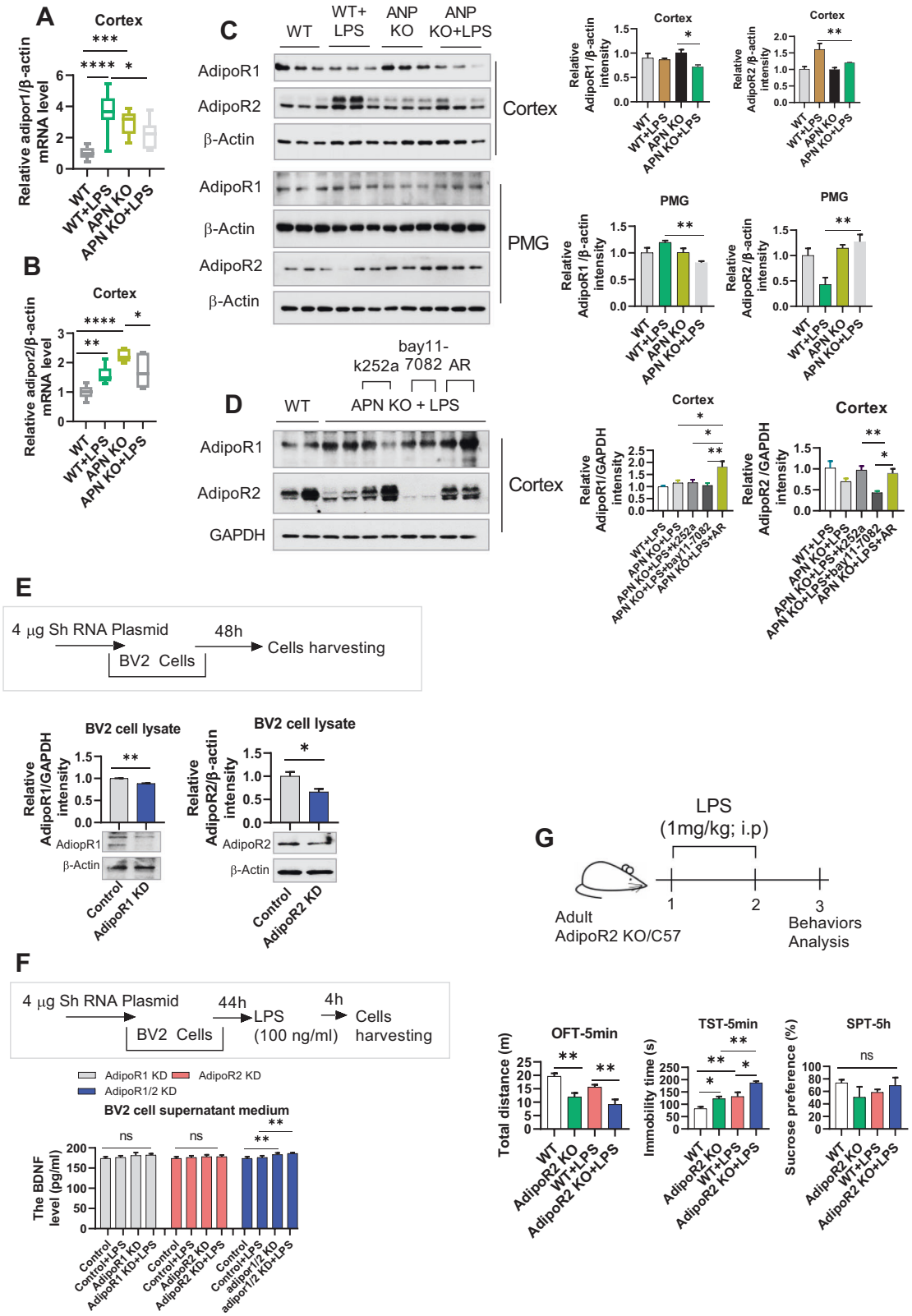


Fig. 5 APN receptors expression in APN KO mice, PMG, and BV2 cells. A–B Bar graphs showing mRNA level of AdipoR1 and AdipoR2, **C** Representative immunoblots with their intensity graphs of AdipoR1/R2 expression in the cortex and PMG of APN KO mice**. D** immunoblots with bar graphs showing AdipoR1/R2 expression in different antagonist treated LPS-administrated APN KO mice**. E** Immunoblots and bar graphs show the knockdown of AdipoR1/R2 in the shRNA-treated BV2 cells. **F** Shows BNDF level in the sole or co-AdipoR1/R2 knockdown in LPS-treated BV2 Cells. **G** LPS-treatment schedule (upper), and OFT, TST, and SPT assays (down) $n = 10–12$). Mean ± SEM, ANOVA, Tukey's test. $p > 0.05$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

indicating the possible ikkα/β/NFκB and TrkB/BDNF signaling crosstalk [56]. Further, the TrkB activator (7,8-DHF) rescued Bay11-7082 reduced APN KO mice antidepressive behaviors. Moreover, we did not find any significant changes in the p-NFκB expression, suggesting that NFκB may act downstream of TrkB/BDNF signaling.

The functional mechanisms of adiponectin in depression are poorly understood, although its receptors, AdiopR1/R2, are widely expressed within the brain [57, 72]. AdipoRon (AR) is the well-established adiponectin agonist, which activates adiponectin receptors (AdiopR1/R2) [57, 73]. Studies have also shown the interrelationship between adiponectin and mitochondrial function, whose dysfunction is an established mechanism participating in different pathologies [27, 51, 52, 74]. Herein, APN KO mice displayed dysregulated mitochondrial proteins including complexes (I, II, III), PGC-1, OPA1, Drp-1 and MFN-1, along with high ATP and mtDNA content. Notably, the phenomenon was modified by Bay11-7082 and AP administration to LPS-treated APN KO mice. In our study, AR-treatment abolished the antidepressive-like behaviors in LPS-treated APN KO mice, as demonstrated by increasing immobility and decreasing sucrose preference. However, AR treatment did not alter mature-BDNF expression in the LPS-treated APN KO mice cortex. Rather than that, it improved the redox defensive mechanism by increasing Nrf2/SOD2 expression, enhanced IL-6 and IL-4 expression, along with TGFβ−1 and IL-10. Thus, it indicates an intricate interplay among redox signaling, neuroinflammation, and neurotrophic factors.

