TITLE: **Delayed memory deficits in males and females long after subchronic immune challenge**

RUNNING TITLE: **Delayed memory deficits after immune challenge**

Daria Tchessalova[1,2] (dtchessa@umich.edu) and Natalie C. Tronson Ph.D[2] (ntronson@umich.edu),

[1] Neuroscience Graduate Program, University of Michigan, Ann Arbor
[2] Department of Psychology, University of Michigan, Ann Arbor

Corresponding Author: Natalie C. Tronson, Ph.D,
Department of Psychology
University of Michigan
530 Church st
Ann Arbor, 48109
United States of America

ABSTRACT

Memory impairments and cognitive decline persist long after recovery from major illness or injury, and correlate with increased risk of later dementia. Here we developed a subchronic peripheral immune challenge model to examine delayed and persistent memory impairments in females and in males. We show that intermittent injections of either lipopolysaccharide or Poly I:C cause memory decline in both sexes eight weeks after the immune challenge. Importantly, we observed sex-specific patterns of deficits. Females showed deficits in object recognition one week after challenge that persisted for at least eight weeks. In contrast, males had intact memory one week after the immune challenge, but exhibited broad memory impairments across object recognition, as well as context and tone fear conditioning tasks eight weeks later. The memory deficits in males and in females were observed without sustained microglial activation or changes in blood-brain barrier permeability. Together, these data suggest that sex differences in transient neuroimmune activity result in differential vulnerabilities of females and males to memory decline after immune challenge. This model will be an important tool for determining the mechanisms in both sexes that contribute to memory impairments that develop over the weeks and months after recovery from illness and provide new insights into the role of chronic inflammation in the pathogenesis of long lasting memory decline and dementias.

INTRODUCTION

Long-lasting memory dysfunction is common after a critical illness or major surgery. More than 25% of patients develop memory impairments that persist for months to years after recovery from major illness (Gharacholou *et al*, 2011; Semmler *et al*, 2013). These memory deficits can progress over time, contributing to neurodegenerative disorders and dementias including Alzheimer's disease (Evered *et al*, 2016). Chronic low-grade inflammatory signaling is also correlated with memory decline (Noble *et al*, 2009, 2010; Yaffe *et al*, 2003), and has been implicated as both a cause and a consequence of dementia (Stephenson et al, 2018). In this study, we examine the causal role of a subchronic peripheral immune challenge in progressive memory decline.

Immune signaling is critically important for normal neural function, and activation of the peripheral immune system modulates memory and other cognitive processes (Marin and Kipnis, 2017; Wilson *et al*, 2002; Yirmiya and Goshen, 2011). In animal models, systemic immune activation results in memory deficits during acute inflammatory signaling (Czerniawski and Guzowski, 2014; Kranjac *et al*, 2011; Pugh *et al*, 1998). Hippocampal-dependent memory tasks including novel object recognition, spatial memory, and context fear conditioning are all sensitive to disruption by an acute systemic inflammatory challenge (Frühauf *et al*, 2015; Kahn *et al*, 2012; Pugh *et al*, 1998) suggesting that the hippocampus is particularly susceptible to dysregulation by immune activation (Williamson and Bilbo, 2013).

An overwhelming immune response has long-lasting consequences for neuroimmune signaling, and for neural and cognitive function. The cecal-ligation and puncture model of sepsis results in impairments of memory that emerge soon after surgery (e.g., Barichello *et al*, 2007) and persist for months (e.g., Huerta *et al*, 2016). Similarly, a single high-dose injection of lipopolysaccharide results in memory deficits at least 5 months post-injection (Ming *et al*, 2015). Whereas neuroimmune activation in the weeks and months after an overwhelming inflammatory insult have been associated with memory deficits (Giustina *et al*, 2017; Semmler *et al*, 2007; Weberpals *et al*, 2009), it is not clear whether sustained neuroimmune activation or immune-triggered changes in neural processes mediates memory deficits long after a systemic immune challenge. Lower intensity immune challenges may be more relevant to modeling progressive memory impairments as a consequence of chronic, low-grade inflammation (e.g., Yaffe *et al*, 2003). Repeated, lower dose immune challenges also cause impairments of memory (Kahn *et al*, 2012; Weintraub *et al*, 2013, 2014) and neural plasticity (Maggio *et al*, 2013) in the first weeks after injection. In this project, we determined whether a moderate, subchronic immune challenge results in persistent memory deficits months after immune challenge, whether memory deficits are associated with lasting neuroimmune challenge, and the differential susceptibility to immune-

mediated memory deficits between sexes.

Given the greater vulnerability of women to memory disorders such as Alzheimer's disease (Snyder et al., 2016), we are particularly interested in the differential impact of subchronic immune challenge on males and females. Sex differences in peripheral responses are well described (Ghosh and Klein, 2017; Scotland *et al*, 2011) and there is growing evidence for neuroimmune responses and function (Acaz-Fonseca *et al*, 2016; Engler *et al*, 2016; Sorge *et al*, 2016; Speirs and Tronson, 2018). It is likely, therefore, that males and females are differentially susceptible to memory decline after subchronic immune challenge. To date, all studies of the lasting consequences of immune challenge on memory have been conducted with male animals.

Here we identified a causal role for subchronic immune activation on memory deficits in the months following recovery in both sexes. We demonstrated that males show delayed memory deficits across hippocampal- and amygdala-dependent tasks eight weeks after immune challenge, and females showed impaired object recognition memory soon after immune challenge that persists for at least eight weeks. These memory deficits were evident despite no long-lasting changes in microglial activation or blood-brain barrier permeability.

MATERIALS AND METHODS

Animals. 9-11 week old male and female C57BL/6N mice from Envigo (Indianapolis, IN) were used in all experiments. Mice were individually housed with mouse chow and water provided *ad libitum* as previously described (Keiser *et al*, 2017). Individual housing in mice prevents fighting-induced stress (Meakin *et al*, 2013) and is ethologically appropriate for males and females (Becker and Koob, 2016). Individual housing is suitable for testing novel object recognition (Vogel-Ciernia and Wood, 2015) and contextual fear conditioning (Keiser *et al*, 2017) and follows the University of Michigan Institutional Care and use Committee policies on managing fighting in mice. The facility is ventilated with constant air exchange (60 m^3/ h), temperature (22 ±1°C), and humidity (55±10%) with a standard 12 h light-dark cycle. Experiments were performed during the light portion of the cycle. Mice were acclimated to the colony room for at least seven days prior to injections. All experimental methods used in these studies were approved by the University of Michigan Committee on the Use and Care of Animals.

Immune stimulants. Lipopolysaccharide (LPS, Escherichia coli, serotype 0111:B4; Sigma-Aldrich, St. Louis) was dissolved in saline (12.5µg/mL) and was injected intraperitoneally (i.p.; 250ug/kg; *Fruhauf et al*, 2015). Polyinosinic:polycytidylic acid (Poly I:C, P9582; Sigma-Aldrich) lyophilized powder was dissolved in distilled deionized water (10mg/ml), heated to 50°C and cooled to allow re-annealing, and injected i.p. (6mg/kg; Cunningham *et al* 2007).

Subchronic Immune Challenge. Mice received five injections of LPS (250ug/kg; n= 8-9), Poly I:C (6 mg/kg; n= 8-9), or saline vehicle control (n= 8-9), spaced three days apart. All injections were performed at the same time of day (Roberts, 2000). Mice were weighed daily throughout the injection period and weekly until testing. Changes in weight were assessed using a repeated measures (Day × ImmuneChallenge) ANOVA.

Behavioral Testing. See Fig. 1A for experimental design. Memory tests began eight weeks (Fig. 2-5) or one week (Fig. 6-7) after the final injection. In experiments in which animals were tested on multiple tasks (Figures 2-4), novel object recognition was always tested first, followed by context fear conditioning.

Novel Object Recognition. The testing arena consisted of two rectangular opaque white chambers, (LWD: 40cm × 32cm × 32.5cm; 45 lux at center). To assess long-term novel object recognition memory, mice were first habituated to testing chambers (10 mins/day for 6 days). Mice received two 10-minute training trials spaced 3 hours apart in which they explored two identical objects. A single test session occurred 24 hours after the first training session, in which mice were replaced in the arena with one familiar object (from training) and one novel object (Vogel-Ciernia and Wood, 2015). Novel and familiar objects were counterbalanced across animals. The time spent exploring each object (animal's nose within 2 cm of object) was measured automatically (Ethovision XT 9.0 tracking software; Ballaz et al. 2007) and corroborated by an experimenter blind to experimental conditions. Novel object preference was calculated as the percent time spent at the novel object {100*[(Time exploring novel object)/(time spent exploring both objects)]}.

