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Pubertal immune stress transiently alters spatial memory processes in adulthood

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ABSTRACT

Pubertal immune challenge can permanently alter hippocampus-dependent memory processes in a sex-specific manner. Although gonadal hormones can influence various cognitive processes, their role in regulating the cognitive sequelae to pubertal immune challenge has not been thoroughly assessed. We examined whether a pubertal immune challenge could affect hippocampus-dependent memory functions in adulthood and whether these effects are regulated by gonadal steroid hormones. We hypothesized that exposure to an immune challenge during puberty would induce sex-specific deficits in the behavioral and cellular correlates of hippocampus-dependent memory during adulthood. At six weeks of age, during the stress-vulnerable pubertal period, male and female CD-1 mice were injected with either saline or the bacterial endotoxin lipopolysaccharide (LPS). Three weeks later, mice underwent either gonadectomy or sham-surgery. At ten weeks of age (i.e., in adulthood), mice began behavioral testing in an open field, Barnes maze, and Morris water maze. Brain tissue was collected at 17 weeks of age and stained for doublecortin and Ki67 to examine migrating neuronal progenitor cells and cellular proliferation in the neurogenic subgranular zone (SGZ) and the cornus ammonis (CA)1 and CA3 regions of the hippocampus. Pubertal LPS treatment impaired learning during adulthood in both sexes and increased cellular proliferation in the CA1 region in castrated males only. Although adult sex hormones did not reliably modulate these changes, gonadectomy impaired learning during the Morris water maze in both sexes. Learning deficits were more prominent during the Barnes maze, which suggests a stress-dependent expression of LPS-induced cognitive deficits. Neurogenesis in the SGZ and cellular proliferation in the CA3 were not affected by pubertal LPS treatment or gonadectomy. These novel findings emphasize the sensitivity of developing cognitive processes during puberty to immune challenges and suggest a possible mechanism for learning-based difficulties in adulthood.

1. Introduction

Puberty is a critical period for sexual maturation characterized by sex-specific changes to behavioral and neuroendocrine systems and their underlying neural circuitry (Brenhouse and Andersen, 2011; McCarthy et al., 2015; Sisk and Foster, 2004). Sexual dimorphisms in the reorganization and restructuring of the pubertal brain are regulated by the influx of gonad-derived hormones (i.e., androgens, estrogens, and progestogens) into the brain that follows pre-pubertal reactivation of the hypothalamic-pituitary-gonadal axis. These sex-specific organizing effects also extend to the body's central stress response system, the hypothalamic-pituitary-adrenal axis (Green and McCormick, 2016; Oyola and Handa, 2017). The extensive neuroplasticity and sex-based distinctions in physiological stress responses during puberty contribute to sex differences in stress reactivity and vulnerability, particularly towards immune challenges (e.g., Girard-Joyal et al., 2015; Sharma et al., 2018). In pubertal mice, immune challenges such as the bacterial endotoxin lipopolysaccharide (LPS) induce the most robust effects at six weeks old, a stress-sensitive peripubertal period (Laroche et al., 2009a,b). Systemic LPS exposure during this stress-sensitive pubertal period permanently impairs various reproductive and non-reproductive functions (e.g., sexual receptivity, anxiety-like and depression-like behaviors, recognition memory, and dopamine-sensitive functions) (Girard-Joyal and Ismail, 2017; Ismail and Blaustein, 2013; Ismail et al.,

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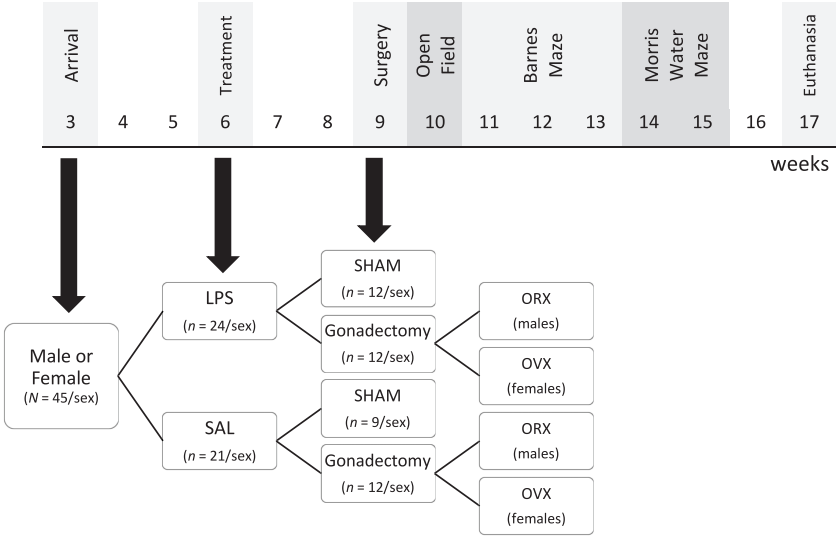

Fig. 1. Experimental timeline and experimental groups. *Note*: SAL = saline; LPS = lipopolysaccharide; SHAM = sham-surgery; ORX = orchiectomy; OVX = ovariectomy.