AdipoR1/R2 are distinctly distributed in the brain and play a significant role in neurological disorders via their downstream signaling [72, 75]. Dysregulation of APN receptors and their associated signaling can impair inflammatory response accompanied by dysregulated BNDF and synaptic processes [38, 52, 73]. In agreement with previous reports, our results of APN KO mice showed altered AdipoR1/R2 expression (at mRNA and protein level) in the cortex and PMG cells, as further modified by LPS-administration and rescued by AdipoRon (AR) treatment [38, 73, 76]. Additionally, impaired expression of BDNF and cytokines detected in the AdipoR1/R2 knockdown BV2 cells indicated a co-relation of APN receptors to neuroinflammatory signaling and synaptic processes. Moreover, AdipoR2 deprived mice displayed depressive behaviors as aggravated by LPS, further supporting the involvement of APN receptors in depression. However, in the background of our findings, APN and APN receptors deprivation may be interdependently involved in neuroinflammation-associated depression.

In conclusion, the interplay of TrkB/BDNF/NFκB signaling might underlie the antidepressant-like behaviors of APN KO mice, independent of AdipoRs.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article [and its supplementary information files.

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AUTHOR CONTRIBUTIONS

WL designed and performed the experiments, TA data analysis, wrote the manuscript, Chengyou Zheng, Kaiwu He, and Zizhen Liu helped in the experiment; Fawad Ali Shah, Ningning Li, and Zhi-Jian Yu helped in manuscript, experimental tools and supported the study. SL endorsed the study, corresponding authors, reviewed and approved the manuscript, and held all the responsibilities related to this manuscript. All authors reviewed and approved the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

According to the protocols approved by the Institutional Animal Care and Use Committee of Peking University Shenzhen Graduate School, all experimental procedures were carried out.

ADDITIONAL INFORMATION

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