Short-term novel object recognition was assessed as a single, 10-minute training session followed 3 hours later by a test session as described above (Ballaz *et al*, 2007).

<u>Data analysis and Statistics</u>. Habituation was analyzed using repeated measures (Day × ImmuneChallenge) ANOVA. Separate repeated measures (ObjExpl × ImmuneChallenge) ANOVA were conducted for each sex (e.g., Darcet *et al*, 2014). Planned comparisons were used (with LSD) to examine comparisons between exploration of novel and familiar objects for each group.

Context Fear Conditioning. The context fear conditioning apparatus consisted of rectangular chambers (LWD: 9.75″ × 12.75″ × 9.75″) containing grid floor rods connected to a shock generator, an enclosed sound-attenuating system, and a NIR camera (VID-CAM-MONO-2A) and Video Freeze software for automatic scoring of freezing behavior (MedAssociates, VT). During background context fear conditioning, mice were placed in the training context (rectangular box with white walls, lights on, an evenly sized grid floor, 70% ethanol odor) for 3 min, after which a 30-sec tone (10 kHz, 75 dB SPL) was presented co-terminating with a 2 sec 0.8mA footshock (Tronson *et al*, 2010). Mice were returned

to their home cages immediately following training. Twenty-four hours later, context fear memory was assessed. Mice were replaced in the training context for 3 mins and freezing behavior was measured (Keiser *et al*, 2017). The following day, mice were tested for fear conditioning to the tone in a novel context (black angled walls, house lights off, staggered grid floors, 1% acetic acid odor). After 90 sec in the novel context, three 30-sec tones separated by 60 second intertrial intervals were presented and freezing to the context and tones were measured. To assess the effect of subchronic immune challenge on late-occurring impairments in memory retrieval, animals were trained on background context fear conditioning one week after immune challenge, and re-tested for context- and tone- fear memory seven weeks later. Foreground context fear conditioning was conducted as above, without presentation of the tone or tone fear tests (Keiser *et al*, 2017).

Data analysis and Statistics. Separate one-way ANOVA were used to assess the effect of prior immune challenge on context fear conditioning for each sex, and repeated measures ANOVA (Tone × ImmuneChallenge) was used to assess freezing to the cue in background fear conditioning. Post hoc tests (with LSD) were used to further assess specific group differences.

Immunohistochemistry. Animals (n = 4 / group) were anesthesized (Avertin 480 mg/kg, i.p.) and transcardially perfused at 12 weeks post last immune challenge with 4% paraformaldehyde. 40 μM sections through the hippocampus were incubated with rabbit anti-mouse Iba-1 (1:10,000, WAKO), goat anti-mouse secondary (1:200, Vector Labs), and DAB chromagen (Sigma Aldrich, St Louis, MO). ImageJ (NIH, Bethesda, MD) was count microglia, and a fractal analysis plugin in ImageJ was used to determine microglial morphology [FracLac V. 2.5, (Karperien *et al*, 2013)]. Decreased pattern complexity and self-similarity (fractal dimension) and increased heterogeneity (lacunarity) were measures of microglia activity. An experimenter blind to the treatment conditions performed microglial counts and morphology analyses in the dentate gyrus molecular layer and CA1.

Data analysis and Statistics. Two-way ANOVA (Sex × ImmuneChallenge) was used to assess the effect of prior immune challenge on microglial activation for both microglia number and morphology. Post-hoc tests were used to further assess specific group differences.

Blood-Brain Barrier Permeability. Sodium fluorescein (2%, i.p.) was injected 20 mins prior to blood collection and transcardiac perfusion with saline. Brain permeability index (BPI) was calculated comparing fluorescence (relative fluorescence units, RFU) in brain to fluorescence in serum [BPI = (RFU brain/brain weight)/ (RFU Serum/serum volume), and normalized to BPI in control animals (Devraj *et al*, 2018). We also used fluorescent microscopy to visualize qualitative differences in sodium fluorescein penetration in brain sections (20μM) (Nikon A1 laser scanning microscope (Devraj *et al*, 2018; Nikolian *et al*, 2018).

Data analysis and Statistics. Independent sample t-tests were used to compare BPI for Poly I:C and saline treated animals.

RESULTS

Systemic effects of Poly I:C and LPS.

To assess acute and long-lasting systemic effects of subchronic Poly I:C and LPS, we measured changes in weight over the eight-week post-challenge period. All mice gained weight across the 15 day treatment period (Injection: Males: $F(4,88) = 43.01$, $p < 0.001$; Females: $F(4,96) = 42.72$, $p < 0.001$). Across all 5 injections, male and female mice treated with LPS or Poly I:C showed decreased weight the day after injection (Males: ImmuneChallenge × Cycle: $F(4,44) = 4.32$, $p < 0.01$; LPS: $p < 0.01$; Poly I:C $p < 0.05$ vs injection day; Females: ImmuneChallenge × Cycle: $F(4,48) = 4.63$, $p < 0.01$; LPS $p <0.05$, Poly I:C $p < 0.01$ vs injection day Fig. 1B,C). For male mice treated with LPS, this weight loss persisted on the second day after injection (LPS: $p < 0.05$; Poly I:C $p = 0.40$ vs injection day). Saline-treated males showed no change in weight on average across the 2 days after injection ($p = 0.22$ and $p = 0.52$, respectively vs injection day) and saline treated females showed a small but significant weight gain ($p < 0.01$, $p = 0.01$, respectively vs injection day). Across the eight week time period, mice gained weight at equivalent rates regardless of prior treatment (Males: ImmuneChallenge × Day: $F(2,22) = 1.93$; $p = 0.17$; Females: $F(2,22) = 1.91$, $p = 0.17$; Fig. 1B,C).

Subchronic immune challenge induced persistent deficits in novel object recognition memory.

We first analyzed locomotor activity during habituation to testing chambers and exploration of objects during training. There were no differences in locomotor activity nor habituation to the testing chamber for either sex (Males: Day: $F(5,110) = 3.95$, $p < 0.01$; Day × Drug: $F(10,110) = 1.29$, $p = 0.27$; Females: Day $F(5, 120) = 21.17$, $p < 0.001$; Day × Drug: $F(10,120) < 1$; Fig. 2C,E). During training, there were no differences in locomotor activity (Males: Drug: $F(2,22) < 1$; Females: $F(2,24) < 1$), and all animals showed similar exploration of objects, regardless of prior treatment (Males: $F(2,24) < 1$; Females $F(2,26) < 1$; Fig. 2D,F). Prior subchronic LPS or Poly I:C treatment did not affect locomotor activity, habituation to a new arena, or object exploration in males or females.

Novel object recognition memory was tested eight weeks after LPS or Poly I:C using a paradigm resulting in a hippocampal-dependent memory 24 hours after training (Vogel-Ciernia & Wood, 2013). During test, we observed a significant detrimental effect of prior immune challenge on exploration of the novel object compared to the familiar object in males (ObjExpl × ImmuneChallenge: $F(2,22) = 3.90$, $p < 0.05$), with saline ($p < 0.01$), but not LPS ($p = 0.76$) nor Poly I:C ($p = 0.93$) treated males showing intact

object recognition memory (Fig. 2A). In females, we observed a trend towards decreased novel object recognition after immune challenge (ObjExpl × ImmuneChallenge: $F(2,14) = 2.97$, $p = 0.07$). Importantly, only saline-treated females showed significantly more exploration of the novel object than the familiar object (saline: $p < 0.05$; LPS: $p = 0.93$; Poly I:C $p = 0.46$; Fig. 2B). Prior immune challenge disrupted novel object recognition in both males and in females.

Male-specific impairment of context fear conditioning 8 weeks after Poly I:C challenge.

In males, prior Poly I:C but not LPS challenge caused deficits in context fear conditioning ($F(2,24) = 3.63$, $p < 0.05$; Poly I:C $p < 0.05$, LPS: $p = 0.97$ cf saline; Fig. 3A). In contrast, females showed no deficits in context fear conditioning after either LPS or Poly I:C ($F(2,24) < 1$; Fig. 3B). Neither locomotor activity during training (Males: $F(2,24) <1$, Females: $F(2,24) < 1$; Fig. 3E,G) nor response to shock (Males: $F(2,22) = 2.08$, $p = 0.15$, Females: $F(2,24) = 1.22$, $p = 0.32$; Fig. 3F,H) differed across groups in either sex, thus deficits in context fear conditioning were not due to a failure to detect the shock US.