2011; Olesen et al., 2011).

Adult hippocampus-dependent cognition is sensitive to these dynamic immune-endocrine interactions during the stress-sensitive pubertal period. In female CD-1 mice, pubertal LPS exposure significantly impairs the recognition of familiar social and object stimuli during adulthood and permanently blocks estradiol's cognition-enhancing properties (Ismail and Blaustein, 2013). Translation of these findings to males, however, is complicated by the apparent male performance bias in rodent models of spatial learning and memory and the potential variability in performance associated with the estrous cycle phase and circulating estrogen levels among pubescent and adult female rodents during long-term hormone-dependent tasks (Duarte-Guterman et al., 2015; Frick et al., 2015; Jonasson, 2005).

One possible mechanism driving these long-term effects of pubertal LPS exposure on hippocampus-dependent cognition may be via LPS-induced changes in hippocampal neurogenesis. Performance on hippocampus-dependent tasks is thought to correlate with the production, survival, integration, and activation of new neurons in the subgranular zone (SGZ) of the dorsal hippocampus (for review see Duarte-Guterman et al., 2015). Neurogenic processes are also sensitive to estrous phase, circulating estrogen levels, and to age and sex (e.g., greater cellular proliferation but lower expression of immature neurons in the hippocampus among adult females compared to adult males) (for reviews see Duarte-Guterman et al., 2015; Frick et al., 2015). Chronic LPS-induced neuroinflammation suppresses basal neurogenesis in the SGZ by decreasing the short-term survival of new neurons (Bastos et al., 2008; Ekdahl et al., 2003; Monje et al., 2003). However, age, sex, and hormonal differences in neurogenesis complicate the generalizability of findings from chronic LPS paradigms to hypothesize how neuroinflammatory agents during puberty impact basal neurogenesis in males and females.

Our understanding of hippocampus-dependent cognitive sequelae to pubertal immune challenge is currently limited to social and object recognition memory in female CD-1 mice. Therefore, we assessed sex differences in how pubertal LPS exposure impacts spatial learning and memory during adulthood in CD-1 mice. Given the association between learning and hippocampal neurogenesis, we also examined how basal neurogenesis is affected by this paradigm. Compared to saline-treated controls, mice treated with LPS during puberty were expected to show poorer spatial learning and memory and reduced hippocampal expression of Ki67 (i.e., cellular proliferation) and doublecortin (DCX) (i.e., neuronal precursor cells). Males were expected to show better spatial learning and memory and greater hippocampal neurogenesis than females, regardless of treatment and surgery. Lastly, mice were gonadectomized or sham-operated during early adulthood to examine whether circulating gonadal hormones regulate the enduring cognitive and neurogenic effects of pubertal LPS treatment in both males and females. Gonadectomy was expected to impair cognitive performance and hippocampal neurogenesis in both sexes.

2. Material and methods

2.1. Animals

Ninety CD-1 male and female mice were shipped from Charles River Laboratories (Saint-Constant, Québec, Canada) at three weeks old, an age that is resistant to the enduring effects of shipping stress (Laroche et al., 2009a,b). Mice were segregated by sex and housed in groups of three in rooms maintained on a reversed light cycle (lights off at 1000 h) under standard conditions (14 h:10 h light/dark cycle; 24 ± 2 °C; relative humidity of 40 ± 5). The polycarbonate Lexan housing cages (17 × 28 × 12 cm [width x length x height]) were bedded with Teklad Corn Cob bedding (Harlan Laboratories, Inc., Madison, WI, USA, .25 in. diameter) and enriched with one square piece of Nestlet (Ancare Corp., Bellmore, NY, USA) and a cardboard refuge hut (Ketchum Manufacturing, Inc., Brockville, ON, Canada). Food (Harlan Laboratories, Inc., Madison, WI, US, T2018 – Global 18% rodent) and water were available ad libitum. All behavioral tests were completed during the dark phase under red light unless otherwise specified. All experiments were approved by the Animal Care Committee of the University of Ottawa.

