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Microglia depletion ameliorates neuroinflammation, anxiety-like behavior, and cognitive deficits in a sex-specific manner in Rev-erbα knockout mice

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Abstract

The circadian system is an evolutionarily adaptive system that synchronizes biological and physiological activities within the body to the 24 h oscillations on Earth. At the molecular level, circadian clock proteins are transcriptional factors that regulate the rhythmic expression of genes involved in numerous physiological processes such as sleep, cognition, mood, and immune function. Environmental and genetic disruption of the circadian clock can lead to pathology. For example, global deletion of the circadian clock gene Rev-erbα (RKO) leads to hyperlocomotion, increased anxiety-like behaviors, and cognitive impairments in male mice; however, the mechanisms underlying behavioral changes remain unclear. Here we hypothesized that RKO alters microglia function leading to neuroinflammation and altered mood and cognition, and that microglia depletion can resolve neuroinflammation and restore behavior. We show that microglia depletion (CSF1R inhibitor, PLX5622) in 8-month-old RKO mice ameliorated hyperactivity, memory impairments, and anxiety/risky-like behaviors. RKO mice exhibited striking increases in expression of pro-inflammatory cytokines (e.g., IL-1β and IL-6). Surprisingly, these increases were only fully reversed by microglia depletion in the male but not female RKO hippocampus. In contrast, male RKO mice showed greater alterations in microglial morphology and phagocytic activity than females. In both sexes, microglia depletion reduced microglial branching and decreased CD68 production without altering astrogliosis. Taken together, we show that male and female RKO mice exhibit unique perturbations to the neuroimmune system, but microglia depletion is effective at rescuing aspects of behavioral changes in both sexes. These

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

results demonstrate that microglia are involved in Rev-erbα-mediated changes in behavior and neuroinflammation.

Keywords

Circadian; Neuroinflammation; Microglia; Rev-Erbα; Mood; Cognition

1. Introduction

Organisms on Earth have evolved in response to 24 h solar cycles. An internal timekeeping system, termed the circadian system, allows organisms to synchronize physiology and behavior to the predictable patterns of light and dark. The circadian system enhances fitness by restricting activities to the optimal temporal niche. Importantly, disruption of the circadian system has become prevalent in modern society with the widespread adoption of electric lighting over the past 150 years. Environmental risk factors can disrupt circadian rhythms. For example, an altered light–dark cycle in transmeridian travel can desynchronize internal clocks; similarly, a disrupted sleep cycle is often experienced during shift work. Circadian disruption is associated with health consequences including increased risk for neuropsychiatric disorders (Chang et al., 2015; Deacon & Arendt, 1996; Espitia-Bautista et al., 2017; Fonken & Nelson, 2014; LeGates et al., 2014).

In mammals, circadian rhythms are controlled by feedback loops composed of transcriptional activators and repressors including Bmal1, Clock, Per1/2, Cry1/2, Rev-erbα/β, and Rorα/β/γ (Partch et al., 2014). These so-called “clock” genes oscillate in the main circadian pacemaker in the suprachiasmatic nucleus of the hypothalamus as well as in tissuespecific clocks located throughout the brain and body. In addition to regulating their own transcription, clock genes act as transcriptional regulators for myriad other gene and protein products. This is a major pathway for how the circadian system generates rhythms in physiological functions based on tissue and system-specific needs. Importantly, disruption to the circadian system can lead to alterations in these clock genes and the downstream clock-controlled genes that they regulate.

Disruption of the circadian system is associated with mood disorders. In humans, circadian patterns of gene expression are much weaker in the brain of individuals with major depressive disorder (Li et al., 2013). Work in animal models supports a causal role for circadian disruption in mood disturbances. For example, environmental disruptions of the circadian system through exposure to light at night or non-24 h light cycles induce changes in mood in rodent models (Ben-Hamo et al., 2016; Daut et al., 2019; Horsey et al., 2019). Affective behavioral changes are also observed with genetic disruption to the circadian clock, for instance, in mice carrying ClockΔ19 mutation and Rev-erbα knockout mice (Chung et al., 2014; Ketchesin et al., 2020; Roybal et al., 2007). Rev-erbα knockout (RKO) mice display higher risk-taking and aggressive behaviors, altered depressive-like responses, and impaired cognitive function (Chung et al., 2014; Jager et al., 2014; Otsuka et al., 2022; Schnell et al., 2014). Genetically deleting Rev-erbα disrupts circadian as well as other downstream targets (Cho et al., 2012), including inflammatory responses (Griffin et al.,

2019; Griffin et al., 2020). Notably, prior work characterizing the behavioral phenotype of RKO mice only involve males: a lack of female data could limit the best interpretation of results as there are sex differences in neuropsychiatric disorders (Green et al., 2019; Thibaut, 2016). Moreover, the mechanisms mediating circadian-induced changes in mood are not fully understood.

Changes in mood are also associated with immune dysfunction. Increases in inflammatory markers in the brain are implicated in neuropsychological conditions such as depression, bipolar disorder, and posttraumatic stress disorder (Fonken & Gaudet, 2022; Goldsmith et al., 2016; Hori & Kim, 2019; Masi et al., 2015; Pereira et al., 2021). The circadian system potently regulates the immune system, including the primary immune cell of the central nervous system, microglia. Therefore, here, we examined whether changes in mood and cognitive-related behaviors induced by circadian disruption are dependent on microglia. To establish whether microglia were necessary for the effects of RKO, we used chow containing a CSF1R inhibitor to deplete microglia from the CNS of wild-type and RKO mice. We reveal that depleting microglia resolved neuroinflammation in a sex-dependent manner and partially ameliorated the behavioral phenotype of RKO mice. Overall, this study demonstrates that RKO shifts the immune profile of the brain towards a pro-inflammatory state, contributing to changes in affective and cognitive behaviors.

2. Materials and methods

2.1. Animals

Animal experiments were approved by the University of Texas at Austin (UT) Institutional Animal Care and Use Committee and were performed according to the local and national guidelines governing animal care. Male and female Rev-erbα$^{-/-}$ mice on a C57BL/6J background (abbreviated as RKO) and wild-type littermates (abbreviated as WT) were bred at Jackson Labs (B6.Cg-Nr1d1tm1Ven/LazJ, strain# 018447) for this study due to COVID-19 disruptions. Upon arrival at the University of Texas, sex- and genotype-matched mice were grouphoused under an average room temperature of 21°C ± 1 °C with corncob bedding, sizzle-nest, disposable houses, and unlimited access to standard lab diet 5LL2 and water. Mice were housed in a ventilated light-controlled Sun Calc rack (Lab Products, Inc.) with 12 h/12 h light–dark cycle (125 lx white light / 0 lx measured inside the cage).

2.2. Microglia depletion

Microglia depletion was accomplished using a CSF1R inhibitor diet as in prior work (Dagher et al., 2015; Elmore et al., 2014). CSF1R inhibitor PLX5622 was ordered from MedChem Express (Monmouth Junction, NJ) and sent to Research Diets (New Brunswick, NJ) to be formulated in standard lab chow AIN-76A (1200 ppm). Adult (~8 mos) RKO mice and WT littermates were placed on the 1200 ppm PLX5622-containing chow (referred to as PLX5622) or the AIN-76 control diet (referred to as control diet or control). To ensure sufficient depletion of microglia, mice were placed on the diets 3 weeks before behavioral testing through the end of the study. Food pellets were replaced every 3 days per manufacturer's instruction, and food consumption was calculated. Mice consumed slightly less food during the first week on the new diet regardless of treatment groups. Microglia

depletion was confirmed in a pilot study (tissue collected after 3 weeks to match the onset of behavioral testing) and was further validated in all experimental mouse brains at the end of the study using immunohistochemistry.

2.3. Behavioral phenotyping

Behavioral tests were conducted after 3 weeks of PLX5622 treatment (summarized in Fig. 1). Unless otherwise specified, behavioral tests were conducted in a room with 100–150 lx illumination (as measured at the mouse's eye level) between Zeitgeber Time 4–8 (ZT0 corresponds to lights on in the housing environment). Behavioral tests were recorded using a ceiling camera and Ethovision XT11 software (Noldus Corp., Leesburg, VA, USA).

2.3.1. Barnes maze—Hippocampal-dependent learning and memory were evaluated with a Barnes maze test as previously described (Fonken et al., 2012). The Barnes maze apparatus is an open round platform (92 cm in diameter) lifted to a height of 72 cm with 20 escape holes (5 cm in diameter) along the edge, one of which was connected to a hidden escape box. Signs were placed on the wall to serve as visual cues throughout habituation, training, and testing, and bright illumination (400 lx at animal eye level) and white noise were used as aversive stimuli to motivate escape. Here we used a 6-day test schedule, consisting of 1 day of habituation, 4 days of acquisition × 3 trials per day, and 1 day of probe trial. On the habituation day, mice were placed in the center of the maze and immediately gently guided into the hidden box. Next, in the acquisition trials, mice were given 2 min to find the hidden box through the escape hole, and the aversive stimuli were terminated once mice entered the goal box. Finally, in the probe trial, the hidden box was replaced by a flat surface identical to those used for the other holes, and mouse behaviors on the maze were recorded for 90 s. Ethovision XT11 software was used to measure latency and path length to the first goal box visit during acquisition and probe trials, and the time the mice spent in the target quartile during the probe trial.