Fear conditioning to the tone was also impaired in males with lower freezing to the first test tone in Poly I:C-treated males (ImmuneChallenge: $F(2,22) = 4.87$, $p < 0.05$; Tone × ImmuneChallenge: $F(4,44) = 2.77$, $p < 0.05$; Tone: $F(2,44) = 1.50$ $p = 0.24$; $p < 0.05$ cf saline; $p < 0.01$ cf LPS; Fig. 3C). There were no differences between groups in freezing to the novel context ($F(2,22) = 2.15$, $p = 0.14$). In contrast, females showed no alterations of tone fear conditioning after either immune challenge (ImmuneChallenge: $F(2,44) = 1.31$, $p = 0.29$; Tone × ImmuneChallenge $F(4,48) = 1.38$; $p = 0.25$; Tone: $F(2,48) = 19.48$, $p < 0.001$ Fig. 3D), with no differences in freezing to the novel context ($F(2,24) < 1$). These finding demonstrate that males, but not females, are sensitive to disruption of fear conditioning eight weeks after subchronic Poly I:C challenge.

Poly I:C caused disruption of novel object recognition and context fear conditioning in males eight weeks after subchronic challenge.

To further examine memory deficits eight weeks after subchronic Poly I:C in males, we used a short-term novel object recognition paradigm (Ennaceur *et al*, 1997) and replicated our context fear conditioning experiment in males. There were no differences between groups in object exploration ($t(14) = 1.19$, $p = 0.25$; Fig. 4D) nor locomotor activity ($t(14) <1$; Fig. 4E) during training. At test, novel object preference was significantly lower in the Poly I:C-treated mice (ObjExpl × ImmuneChallenge: $F(1,14) = 21.55$, $p < 0.01$; Fig. 4A), with significantly greater novel object exploration after saline ($p < 0.05$) and significantly lower novel object exploration after Poly I:C ($p < 0.01$). Thus, consistent with our previous

experiment, short-term novel object recognition was impaired after subchronic Poly I:C challenge.

We replicated the finding that Poly I:C induces disruption of context fear conditioning at 8 weeks in a separate cohort of animals. Poly I:C-treated males also displayed significantly lower context fear conditioning compared with saline controls ($t(14) = 2.75$, $p < 0.05$; Fig. 6B), with no changes in locomotor activity ($t(14) < 1$; Fig. 4F) or to reactivity to the shock ($t(14) < 1$; Fig. 4G). Tone fear conditioning was also impaired after Poly I:C (ImmuneChallenge $F(1,14) = 6.67$, $p < 0.05$; Tone: $F(2, 28)$, $F < 1$; ImmuneChallenge × Tone $F(2,28) = 1.27$, $p = 0.23$), with no differences in freezing to the novel context ($t(14) = 1.15$, $p = 0.27$; Fig. 4C).

LPS caused disruption of novel object recognition eight weeks after subchronic challenge.

Similarly, short-term novel object recognition was impaired in both sexes eight weeks after subchronic LPS challenge compared with saline-treated controls (Males: ObjExpl × ImmuneChallenge $F(1,14) = 8.03$, $p < 0.05$; Females: (ObjExpl × ImmuneChallenge $F(1,14) = 32.88$, $p < 0.01$; Fig. 5A,B). Only saline-treated animals showed preference for the novel object (Males: $p < 0.05$; Females $p < 0.01$), and LPS animals showed no preference (Males: $p = 0.23$). Prior LPS had no effect on object exploration (Males: $t(14)= 1.04$, $p = 0.31$; Females: $t(14) = 1.47$, $p = 0.17$; Fig. 5C) or locomotor activity during training (all $t(14) <1$; Fig. 5 D,F).

Consistent with our earlier experiment (Fig. 3), we observed no disruption of foreground context fear conditioning eight weeks after subchronic LPS in either sex, although increased freezing was observed in females (ImmuneChallenge $F(1,28) = 3.56$, $p = 0.07$; Sex $F(1,28) < 1$; ImmuneChallenge × Sex $F(1,28) < 1$; Fig. 5 G,H).

Female-specific object recognition memory deficits one week after subchronic immune challenge.

No decreases in body weights were observed in Poly I:C-treated mice prior to habituation one week or two weeks after the last injection, prior to novel object recognition training (Males: ImmuneChallenge × Day: $F(2,28) <1$; Females: $F(2,28) < 1$). Similarly, there were no differences in habituation to the testing chambers (Males: Day $F(5,70) = 5.75$; $p < 0.01$; Day × Drug: $F(5,70) < 1$; Females: Day $F(5,70) = 5.75$, $p < 0.01$; Day × Drug $F(4,48) = 1.07$, $p = 0.37$; Fig. 6C,E). All groups also showed similar locomotor activity (Males: $t(14) <1$; Females: $t(14) < 1$) and object exploration during training (all $t < 1$; Fig. 6D,F).

In males, we observed preference for exploration of the novel compared to the familiar object in both saline- and Poly I:C-treated groups (ObjExpl*:* $F(1,14) = 7.82$, $p < 0.05$) and no differences between

treatments (ObjExpl × ImmuneChallenge: $F(1,14) < 1$; Fig. 6A). Therefore, males showed no deficit in object recognition one week after immune challenge. In contrast, subchronic immune challenge caused impairments of novel object recognition in females (ObjExpl × ImmuneChallenge: $F(1,14) = 7.96$, $p < 0.05$; Fig. 6B). Whereas saline-treated mice showed significantly greater exploration of the novel vs the familiar object ($p < 0.01$), Poly I:C-treated mice showed no such preference ($p = 0.46$). Thus females, but not males, are sensitive to impairments in memory in the first weeks after a subchronic immune challenge.

No impairments in context fear conditioning one week after immune challenge.

Poly I:C-treated mice did not show ongoing decreases in weight at one week post challenge (Males: ImmuneChallenge × Day: Males: $F(1,13) = 1.39$, $p = 0.19$; Females: $F(1,14) < 1$), and there were no differences in locomotor activity (Males: $t(13) = 1.13$, $p = 0.28$; Females: $t(13) < 1$; Fig. 7E,G) or response to the footshock during training (Males: $t(13) = 1.57$, $p = 0.14$; Females: $t(13) < 1$; Fig. 7F,H).

We next examined whether deficits in fear conditioning emerged one week after the immune challenge and we re-tested animals seven weeks after context fear conditioning to determine whether the memory impairments observed in previous experiments were due to disruption of memory retrieval processes. In males, we found no memory deficits at either time point, and contrary to expectations, observed an increase in freezing one week after Poly I:C (ImmuneChallenge $F(1,13) = 5.17$, $p < 0.05$; Time × Immune Challenge: $F(1,13) = 2.19$, $p = 0.16$; Time: $F(1,13) < 1$; 1week: $p < 0.01$; 8 weeks: $p = 0.67$; Fig. 7A). Subchronic immune challenge did not result in either memory deficits one week later, or in retrieval deficits eight weeks after the final injection. In females, there were no effects of prior Poly I:C on context fear conditioning (ImmuneChallenge: $F(1,14) = 2.1$, $p = 0.17$; Time × ImmuneChallenge: $F(1,14) < 1$), however females in both groups showed a dramatic decrease in freezing to the context seven weeks after training (Time: $F(1,14) = 27.30$, $p < 0.001$; Fig. 7B), suggesting a decrease in memory retrieval at the remote time point in females.

We observed no effect of Poly I:C on freezing to the tone at either one week or eight weeks after the final injection (Males: ImmuneChallenge: $F(1,13) < 1$; ImmuneChallenge × Time $F(1,13) < 1$; ImmuneChallenge × Time × Tone $F(2,26) < 1$; Fig. 7C; Females: ImmuneChallenge: $F(1,14) = 3.32$, $p = 0.09$; ImmuneChallenge × Time × Tone $F(2,28) < 1$; Fig. 7D) demonstrating that Poly I:C does not cause short-term changes in auditory fear conditioning or long lasting changes in memory retrieval.