2.2. Experimental procedures

Mice were exposed to LPS or saline at the stress-sensitive age of six weeks. Nine-week-old mice underwent gonadectomy (i.e., orchiectomy [ORX] and ovariectomy [OVX]) or sham-surgery (SHAM) to examine the role of gonad-derived hormones. Following one week of recovery, adult (i.e., ten-week-old) mice completed the open field test, the Barnes maze (BM), and the Morris water maze (MWM) over five weeks to limit carryover effects (Fig. 1). Vaginal smears were collected during the learning (every other day) and probe phases of the BM and MWM. Brain tissue was collected two weeks after behavioral testing to examine group differences in basal hippocampal neurogenesis and cellular proliferation.

2.3. Pubertal immune stress

2.3.1. Systemic exposure to lipopolysaccharide and saline

Mice were treated intraperitoneally (*ip*) with either 0.9% sterile saline control ($n = 42$) or LPS (from *Escherichia coli* serotype O26:B6; #L3755; Sigma Chemical Co., St. Louis, MO, USA; 1.5 mg/kg body weight; $n = 48$) diluted in 0.2 mg/mL sterile saline. This LPS dosage during puberty elicits mild sickness symptoms for approximately 48 h in both sexes and impairs hippocampus-dependent cognition in adult females (Cai et al., 2016; Ismail and Blaustein, 2013). Injections were performed towards the end of the light phase.

2.3.2. Sickness monitoring

Behavioral signs of sickness were assessed at 30 min and 2, 4, 8, 12, 24 and 48 h following saline or LPS exposure by two experienced observers blind to the treatment conditions. As described by Kolmogorova et al. (2017), all mice received a score between 0 and 4 at each time point to reflect the number of sickness symptoms (lethargy, huddling, piloerection, and ptosis) observed. Final sickness scores were calculated as the average of the two raters' individual scores.

2.3.3. Body weight analyses

The body weights were recorded at the time of injection (i.e., baseline) and at 12, 24, and 48 h after injection. Percentage changes in body weight from baseline were calculated as described by Kolmogorova et al. (2017).

2.4. Sham-surgery and gonadectomies

Gonadectomy and sham-surgeries for both sexes were completed as described by Cai et al. (2016). All mice were provided ad libitum access to water bottles filled with 3% children's Tylenol® (acetaminophen) during the 48 h before surgery and for three days after surgery. Post-operative care included a subcutaneous injection of Carprofen (5 mg/kg body weight) and topical application of bupivacaine to the sutured skin. The cages were placed on Gaymar® T/Pump® classic heating pads set to 38 °C (± 1 °C) for two days post-surgery.

2.5. Behavioral testing

2.5.1. Open field test

General locomotor activity was assessed by examining free exploration of an open Plexiglass arena (90 cm x 90 cm x 45 cm walls) over five minutes. Olfactory cues were reduced by wiping the arena with 70% alcohol before introducing each mouse. Video recordings captured by a closed-circuit Panasonic video camera were analyzed for distance travelled (cm) and speed (cm/s) with Noldus EthoVision® XT software. Noise in the tracking analyses (e.g., body wobble and precision-level noise) was smoothed by a robust Locally Weighted Scatterplot Smoothing (LOWESS) with a 10-second half-window (Benjamini et al., 2010).

2.5.2. Barnes maze

Hippocampus-dependent spatial learning and memory were examined with the BM (Sunyer et al., 2007). In order to reduce potential anxiety, mice were first familiarized with the escape box and the elevated circular arena (120 cm diameter, 90 cm high). Mice were then provided four days (four daily trials with a 15-minute inter-trial interval [ITI]) to learn to distinguish the entrance into the escape box amongst 15 other identical holes (9 cm diameter) evenly spread out along the perimeter. Reference cues were available on the periphery. Briefly, mice were placed in an opaque chamber in the arena center. The chamber was removed after 10 s and the mouse was exposed to two aversive stimuli – an overhead 27 W bright white bulb and an 85-dB piezo buzzer (3500 ± 500 Hz; Nexxtech, Barrie, Ontario, Canada). The trial ended either upon entry into the target box (i.e., minimum three paws touching the ramp into the target box) or after 3 min had elapsed. Mice were allowed 1 min in the target box. Short-term and long-term spatial memory (days 5 and 12, respectively) were evaluated from 90-second probe trials during which the target hole was closed.

