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Microglia depletion in early life programs persistent changes in social, mood-related, and locomotor behavior in male and female rats

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Abstract

Microglia, the innate immune cells of the central nervous system, regulate brain development by promoting cell genesis, pruning synapses, and removing dying, newly-born or progenitor cells. However, the role of microglia in the early life programming of behavior under normal conditions is not well characterized. We used central infusion of liposomal clodronate to selectively deplete microglia from the neonatal rat brain and subsequently assessed the impact of microglial depletion on programming of juvenile and adult motivated behaviors. Liposomal clodronate treatment on postnatal days one and four led to greater than 70% loss of forebrain microglia by postnatal day 6 that lasted for approximately ten days. Neonatal microglia depletion led to reduced juvenile and adult anxiety behavior on the elevated plus maze and open field test, and increased locomotor activity. On a test of juvenile social play, microglial depletion led to decreased chase behaviors relative to control animals. There was no change in active social behavior in adults on a reciprocal social interaction test, but there was decreased passive interaction time and an increased number of social avoidance behaviors in clodronate treated rats relative to controls. There was an overall decrease in behavioral despair on the forced swim test in adult rats treated neonatally with clodronate. Females, but not males, treated neonatally with clodronate showed a blunted corticosterone response after acute stress in adulthood. These results show that microglia are important for the early life programming of juvenile and adult motivated behavior.

1. Introduction

Microglia are the innate immune cells of the central nervous system. Microglia colonize the rodent brain beginning on embryonic day 9.5 and reach peak numbers by the third postnatal

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week (Ginhoux et al., 2010; Kim et al., 2015; Nikodemova et al., 2015). They release a variety of diffusible factors, such as chemokines and cytokines, and express a variety of receptors that allow them to respond to and modulate events under normal and abnormal circumstances in the brain. In the developing brain, microglia have been shown to prune synapses and regulate neurogenesis, apoptosis, and axonal innervation (Cunningham et al., 2013; Paolicelli et al., 2011; Schafer et al., 2012; Shigemoto-Mogami et al., 2014; Squarzone et al., 2014). While many of these studies have investigated microglia-regulated processes in a cellular developmental context, few studies have examined whether microglia influence behavioral development in the absence of inflammation, stress or other pathology.

Previous studies have investigated the behavioral effects of depleting microglia from the adult rodent brain, and these studies have found very limited and transient effects of microglial depletion on social and anxiety behaviors (Elmore et al., 2014; Torres et al., 2016). In development, models that are thought to activate microglia or alter their function, such as perinatal stress or inflammatory challenge, show changes in social, anxiety and despair-like behavior, as well as stress reactivity (Choi et al., 2016; Hellwig et al., 2016; Lin et al., 2012; Majidi-Zolbanin et al., 2013; Meyer, 2006; Vallée et al., 1997; Wei et al., 2010; Zhan et al., 2014). These results suggest that microglia may be more important for developmental programming of behavior than the maintenance of behavior in adulthood. However, it is still unclear whether basal microglial function during development directly impacts the development of these behaviors and their expression later in life.

One strategy to determine the role of microglia on the development of later life behavior is to selectively deplete microglia through the use of liposomal clodronate. Clodronate is a cytotoxic drug that, when encapsulated in lipid droplets, is selectively taken up by phagocytic cells, where it subsequently induces apoptosis (van Rooijen et al., 1996). Liposomal clodronate specifically depletes macrophages while sparing other cell types, such as neurons, oligodendrocytes and astrocytes, when centrally injected into the central nervous system (Faustino et al., 2011; Lee et al., 2012; van Rooijen and Hendrikx, 2010). In the current studies we used central infusion of liposomal clodronate to deplete microglia from the developing brain and test whether microglia regulate the development of motivated behaviors in male and female rats. We found that microglia depletion in the early postnatal period led to decreased anxiety behavior and depressive-like behavior and increased locomotor activity in male and female rats, and reductions in stress-induced corticosterone release in females. The current studies underscore that microglia are necessary for the normal development of several motivated behaviors.

2. Materials and Methods

2.1 Animals

All procedures were conducted in accordance with The Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by The Ohio State University Institutional Animal Care and Use Committee. One cohort of age matched juvenile and virgin adult male and female Sprague Dawley rats from four separate litters were used for all behavioral testing. Adult Sprague Dawley rats (Harlan) were mated in our facilities, or timed pregnant animals were ordered to deliver within a week of arrival

at the animal facility (Harlan). Two experimental litters yielded from in house breeding and two litters yielded from ordered timed-pregnant females. Animals were housed in sex-matched pairs in a temperature and humidity controlled room with *ad libitum* access to food and water, and the room was maintained on a 12h/12h light/dark cycle (lights on at 20 hrs). Pregnant females were allowed to deliver naturally and the day of birth was designated as postnatal day (P) 0.

2.2 In vivo manipulations

Bilateral intracerebroventricular (icv) injections were performed on neonates on P1 and P4 under brief cryoanesthesia. A 23 gauge Hamilton syringe attached to a stereotaxic manipulator was placed 1 mm caudal to Bregma and 1 mm lateral to the midline, lowered 3 mm into the brain, and then backed out 1 mm. A total of 1µl of liposomal clodronate (Encapsula NanoSciences, Cat. 8092) or control liposomes (vehicle) was infused over 60s, and the procedure was repeated on the other hemisphere. For all procedures, the separation of pups from the dam was kept to a for the duration of time spent away from the dam. Other than icv treatment of pups on P1 and P4, experimental animals were otherwise left undisturbed with the maternal dam until sacrifice or weaning. The experimental timeline is shown in Figure 1.

2.3 Immunohistochemistry

A time-course study of microglial numbers following injection of liposomal clodronate was performed to 1) verify that liposomal clodronate effectively depleted microglia and 2) to determine the time course of microglial depletion and repopulation. After liposomal clodronate treatment, rats were sacrificed on either P2 (after receiving only one injection on P1), P6, P12, or P22 (after receiving injections on P1 and P4). Rats were deeply anesthetized with FatalPlus (Vortech Pharmaceuticals), transcardially perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were then removed, postfixed overnight in the same fixative, and cryoprotected in 30% sucrose in 0.1M PBS until they sank. Brains were coronally sectioned on a cryostat at a thickness of 45 µm and mounted on charged slides into two alternate series. Brain sections underwent immunofluorescence staining for the microglia/macrophage specific marker, ionized calcium-binding adaptor molecule 1 (Iba1; Wako Chemicals). Slide mounted sections were extensively rinsed with 0.1M PBS then incubated for 20 minutes in 50% methanol. Sections were rinsed in 0.1M PBS then treated in 10 mM sodium citrate solution (pH 9.5) heated to 70° for 20 minutes, rinsed in 0.1M PBS then blocked for 1 hr in 0.1M PBS + 0.4% Triton-X + 5% normal donkey serum (NDS). Sections were then incubated at 4°C overnight in antiserum to Iba1 (1:1000) in 0.1M PBS + 0.4% Triton-X + 2.5% NDS. On day 2, sections were rinsed in 0.1M PBS and incubated in the dark for 2 hr at room temperature with anti-rabbit AlexaFluor 488 (ThermoFisher Scientific, 1:333) in 0.1M PBS + 0.4% Triton-X + 2.5% NDS, rinsed in the dark in 0.1M PBS, counterstained with DAPI, and coverslipped with VectaShield HardSet mounting medium (Vector Laboratories). Sections were then imaged using a Zeiss AxioImager.M2 microscope, Zeiss AxioCam MRm camera, and StereoInvestigator software (MBF Biosciences).