Subchronic Poly I:C challenge does not induce sustained neuroimmune activation

We observed no differences in microglia number (CA1: all $F(1,12) < 1$; Fig. 8A-B; DG: ImmuneChallenge: $F(1,12) = 1.94$, $p = 0.19$, Sex × ImmuneChallenge: $F(1,12) < 1$; Fig. 8D-E) or morphology (CA1: fractal dimension: ImmuneChallenge: $F(1,12) = 2.63$, $p = 0.13$, Sex × ImmuneChallenge: $F(1,12) < 1$, lacunarity: ImmuneChallenge: $F(1,12) = 1.78$, $p = 0.21$, Sex × ImmuneChallenge: $F(1,12) < 1$, Fig. 8C-D; DG: fractal dimension: all $F(1,12) < 1$, lacunarity ImmuneChallenge: $F(1,12) = 2.16$, $p = 0.17$, Sex × ImmuneChallenge: $F(1,12) < 1$, Fig. 8G-H) in the dorsal hippocampus months after Poly I:C challenge. Similarly, blood-brain barrier showed no increase in permeability long after Poly I:C treatment. Poly I:C did not alter the brain permeability index (ImmuneChallenge: $t(1,12) = 1.06$, $p = 0.31$; Fig. 8I) and there was no increases in sodium fluorescein staining (data not shown) of saline and Poly I:C treated males or females. Together, these findings demonstrate that prior subchronic Poly I:C does not cause persistent increase in neuroimmune activation.

DISCUSSION

These findings are the first to demonstrate sex-specific patterns of memory deficits after a subchronic, systemic immune challenge. Object recognition memory was impaired in both sexes eight weeks after immune challenge, but females also exhibited early deficits on novel object recognition. Furthermore, males but not females showed delayed deficits in context and tone-dependent fear conditioning after Poly I:C. Retrieval of an established memory was unaffected. Thus, prior immune challenge specifically caused dysregulation of memory formation. Importantly, memory deficits were independent of sustained sickness or neuroimmune activation as indicated by normal weight gain, microglial activation, and blood-brain barrier function. Together, these findings demonstrate progressive changes in neural function and sex-specific patterns of memory deficits emerging over the weeks and months following a mild, subchronic immune challenge.

The delayed emergence of memory deficits in the absence of ongoing neuroimmune activity suggests that, transient immune response exerts lasting indirect effects on neural function (Tchessalova *et al*, 2018). Previous studies have identified several processes that are persistently dysregulated after a transient immune challenge. For example, a single high dose of LPS results in persistent alterations of cholinergic function (Ming *et al*, 2015), impairments in neurogenesis (Ormerod *et al*, 2013), epigenetic modifications (Schaafsma *et al*, 2015), and late occurring striatal neurodegeneration (Liu *et al*, 2008; Qin *et al*, 2007). Other studies have demonstrated decreases in spine density in hippocampus (Chavan *et al*, 2012; Volpe *et al*, 2015) and amygdala (Huerta *et al*, 2016) one month after such as cecal ligation

and puncture. In other models, activation of astrocytes persists at least one month after surgery (Jin *et al*, 2014). In the subchronic immune challenge model described here, we observed no lasting neuroimmune activation. As such, this model will be an important tool for distinguishing the direct immune mechanisms and indirect impact on neural function that contribute to early and progressive memory impairments after neuroimmune activation in both sexes.

In males, both LPS and Poly I:C disrupted object recognition, however only Poly I:C resulted in deficits in context and auditory fear conditioning. This suggests that specific patterns of immune activation lead to different patterns of neural dysfunction. Indeed, distinct types of immune responses are observed in various animal models of inflammation (Heremans *et al*, 1989). Here, LPS and Poly I:C bind to different toll-like receptors (TLR4 and TLR3, respectively), recruit different signaling pathways, and thereby exert differential effects on the brain (Doyle *et al*, 2003). Acutely, both LPS and Poly I:C cause memory impairments (Cloutier *et al*, 2012), but LPS causes greater decreases in wheel running and locomotor activity (Hopwood *et al*, 2009), and Poly I:C causes more deleterious effects in developmental models (Arsenault *et al*, 2014). We observed that prior Poly I:C, but not LPS, disrupted fear conditioning, suggesting that viral mimetics induce a greater long-lasting impact on fear-related circuitry, including amygdala. Identifying how each kind of immune challenge (e.g., polymicrobial sepsis, gram-negative and gram-positive bacteria, viral mimics, injury/surgery/heart attack, etc) targets different neural structures and functions will be critical for understanding dysregulation of cognitive and affective processes that persist long after a transient immune event.

In contrast to the delayed memory deficits in males, females showed greater vulnerability to memory impairments in the first week after the immune challenge. This pattern of early and persistent deficits in females in contrast to delayed, broader memory impairments in males is consistent with recent findings from our lab showing more rapid activation and resolution of neuroinflammatory signaling after systemic immune challenge in females compared with males (Speirs and Tronson, 2018). These findings are consistent with sex differences in peripheral immune responses (Furman et al. 2014; Klein et al. 2015), and differential regulation of neuroimmune activation (Acaz-Fonseca *et al*, Arevalo, 2015; Bodhankar *et al*, 2015; Santos-Galindo *et al*, 2011). Sex differences in susceptibility to memory dysfunction may therefore be due to differences in pattern, time course, or strength of immune activation.

That males, but not females show deficits in fear conditioning after a subchronic immune challenge also suggest sex differences in susceptibility to immune modulation across brain regions. This is consistent with previous studies of sex differences in neuroimmune activity, for example, after chronic stress, males but not females showed ongoing microglial activation in basolateral amygdala (Bollinger *et*

al, 2017). Alternatively, sex differences in strategy and mechanisms of fear memory formation may mediate differential susceptibility of males to disruption by prior Poly I:C (Keiser *et al*, 2017; Shansky, 2018). Fear conditioning triggers sex-specific patterns of activation across brain regions (Keiser *et al*, 2017; Lebron-Milad *et al*, 2012) and signaling mechanisms (Keiser and Tronson, 2015; Mizuno and Giese, 2010). Thus male-specific disruption of fear conditioning may be due to sex differences in neuroimmune signaling, and/or to disruption of circuits or mechanisms required for fear conditioning in males but not females.

This model of subchronic immune activation is an important new tool for identifying the neural mechanisms underlying progressive cognitive deficits in the weeks and months after recovery from illness. Long-lasting memory deficits have previously been described after serious illness in humans, and in models of sepsis including high-dose LPS stimulation or cecal ligation and puncture (Huerta *et al*, 2016; Ming *et al*, 2015). Here we demonstrated that repeated administration of a lower dose of LPS or Poly I:C is sufficient to cause delayed and persistent memory deficits without ongoing neuroimmune activation. This emphasizes the importance of identifying the impact of transient neuroimmune signaling on neuronal signaling and neural function. Importantly, this model is sensitive to sex differences in vulnerability to and time course of emergent memory deficits, and differential effects of bacterial and viral-like immune triggers. These findings thus provide a basis from which to identify immune and non-immune mechanisms that drive memory deficits long after recovery from illness, surgery, or injury.

Together, our findings demonstrate that subchronic systemic inflammation causes sex-specific patterns of memory decline over the following months. Both males and females are susceptible to impairments in memory after immune challenge, but females are more vulnerable to early-onset memory deficits, whereas males are more susceptible to amygdala-dependent memory impairments. Surprisingly, these memory impairments are not associated with persistent neuroimmune mechanisms. Additional studies will be required to identify the long-lasting neural changes that mediate such delayed and persistent memory impairments. This subchronic immune challenge model is thus a valuable tool for identifying how systemic inflammation initiates memory decline and memory-related disorders. Understanding the persistent neural changes as a consequence of transient neuroimmune activation will be critical for development of strategies to prevent cognitive decline after major illness or chronic inflammation in women and in men.