2.3.2. Open field & juvenile social exploration—An open field and juvenile social exploration test were performed as previously described (Chen et al., 2021b). Mice were placed at a random corner of a 50 cm × 50 cm open-top plexiglass box, and exploratory behaviors were recorded for 5 min. Locomotion as well as time spent in the inner 66% of the box were automatically analyzed by Ethovision software. Immediately after the open field test, a sex-matched juvenile mouse was introduced into the apparatus, and behaviors were recorded for 5 min. Social interaction that was initiated by the experimental mouse was scored by an experienced observer blinded to the experimental groups using Ethovision. The apparatus was cleaned with 70% ethanol to disinfect and eliminate odor between mice.

2.3.3. Elevated plus maze—The elevated plus maze test was performed as previously described (Chen et al., 2021b). Mice were placed in the center of a plus-maze lifted to a height of 39 cm that consisted of two closed arms (30.5 cm × 5 cm) surrounded by 18.5 cm high walls and two open arms (30.5 cm × 5 cm), and exploratory behaviors were recorded for 5 min. The apparatus was cleaned with 70% ethanol to disinfect and eliminate odor between mice. Arm entries and time spent on each arm were automatically analyzed by Ethovision software. To minimize the differential locomotion as a confounding effect, both

open arm time and arm preference are reported: arm preference = [(open arm time −closed arm time) / (open arm time + closed arm time)] × 100%.

2.3.4. Nest building—Nesting behavior is a natural behavior reflecting the general wellness of rodents (Gaskill et al., 2013; Jirkof, 2014) and can be used as an indicator of behavioral deficits (Gaskill et al., 2013; Neely et al., 2019). Mice were singly housed during the nest-building test. 2 g of square cotton nestlets were provided as nesting material to each cage prior to lights off. Nest scores were taken 3 times throughout the following day on a scale of 1 to 5: 1 = almost intact nestlet (<10% torn); 2 = partially torn nestlet (10% – 50% torn); 3 = mostly torn (50% – 90% torn) but not gathered nestlet (spreading in > ¼ of the cage); 4 = almost perfect (>90% torn) but flat nest; 5 = perfect nest (>90% torn) that is higher than a sitting mouse. Scores of 3.5 and 4.5 were given if the nests met the criteria for an almost perfect or perfect nest but had > 10% of nestlets untorn.

2.3.5. Sucrose anhedonia—Anhedonia, or the decrease in the ability to experience pleasure, is a core symptom of depression. A two-bottle sucrose preference test was conducted to assess the preference of mice for rewarding sucrose solution (Chen et al., 2021b). In brief, mice were provided with 2 bottles with either 2% sucrose solution or plain water for 6 h, starting immediately after lights off. Bottles were weighed before and afterward to calculate total liquid consumption as well as the preference for sucrose solution (sucrose preference = sucrose solution consumption / total liquid consumption × 100%). A habituation trial was conducted before the test day to familiarize the mice with the two-bottle choice. Data points with a total liquid consumption > 6.0 g were excluded for bottle leakage.

2.3.6. Spray test—Rodent self-grooming is an innate behavior important for hygiene maintenance and other physiological processes, and changes in self-grooming may reflect pathophysiology or a depressive-like sate (Kalueff et al., 2016; Kalueff & Tuohimaa, 2004). A spray test was conducted to assess self-grooming. Mice were singly housed overnight before the spray test. The next morning, mice were sprayed with water to wet their back coat to initiate self-grooming behaviors. Mice received 2 sprays from 15 cm away and were then immediately placed back in their single-house home cages. Grooming behaviors were recorded for 15 min. The duration of self-grooming behaviors including paw licking, nose and face wash, head wash, body grooming, leg licking, and tail/ genitals grooming were scored and analyzed with Ethovision software by an experimenter blinded to experimental groups. In addition, home cage locomotion during the 15 min trial was automatically analyzed by Ethovision. The percentage of distance traveled per zone over total distance traveled was reported instead of the time spent in zones to avoid the confounding effect of mice grooming in the center.

2.4. Immunohistochemistry

Mice were euthanized by rapid decapitation under deep anesthesia (5% isoflurane). One hemisphere of each brain was fixed in 4% paraformaldehyde followed by 30% sucrose, and 20 μm sections were cut on a cryostat. Immunohistochemistry was performed as previously described (Fonken et al., 2013a). In brief, slides were blocked with 10% goat serum and

incubated overnight with primary antibodies Iba1 (rabbit anti-mouse, 1:1000, Bio-Rad, Cat. No. MCA1957GA,) and CD68 (rat anti-mouse, 1:200, Wako Lab Chemicals, Cat. No. 019–19741), and then incubated for 2 h with secondary antibodies Alexa 568 (goat anti-rabbit, 1:500, Invitrogen, Cat. No. A11011) and Cy5 (goat anti-rat, 1:800, Invitrogen, Cat. No. A10525), followed by 15 min in Hoechst 33,342 (1:100,000, Invitrogen, Cat. No. H3570). For astrocyte staining, a separate set of slides were processed with the primary antibody GFAP (rabbit anti-GFAP, 1:2000, Agilent, Cat. No. Z0334) and secondary antibody Alexa 488 (goat anti-rabbit, 1:500, Invitrogen Cat. No. A11034). Images of the CA1 region of the hippocampus and the prefrontal cortex were captured at 20X using an Olympus Fluoview FV3000 confocal microscope. Iba1 + cells were manually counted in the regions of interest using Fiji. CD68 and Iba1 colocalization was performed using manual thresholding in Imaris software. Hippocampal CA1 GFAP percent area coverage was quantified in Fiji by thresholding and counting the number of GFAP + pixels per image. The hippocampal CA1 region was additionally imaged at 60X, and image stacks were used to create skeletal reconstructions in Fiji as previously described (Sanchez et al. 2022). 6–8 microglia were reconstructed from each mouse, with 4–6 mice per experimental group. Sholl analyses were performed on all microglia. The experimental unit was the individual animal, so measurements from all the microglia from an individual animal were averaged to give one data point.

2.5. Gene expression

The hippocampus was freshly dissected from the other hemisphere of the brain during euthanasia, flash-frozen on dry ice, and stored at −80 degrees Celsius until RNA extraction. RNA was extracted using a mechanical homogenizer and TRIzol/chloroform extraction method. 1.5 μg of RNA was reverse-transcribed to cDNA using a thermocycler. Gene expression in brain regions of interest was determined with quantitative PCR (qPCR) as previously described in Chen et al., (2021b). Inventoried primers for TaqMan gene expression assays were ordered from Invitrogen Life Technology, Cat. No. 4,331,182 (Assay IDs include IL-1β: Mm00434228_m1, IL-6: Mm00446190_m1, CD68: Mm03047343_m1, Ym1: Mm00657889_m1).

2.6. Statistics

Data are presented as mean ± SEM. Data analysis was performed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA), analyzed with a two-way analysis of variance (ANOVA) with a post hoc Tukey's test, or a three-way ANOVA with repeated measurements and a post hoc Bonferroni's test on the consolidated data. 6–9 male and 3–9 female mice were used for each genotype and each diet group (see below for more details). Female estrous phase was not assessed, and a lower n was included in female groups due to the limited availability of female offspring and breeding challenges during the COVID-19 pandemic. The significance level was set as $p < 0.05$. Sex was analyzed as an independent variable for each dataset; if no sex difference was detected, male and female data were combined and labeled with different colors. Males and females were analyzed separately for gene expression due to substantial sex differences. 1 male RKO-PLX5622 mouse (elevated plus maze test) and 1 female RKO-PLX5622 mouse (Barnes maze, open field, and juvenile social exploration tests) were excluded from behavioral analyses for irregular behaviors

during tests. In addition, Grubb's test for outliers was used in an unbiased manner to identify and exclude outliers if relevant (noted in results if an animal was excluded).

3. Results

3.1. CSF1R inhibitor treatment depleted microglia and ameliorated neuroinflammation in a sex-specific manner in the hippocampus of Reverbα knockout mice

First, we assessed how RKO and microglial depletion affected neuroimmune dynamics. RKO mice had increased numbers of Iba1 + microglia in hippocampus and prefrontal cortex, as compared to WT littermates (hippocampus: RKO × PLX interaction, $F_{(1, 28)} = 43.98$, PFC: RKO × PLX interaction, $F_{(1, 38)} = 12.03$, post hoc, $p < 0.05$, Fig. 1B,C). PLX5622 significantly reduced $Iba1^{+}$ microglia cell numbers in both regions of WT and RKO mice to comparable levels (ã 70% depletion in WT mice and a ~ 90% depletion in RKO mice, post hoc, $p < 0.05$, Fig. 1B,C).