2.4 Densitometry and microglia cell counts

Six animals from each treatment group (three male, three female) were imaged for each time point, except one clodronate treated male was removed from the P22 time point due to low body weight. To quantify gross changes in microglia within the brain following liposomal clodronate treatment, digital image analysis (DIA) of Iba-1 staining was performed (Donnelly et al. 2009). The amygdala and medial prefrontal cortex (mPFC) were chosen for analysis because they are involved in regulating the motivated behaviors assayed in these studies and are relatively distant from one another on both dorsal-ventral and anterior-posterior axes. For densitometric analysis four images were taken from four separate sections mPFC and amygdala in one hemisphere at 10× magnification. The hemisphere used for analysis was counterbalanced within and across conditions. A threshold for positive staining was determined for each image and was processed by densitometric scanning of the threshold targets using ImageJ software (NIH) following a previously reported protocol (Donnelly et al., 2009). In brief, a threshold for positive labeling was determined for each image that included all cell bodies and processes, but excluded background staining. The proportional area was reported as the average percentage area in the positive threshold for all representative images (Wohleb et al., 2013). In addition to microglial densitometry, the number of microglia per region of interest was also determined in the mPFC and amygdala. To quantify the number of microglia, image stacks were taken at 20x for all time points and the total number of microglia was counted within a region of interest defined as $448\mu\text{m} \times 336\mu\text{m}$ (area = $0.15\mu\text{m}^2$) for four sections with a physical distance of $45\mu\text{m}$ between each section. The area of each region of interest was the same for all animals and time points, thus data is expressed as the number of microglia per region of interest.

2.5 Behavior

All behavioral testing was performed by one experimenter. Behavioral testing was videotaped for later analysis, and all testing and scoring of behavior was done blind to treatment and sex. Juvenile rats were weaned at P24 and housed in same-sex groups of 2–3. Behavioral testing was performed on the same cohort of animals in the juvenile and adult periods in order to assess any changes in behavior over development. All behavioral testing for adult females was performed on diestrus except for social interaction, which has been reported to be constant across estrous phase under low light levels (Stack et al., 2010). Diestrus is the phase of the estrous cycle when females have low levels of progestins and estrogens, and experience higher levels of anxiety in the open field and elevated plus maze compared to proestrus and estrous (Hiroi and Neumaier, 2006; Marcondes et al., 2001; Walf and Frye, 2007). Adult females were vaginally swabbed to determine the day of estrous, beginning seven days prior to initial testing to habituate animals to the procedure. Following initial habituation, females were swabbed 2–3 days prior to testing when there was a break following previous behavioral tests. On the day of behavioral testing, females were swabbed approximately 1 hr before testing began. For behavioral testing, group sizes were as follows: Vehicle treated males $n = 10$, clodronate treated males $n = 9$, clodronate treated females $n = 11$, and vehicle treated females $n = 11$. Due to technical difficulties with videotaping, two behavioral tests had modified animal numbers. For the juvenile elevated plus maze task: Vehicle treated males $n = 10$, clodronate treated males $n = 8$, vehicle treated females $n = 11$, and clodronate treated females $n = 10$, and for the adult open field test: Vehicle treated males

n = 9, clodronate treated males n = 9, clodronate treated females n = 11 and vehicle treated females n = 11.

2.6 Juvenile social play

Juvenile social play testing was performed on P28–29. All juvenile social play was performed without prior social isolation. Juvenile paired play and group play were both assessed as there can be sex differences in number of play behaviors depending on the paradigm used (Argue and McCarthy, 2015; Auger and Olesen, 2009). Paired play consisted randomly assigned age-matched, same-sex pairs. Group play consisted of mixed treatment groups of 4–5 animals, consisting of 2–3 pair-housed males and 2–3 pair-housed females. All social play tests were conducted for 13 minutes and the last 10 minutes was used for analysis. Animals were marked with a sharpie to differentiate between them during testing. For both paired play and group play, we assessed the frequency of chasing, pouncing, wrestling, boxing, pinning, and social exploration (e.g., sniffing conspecifics). Behaviors were divided into rough and tumble play (e.g., wrestling, pinning, boxing, and pouncing), chase behaviors, and number of social explorations for statistical analysis.

2.7 Juvenile open field test

Juvenile open field was performed on P30–32. The open field arena was a Plexiglas cage (60 cm x 60 cm x 40 cm) divided by gridlines into 36 10 x 10 cm squares. The inner square consisted of 16 10 x 10 cm squares. Open field behavior was run under dim red light and analyzed for 5 minutes. Data collected included the number of lines crossed, center entries, time spent in the center, and number of rears.

2.8 Juvenile elevated plus maze

The elevated-plus maze (EPM) consisted of a cross-shaped platform (height: 50 cm) with four arms (width: 10 cm, length: 50 cm), two of which were enclosed by walls 50 cm in height. The EPM test was performed under bright white light during the dark phase of the light cycle. Rats were placed in the center of the platform (10 x 10 cm), facing a junction between an open and closed arm and allowed to explore for 10 min. The number of open arm entries and the time spent in the open arms were quantified. There were four clodronate males and three clodronate females that fell or jumped off the EPM, thus data for the juvenile EPM is expressed as the percent of total time on open arms.

2.9 Adult social interaction

The social interaction test was performed from P80–90 in the same arena as used for open field behavior detailed above. Stimulus animals were novel sex- and age-matched rats that had not previously been used in prior behavioral testing. Stimulus animals were used for no more than five pairings and were not used for consecutive tests. Stimulus animals were habituated to the open field for 30 minutes 24 hr before testing. The social interaction test was run under dim red light conditions, because pre-habituation and dim red light conditions elicit the lowest amount of anxiety and the highest amount of social interaction, thus it is best for detecting changes in sociability rather than anxiety (File and Seth, 2003). Females were run regardless of estrous cycle as female social behavior has been reported to be

constant across estrous phase under low light levels (Stack et al., 2010). The test consisted of placing a stimulus animal in one corner, and placing an experimental animal in the opposite corner. Open-ended interaction was observed for 10 min. Data collected included total active interaction time, number of active bouts, passive interaction time, and number of social avoidance behaviors. Active interactions include sniffing, climbing on, or playing with the stimulus rat. Active bouts had to be separated by a least 5 seconds to be considered a separate bout. Passive interaction consists of a rat being within 5 cm of the stimulus rat, but not actively interacting with or investigating the stimulus rat. Social avoidance behaviors were counted when the stimulus rat attempted to actively interact with the experimental rat, but the experimental rat moved away from the stimulus rat thereby avoiding interaction. Animals were marked with a sharpie to differentiate between them during testing.