All trials were recorded by an overhead closed-circuit Panasonic video system. One observer blind to group conditions analyzed the videos for primary and total latency (s), primary and total errors, search strategies (direct, mixed, and serial), and head pokes into the target and opposite holes according to Sunyer et al. (2007). Total latency and search strategies were assessed only for the acquisition phase, whereas frequency of head pokes in the target and opposite holes were examined during the memory probes. Acquisition parameters are reported as daily averages ($M \pm S.E.M.$).

2.5.3. Morris water maze

Spatial memory (acquisition and retention) and cognitive flexibility were assessed using the MWM (Vorhees and Williams, 2006). The arena, a circular pool (120 cm diameter, 50 cm tall) filled with water (35 cm deep; 21 ± 1 °C), was placed in a room dimly illuminated by an 8 W white bulb. Spatial cues were available on the pool edge. The pool was arbitrarily divided into north, east, south, and west quadrants. To habituate to the arena and the escape platform (5 cm diameter), mice first completed three 60-second sessions in clear water. The remainder of the MWM was performed in water made opaque by non-toxic tempera paint. The acquisition phase (days 1–4) involved four 60-second trials (30-second ITI) daily with the objective of locating the unmarked platform submerged 0.5 cm below water level in the target quadrant (north). Trial starting positions were semi-randomized to different non-target quadrants opposite the platform. Spatial retention was assessed 24 h after the last acquisition trial using a 60-second trial (day 5) during which the platform was removed. To assess cognitive flexibility (days 7–11), starting locations were reversed and the platform was moved to the south quadrant.

All trials were recorded by an overhead closed-circuit Panasonic video system. Total distance (cm) travelled in the arena and in the target quadrants, duration (s) in the target and opposite quadrants, and average distance (cm) to the platform in each phase were obtained from videos analyzed with Noldus EthoVision® XT software. Mice were tracked using the whole body "differencing" detection method with a 10-second half-window LOWESS to reduce noise (Benjamini et al., 2010). Latency (s) to reach the platform and distance (cm) travelled in the target quadrant were also examined during the learning phases and probes, respectively. Non-probe data are reported as daily averages ($M \pm S.E.M.$).

2.6. Estrous cycling

Estrous stage (i.e., proestrus, estrus, metestrus, and diestrus) was determined from vaginal smears collected as described by Byers et al. (2012). A trained rater examined all samples at 10 x and 40 x magnification under a light microscope (Olympus BX61).

2.6.1. Perfusion and tissue collection

All mice were deeply anesthetized with Euthanyl (40 mg/kg body weight, *ip*) at 17 weeks of age. Upon confirmation of deep anesthesia, mice were intracardially perfused with 20 mL saline followed by 20 mL of 4% paraformaldehyde (PFA). The excised brains were post-fixed in 4% PFA for 2 h and then placed in fresh 30% sucrose solution at 24 and 48 h after extraction. The brain tissue was sliced by vibratome into 40 μm free-floating sections (1 in 3 series) and then stored in Eppendorf tubes filled with cryoprotectant solution at −20 °C.

2.6.2. Immunocytochemistry

All washes and incubations were performed at room temperature with gentle agitation, unless otherwise specified. Briefly, free-floating sections were washed with 1X phosphate-buffered saline (PBS; three x