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FIGURES

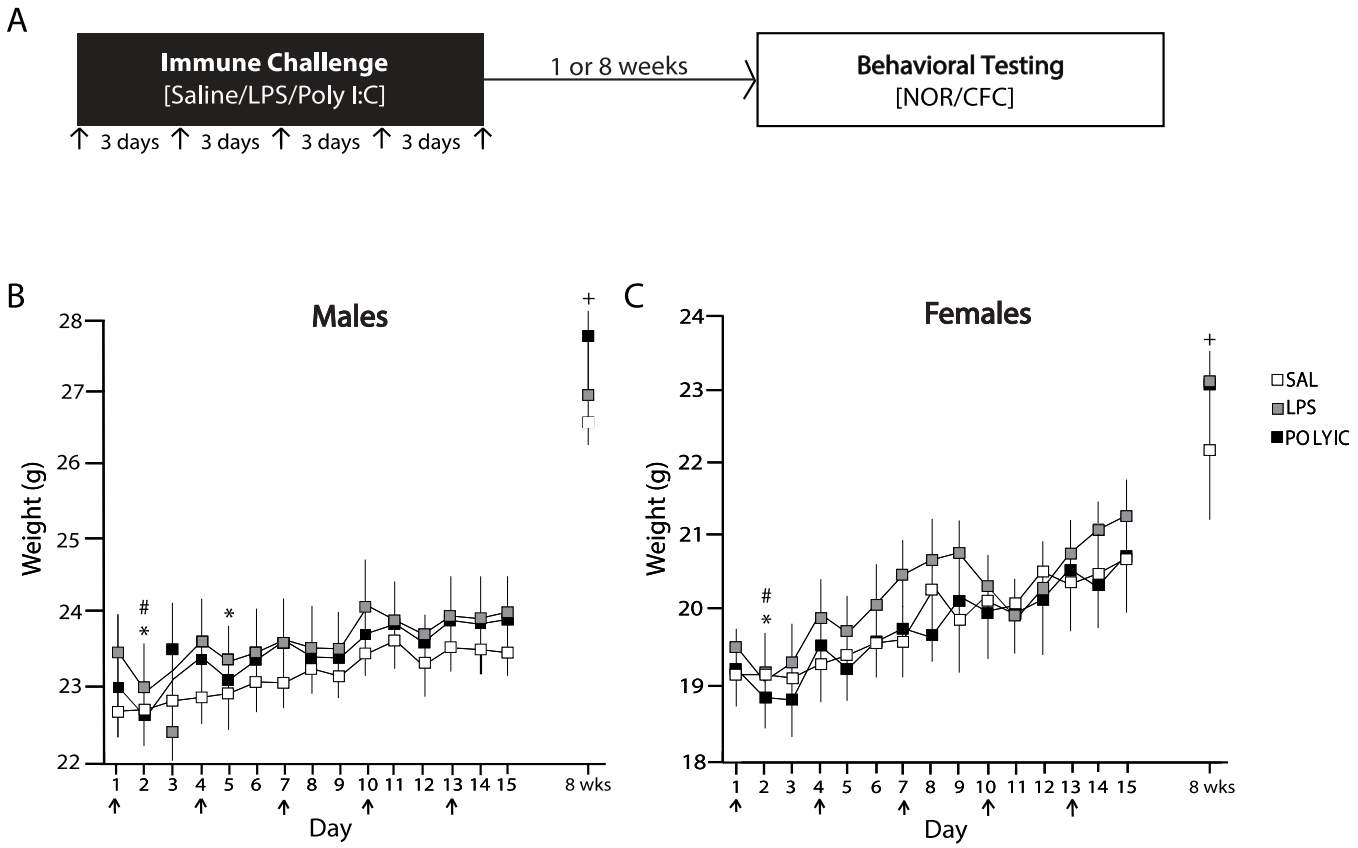

Figure 1. Experimental design and mouse weights after immune challenge. (A) Experimental Design. (B) Males and (C) females showed weight loss after LPS and Poly I:C injections, but regained weight by 8 weeks after final injection. Arrows indicate injection days. SAL: saline; LPS: lipopolysaccharide; Poly I:C: Polyinosinic:polycytidylic acid. *$p < 0.05$ compared with injection day LPS group; #$p < 0.05$ compared with injection day Poly I:C group, +$p < 0.05$ Poly I:C vs Saline.

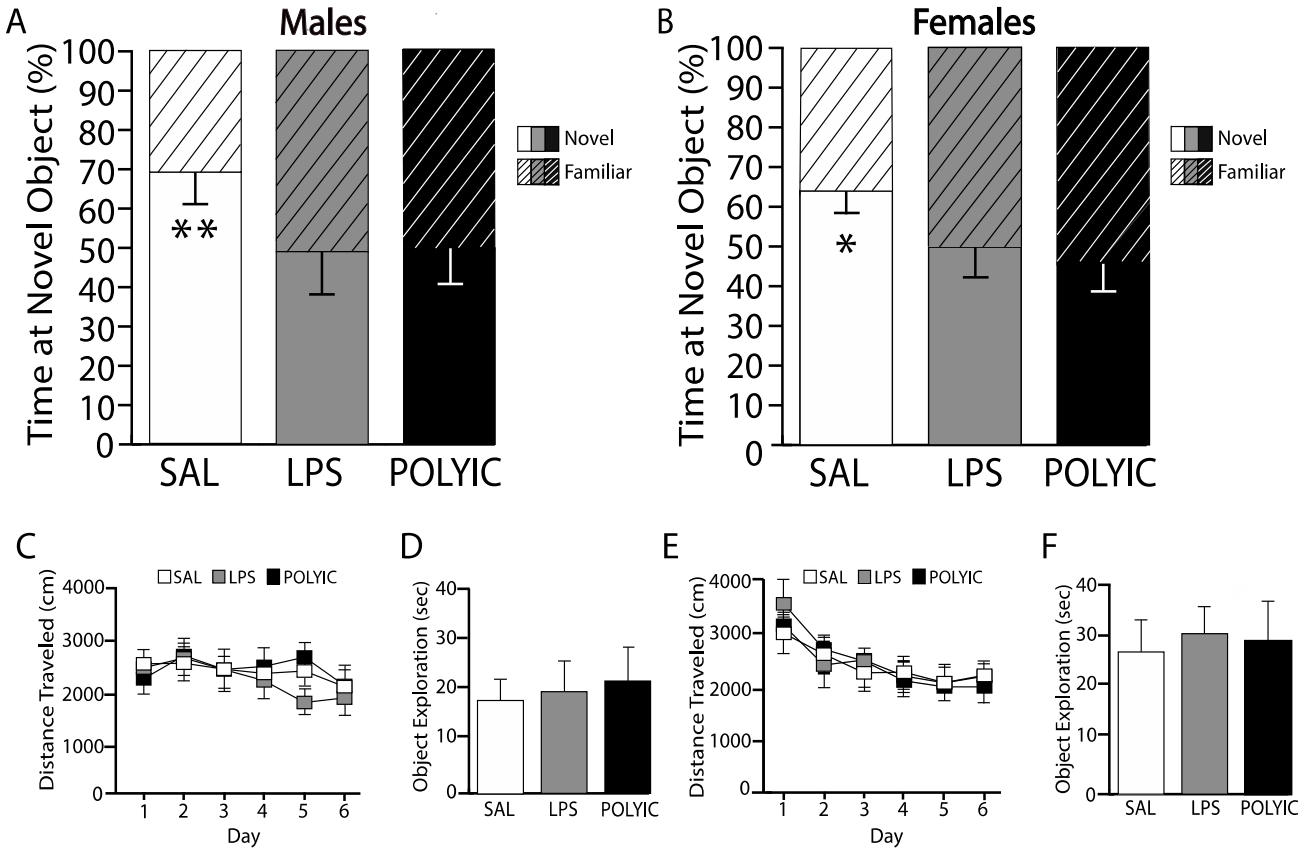

Figure 2. Novel object recognition memory is disrupted eight weeks after subchronic immune challenge. (A,B) Saline-treated males and females spent more time exploring the novel object (solid bar) compared with the familiar object (striped bar). This was disrupted in LPS- and Poly I:C treated mice (n = 8-9 animals/ group). (C,E) Neither locomotor activity nor habitation was affected by prior immune challenge. (D,F) There were no differences in total object exploration during training. SAL: saline; LPS: lipopolysaccharide; Poly I:C: Polyinosinic:polycytidylic acid. *$p < 0.05$, **$p < 0.01$ cf Familiar Object.