2.10 Adult open field test

The open field arena used had the same dimensions and layout as the open field used for juvenile open field (section 2.4). The open field was performed from P70–80. Open field behavior was run under dim red light and analyzed for 5 minutes. Analysis consisted of number of lines crossed, center entries, time spent in the center, and number of rears.

2.11 Adult elevated plus maze

The EPM apparatus was the same to that used for juvenile EPM behavior (section 2.5). The elevated plus maze was performed from P60–70. EPM was run under bright white lights. Rats were placed in the center of the platform, facing a junction between an open and closed arm and allowed to explore for 10 min. The number of open arm entries, open arm time, closed arm entries and closed arm time was analyzed.

2.12 Adult forced swim test

The forced swim test (FST) was used to assess behavioral despair on P90–100. Females were assessed during diestrus. Plexiglas cylinders (diameter: 30.5 cm, height: 49 cm) were filled to a depth of 30 cm with $25 \pm 0.5^{\circ}\text{C}$ water. FST was run under bright white lights. Rats were placed individually into the FST cylinders for 10 min, towel dried, and returned to their home cage. 24 h later, rats were returned to the same apparatus for 5 min and the session digitally recorded. The percentage of time spent immobile $[(\text{time spent floating in the water only making movements necessary to maintain the head above water} / \text{total test time}) \times 100]$ was later measured blind to condition by a trained observer.

2.13 Adult restraint stress and serum corticosterone assay

To determine acute stress reactivity, adult rats were restrained in ventilated clear Plexiglas cylinders (21 cm long, 6 cm internal diameter; Plas-Labs 554-BSRR) for 30 min under bright light illumination immediately prior to sacrifice on P120. Females were stressed on diestrus. Restraint cylinders were thoroughly cleaned after each use with soap and water followed by 70% ethanol and dried. Immediately after restraint, rats were deeply anesthetized with FatalPlus (Vortech Pharmaceuticals) and blood was obtained via cardiac puncture. Blood samples were placed on ice and serum was collected after centrifuging samples at $4,000 \times \text{rpm}$ for 15 min. Serum was stored at -80°C until assay. A corticosterone

ELISA (Arbor Assays Cat. K014-H1) was used to determine serum concentrations of corticosterone and run according to the manufacturer's instructions. All standards and samples were assayed in duplicate, and corticosterone levels were assayed on two separate plates due to the large cohort of animals. Corticosterone levels were normalized using a control animal sample run on both plates to account for any inter-plate variability.

2.14 Statistics

All data were analyzed using two-way ANOVA, with treatment (vehicle or clodronate) and sex (male or female) as the two main factors, yielding a 2×2 design. When a significant main effect was obtained in ANOVA, planned post-hoc comparisons were made using Bonferroni post-hoc tests with p values corrected for multiple comparisons. Male and females that were analyzed separately for all experiments except were grouped for analysis of microglia colonization of the brain due to lack of sex differences seen in the clodronate analysis and low power to detect sex differences. When a significant interaction effect was obtained, Tukey post hoc analyses were performed. For all analyses, differences were considered significant when the corrected $p < 0.05$. All data are expressed as mean with standard error of the mean (SEM). Statistical analyses were conducted in Prism 6.0 for Mac/PC.

3. Results

3.1 Iba1 immunofluorescence analysis

Figure 2 shows representative pictures of microglia from each time point in the mPFC (left) and amygdala (right), and figure 3 summarizes microglial counts and densitometry across development following neonatal treatment with liposomal clodronate or vehicle.

In the mPFC, microglia counts per region of interest (ROI) indicated that microglia numbers were significantly reduced by 87.3% in clodronate treated rats compared to control treated rats at P2 (Fig. 3a; treatment: $F_{1,8} = 85.4$, $p < 0.001$; sex: $F_{1,8} = 0.318$, ns; interaction: $F_{1,8} = 0.5443$, ns) and multiple comparisons showed that clodronate treated males and females had significantly fewer microglia than vehicle treated males and females. The number of microglia was significantly reduced by 72.4% in clodronate treated rats compared to control treated rats at P6 (Fig. 3a; treatment: $F_{1,8} = 12.07$, $p = 0.0084$; sex: $F_{1,8} = 1.05$, ns; interaction: $F_{1,8} = 0.00222$, ns). The number of microglia were significantly increased by 61.3% in the mPFC of clodronate treated rats compared to vehicle treated rats at P12 (Fig. 3a; treatment: $F_{1,8} = 29.3$, $p = 0.0006$; sex: $F_{1,8} = 0.0609$, ns; interaction: $F_{1,8} = 3.66$, ns) and multiple comparisons showed that clodronate treated females had significantly more microglia than vehicle treated females. Microglia density in clodronate treated rats returned to control levels at P22 (Fig. 3a; treatment: $F_{1,7} = 0.421$, ns; sex: $F_{1,7} = 0.0362$, ns; interaction: $F_{1,7} = 6.84$, $p = 0.0346$).

Densitometric analysis indicated that the percent area of Iba1 staining was significantly reduced in clodronate treated rats compared to control treated rats at P2 in the mPFC (Fig. 3c; treatment: $F_{1,8} = 94.2$, $p < 0.0001$; sex: $F_{1,8} = 0.096$, ns; interaction: $F_{1,8} = 0.399$, ns) and multiple comparisons showed that clodronate treated males and females had less Iba1

staining compared to vehicle males and females respectively. Iba1 staining was significantly reduced in clodronate treated rats compared to control treated rats at P6 (Fig. 3c; treatment: $F_{1,8} = 187$, $p < 0.0001$; sex: $F_{1,8} = 5.02$, ns; interaction: $F_{1,8} = 2.39$, ns) and multiple comparisons showed that clodronate treated males and females had significantly less area stained by Iba1 compared to vehicle treated males and females respectively. There was no difference in the percent area stained by Iba1 at P12 (Fig. 3c; treatment: $F_{1,8} = 4.31$, ns; sex: $F_{1,8} = 0.267$, ns; interaction: $F_{1,8} = 2.59$, ns), and P22 (Fig. 3c; treatment: $F_{1,7} = 0.142$, ns; sex: $F_{1,7} = 0.814$, ns; interaction: $F_{1,7} = 0.812$, $p = 0.0346$).