Male and female mice exhibited differential hippocampal inflammatory gene expression responses to RKO and PLX treatment (IL1β: sex × RKO, $F_{(1,46)} = 182.9$ and RKO × PLX, $F_{(1,46)} = 7.6$; IL6: sex × RKO × PLX, $F_{(1,47)} = 57.5$; CD68: sex × RKO × PLX, $F_{(1,47)} = 9.3$; Ym1: sex × RKO, $F_{(1,45)} = 97.5$, $p < 0.05$ in all cases, Fig. 2). In male mice, RKO increased hippocampal expression of the pro-inflammatory cytokine Il1β (RKO × PLX interaction, $F_{(1, 25)} = 9.76$, [control diet]: WT vs RKO, $p < 0.001$, Fig. 2A), Il6 (RKO × PLX interaction, $F_{(1, 26)} = 10.98$, [control diet]: WT vs RKO, $p < 0.001$), and phagocytic marker CD68 (RKO × PLX interaction, $F_{(1, 26)} = 18.98$, [control diet]: WT vs RKO, $p < 0.001$) by ~ 2-fold, suggesting increased hippocampal inflammation. This higher neuroinflammatory phenotype was reversed by depleting microglia with PLX5622 treatment (in Il1β, Il6, and CD68, [RKO]: control diet vs PLX, $p < 0.001$, Fig. 2A). The anti-inflammatory gene Ym1 (a.k.a. chitinase-like protein 3 (Chil3)) was also suppressed by PLX treatment (main effect of PLX, $F_{(1, 24)} = 10.16$, $p < 0.01$, Fig. 2A). Taken together, in male mice, RKO activated neuroimmune pathways and induced a pro-inflammatory phenotype, which was suppressed by microglia depletion with PLX5622 treatment.

Similar neuroinflammatory patterns were observed in the hippocampus of female RKO mice; however, the relative expression levels of neuroinflammatory markers were massively higher in females compared to males (~10-fold increase in female RKOs vs ~2-fold increase in male RKOs). Female RKO mice had increased expression of Il1β (main effect of RKO, $F_{(1, 21)} = 177.3$, $p < 0.001$, Fig. 2B), Il6 (RKO × PLX interaction, [control diet]: WT vs RKO, $p < 0.001$), CD68 (RKO × PLX interaction, $F_{(1, 21)} = 12.83$, [control diet]: WT vs RKO, $p < 0.001$), and Ym1 (main effect of RKO, $F_{(1, 21)} = 79.45$, $p < 0.001$). Surprisingly, although PLX5622 treatment similarly depleted microglia in the RKO males and the RKO females, it did not fully reverse neuroinflammation in the hippocampus of female mice (Fig. 2B). PLX5622 treatment reduced Il1β expression (main effect of PLX, $F_{(1, 21)} = 5.34$, $p < 0.05$) and reduced Il6 expression specifically in RKO mice (RKO × PLX5622 interaction, $F_{(1, 21)} = 54.39$, [RKO]: control diet vs PLX5622, $p < 0.001$). However, Il1β and Il6 expression were still substantially elevated in the female RKO mice ([PLX5622]: WT vs RKO, $p < 0.05$) as was the expression of the anti-inflammatory marker Ym1. Taken together, RKO increased neuroinflammation to a greater extent in females compared to males and

induced higher expression of both pro- and anti-inflammatory markers, which was partially suppressed by microglia depletion with PLX5622 treatment.

3.2. Exaggerated neuroinflammation in Rev-erbα knockout mice is associated with altered locomotion and exploratory and cognitive behaviors

During several months of husbandry care, RKO mice were noticeably more reactive to routine care and did not fully acclimate to handling. To assess the behavioral outcomes of deleting Rev-erbα, we conducted a battery of behavioral tests (summarized in Fig. 1). In line with previous reports conducted in male mice (Chung et al., 2014; Jager et al., 2014; Otsuka et al., 2022), here we report behavioral changes associated with a heightened risk-taking and anti-anxiety-like phenotype in both male and female RKO mice.

Locomotor and exploratory behaviors were characterized in an elevated plus maze test. RKO mice showed hyperlocomotion compared to WT littermates (RKO × PLX5622 interaction, $F(1, 48) = 7.429$, [control diet]: WT vs RKO, $p < 0.001$, Fig. 3A). This hyperactive phenotype was partially reversed by microglia depletion ([RKO]: control diet vs PLX5622, $p < 0.001$; [PLX5622]: WT vs RKO, $p < 0.001$, Fig. 3A). RKO also increased time spent in the open arms (main effect of RKO: $F_{(1, 44)} = 62.47$, $p < 0.001$, main effect of sex: $F_{(1, 44)} = 9.370$, $p < 0.01$, [males, control diet]: WT vs RKO, $p < 0.01$, [females, control diet]: WT vs RKO, $p < 0.01$, [females, PLX5622]: WT vs RKO, $p < 0.001$, Fig. 3A). This higher preference for open arms (main effect of RKO: $F_{(1, 44)} = 62.49$, $p < 0.001$, Fig. 3A) suggests a higher willingness to take risks (Laviola et al., 2003; Macrìèt al., 2002).

Increased locomotor activity was also observed in the open field in RKO mice as compared to WT controls (main effect of RKO, $F_{(1, 45)} = 242.8$, $p < 0.001$, Fig. 3B). Interestingly, RKO mice spent more time in the periphery of the open field despite higher locomotion (PLX5622 × sex × RKO interaction, $F_{(1, 45)} = 5.69$, post hoc, $p < 0.05$, Fig. 3B). This was particularly apparent in female RKO mice on a control diet. However, this higher peripheral tendency was not specific to a novel environment. In the spray test, where mice were tested in their home cage, RKO mice also demonstrated a preference to stay along the walls (main effect of RKO: $F_{(1, 42)} = 24.63$, $p < 0.05$, Fig. 3C) but had comparable locomotor activity to WT controls, suggesting that the peripheral tendency was not due to differential locomotor activity. Compared to the effects of the PLX5622 diet on behavior in the EPM, PLX5622 did not alter distance traveled or time in the center in the home cage, indicating that microglia depletion may have specifically ameliorated novel, goal-directed locomotive behaviors.

Social and additional mood-related behaviors were also evaluated using a juvenile social exploration test, a sucrose anhedonia test, a spray test, and a nest-building test. RKO decreased social interaction (main effect of RKO, $F_{(1, 50)} = 79.62$, $p < 0.001$, Fig. 4A), sucrose preference (main effect of RKO, $F_{(1, 46)} = 27.32$, $p < 0.001$, Fig. 4B), and nest building (males: time × RKO interaction, $F_{(2, 52)} = 4.96$, females: main effect of RKO, $F(1, 21) = 134.5$, $p < 0.001$, Fig. 4C) suggesting a depressive-like phenotype and decreased general wellness. RKO mice also showed excessive self-grooming (main effect of RKO, $F_{(1, 43)} = 16.12$, $p < 0.001$, Fig. 4D). PLX5622 treatment did not alter these behaviors. Collectively, in both male and female mice, deletion of the Reverbα gene induced a mixed anxiety-like and depressive-like phenotype, with hyperlocomotion, higher

risk-taking, sucrose and social anhedonia, and evidence of decreased general wellness. Microglia depletion with PLX5622 treatment ameliorated aspects of the RKO anxiety- but not depressive-related behavioral phenotype.

3.3. Memory impairments in Rev-erbα knockout mice are ameliorated by microglia depletion

Altered microglial activity and neuroinflammation are implicated in cognitive decline in neurodegenerative diseases such as Alzheimer's disease (Hansen et al., 2018). A hippocampal-dependent learning and memory task, the Barnes maze test, was used to assess cognitive function in RKO mice. The hyperlocomotion phenotype was observed in RKO mice during all the acquisition and probe trials; thus, here we report the path length in addition to the latency to the target zone.