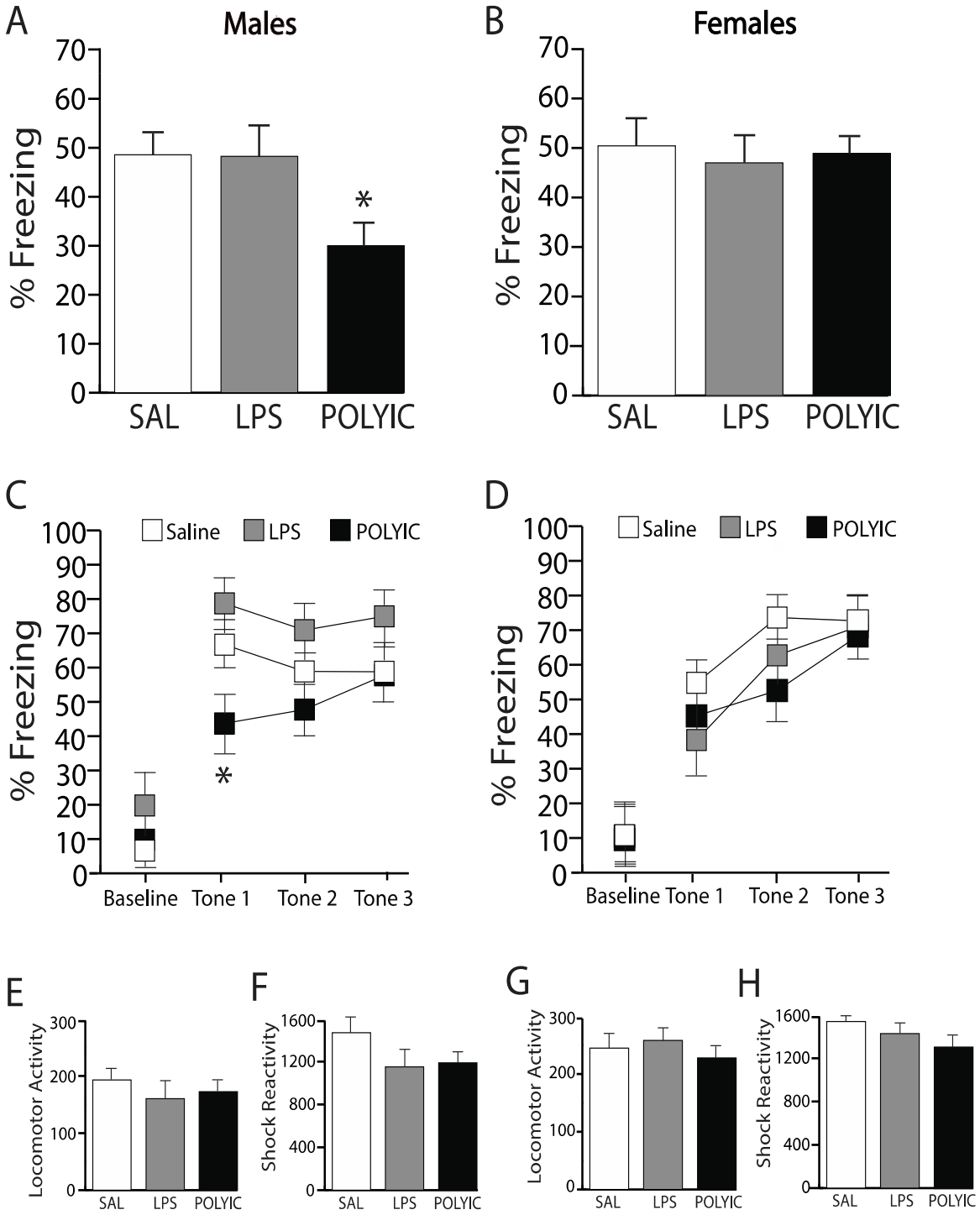

Figure 3. Context fear conditioning is impaired in males eight weeks after subchronic Poly I:C. (A) Freezing to the training context in males is decreased eight weeks after Poly I:C but not LPS (n = 8-9 per group). (B) Context fear conditioning was not impaired in females after LPS or Poly I:C (n = 9 per group). (C) In males, Poly I:C, but not LPS, resulted in a mild impairment in tone fear conditioning. (D) Females showed no disruption of cued-fear conditioning eight weeks after either immune challenge. (E,F) Locomotor activity and (F,H) shock reactivity during training was not altered after LPS or Poly I:C either sex. SAL: saline; LPS: lipopolysaccharide; Poly I:C: Polyinosinic:polycytidylic acid. *$p < 0.05$ cf Saline.

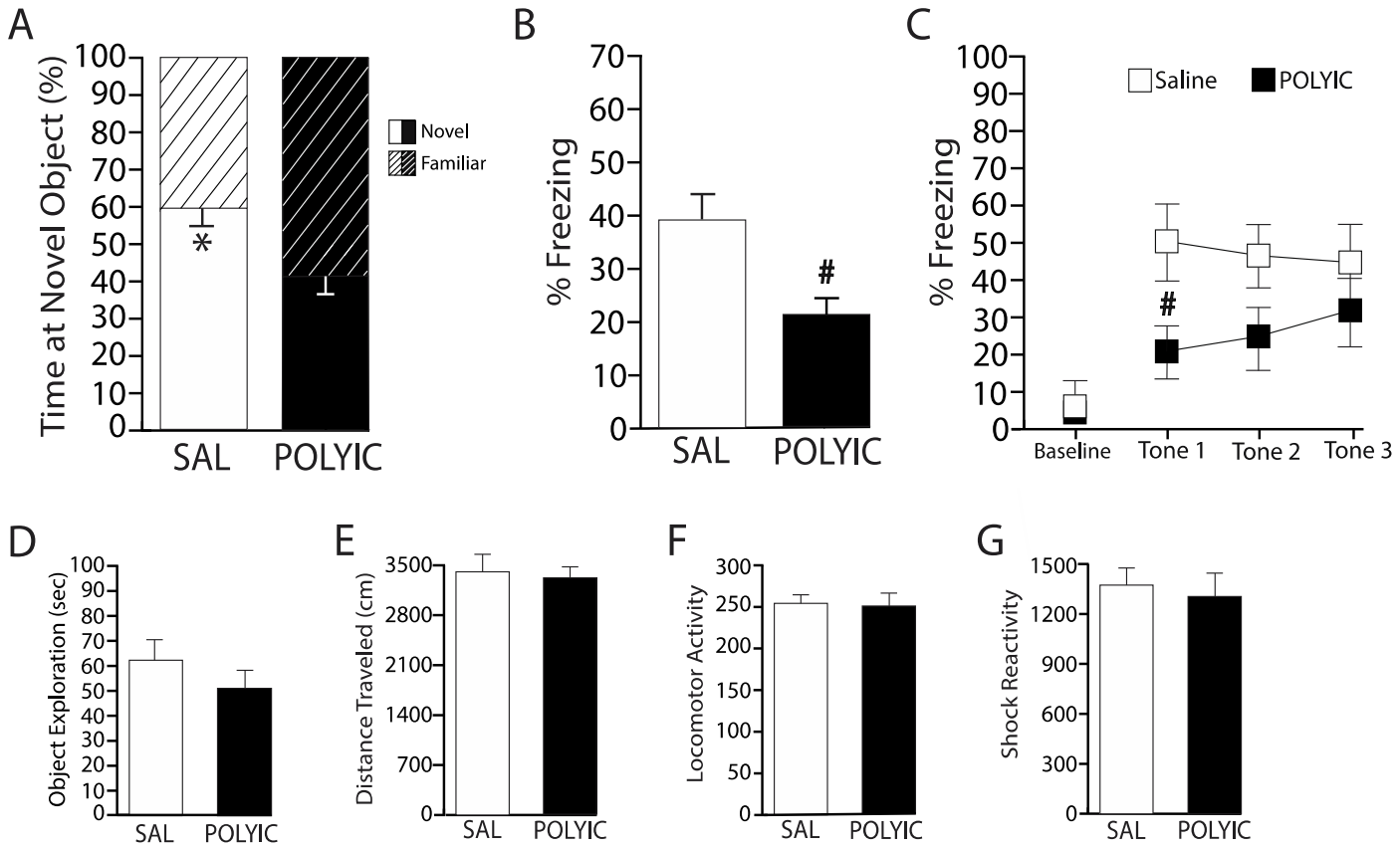

Figure 4. Novel object recognition and contextual fear conditioning are impaired in males 8 weeks after subchronic Poly I:C challenge. A) Saline-treated mice show preference for the novel object when tested three hours after training, and this is disrupted eight weeks after Poly I:C. (B) Context fear conditioning and (C) tone fear conditioning is decreased in males eight weeks after subchronic Poly I:C challenge. (D,E) Total object exploration and locomotor activity during novel object training are similar between groups. (F,G) In context fear conditioning, locomotor activity and reactivity to the shock is not altered by subchronic Poly I:C. SAL: saline; Poly I:C: Polyinosinic:polycytidylic acid. *$p<0.05$ *cf* Familiar object, # $p<0.01$ *cf* saline.