In the amygdala (Figures 2 and 3, right panels). microglia counts were significantly reduced by 39.6% compared to control injected animals at P2 (Fig. 3b; treatment: $F_{1,8} = 15.2$, $p = 0.0046$; sex: $F_{1,8} = 0.0224$, ns; interaction: $F_{1,8} = 1.04$, ns) and multiple comparisons showed that clodronate treated males had significantly fewer microglia compared to vehicle treated microglia. The number of microglia was significantly reduced by 83.7% compared to control injected animals at P6 (Fig. 3b; treatment: $F_{1,8} = 71.4$, $p < 0.0001$; sex: $F_{1,8} = 6.99$, $p = 0.0295$; interaction: $F_{1,8} = 0.0128$, ns). Multiple comparisons showed that clodronate treated males and females had significantly fewer microglia than vehicle treated males and females respectively. There were no differences in number of microglia between treatments at P12 (Fig. 3b; treatment: $F_{1,8} = 0.494$, ns; sex: $F_{1,8} = 0.193$, ns; interaction: $F_{1,8} = 0.0774$, ns), and P22 (Fig. 3b; treatment: $F_{1,7} = 2.29$, ns; sex: $F_{1,7} = 4.29$, ns; interaction: $F_{1,7} = 0.684$, ns).

Densitometric analysis indicated that Iba1 staining area was significantly reduced in clodronate treated rats compared to control treated rats at P2 in the amygdala (Fig. 3d; treatment: $F_{1,8} = 45.7$, $p = 0.0001$; sex: $F_{1,8} = 0.676$, ns; interaction: $F_{1,8} = 3.58$, ns) and multiple comparisons showed that clodronate treated males and females had significantly less Iba1 staining than vehicle treated males and females respectively. Iba1 staining area was significantly reduced in clodronate treated rats compared to control treated rats at P6 (Fig. 3d; treatment: $F_{1,8} = 85.5$, $p < 0.0001$; sex: $F_{1,8} = 2.03$, ns; interaction: $F_{1,8} = 0.256$, ns). Multiple comparisons showed that clodronate treated males and females had significantly reduced Iba1 staining compared to vehicle treated males and females respectively. There were no differences in Iba1 staining area at P12 (Fig. 3d; treatment: $F_{1,8} = 1.82$, ns; sex: $F_{1,8} = 0.666$, ns; interaction: $F_{1,8} = 0.706$, ns), or P22 (Fig. 3d; treatment: $F_{1,7} = 1.11$, ns; sex: $F_{1,7} = 0.0995$, ns; interaction: $F_{1,7} = 0.0324$, ns).

To better understand the normal trajectory microglial colonization in the mPFC versus amygdala, we compared microglial number and Iba1 staining density between these two brain regions in control animals. Differences between males and females were not tested due to low power ($n = 3$ per sex per time point). There was a significant effect of age, area, and a significant interaction in the number of microglia in the mPFC and amygdala (Age: $F_{3,40} = 47.4$, $p < 0.0001$; brain region: $F_{3,40} = 35.8$, $p < 0.0001$; interaction: $F_{3,40} = 4.77$, $p = 0.0062$). Post-hoc analyses showed that there was an increase in the number of microglia in the mPFC from P6 to P12 and a decrease from P12 to P22. There was a significant increase in number of microglia in the amygdala from P2 to P6 and a decrease from P12 to P22. There were significantly more microglia in the amygdala compared to the mPFC at P2 and P6.

There was a significant effect of age and a significant interaction in the percent area stained by Iba1 in the mPFC and amygdala (Age: $F_{3,40} = 139$, $p < 0.0001$; brain region: $F_{3,40} = 2.37$, ns; interaction: $F_{3,40} = 21.9$, $p < 0.0001$). Post-hoc analyses showed that there was a significant increase in the percent area stained by Iba1 from P2 to P6 and from P6 to P12, but not from P12 to P22 in the mPFC. There was a significant increase in the percent area stained by Iba1 in the amygdala from P2 to P6, but from P6 to P12 and P12 to P22. There was significantly more area stained by Iba1 in the amygdala compared to the mPFC at P2 and P6. At P12 there was significantly more area stained by Iba1 in the mPFC compared the amygdala. There was no difference in Iba1 staining between the two brain regions at P22.

3.2 Juvenile paired play

Clodronate treated rats chased partner rats less than vehicle treated rats (Fig. 4a; treatment: $F_{1,37} = 17.9$, $p = 0.0001$; sex: $F_{1,37} = 1.47$, ns; interaction: $F_{1,37} = 2.41$, ns). Post-hoc tests showed that clodronate treated males chased significantly less than vehicle treated males. There were no significant differences in the number of rough and tumble behaviors (Fig. 4b; treatment: $F_{1,37} = 1.25$, ns; sex: $F_{1,37} = 0.093$, ns; interaction: $F_{1,37} = 1.24$, ns). Clodronate treated animals displayed significantly more social exploration behaviors than vehicle treated animals (Fig. 4c; treatment: $F_{1,37} = 4.72$, $p = 0.036$; sex: $F_{1,37} = 1.83e-005$, ns; interaction: $F_{1,37} = 2.13$, ns). Post-hoc tests showed that clodronate treated females made significantly more social explorations than vehicle treated females.

3.3 Juvenile group play

Clodronate treated rats chased conspecifics significantly less than vehicle treated rats (Fig. 4d; treatment: $F_{1,37} = 34.5$, $p = 0.0001$; sex: $F_{1,37} = 0.20$, ns; interaction: $F_{1,37} = 0.23$, ns). Multiple comparisons showed that clodronate treated males and clodronate treated females chased conspecifics significantly less than vehicle treated males and vehicle treated females respectively. There were no significant differences in the number of rough-and-tumble behaviors (Fig. 4e; treatment: $F_{1,37} = 0.92$, ns; sex: $F_{1,37} = 0.29$, ns; interaction: $F_{1,37} = 0.92$, ns) or in the number of social explorations (Fig. 4f; treatment: $F_{1,37} = 4.21$, ns; sex: $F_{1,37} = 1.90$, ns; interaction: $F_{1,37} = 1.72$, ns).

3.4 Adult social interaction

Female rats actively investigated the stimulus rat for a significantly longer period of time than male rats regardless of treatment (Fig. 5a; treatment: $F_{1,37} = 0.15$, ns; sex: $F_{1,37} = 6.49$, $p = 0.015$; interaction: $F_{1,37} = 0.11$, ns). Female rats performed more bouts of active investigation than male rats regardless of treatment (Fig. 5b; treatment: $F_{1,37} = 0.023$, ns; sex: $F_{1,37} = 7.49$, $p = 0.0095$; interaction: $F_{1,37} = 0.11$, ns). Clodronate treated rats spent significantly less time passively interacting with the stimulus rat than vehicle treated rats (Fig. 5c; treatment: $F_{1,37} = 24.4$, $p < 0.0001$; sex: $F_{1,37} = 2.03$, ns; interaction: $F_{1,37} = 8.59$, $p = 0.0058$). Vehicle treated females passively interacted more than vehicle treated males, and clodronate treated females. Clodronate treatment induced a significant increase in the number of social avoidance behaviors in adulthood relative to vehicle treatment (Fig. 5d; treatment: $F_{1,37} = 7.21$, $p = 0.0108$; sex: $F_{1,37} = 1.67$, ns; interaction: $F_{1,37} = 0.10$, ns). Multiple comparisons showed that vehicle treated females and clodronate treated females

passively interacted more than vehicle treated males and clodronate treated males respectively.