During acquisition, all mice showed a reduction in path length over time suggesting learning. However, the path length for RKO mice was double that of the WT mice in both males (training day × RKO interaction, $F_{(3, 84)} = 3.73$, [day 1, 2, 4]: WT vs RKO, $p < 0.05$, Fig. 5A) and females (training day × RKO interaction, $F_{(3, 66)} = 10.38$, [day 1, 2]: WT vs RKO, $p < 0.05$, Fig. 5A), suggesting that RKO impaired learning. Latency to target during acquisition was also longer in RKO male (main effect of RKO, $F_{(1,25)} = 5.129$, $p < 0.05$, Fig. 5A) and female mice (main effect of training day, $F_{(3, 63)} = 7.013$, $p < 0.001$, main effect of RKO, $F_{(1, 21)} = 6.364$, $p < 0.05$, Fig. 5A). In the probe trial, RKO mice also traveled further before first visiting the target zone (RKO × PLX interaction, $F_{(1, 49)} = 7.56$, [control]: WT vs RKO, $p < 0.001$, Fig. 5B). This higher path length was partially ameliorated by depleting microglia ([RKO]: control diet vs PLX5622, $p < 0.05$). Latency to target followed the same pattern, with RKO mice on the control diet taking longer to find the target (RKO × PLX interaction, $F_{(1, 49)} = 6.650$, $p < 0.05$, [control]: WT vs RKO, $p < 0.001$, Fig. 5B). RKO mice on either control or PLX diet spent half as much time in the target quartile compared to WT mice (main effect of RKO, $F_{(1, 45)} = 58.29$, $p < 0.001$, main effect of sex, $F_{(1, 45)} = 19.07$, $p < 0.001$). Taken together, RKO mice exhibited impaired cognitive function using the Barnes maze, and depleting microglia for three weeks in RKO mice ameliorated aspects of the cognitive dysfunction.

3.4. Sex-dependent alterations in microglia and astrocytes in Rev-erbα knockout mice

Both male and female RKO mice exhibited hyperlocomotion and deficits in spatial learning that were ameliorated by microglia depletion. However, RKO mice showed sex-dependent differences in levels of inflammatory cytokines, with PLX5622 rescuing pro-inflammation in males to a greater extent than females. Furthermore, PLX5622 differentially improved risk-taking behavior in male but not female RKO mice. Together these data suggest Rev-erbα knockout differentially affected the neuroimmune system of male and female mice. Microglia are the primary phagocytic cell of the central nervous system, clearing debris and toxic byproducts from *peri*-synaptic spaces. They additionally phagocytose synaptic elements, ensuring efficient synaptic transmission and facilitating learning and memory processes (Wu et al. 2015). Differential phagocytic activities could therefore mediate the sex differences we observed in RKO mice. Prior work has demonstrated that male RKO mice exhibit elevated colocalization of Iba1 and CD68, which signifies increased

phagolysosome activity (Griffin et al. 2019). To add to the previous literature, we performed immunohistochemical staining and measured Iba1/CD68 colocalization in both male and female mice. We observed an increase in colocalized Iba1 and CD68 in the RKO males compared to wild-types, consistent with previously published results (Griffin et al., 2019). In contrast, there was only a subtle increase of CD68 in female RKO mice compared to wild-types, which did not rise to the level of statistical significance (sex × genotype interaction: $p < 0.05$, $F_{(1,32)} = 5.726$; genotype × diet interaction: $p < 0.01$, $F_{(1,32)} = 14.84$; [male control diet]: wild-type vs. RKO, $p < 0.001$; Fig. 6B). PLX5622 decreased total colocalized CD68 and Iba1 in both sexes, as was expected since microglial counts were heavily reduced (main effect of PLX5622, $F_{(1, 32)} = 71.48$, $p < 0.001$; [male RKO]: control diet vs. PLX5622, $p < 0.001$; [female RKO]: control diet vs. PLX5622, $p < 0.01$, Fig. 6B). In the remaining microglia of both male and female mice on the PLX5622 diet, we observed increased CD68 colocalization, which may indicate heightened phagocytic activity (Main effect of diet, $F_{(1, 32)} = 13.93$, $p < 0.001$, Fig. 6B).

We additionally performed immunohistochemical staining of microglia and reconstructed the skeletons of 6–8 microglia per mouse in the CA1 region of the hippocampus (Fig. 6C). We then used a Sholl analysis to visualize the number of branches along the radial distance from the soma. Increased branching is associated with homeostatic surveillance, while decreased branching can indicate a proinflammatory state. We observed that male RKO microglia had decreased branching along the dendritic arbor compared to wild-type controls (distance × genotype interaction: $p < 0.001$, $F_{(59,900)} = 3.250$, Fig. 6C). On the PLX5622 diet, the remaining microglia from both genotypes showed even further reductions in microglial branching (distance × diet interaction: $p < 0.001$, $F_{(59,900)} = 252.7$, Fig. 6C,D). In contrast, female RKO mice did not show changes in microglial morphology compared to wild-type controls. Females of both genotypes showed similar arborization, with the PLX5622 diet reducing microglial branching in both genotypes, like the males (distance × diet interaction: $p = 0.0223$, $F_{(59,780)} = 1.426$, Fig. 6C). Sex- and diet-dependent differences in branching were most evident at approximately 25 μm from the soma, where male RKO showed the largest decrease in branching compared to the wildtypes, while females showed no difference (main effect of genotype: $p < 0.05$, $F_{(1,28)} = 5.438$; main effect of diet: $p < 0.001$, $F_{(1,28)} = 14.85$. [male control diet]: wild-type vs RKO, $p < 0.05$. [male wild-type]: control diet vs. PLX5622, $p < 0.05$, Fig. 6D). Collectively, these data implicate Rev-erbα as regulating male and female microglial functions in different ways.

The neuroimmune system involves the coordinated activities of several different cell types, and microglia can influence the astrocytic neuroprotection of excitatory synapses (Vainchtein & Molofsky, 2020). Previous work indicated that RKO mice have elevated astrocytic GFAP expression (Griffin et al. 2019), but it is unknown whether male and female RKO mice are similarly affected. We performed a GFAP stain (Fig. 6E) and quantified the percent area of GFAP coverage in area CA1 of hippocampus. Both female and male RKO showed elevated GFAP coverage compared with wild-type controls (Main effect of genotype: $F_{(1,28)} = 16.55$, $p < 0.001$, Fig. 6F), indicating increased astrocytic activation in the knockout. Microglia depletion did not ameliorate GFAP coverage, indicating that astrocytic activation in RKO mice may be caused by other factors involved in the systemic loss of Rev-erbα, and not immediately influenced by microglial functions. It is important

to recognize, though, that our test was limited to GFAP expression, and a more detailed examination of astrocytes could reveal alterations in astrocytic function with microglia depletion.

4. Discussion

Environmental and genetic disruptions of the circadian clock can lead to pathological conditions. Previous studies show that global deletion of the circadian clock gene Rev-erbα (RKO) leads to hyperlocomotion, along with mood and cognitive impairments. However, the mechanisms underlying the changes in behavior remain unclear. We hypothesized that RKO alters microglia function, leading to neuroinflammation and changes in mood and cognition and that microglia depletion would ameliorate behavioral changes. Overall, this manuscript shows that microglia play a role in Rev-erbα-mediated neuroinflammation and animal behaviors. Furthermore, our work reveals that male and female RKO mice exhibit some differences in behavior, cytokine expression, microglial morphology, and phyagolysosomal protein expression. Our findings suggest that Rev-erbα is a crucial connection between the circadian system, the immune system, and behavior regulation.

Here we demonstrate that RKO increases neuroinflammation in the hippocampus of male and female mice. Male and female RKO mice had increases in microglia counts as well as increases in pro-inflammatory cytokine expression. This parallels prior work showing that REV-ERBα modulates inflammatory cytokine expression and microglial responses (Gibbs et al., 2012; Griffin et al., 2019; Griffin et al., 2020). Furthermore, we demonstrate that microglia are involved in exaggerated neuroinflammation in RKO mice. Depleting microglia using the CSF1R inhibitor PLX5622 ameliorated neuroinflammatory changes in a sexspecific manner. Indeed, neuroinflammation was completely resolved in male RKO mice by PLX5622 treatment. In contrast, microglia depletion did not resolve neuroinflammation in female RKO mice to the same extent as in males.

In addition to changes in neuroinflammation, we show that RKO induces behavioral deficits. Both male and female RKO mice had a mixed anxiety-like and depressive-like phenotype. They also displayed cognitive deficits and decreased general wellness. This is in line with previous work showing mood and cognitive deficits in male RKO mice (Chung et al., 2014; Jager et al., 2014; Otsuka et al., 2022; Schnell et al., 2014). Furthermore, we demonstrate that microglia depletion ameliorated goal-directed hyperactivity in a novel environment, without affecting baseline locomotion. This adds to the growing literature on circadian disruption and mood by demonstrating that behavioral changes are mediated by the neuroinflammatory environment.