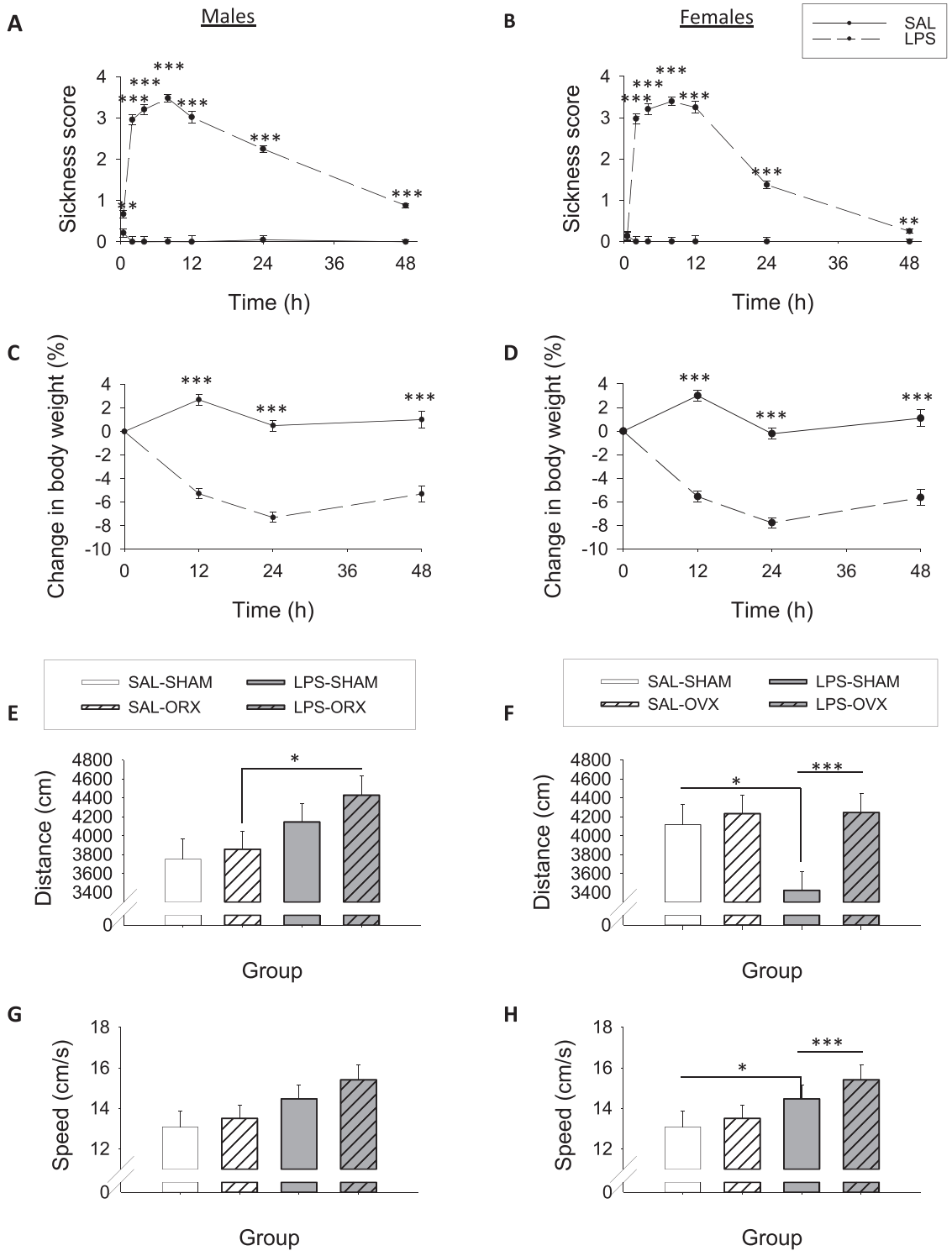

Fig. 2. Acute sickness responses to saline or LPS treatment and open field locomotor activity. Figures A and B refer to acute changes in sickness behaviour following saline or LPS treatment in males and females, respectively. Figures C and D refer to acute changes in percent body weight change from baseline following saline or LPS treatment in males and females, respectively. Figures E and F refer to treatment (saline versus LPS) and surgery (intact versus gonadectomized) differences in distance (cm) travelled in the open field in males and females, respectively. Figures G and H refer to treatment and surgery differences in velocity (cm/s) in the open field in males and females, respectively. *Note*: SAL = saline; SHAM = sham-surgery; ORX = orchiectomy; OVX = ovariectomy; * = $p < .05$, ** = $p < .01$, *** = $p < .001$.

5 min.) and then blocked with 1% bovine serum albumin / .3% Triton X-100 / 1X PBS for one hour. The tissue was incubated for 24 h with goat DCX (1:1000; Santa Cruz Biotechnology; cat: SC-806) and rabbit Ki67 (1:5000; Abcam; cat: ab15580) in a solution of 1X PBS / .3% Triton X-100. The sections underwent another 1X PBS wash (three x 5 min.) before a 30-minute incubation in a solution of donkey anti-rabbit Alexa Fluor 488 (1:2000; ThermoFisher Scientific; cat: A21206), donkey anti-goat Alexa Fluor 594 (1:1000; ThermoFisher Scientific; cat: A11058), and 1X PBS / .3% Triton X-100 at 30 °C in the dark. After a final set of three five-minute washes in 1X PBS, hippocampal sections

were mounted onto slides coated with anti-fade (.1% P-phenylenediamine in 90% glycerol) and sealed with nail polish.