3.5 Juvenile open field test

There was a significant effect of treatment on activity in the open field, with clodronate treated rats crossing significantly more gridlines than vehicle treated rats, as well as a significant sex difference in activity, with females crossing significantly more gridlines than males (Fig. 6a; treatment: $F_{1,37} = 81.4$, $p < 0.0001$; sex: $F_{1,37} = 6.63$, $p = 0.014$; interaction: $F_{1,37} = 3.38$, ns). Multiple comparisons showed that clodronate treated females crossed significantly more lines than vehicle treated females and clodronate treated males, and clodronate treated males crossed significantly more lines than vehicle treated males. Clodronate treated rats entered the center area more frequently than vehicle treated rats (Fig. 6b; treatment: $F_{1,37} = 21.1$, $p < 0.0001$; sex: $F_{1,37} = 2.22$, ns; interaction: $F_{1,37} = 4.64$, $p = 0.038$). Multiple comparisons showed that clodronate treated females entered the center area significantly more than vehicle treated females. There were no significant differences for time spent in the center of the open field (Fig. 6c; treatment: $F_{1,37} = 2.32$, ns; sex: $F_{1,37} = 3.11$, ns; interaction: $F_{1,37} = 1.25$, ns). Clodronate treated rats reared significantly less than vehicle treated rats irrespective of sex (Fig. 6d; treatment: $F_{1,37} = 8.01$, $p = 0.0075$; sex: $F_{1,37} = 0.0042$, ns; interaction: $F_{1,37} = 0.26$, ns).

3.6 Adult open field test

There was a significant effect of treatment on activity in the open field, with clodronate treated rats crossing significantly more gridlines than vehicle treated rats, as well as a significant sex difference in activity, with females crossing significantly more gridlines than males (Fig. 7a; treatment: $F_{1,36} = 98.0$, $p < 0.0001$; sex: $F_{1,36} = 12.38$, $p = 0.0012$; interaction: $F_{1,36} = 1.16$, ns). Clodronate treated male and female rats crossed significantly more gridlines than vehicle treated male and female rats respectively. Clodronate treated female rats also crossed more gridlines than clodronate treated male rats. Clodronate treated rats entered the center significantly more than vehicle treated rats (Fig. 7b; treatment: $F_{1,36} = 10.3$, $p = 0.0028$; sex: $F_{1,36} = 3.29$, ns; interaction: $F_{1,36} = 0.033$, ns). Females spent significantly more time in the center of open field compared to males (Fig. 7c; treatment: $F_{1,36} = 0.27$, ns; sex: $F_{1,36} = 10.4$, $p = 0.0027$; interaction: $F_{1,36} = 1.25$, ns). Multiple comparisons showed that clodronate treated females spent significantly more time in the center of the open field than clodronate treated males. There was a significant effect of treatment and significant interaction for number of rears in the open field (Fig. 7d; treatment: $F_{1,36} = 27.3$, $p < 0.0001$; sex: $F_{1,36} = 0.95$, ns; interaction: $F_{1,36} = 12.8$, $p = 0.001$). Multiple comparisons showed that clodronate treated males reared significantly more than vehicle treated males and vehicle treated females. Vehicle treated males also reared significantly less than vehicle treated females.

3.7 Juvenile elevated plus maze

Clodronate treated rats spent significantly more time on the open arms than vehicle treated rats (Fig. 8a; treatment: $F_{1,34} = 36.9$, $p < 0.0001$; sex: $F_{1,34} = 0.43$, ns; interaction: $F_{1,34} = 0.48$, ns). Multiple comparisons showed that clodronate treated males and clodronate treated females spent significantly more time on the open arms than vehicle treated males and

vehicle treated females respectively. Clodronate treated rats entered the open arms significantly more than vehicle treated rats (Fig. 8b; treatment: $F_{1,34} = 19.9, p < 0.0001$; sex: $F_{1,34} = 0.0099$, ns; interaction: $F_{1,34} = 0.039$, ns). Multiple comparisons showed that clodronate treated males and clodronate treated females entered the open arms significantly more often than vehicle treated males and vehicle treated females respectively. Additionally, four of nine clodronate treated males and three of ten clodronate treated females fell or jumped off the EPM, and zero vehicle treated animals fell or jumped off the EPM.

3.8 Adult elevated plus maze

There was a significant effect of treatment and sex on the time spent in open arms (Fig. 9a; treatment: $F_{1,37} = 11.9, p = 0.0014$; sex: $F_{1,37} = 4.23, p = 0.047$; interaction $F_{1,37} = 0.35$, ns) with clodronate treated rats spending more time on the open arms compared to vehicle treated rats. Multiple comparisons showed that clodronate treated males and vehicle treated females spent significantly more time on the open arms than vehicle treated males and vehicle treated females respectively. Clodronate treated rats entered the open arms significantly more than vehicle treated rats (Fig. 9b; treatment: $F_{1,37} = 8.74, p = 0.0054$; sex: $F_{1,37} = 3.13$, ns; interaction: $F_{1,37} = 0.077$, ns). Multiple comparisons showed that clodronate treated females entered the open arms more than vehicle treated females. Clodronate treated rats spent significantly less time in the closed arms compared to vehicle treated rats (Fig. 9c; treatment: $F_{1,37} = 7.13, p = 0.011$; sex: $F_{1,37} = 1.93$, ns; interaction: $F_{1,37} = 0.16$, ns). There were no differences in the number of closed arm entries (Fig. 9d; treatment: $F_{1,37} = 3.29$, ns; sex: $F_{1,37} = 0.019$, ns; interaction: $F_{1,37} = 0.0027$, ns).

3.9 Adult forced swim test

Clodronate treated rats spent significantly less time immobile in the forced swim test than vehicle treated rats, and females spent significantly less time immobile than males regardless of treatment (Fig. 10a; treatment: $F_{1,37} = 10.1, p = 0.0031$; sex: $F_{1,37} = 10.9, p = 0.0021$; interaction: $F_{1,37} = 0.83$, ns). Multiple comparisons showed that vehicle treated males spent significantly more time immobile than vehicle treated females and clodronate treated males. Clodronate treated rats spent significantly more time swimming than vehicle treated rats and females spent significantly more time swimming than males (Fig. 10b; treatment: $F_{1,37} = 13.3, p = 0.0008$; sex: $F_{1,37} = 11.3, p = 0.0018$; interaction: $F_{1,37} = 0.46$, ns). Multiple comparisons showed that vehicle treated females and clodronate treated males spent significantly more time swimming compared to vehicle treated males. Clodronate treated rats spent significantly less time climbing the walls of the testing chamber than vehicle treated rats (Fig. 10c; treatment: $F_{1,37} = 5.57, p = 0.024$; sex: $F_{1,37} = 1.72$, ns; interaction: $F_{1,37} = 0.19$, ns).