4.1. Rev-erbα-knockout-induced immune activation

Changes in the immune system can alter brain function. For example, immune activation triggers pro-inflammatory cytokine production in the brain that results in adaptive (e.g., sickness behavior) and maladaptive (e.g., depression) behavioral changes (Dantzer, 2009; Hart, 1990). Importantly, the immune system is regulated by the circadian system (Labrecque & Cermakian, 2015). Indeed, REV-ERBs bind to response elements of pro-inflammatory genes such as Nlrp3 and Il1β and inhibit their expression and activation (Duez

& Pourcet, 2021; Griffin et al., 2019; Pourcet et al., 2018). Therefore, daily oscillations in REV-ERBs tightly regulate immune activities, and alterations in the circadian neuroimmune pattern may lead to exaggerated neuroimmune responses: a loss of REV-ERBα leads to dysregulated and hyperactive immune responses (Fig. 2) (also reported by Gibbs et al., 2012). Consistent with what we observed, dampened circadian patterns in cytokine production and glymphatic drainage in aging brains are often accompanied by higher baseline neuroinflammation and impaired neuronal functions (Fonken & Gaudet, 2022; Gan et al., 2018; Zeppenfeld et al., 2017).

A substantial difference in neuroinflammation was observed in male versus female RKO mice. Female RKO mice had an ~ 10-fold increases in pro-inflammatory cytokine expression compared to WT littermates, while the increase was only ~ 2-fold in male RKO mice (there were no sex differences in cytokine expression in the WT mice). Furthermore, depleting microglia with 5 weeks of PLX5622 treatment did not ameliorate neuroinflammation in RKO female mice to the same extent as in males, possibly due to their higher baseline inflammation. These results agree with previous findings suggesting environmental and genetic circadian disruption can alter neuroimmune pathways (Chen et al., 2021a, 2021b; Fonken et al., 2013b; Fonken et al., 2013c; Gibbs et al., 2012; Narasimamurthy et al., 2012; Nejati Moharrami et al., 2018; Nguyen et al., 2013; Pearson et al., 2020; also see review by Scheiermann et al., 2013). Indeed, prior work by Griffin et al (2019), suggested RKO increased astrogliosis and microgliosis through changes in NFκB signaling (although the sex of the mice was not reported). We observed that both male and female RKO mice had increased astrogliosis, indicating an overall heightened pro-inflammatory state in both sexes. Importantly, microglia depletion did not improve astrogliosis in the RKO mice. Additionally, while microglia depletion ameliorated some of our behavioral phenotypes, it did not restore social interactions or alleviate anhedonia. These data suggest that, while microglia play a role in some behaviors in RKO mice, there are additional, microglia-independent mechanisms mediating these behaviors.

Our findings add to the existing literature supporting the idea that circadian disruption alters immune function, and it further revealed important differential effects of RKO on male and female mice. Our work showed increased expression of the phagocytic gene, CD68, in both male and female RKO mice, but only increased colocalized expression of CD68 and Iba1 in male, and not female, RKO mice. Indeed, microglia regulate neuronal activity via synaptic pruning and phagocytosis in a circadian manner (Choudhury et al., 2020; Hayashi et al., 2013; Iweka et al., 2022). Our findings suggest that Rev-erbα deficiency may increase phagocytosis-related pathways, which may contribute to defects in neuronal activity in male mice.

Similarly, we observed greater morphological changes in male RKO microglia than in females, possibly indicating differential shifts in microglial functions in male, compared to female, RKO mice. The PLX5622 diet decreased microglial branching in both wild-type and RKO mice, indicating a more activated phenotype in remaining microglia. The reduction in male RKO microglial branching could indicate a neuroprotective homeostatic response to global inflammation.

Male and female RKO mice exhibited slightly different alterations to the neuroimmune system, but it is unclear whether these effects were the result of male-to-female differences in clock gene regulation, or whether they stem from broader sex differences in neuroimmune responses. Ikeda et al. (2019) revealed that while double knockout of Reverbα/β in embryonic stem cells does not abrogate Per2 rhythms, it alters circadian expression of other Rev-erb target genes, such as Bmal1 and Npas2. It is possible that Rev-erb knockout affects circadian gene expression in a sex-dependent manner. Considering our results, it would be insightful to explore whether clock gene phase and amplitude are altered differently in male or female RKO mice.

4.2. Aberrant locomotion in Rev-erbα knockout mice is ameliorated by microglia depletion

We show that RKO induced hyperlocomotion in both male and female mice. Prior work has also reported a hyperlocomotion phenotype of male RKO mice (Chung et al., 2014; Jager et al., 2014; Otsuka et al., 2022). Hyperlocomotion in RKO mice is associated with aberrant activity in the midbrain dopaminergic system (Jager et al., 2014); however, resetting the midbrain dopaminergic activity with tyrosine hydroxylase inhibitor only partially rescued the hyperlocomotion in RKO mice (Jager et al., 2014). This prompted us to investigate other possible mechanisms that influence the aberrant neuronal activity in the loss of Rev-erbα which ultimately results in hyperlocomotion. Synaptic strength is modulated by the neuroimmune system. Thus, we hypothesized that microglia may contribute to aberrant neuronal activity leading to hyperlocomotion in RKO mice. Resolving neuroinflammation in both male and female RKO mice ameliorated goal-directed hyperactivity in a novel environment, without altering basal locomotion patterns (i.e., in the home cage) (Fig. 3). Our findings in part support our hypothesis and add to the existing literature emphasizing the importance of the immune system in mood-related behaviors (Hodes et al., 2015; Jager et al., 2014; Kwon et al., 2022).

While microglia depletion was a useful strategy in ameliorating neuroinflammation in our mouse model, further studies are needed to elucidate how this treatment induces changes in the neuronal activity that underlie animal behaviors. In addition to altering neuroimmune activities, microglial depletion has been shown to directly alter circadian gene expression. Sominsky et al., (2021) recently showed that microglia depletion shifted the expression of many circadian genes, including Bmal1, Per1, and Per2 in the rat hippocampus. In our study, microglia depletion may have altered clock gene expression in the RKO mice in a manner that broadly impacted neuronal functioning. While we did not assay circadian expression of clock genes, it is possible that our behavioral improvements with microglia depletion stem, in part, from global impacts to circadian gene transcription.

4.3. Rev-erbα-knockout-induced changes in affective and cognitive behaviors

RKO induced mixed changes in anxiety-related behavioral assays. In an elevated plus maze, RKO mice had a higher preference for open arms than WT controls, suggesting a reduced anxiety-like phenotype, or a higher willingness to take risks (Laviola et al., 2003; Macrièt al., 2002). In contrast, in an open field, RKO mice showed increased peripheral tendency suggesting an anxiety-like phenotype. This differential anxiety-like phenotype in RKO mice

was also reported by (Otsuka et al., 2022). Of note, changes in locomotion in the open field and elevated plus maze tests could be a confounding effect in interpreting anxiety-like phenotypes. To minimize the confounding effect of hyperactivity, the preference to open arms (the ratio of the difference between time spent in open versus closed arms) was reported in addition to the absolute duration spent exploring open arms.

Differential anxiety-like behaviors in the open field and elevated plus maze demonstrate the need to develop more consistent and improved anxiety-related assays. Indeed, many publications report inconsistent anxiety-like behaviors across several anxiety tests (Goto et al., 1993; Holmes et al., 2003; Silva et al., 1997; Tsujimura et al., 2008) and developing alternative anxiety-test, such as conflict test, could more effectively delineate anxiety-related behaviors (e.g., Lee et al., 2023). Alternatively, this may imply that these two behavioral tests could be measuring different aspects of anxiety-like behaviors. For example, the peripheral tendency in RKO mice in the open field may result from overactive tactile whiskers, as mice with brain injury can be overactive to tactile stimuli (Learoyd & Lifshitz, 2012). This is supported by the fact that RKO mice also had a stronger preference for the periphery compared to WT controls in a non-anxiogenic home cage environment (Fig. 3C), where novelty-induced exploration was not observed. If this is the case, then the peripheral tendency potentially suggests a sensationseeking behavior, which, together with the risk-taking behavior observed in the elevated plus maze, represents a more impulsive behavioral phenotype of RKO mice (Laviola et al., 2003).

Contrary to previous studies that report decreased depressive-like behaviors in RKO mice, here we report consistent increases in depressive-like responses using sucrose preference, social interaction, and nest-building tasks. Importantly, prior work used locomotion-based tests (forced swim and tail suspension tests) (Chung et al., 2014; Otsuka et al., 2022; Schnell et al., 2014), in which the hyperactivity of RKO mice could be a confounding factor in interpreting results. A mixed depressive-like and “mania-like” phenotype (anhedonia, hyperactivity, and decreased anxiety-like behaviors) have been reported in ventral tegmental area (VTA)-specific Clock knockdown mice that have aberrant dopaminergic activity in the VTA (Mukherjee et al., 2010), implying a potential involvement of VTA dopaminergic signaling in clock mutant-induced depressive-like behaviors.