2.6.3. Cell quantification

All analyses were performed manually on one representative section of the dorsal hippocampus for each mouse (i.e., bregma: -2.18 mm, Franklin and Paxinos, 2007) by two raters blind to experimental conditions. DCX^+, $Ki67^+$, and $DCX^+/Ki67^+$ cell expression in the SGZ were examined from the middle 11 images of z-stacks (.95 μm step intervals) collected over the entire 40 μm section (Oishi et al., 2016). Images were captured by sequential excitation at 488 nm and 559 nm at 40 x magnification using an Olympus FV1000 confocal laser microscope coupled with Olympus FluoView FV10-ASW (version 4.2a) software (Center Valley, PA, USA). Emission was collected by spectral detectors in separate channels with user-defined minimum and maximum wavelengths. Fluorescing DCX^+ and $Ki67^+$ cells in the SGZ were counted if a clear circular cell body was visible and the cell bordered the SGZ. Cells that met both criteria and showed clear overlap in morphology were considered double-labelled ($DCX^+/Ki67^+$). $Ki67^+$ cell expression was similarly assessed in the CA1 and CA3 regions of the dorsal hippocampus using representative images captured at 20 x by an Olympus BX61 microscope.

2.7. Statistical analyses

LPS-treated mice that did not show the characteristic sickness behavior response and body weight loss ($n_{males} = 3$; $n_{females} = 2$) were excluded from all analyses to eliminate the possibility of outliers due to improper treatment dosage and/or uncharacteristic LPS tolerance. The remaining dataset was then screened for statistical outliers. In order to maintain sample sizes and to reduce the effects of extreme outliers, cases that fell outside the 1.5 interquartile range in boxplots were adjusted using winsorization (Lotan et al., 2017; Pollet and van der Meij, 2017). A saline-treated [SAL]-OVX female and a SAL-ORX male had died during the study; therefore, sickness monitoring data was available for both mice, whereas open field activity was only available for the male.

Group differences in sickness-related parameters were analyzed with a three-way (sex x treatment x time) mixed-design repeated-measures analysis of variance (ANOVA). Acquisition parameters in the BM and MWM were analyzed with a four-way mixed-design repeated-measures ANOVA to examine the effects of Sex, Treatment, and Surgery over the four learning days. *F*-values were adjusted with the Greenhouse-Geisser correction when sphericity was violated according to Mauchly's test (i.e., $\varepsilon_{Greenhouse\text{-}Geisser} < .75$). The remaining behavioral and cellular data were examined using three-way (Sex x Treatment x Surgery) between-subjects ANOVAs. Measures of effect size are reported using partial eta-squared (η_p^2). ANOVAs were followed by Bonferroni-corrected pairwise comparisons when appropriate. Statistical significance was set to $p < .05$. All statistical analyses were conducted with IBM® SPSS® (version 20.0.0) software.

3. Results

3.1. Sickness behavior and body weight changes

As expected, LPS treatment induced more sickness behaviors than saline treatment ($F_{(1, 86)} = 942.39$, $p < .001$, $\eta_p^2 = .916$) and elicited greater sickness behavior responses among males compared to females ($F_{(1, 86)} = 4.50$, $p = .037$, $\eta_p^2 = .050$) (Fig. 2A and B). The expression of sickness behaviours changed significantly over time ($F_{(4.17, 358.89)} = 211.83$, $\eta_p^2 = .711$) and was influenced by time-point, sex, and treatment ($F_{(4.17, 358.89)} = 4.45$, $\eta_p^2 = .049$) (all $p \leq .001$) such that LPS-treated males displayed more sickness behaviors than females at 30 min (*MD* = .54, *SE* = .14) and at 24 and 48 h (*MD* = .88, *SE* = .13 and *MD* = .63, *SE* = .07, respectively) (all $p < .001$).

Main effects of Time ($F_{(2.24, 192.77)} = 71.38$, $\eta_p^2 = .454$) and Treatment ($F_{(1, 86)} = 294.81$, $\eta_p^2 = .774$) and a significant Time x Treatment interaction ($F_{(2.24, 192.77)} = 94.57$, $\eta_p^2 = .524$) (all $p < .001$) were also seen for percentage change in body weight (Fig. 2C and D).

3.2. Locomotor activity in open field

Gonadectomized mice were faster than intact mice ($F_{(1, 76)} = 5.32$, $p = .024$, $\eta_p^2 = .065$). Velocity and distance travelled varied significantly by sex and treatment ($F_{(1, 76)} = 6.54$, $\eta_p^2 = .079$ and $F_{(1, 76)} = 8.22$, $\eta_p^2 = .098$, all $p < .05$), where LPS-SHAM females were slower and travelled less than SAL-SHAM and LPS-OVX females and their male counterparts (*MD* = -2.60, *SE* = .97 and *MD* = -720.87, *SE* = 279.38, respectively, all $p < .05$), and LPS-ORX males travelled further than SAL-ORX males (Fig. 2E – H).