3.10 Adult acute stress response

There was no significant main effect of clodronate treatment on corticosterone levels after acute restraint stress, but there was a significant effect of sex and significant interaction (Fig. 10d; treatment: $F_{1,37} = 1.13$, ns; sex: $F_{1,37} = 23.1, p < 0.0001$; interaction: $F_{1,37} = 6.25, p = 0.017$). Multiple comparisons showed vehicle treated females had significantly higher levels of corticosterone compared to vehicle treated males and that this sex difference was prevented by clodronate treatment.

4. Discussion

In the current study, we assessed the role of neonatal microglial depletion on behavioral development in male and female rats, and found that temporary neonatal loss of microglia perturbs the development of anxiety, social, and locomotor behaviors as well as the adult stress response. We found few sex differences in the effects of neonatal microglial depletion, suggesting that microglia during the neonatal period do not regulate baseline sex differences in the development of motivated behaviors. Overall, our studies support a role for microglia in the normal development of motivated behaviors.

4.1 Normal microglial dynamics in the developing mPFC and amygdala of males and females

We found a peak in microglial density in the mPFC and amygdala at P12. Our results corroborate the findings of previous studies showing that microglial density peaks brain-wide at P14 and the hippocampus, specifically, at P15 in mice (Kim et al., 2015; Nikodemova et al., 2015). Our data provides novel insight into the normal developmental time course of microglial colonization of the prefrontal cortex and amygdala across the early to mid-postnatal period, which has not previously been examined closely. Interestingly, at P2 and P6 microglia density was lower in the mPFC compared to the amygdala. There was also a larger increase in microglia density and a larger peak in microglial density in the mPFC. These data show different colonization kinetics between different the mPFC and amygdala. While microglial density did not differ between the amygdala and mPFC at P12 and P22, the area stained by Iba1 was lower in the amygdala compared to the mPFC at P12. These results suggest that microglia assume a more mature morphology at an earlier time in the amygdala. The differences in density and microglia morphology might indicate developmental differences between these brain areas or sensitivity to adverse events that change microglial function such as stress or inflammation.

4.2 Microglial depletion strategy

Liposomal clodronate selectively depletes resident tissue macrophages from various organs, as well as the brain, while sparing non-phagocytic cells (van Rooijen and Hendrikx, 2010). Liposomal clodronate induces apoptosis in macrophages that have phagocytized the liposomes (van Rooijen et al., 1996). Other recent studies have used central injection of liposomal clodronate to selectively deplete microglia from the early prenatal brain and adult brain, while leaving other cell types intact (Faustino et al., 2011; Kreisel et al., 2014; Torres et al., 2016). While another technique for depleting microglia, antagonism of colony stimulating factor 1 (CSF1), allows for non-invasive depletion of microglia via oral administration in adulthood, CSF1-receptor signaling is a crucial regulator of proliferation and differentiation of neural progenitor cells during development (Nandi et al., 2012). Thus liposomal clodronate is a more viable strategy to target microglia in the developing rat brain than CSF1-R antagonism. While it is possible that liposomal clodronate may induce an inflammatory reaction as microglia die, studies using diphtheria, and CSF1R antagonists to deplete microglia found no increase in inflammatory cytokines after microglia depletion (Asai et al., 2015; Elmore et al., 2014; Parkhurst et al., 2013). Increased GFAP expression has been noted following microglial depletion in these models, which suggests possible

compensation by astrocytes due to loss of microglia or astrogliosis in response to microglial death (Asai et al., 2015; Elmore et al., 2014; Parkhurst et al., 2013). Increases in GFAP have similarly been found after microglia depletion using liposomal clodronate (Torres et al., 2016). These studies suggest that if there is any change in microglia cytokine release before the microglia are depleted it is likely transient. In our studies, microglia were depleted for about 1.5–2 weeks, at which point the microglia repopulated the brain. However, the percent area stained by Iba1 did not return to control levels until 3 weeks after initial injections, thus the repopulating microglia do not attain normal morphology until 1 week after repopulation. The clodronate depletion strategy employed here therefore is likely to have interfered with normal microglial function in the brain for approximately 3 weeks of development.

4.3 Microglia are important for the development of later-life behavior

Depleting microglia during the early postnatal period had subtle effects on juvenile and adult social behavior. Juvenile clodronate treated rats chased conspecifics less than vehicle treated rats during paired social play testing, while there were no differences in other active play behaviors. On the adult reciprocal social interaction test, adult clodronate treated rats actively avoided conspecifics more, and passively interacted less with conspecifics. Thus social behavior was altered by neonatal clodronate treatment, but the behavioral phenotype observed cannot be characterized as unilaterally hypersocial or antisocial in nature. While we saw no change in active social interaction on the reciprocal social interaction task, future studies will be necessary to determine whether social preference or social memory might be altered in the three chamber social choice task.

Interestingly, we found large changes in mood-related behaviors in the clodronate treated animals. Depleting microglia in the early postnatal period induced a large decrease in anxiety-like behavior in adolescence and adulthood. Clodronate treated rats spent more time in the open arms and entered the open arms more on the EPM than vehicle treated rats. We also found that neonatal microglial depletion led to decreased behavioral despair on the forced swim test. Specifically, neonatal clodronate treatment produced a decrease in immobility time, an increase in time spent swimming, and a decrease in time spent climbing the walls of the testing apparatus. Increases in anxiety often coincide with increases in depressive-like behavior as part of overall change in affective behavior (Lapiz-Bluhm et al., 2008). Thus, the results of the forced swim test align with the decreased anxiety behavior seen in the clodronate treated rats.

We also observed increased locomotor behavior on the open field test (number of grids crossed) and in the forced swim test (time spent swimming). These increases in locomotion could potentially influence the results observed in several other behavioral measures. The increase time spent swimming seen in the clodronate treated rats could be a side effect of increase locomotor activity and not necessarily reflect differences in response to behavioral despair. However, time spent immobile has been found to be negatively correlated with locomotor activity and center entries in the open field, and is suggested to be related to changes in exploratory behavior rather than locomotor behavior (Ho et al., 2002). This dissociation suggests that the observed locomotor effects are mechanistically separate from the effects on depressive-like behavior. Future testing using non-motor anhedonia measures,

such as the sucrose preference test, would be useful to dissociate changes in locomotion from changes in mood. Increased locomotion could potentially explain the decrease in passive interaction time on the social interaction test, but increases in locomotor activity have also been shown to change under a variety of conditions without affecting social behavior (File and Seth, 2003). Moreover, active social interaction was not decreased in our studies, suggesting that locomotor changes alone cannot account for the social behavior effects in our experiment. Increased locomotor activity is a hallmark trait of several neurodevelopmental disorders, including attention deficit/hyperactive disorder and schizophrenia, and can indicate important changes in underlying brain function relevant to neuropsychiatric disorders (Bayless et al., 2015; van den Buuse, 2010; Wong and Josselyn, 2016). Thus, the hyperactive phenotype observed following our early life microglial manipulations warrants future mechanistic investigation.