Prior work demonstrated that RKO impairs cognition in male mice (Jager et al., 2014; Schnell et al., 2014). Here, we build on these findings and demonstrate that impairments in cognitive function occur in both male and female RKO mice in the Barnes maze task. In line with reduced neuroinflammation and rescued hyperlocomotion following PLX5622 treatment, PLX5622 treatment reduced the latency and path length that the RKO mice traveled before first visiting the target zone, indicating rescued learning and memory in RKO mice. It has been previously reported in neurodevelopmental and neurodegeneration models that reducing neuroinflammation may rescue impaired cognitive function (Fielder et al., 2020; Lonnemann et al., 2022; Pinto et al., 2020).

Considering the nuanced sex-dependent differences in the RKO neuroimmune system, with females maintaining heightened proinflammatory cytokine levels after microglia depletion, it was surprising that PLX5622 diet ameliorated behavioral alterations in both male and

female mice. Our immunohistochemical data suggest that, regardless of how RKO induces behavioral impairments, reduced microglial counts, reduced microglial ramification and decreased CD68 may be broadly beneficial for decreasing risk-taking behaviors, mitigating hyperlocomotive activity, and restoring cognitive function in pathological conditions for both males and females. Our findings add to the existing literature supporting that resolving chronic neuroinflammation may be beneficial for improving cognitive deficits.

5. Conclusion

In conclusion, this manuscript shows that RKO mice display a mixed anxiety-like and depressive-like behavioral phenotype, in parallel with exacerbated hippocampal neuroinflammation. Microglia depletion in RKO mice ameliorated neuroinflammation and some behavioral deficits. Male and female mice exhibit some differences in responsiveness to microglial depletion, which may stem from our observed sex differences in microglial surveillance or phagocytosis. Future research should explore immune cell trafficking and specific mediators involved in microglial inflammatory processes, to further determine the mechanisms underlying RKO-induced behavioral changes and sex differences in RKO-induced neuroinflammation. Uncovering these neuroinflammatory mechanisms will help elucidate the role of the circadianneuroimmune axis in neuropsychiatric, neurodevelopmental, and neurodegenerative diseases.

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Data availability

Data will be made available on request.

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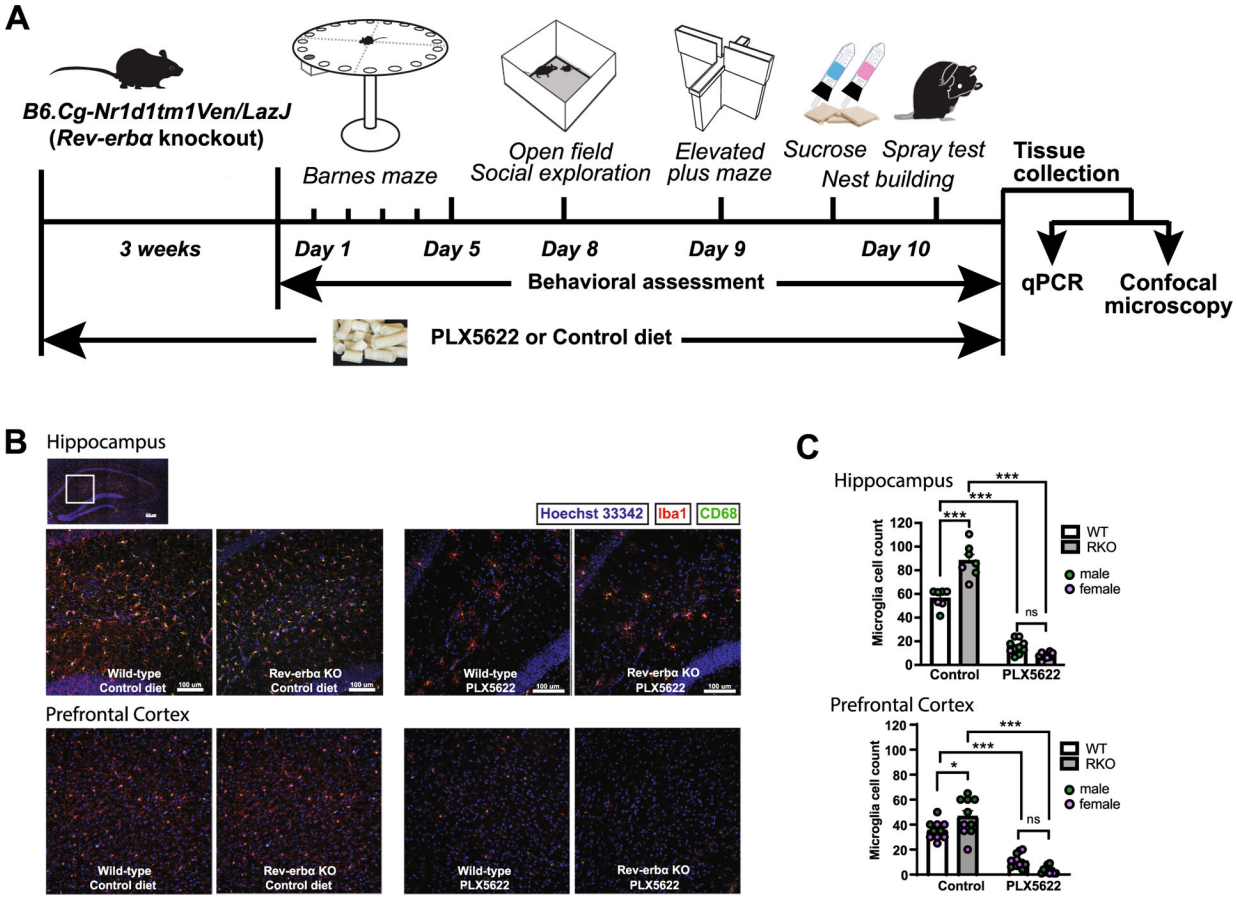

Fig. 1. Schematic timeline for the behavioral assays and tissue collection in Rev-erbα knockout mice.

A. To deplete microglia, adult (~8 mos) RKO mice and WT littermates were treated with a small-molecule CSF1R inhibitor PLX5622-containing diet (1200 ppm) or a standard lab diet starting 3 weeks before behavioral assessment and throughout the remainder of the experiment. Behavioral tests were conducted in the following sequence: Barnes maze, open field, juvenile social exploration, elevated plus maze, sucrose anhedonia, nest building, and spray test. Mice were given a day to recover from the behavior test before tissue collection. B. Representative images of microglia from all experimental groups, after Iba1 immunostaining in the CA1 region of the hippocampus and the Prefrontal Cortex. C. Microglia cell counts in hippocampus and prefrontal cortex. N = 3–7 mice per group, 32 mice total. Data are presented as mean ± SEM, post hoc *p < 0.05, *** p < 0.001, ns: not significantly different. Dark green dots: males. Light purple dots: females.

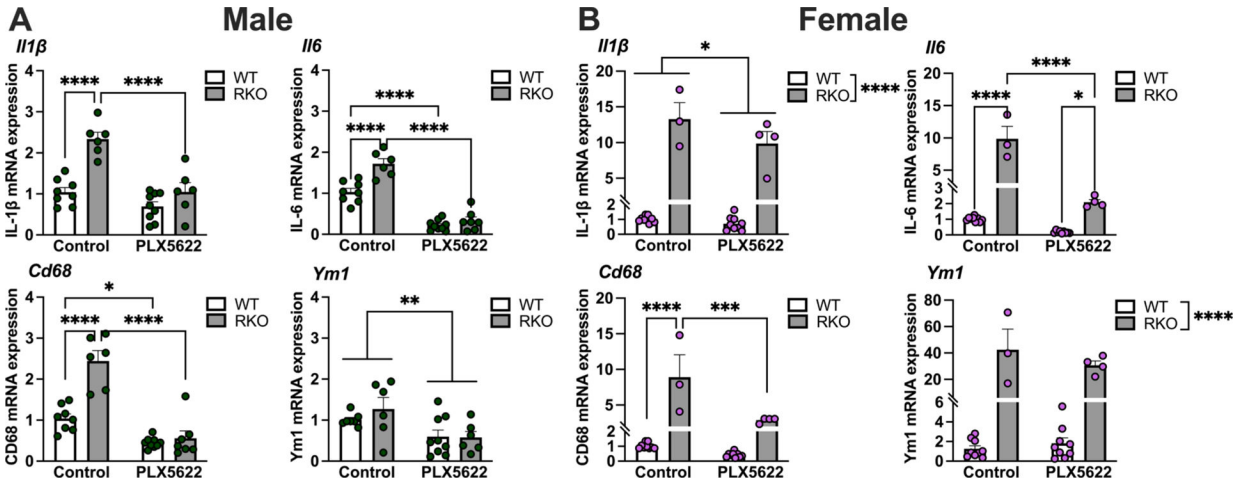

Fig. 2. Rev-erbα knockout induced increases in hippocampal inflammation are ameliorated in a sex-specific manner by microglia depletion.