3.3. Hippocampus-dependent learning and memory

3.3.1. BM acquisition

All groups appeared to learn the location of the target hole. Consistent with these observations, there was a significant main effect of Time for primary and total latencies ($F_{(3, 225)} = 30.92$, $\eta_p^2 = .292$ and $F_{(2.61, 195.97)} = 43.76$, $\eta_p^2 = .368$, respectively) and primary and total errors ($F_{(2.50, 187.83)} = 52.74$, $\eta_p^2 = .077$ and $F_{(2.21, 165.82)} = 47.39$, $\eta_p^2 = .387$, respectively) (all $p < .001$). Intact mice made more errors than gonadectomized mice before finding the target hole (primary errors: $F_{(1, 75)} = 6.78$, $\eta_p^2 = .083$), whereas LPS-treated mice took longer than saline-treated mice to find the target hole (primary latency: $F_{(1, 75)} = 7.68$, $\eta_p^2 = .093$) (all $p < .05$). LPS-treated mice were also slower to escape relative to saline-treated mice (total latency: $F_{(1, 75)} = 7.61$, $\eta_p^2 = .092$) (Fig. 3C and D). The analyses also revealed significant interactions of Time x Surgery for primary latency ($F_{(3, 225)} = 62.90$, $\eta_p^2 = .037$) and primary errors ($F_{(2.50, 187.83)} = 2.92$, $\eta_p^2 = .037$), Time x Treatment for primary and total latencies ($F_{(3, 225)} = 6.30$, $\eta_p^2 = .077$ and $F_{(2.61, 195.97)} = 6.23$, $\eta_p^2 = .077$, respectively), and Time x Sex ($F_{(2.61, 195.97)} = 2.94$, $\eta_p^2 = .038$) and Time x Treatment x Surgery ($F_{(2.61, 195.97)} = 3.03$, $\eta_p^2 = .039$) for total latency (all $p < .05$).

On the first day, saline-treated sham-operated (i.e., control) males took longer to escape relative to SAL-ORX males (Fig. 3A). LPS-ORX males and LPS-SHAM females had longer primary latencies on the second day than LPS-SHAM males (*MD* = 11.23, *SE* = 4.89, and *MD* = 11.99, *SE* = 5.01, respectively) (all $p < .05$). On the third day, LPS-SHAM females had longer primary latencies and made more primary errors compared to their saline-treated counterparts (*MD* = 1.51, *SE* = .62 and *MD* = 20.60, *SE* = 7.89, respectively, all $p < .05$). Relative to their saline-treated counterparts, LPS-intact females and males also committed more errors overall (Fig. 3C and D). On the final day, female controls made fewer total errors than LPS-SHAM females (Fig. 3D) and control males (*MD* = -7.53, *SE* = 2.88, $p = .011$).

Although mice adjusted their search strategies to locate the target hole over time, LPS-treated groups implemented more disorganized, random searches than saline-treated mice (Fig. 3E and F). Accordingly, we observed significant main effects of Time for direct, serial, and mixed searches ($F_{(3, 225)} = 7.90$, $\eta_p^2 = .095$; $F_{(3, 225)} = 6.17$, $\eta_p^2 = .075$; and, $F_{(3, 225)} = 10.36$, $\eta_p^2 = .121$, respectively, all $p \leq .001$) and a significant main effect of Treatment for mixed searches ($F_{(1, 75)} = 6.75$, $p = .011$, $\eta_p^2 = .083$). The ANOVAs also revealed significant interactions of Treatment x Surgery ($F_{(1, 75)} = 4.16$, $p = .045$, $\eta_p^2 = .052$) for direct approaches, Time x Treatment and Time x Sex x Treatment for serial searches ($F_{(3, 225)} = 6.23$, $\eta_p^2 = .077$ and $F_{(3, 225)} = 4.03$, $\eta_p^2 = .051$, respectively, all $p \leq .001$), and Time x Sex x Treatment x Surgery for mixed searches ($F_{(3, 225)} = 2.72$, $p = .045$, $\eta_p^2 = .035$). On the first day, control females used direct searches more often than LPS-SHAM females (*MD* = .19, *SE* = .09, $p = .030$).