4.4 Relevance to other models with microglial dysfunction

Interestingly, several models of early-life perturbations, thought to activate microglia, show opposite behavioral phenotypes compared to the clodronate treated rats. Prenatal immune challenge changes microglial morphology, number, distribution, maturation, and induce inflammatory signaling (Cunningham et al., 2013; Juckel et al., 2011; Manitz et al., 2016; Matcovitch-Natan et al., 2016; Van den Eynde et al., 2014). These challenges also are associated with increased anxiety, increased despair-like behavior, decreased social interaction, increased stress reactivity, and decreased locomotor behavior later in life (Choi et al., 2016; Enayati et al., 2012; Lin et al., 2012; Meyer, 2006; Rayen et al., 2011; Sominsky et al., 2012; Zhu et al., 2014). However, it is still unclear how and whether microglia function is being changed in these other models, thus making it hard to directly compare results (Cunningham et al., 2013; Giovanoli et al., 2015; Manitz et al., 2016; Smolders et al., 2015). Neonatal and prenatal stress also impacts microglial morphology, distribution, and function in both the short and long term following stress exposure, generally leading to increased microglial activation and signaling (Delpech et al., 2016; Roque et al., 2015; Œlusarczyk et al., 2015). Prenatal and early-life stress also increase anxiety, increase despair-like behavior, increase stress reactivity, and decrease social interactions later in life (Holland et al., 2014; Rayen et al., 2011; Vallée et al., 1997; Wei et al., 2010). Lastly, fractalkine knockout mice, a model with disrupted microglial physiology and lower microglial density during the early postnatal period, showed decreased reactivity to stress and social behavior deficits in adulthood (Hellwig et al., 2016; Zhan et al., 2014). However, the knockout effects are lifelong thus making it hard to determine what effects are developmental in origin. Overall, these previous results in comparison with our current study consistently suggest that microglial dysfunction during development leads to altered behavior and that type of dysfunction is important for determining the effect on later-life behavior.

4.5 Sex differences in response to microglial loss

Surprisingly, we saw few sex-specific effects of clodronate treatment. Previous studies have found that during the early postnatal period there are more amoeboid microglia in the male rat brain than the female brain, in the hippocampus, amygdala, and preoptic area of the hypothalamus (Lenz et al., 2013; Schwarz et al., 2012). Microglia can also regulate sex

differences in brain development and program lifelong sex differences in behavior (Lenz et al., 2013). There is also evidence to indicate that there is higher expression of microglial genes in the male human prenatal brain suggesting that males may be more sensitive to changes in microglial function (Werling et al., 2016). Surprisingly, in our study we found limited sex differences on behavioral outcomes. It is possible that sex differences in behavior due to changes in microglial function in the developing brain may only be apparent under specific circumstances such as early life inflammation or stress. In either case, future in-depth study of microglial signaling and function is still needed to determine the timing and extent of sex differences in microglia in the developing brain.

We did observe a sex difference in the acute hypothalamic pituitary adrenal (HPA) axis response to acute stress, wherein clodronate treated females showed a decreased corticosterone response and males showed no effects of neonatal clodronate treatment. Interestingly, sex steroid hormones do not have an organizational effect on the acute stress response (Goel and Bale, 2008; Handa and Weiser, 2014) suggesting that microglia may organize sex differences in the HPA axis independently of sex hormones. However, since corticosterone levels were not measured at baseline in our study, we do not know the full extent of HPA axis dysregulation. Further experiments are needed to clarify to what extent microglia may influence the development of the HPA axis. Lastly, hormonally-driven sexual differentiation of the rodent brain starts at embryonic day 18 (McCarthy, 2008) and developmental sex differences may be harder to inhibit or change by the time microglia were depleted in the current studies. In order to assess sex-specific consequences of microglial loss, future studies should focus on behaviors that are sex specific such as maternal care or reproductive behavior, other behaviors with known sex differences, such as specific spatial memory tasks, or perform prenatal microglial depletion to better target the critical period for sexual differentiation.

4.6 Developmental versus adult effects of microglial loss

Our data supports a strong developmental role for microglia compared to acute microglial effects on behavior in adulthood. Past studies have shown that depleting microglia in juveniles results in impaired motor learning, fear-conditioning, and novel object recognition (Parkhurst et al., 2013). However, depleting microglia during adulthood has transient, but no long-term effects on anxiety behavior, locomotion or other cognitive measures (Elmore et al., 2014; Torres et al., 2016). Our data is also unique in that few developmental immune model experiments have documented the developmental time course of motivated behavior following microglial manipulations from the juvenile period to adulthood. Our results show that changes in anxiety, locomotion, and social behavior are evident from an early age suggesting that microglia are critical to early developmental processes and the onset of motivated behaviors in adolescence.

4.7 Potential mechanisms mediating the behavioral effects of microglial loss

Our current results suggest that microglia during this critical postnatal period are regulating the development of brain circuits involved in regulating motivated behavior. There are many possible cellular and molecular mechanisms through which microglia could affect behavioral development. Several studies point to the critical role of microglia in regulating

normal processes of brain development, including cell genesis, progenitor pool size, synaptogenesis, synaptic pruning, and axonal innervation (Cunningham et al., 2013; Lenz et al., 2013; Paolicelli et al., 2011; Shigemoto-Mogami et al., 2014; Squarzone et al., 2014). Microglial number, density, and morphology all change dramatically during the first three weeks of life (Bennett et al., 2016; Kim et al., 2015; Nikodemova et al., 2015). Thus, depleting microglia during the first postnatal weeks may inhibit one or more of several important early life microglial functions, or may delay or otherwise alter the timing of these critical processes. Future studies should focus on determining which microglial functions during the first weeks of neonatal life, such as phagocytosis of synapses or supporting cell proliferation through diffusible factors, are important for development of anxiety-like and depressive-like behaviors. Additionally, differences in microglia colonization in the neonatal period may indicate different microglia developmental processes or the sensitivity of these brains areas to experiences that alter microglial function.

4.8 Conclusions

Mood disorders have developmental origins and are accompanied by microglial dysfunction (Green et al., 2010; Yirmiya et al., 2015). Several other psychiatric disorders with developmental origins that can also have mood and affect dysregulation, such as autism spectrum disorder, Tourette's syndrome, and schizophrenia, are similarly accompanied by dysregulation in microglial function (Fillman et al., 2013; Lenington et al., 2016; Morgan et al., 2012; Takano, 2015; Tetreault et al., 2012; Vargas et al., 2005). However, the exact developmental contribution of microglia to psychiatric disorders is not well known, especially for mood-related and affective behaviors. Our data suggest that early life programming of mood-related behavior is dependent upon the normal function of innate immune cells in the brain. Determining the function of microglia in normal brain development could also lead to a better understanding of how immune modulating experiences, such as maternal immune activation or maternal stress, disrupt or perturb processes of normal brain development that depend on microglia.