Gene expression in the hippocampus of (A) male and (B) female WT or RKO mice, on a control diet or a diet containing CSF1R inhibitor, PLX5622. Genes tested included pro-inflammatory cytokines IL-1β and IL-6, phagocytic marker CD68, and anti-inflammatory marker Ym1. N = 3 – 9 per group, 54 mice total. Data are presented as mean ± SEM, post hoc *p < 0.05, ** p < 0.01, ***p < 0.005, **** p < 0.001. Dark green dots: males. Light purple dots: females.

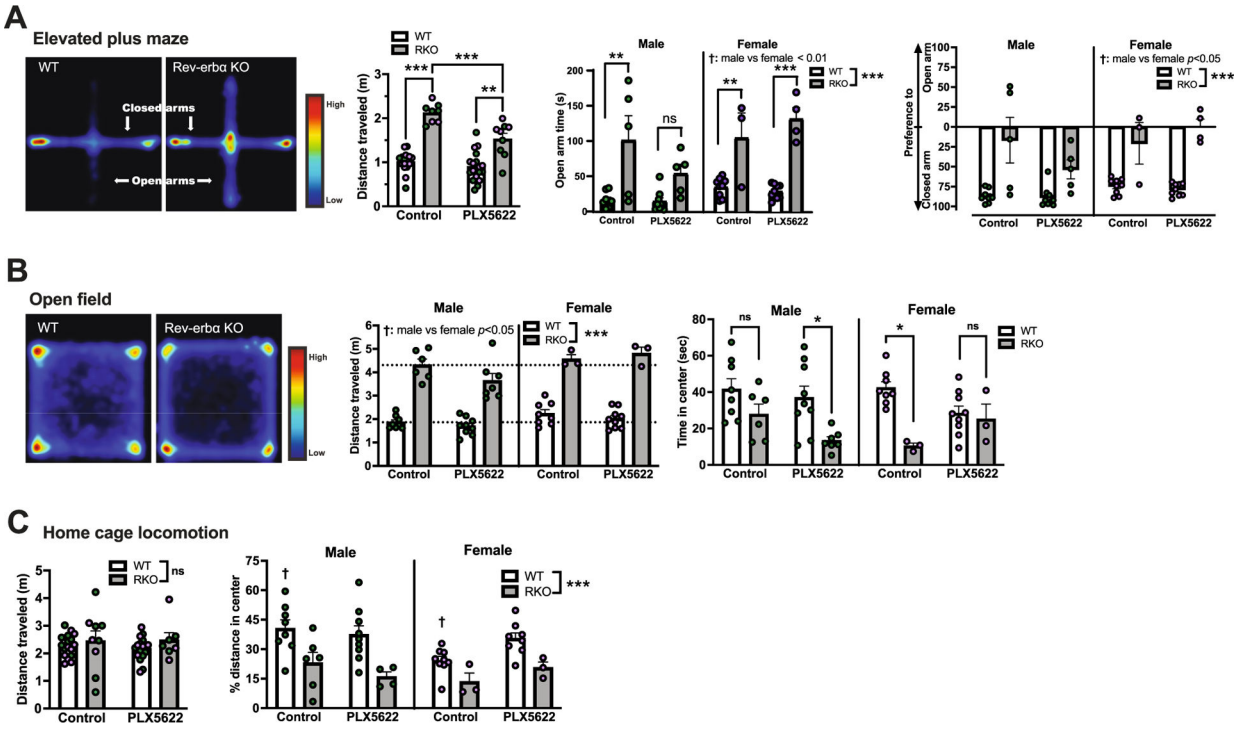

Fig. 3. Microglia depletion ameliorates some anxiety-related behaviors in Rev-erbα knockout mice.

Exploratory and anxiety-like behaviors were assessed in an elevated plus maze and an open field task. (A) Group average heatmaps of the activity of WT and RKO mice in the elevated plus maze. Time spent in open arms in a 5-minute trial. Open arm preference = [(open arm time − closed arm time) / (open arm time + closed arm time)] × 100% (B) Group average heatmaps of the activity of WT and RKO mice in the open field task. Distance traveled, and time spent in middle 66% of the open field. (C) Home cage locomotor activity of WT and RKO mice. Distance traveled and time spent in center 66% of the cage. N = 3–9 per group, 53 mice total. Data are presented as mean ± SEM, post hoc *p < 0.05, ** p < 0.01, *** p < 0.001. Dark green dots: males. Light purple dots: females. †: sex difference.

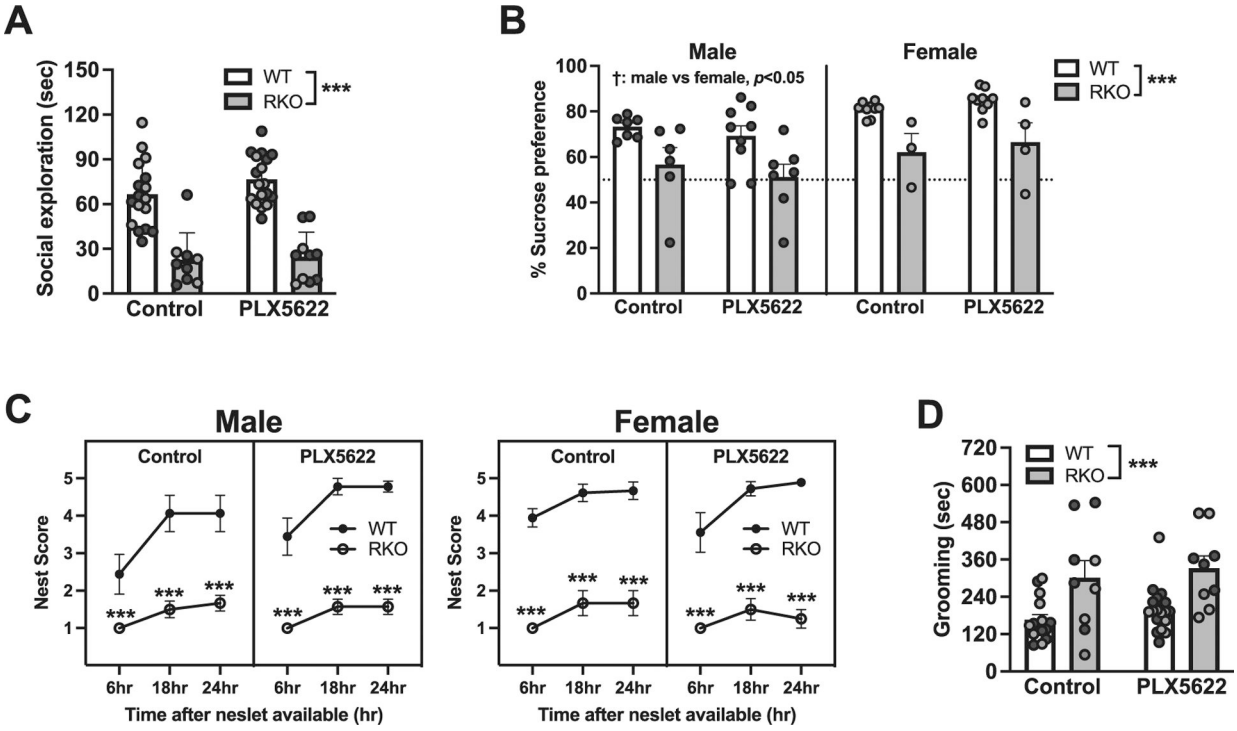

Fig. 4. Rev-erbα knockout induced depressive-like responses that were not ameliorated by microglia depletion.

(A) Time spent exploring a novel, sexmatched juvenile mouse in a 5-minute juvenile social exploration task. (B) Sucrose preference, calculated as the amount of sucrose solution consumed as a percent of total liquid consumed over a 6 h trial. (C) Nest score, ranging from 1 to 5, with 1 indicating almost no nest-building, and 5 indicating a perfect nest. (D) Number of seconds spent grooming in a 15-minute "splash" test. N = 3–9 per group, 54 mice total. Data are presented as mean ± SEM, post hoc *p < 0.05, ** p < 0.01, *** p < 0.001. Dark green: males. Light purple: females. †: sex difference.