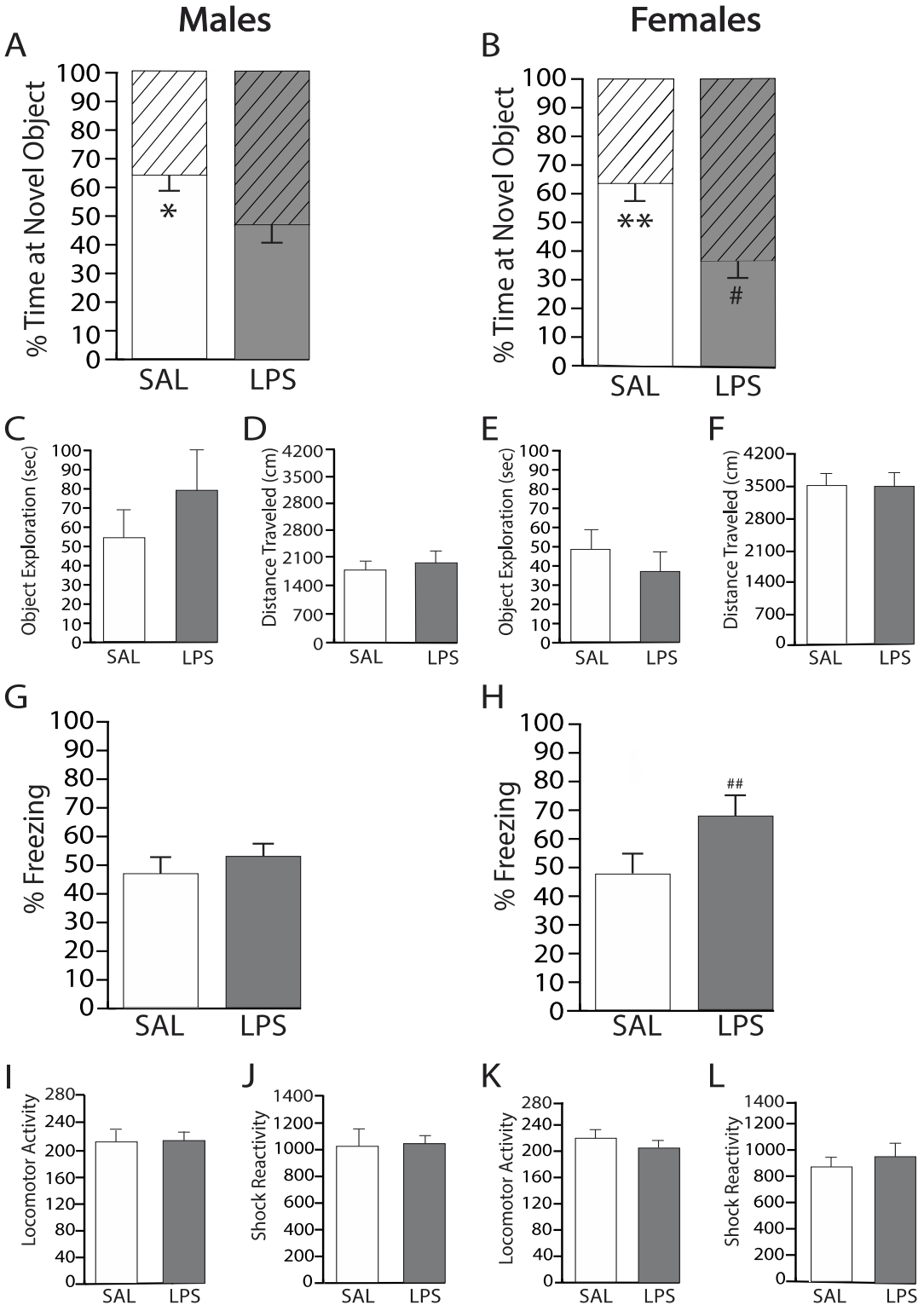

Figure 5. Novel object recognition memory is disrupted 8 weeks after subchronic LPS. (A,B) Novel object recognition was impaired in males and females when tested 3 hours after training. Neither (C,E) Total exploration during training or (D,F) locomotor activity were altered by prior LPS. (G,H) Foreground context fear conditioning was intact in both sexes. (I,K Locomotor activity and (J,L) shock reactivity were not different between groups. SAL: saline; LPS: lipopolysaccharide. *$p < 0.05$, **$p < 0.01$ *cf* Familiar Object, ##$p < 0.01$ *cf* saline.

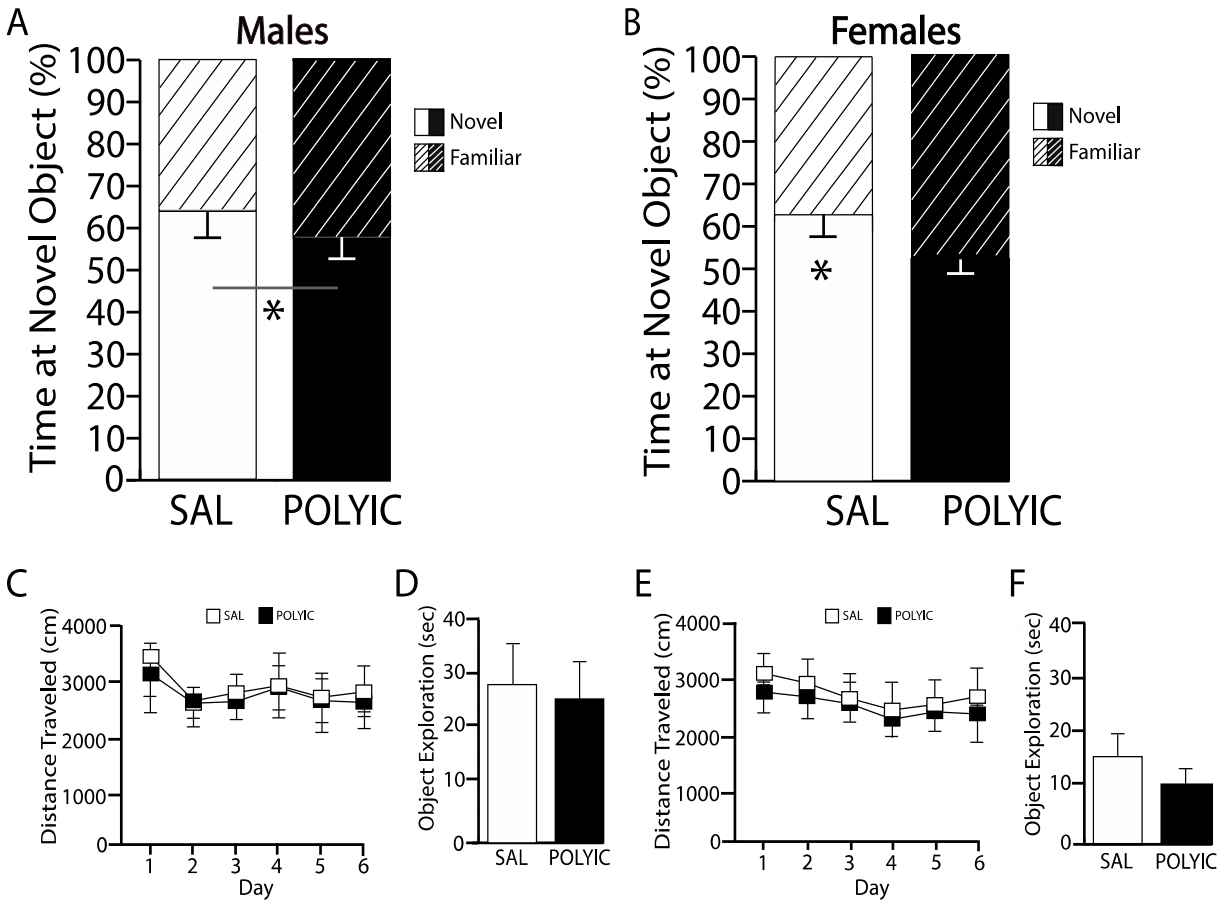

Figure 6. Female-specific impairment of novel object recognition memory one week after subchronic Poly I:C. (A) Intact novel object recognition in males one week after Poly I:C (n = 8). (B) Subchronic Poly I:C treatment disrupted novel object recognition in females one week after the final injection (n = 8). (C, E) Neither habituation nor locomotor activity were affected one week after Poly I:C. (D, F) Total object exploration is similar amongst experimental groups during training in males and in females. SAL: saline; Poly I:C: Polyinosinic:polycytidylic acid. *$p < 0.05$ *cf* Familiar Object.