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Highlights

- We used liposomal clodronate to deplete microglia in neonatal rats and study the lifelong effects on motivated behavior.
- Microglia were depleted from the forebrain for approximately two weeks after central liposomal clodronate injection at postnatal days 1 and 4.
- Early-life depletion of microglia led to hyperactivity, decreased anxiety and depressive behavior, and altered social behavior in adolescence and adulthood.
- Females that had microglia depleted neonatally had a blunted corticosterone response to acute stress in adulthood.

Figure 1.

Schematic of the treatment time, length of microglia depletion, time when microglia recolonize the brain, when microglia density and morphology resembles control animals, and time when behavioral testing was carried out at.

Figure 2. Time course of depletion and recolonization following neonatal treatment with liposomal clodronate
 10x and 20x images of Iba1 (green) from representative areas of the mPFC and amygdala in male vehicle and clodronate treated rats at a) P2, b) P6, c) P12, and d) P22 (P = postnatal day). All sections were counterstained with DAPI (blue). Scale bar = 100 μ m for 10 x and scale bar = 50 μ m for 20x images.

Figure 3. Microglia repopulation dynamics

a) Number of microglia per region of interest in the mPFC at four time points, b) % area of Iba1 staining in the mPFC per region of interest at four time point, c) the total number of microglia per region of interest in the amygdala at four time points, and d) % area of Iba1 staining in the amygdala per region of interest at four time points. Clodronate treated rats had significantly decreased microglia density and % area stained by Iba1 at P2 and P6 in both the mPFC and amygdala (a–d). Multiple comparisons showed that clodronate treated males and females had less microglia at P2 in the mPFC and amygdala (a and c). Clodronate treated males and females had significantly reduced % area stained by Iba1 at P2 and P16 in the mPFC and amygdala (b and d). Clodronate treated rats had significantly increased microglia density at P12 in the mPFC (a). Clodronate females had significantly more microglia compared to controls at P12 in the mPFC. Clodronate treated rats had significantly increased microglia density at P12 in the mPFC (a). P = postnatal day. Data represented as $\pm$ SEM, n = 2–3 for each group, ## = $p < 0.01$, ### = $p < 0.001$, and #### = $p < 0.0001$.

Figure 4. Juvenile paired play and group play behavior

Pair play behaviors included a) number of chase behaviors, b) number of rough and tumble behaviors, and c) number of social explorations. Clodronate treated males chased significantly less than vehicle treated males (a). There was no significant difference for number of rough and tumble play behaviors (b). Clodronate treated females made more social explorations than vehicle treated females (c). Group play outcomes included d) number of chase behaviors, e) number of rough and tumble behaviors, and f) number of social explorations. Both clodronate treated males and clodronate treated females chased significantly less than vehicle treated males and vehicle treated females (d). There were no

significant differences in the number of rough and tumble play behaviors (e) or number of social explorations (f). Data are presented as $\pm$ SEM, ** $p < 0.01$ and *** $p < 0.001$, $n = 9$ for clodronate treated male, $n = 10$ for vehicle treated male, $n = 11$ for clodronate treated and vehicle treated females.

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Figure 5. Adult social interaction

Behaviors measured included a) active investigation time, b) number of active bouts, c) passive interaction time, and d) number of social avoidance behaviors. Female rats actively investigated more times than male rats regardless of treatment (a and b). Vehicle female rats passively interacted more than vehicle treated males and clodronate treated females (c). Clodronate treated rats showed more social avoidance behaviors than vehicle treated rats (d). Data are presented as $\pm$ SEM, * $p < 0.05$, **** $p < 0.0001$, and # $p < 0.05$ between sex or between treatment. $n = 9$ for clodronate treated males, $n = 10$ for vehicle treated males, $n = 11$ for clodronate treated and vehicle treated females.

Figure 6. Juvenile open field behavior

Open-field behaviors consisted of a) the number of gridlines crossed, b) the number of center entries, c) time spent in the center area, and d) the number of rears. Clodronate male and female rats crossed more lines than vehicle male and rats, and clodronate female rats crossed more lines than vehicle female rats (a). There were no differences in time spent in the center area (b). Clodronate female rats entered the center area more than vehicle female rats (c). Clodronate rats rear significantly less than vehicle rats (d). * $p < 0.05$, *** $p < 0.0005$, **** $p < 0.0001$, ## $p < 0.01$ between treatments, $n = 9$ for clodronate male, $n = 10$ for vehicle male, $n = 11$ for clodronate and vehicle female.

Figure 7. Adult open field behavior

Adult open field measures include a) the number of grids crossed, b) the number of center entries, c) time spent in the center area, d) the number of rears. Clodronate treated females and males crossed more grids than vehicle treated females and males respectively (a). Clodronate treated females crossed more grids than clodronate treated males (a). Clodronate treated rats enter the center area more than vehicle treated rats (b). Clodronate treated females spent more time in the center than clodronate treated males (c). Clodronate treated males and vehicle treated females reared significantly more than vehicle treated males (d). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, ## $p < 0.01$, $n = 9$ for clodronate treated males, $n = 9$ for vehicle treated males, $n = 11$ for clodronate treated and vehicle treated females.

Figure 8. Juvenile elevated plus maze behavior

Assessed behaviors included a) open arm entries and b) time spent in the open arms.

Clodronate treated males and clodronate treated females spent more time in the open arms than vehicle treated males and vehicle treated females (b), and entered the open arms more often (a). ** $p < 0.01$, *** $p < 0.001$ $n = 8$ for clodronate treated males, $n = 9$ for vehicle treated males, $n = 10$ clodronate treated females, and $n = 11$ for vehicle treated females.

Figure 9. Adult elevated plus maze behavior

Adult behavioral measures included a) open arm time, b) open arm entries, c) closed arm time, and d) closed arm entries. Clodronate treated females spent more time on the open arms (a) and entered the open arms (b) more than vehicle treated females. Clodronate treated rats spent significantly less time than vehicle treated rats in the closed arms (c). There were no differences in number of closed arm entries (d). * $p < 0.05$, # $p < 0.05$ between treatmentd, $n = 9$ for clodronate treated males, $n = 10$ for vehicle treated males, $n = 11$ for clodronate treated and vehicle treated females.

Figure 10. Adult forced-swim test and acute stress responsivity

Forced-swim test behavioral measures included a) time spent immobile, b) time swimming, c) time climbing. Vehicle male rats spent more time immobile than vehicle female rats and clodronate male rats (a). Clodronate rats spent less time climbing than vehicle rats (b). Vehicle male rats spent significantly less time swimming than vehicle female rats and clodronate male rats (c). Corticosterone levels after acute restraint stress (d). Corticosterone levels were normalized to one sample that was run on both plates for a cross-plate comparison. Vehicle treated females had higher levels of corticosterone compared to vehicle treated males after acute restraint stress. $*p < 0.05$, $****p < 0.0001$, $#p < 0.05$ between treatment, $n = 9$ for clodronate treated males, $n = 10$ for vehicle treated males, $n = 11$ for clodronate treated and vehicle treated females.