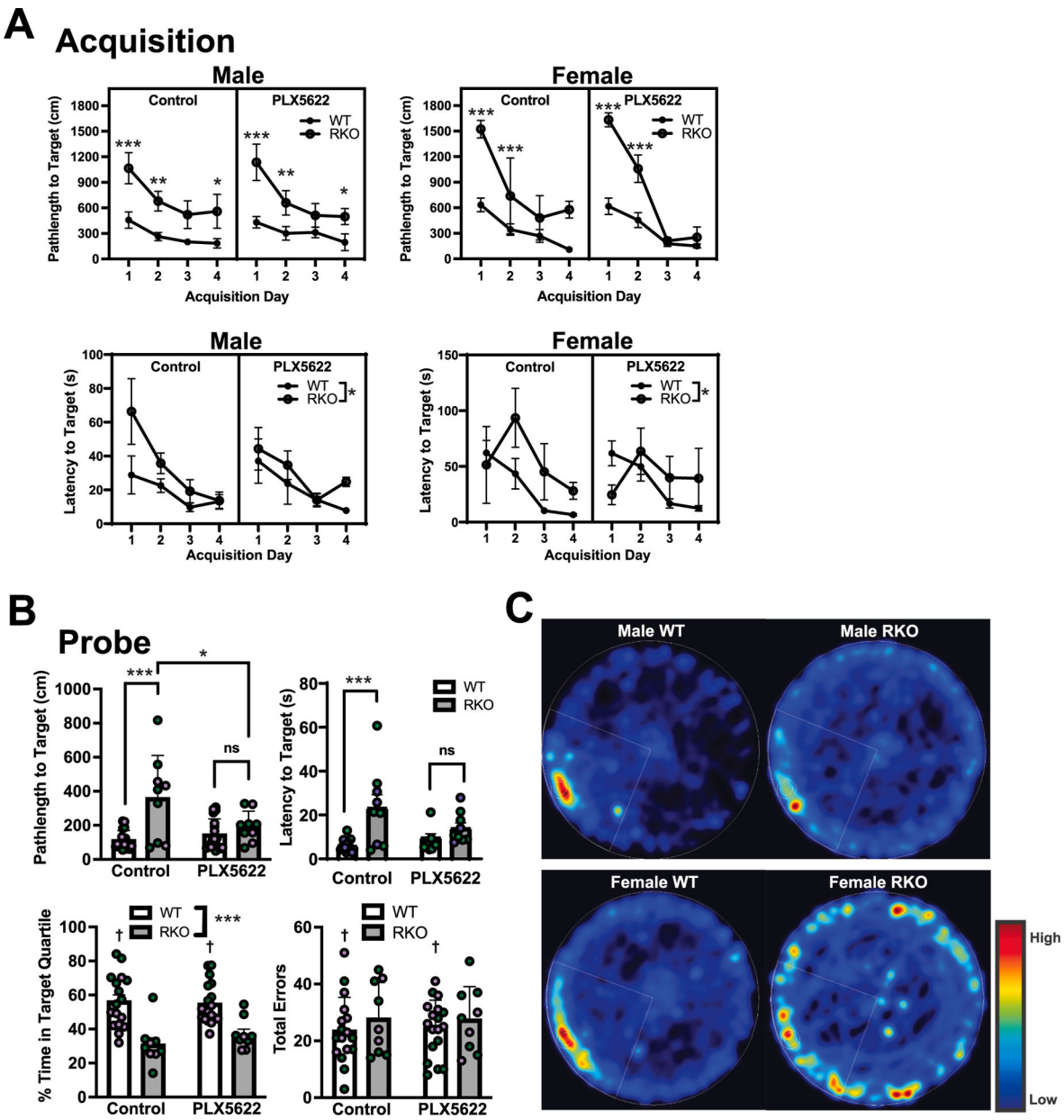

Fig. 5. Rev-erbα knockout induced cognitive impairments in a Barnes maze test are ameliorated by microglia depletion.

(A) WT and RKO mice were trained for 4d on a Barnes Maze (3 trials/day), and spatial learning was tracked during acquisition by measuring pathlength and latency to the target hole across successive training days. (B) On day 5, the escape hole was removed, and a probe trial was run to assess spatial memory. Pathlength and latency to the target are reported, as well as preference for the target quadrant. (C) Group average heatmaps of the activity of WT and RKO mice in the Barnes Maze probe trial. N = 3——9 mice per group, total of 54 mice. Data are presented as mean ± SEM, post hoc *p < 0.05, ** p < 0.01, *** p < 0.001, ns: not significantly different. Dark green: males. Light purple: females. †: sex difference.

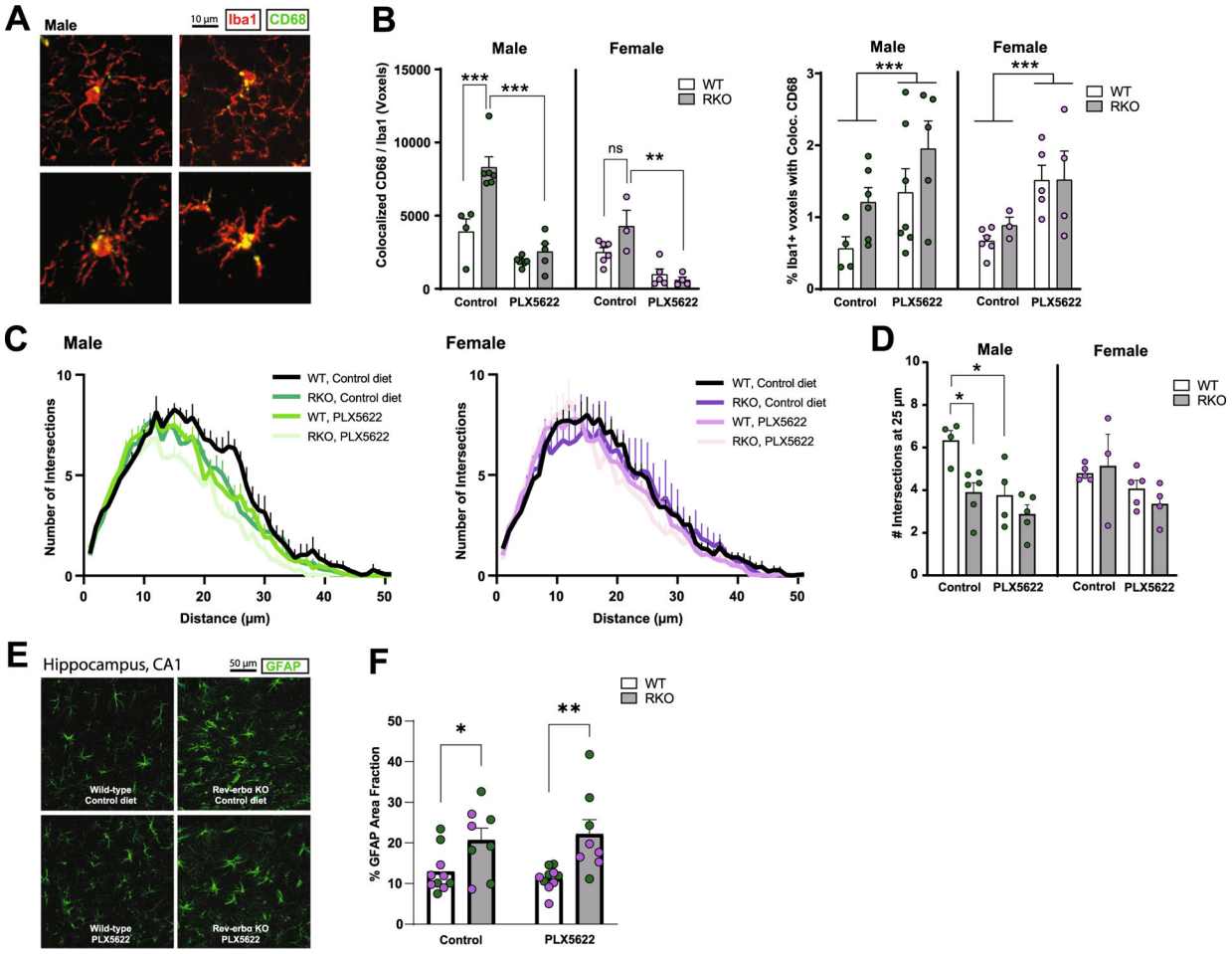

Fig. 6. Rev-erbα knockout alters microglial morphology and astrocytic activation.

(A) Representative images of Iba1 + microglia and CD68 colocalization in male and female WT and RKO mice, on control and PLX5622 diets. (B) Number of voxels of colocalized Iba1 and CD68 (left) and number of colocalized voxels expressed as a percentage of total Iba1 + voxels (right) N = 3–7 per group, 35 mice total. (C) Microglia were morphologically reconstructed from 60x images, and skeletonized to analyze branching complexity. Sholl analysis of number of intersections of the dendritic arbor at progressive distances from the soma is reported. N = 3–6 per group, 36 mice total. (D) Number of Sholl intersections 25 µm from the soma. (E) Representative 20x images of GFAP staining of astrocytes in hippocampal area CA1 in WT and RKO mice on control and PLX5622 diets. (F) Fractional area with a positive GFAP signal. N = 3–6 per group, 36 mice total. Data are presented as mean ± SEM, post hoc *p < 0.05, ** p < 0.01, *** p < 0.001, ns: not significantly different. Dark green: males. Light purple: females. †: sex difference.