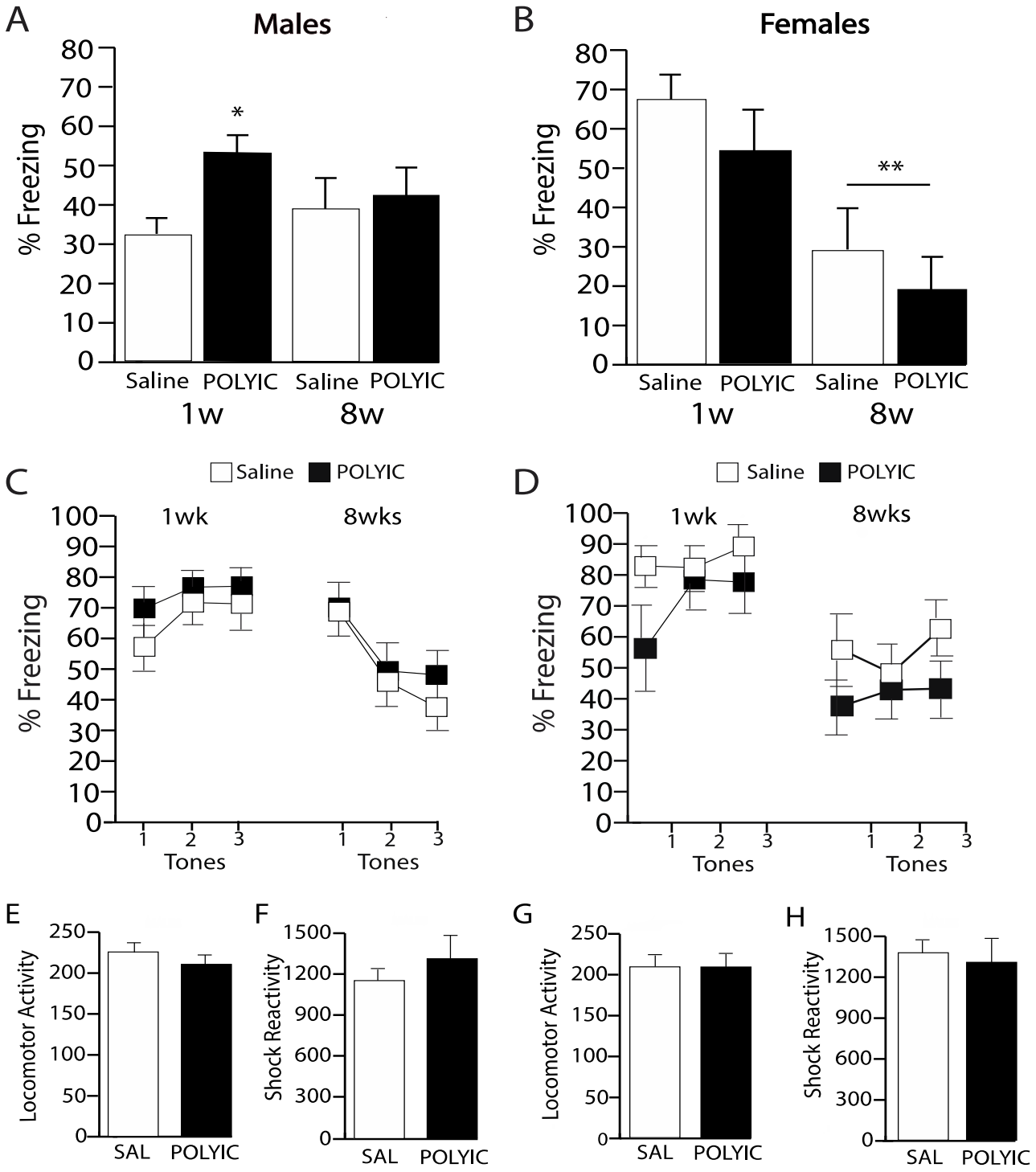

Figure 7. Subchronic Poly I:C does not cause early context fear memory impairments or retrieval deficits. (A) Prior Poly I:C challenge does not disrupt freezing to the training context one week after immune challenge, or during memory retrieval seven weeks later in males (n = 7-8). (B) In females, no disruption of context fear conditioning is observed one week after immune challenge; and, all females showed decreased freezing at the remote test. (C,D) Tone fear conditioning remained intact one week and eight weeks after last injection in both sexes. (E,G) Locomotor activity and (F,H) shock reactivity were equivalent across all groups. SAL: saline; Poly I:C: Polyinosinic:polycytidylic acid. *$p < 0.05$ *cf* Saline; #$p < 0.001$ *cf* 1week.

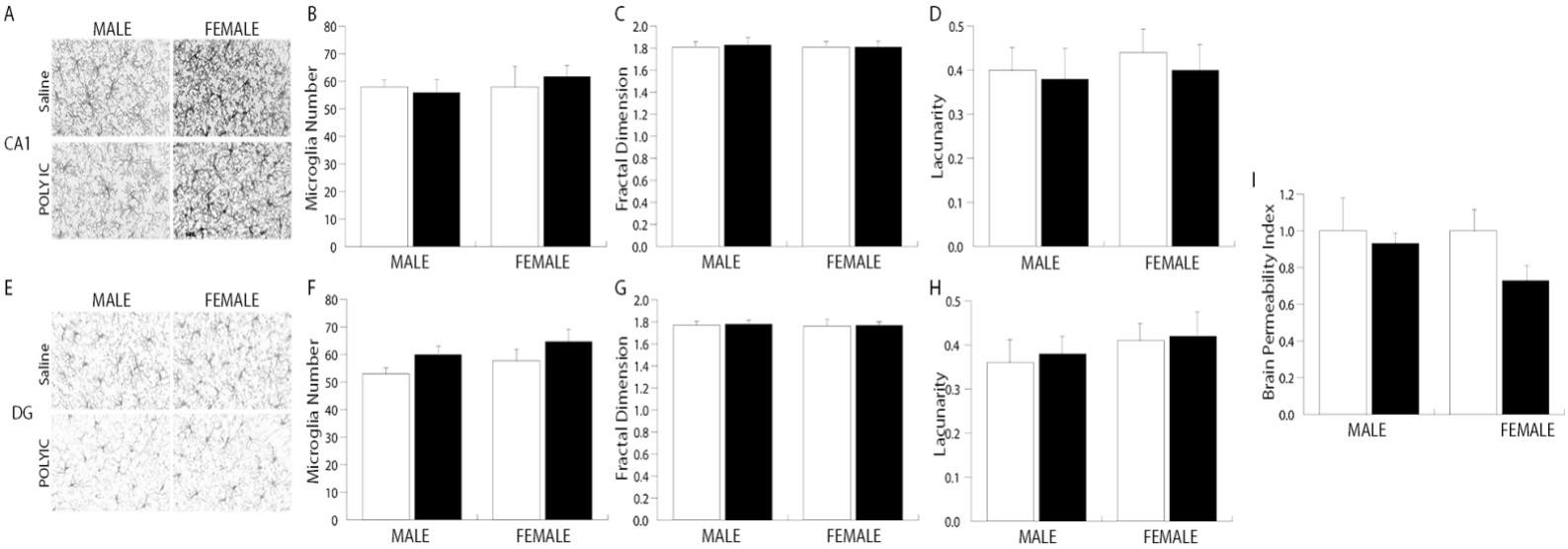

Figure 8. Microglial number and morphology in hippocampus, and blood brain barrier permeability are not altered long after subchronic immune challenge. Representative images of microglia (40x) in males and females are shown in (A) CA1 and (E) dentate (molecular layer) of dorsal hippocampus. Microglial number (B, F) or morphology (C-D, G-H) are not changed by prior subchronic Poly I:C. (I) We observed no increase in blood-brain permeability at this timepoint.