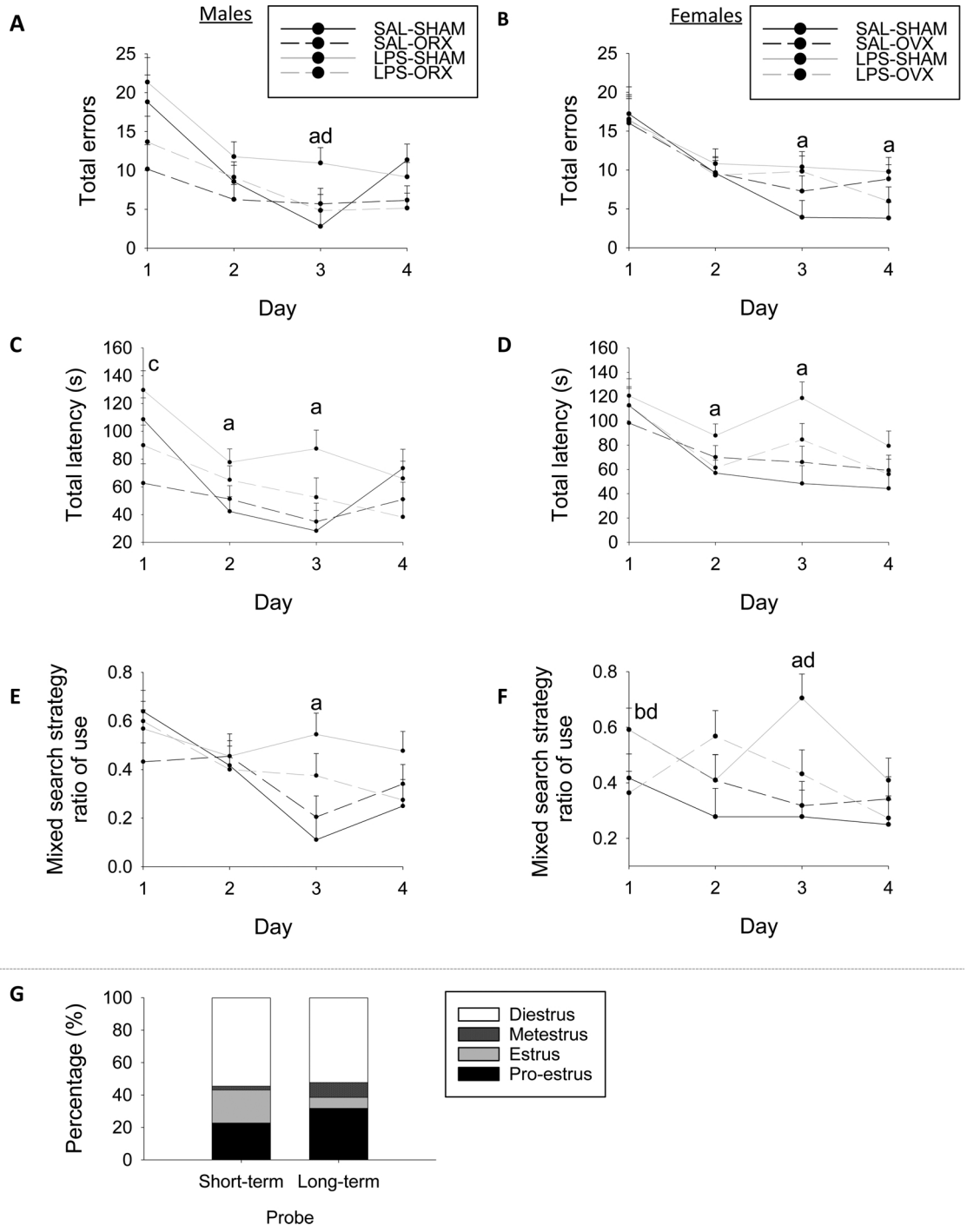

Fig. 3. Treatment and surgery differences in Barnes maze acquisition among males and females. Figures A and B refer to treatment (saline versus LPS) and surgery (intact versus gonadectomized) differences in total errors made by males and females, respectively. Figures C and D refer to treatment and surgery differences in total latencies (s) among males and females, respectively. Figures E and F refer to treatment and surgery differences in mixed search strategy use among males and females, respectively. Figure G depicts percentage of female mice in pro-estrus, estrus, metestrus, and diestrus during the short-term and long-term memory probes. *Note*: SAL = saline; SHAM = sham-surgery; ORX = orchiectomy; OVX = ovariectomy; *a* = significant treatment difference ($p < .05$) among intact subjects, *b* = significant treatment difference ($p < .05$) among gonadectomized subjects, *c* = significant surgery difference ($p < .05$) among saline-treated subjects, *d* = significant surgery difference ($p < .05$) among LPS-treated subjects.

Compared to LPS-OVX females, mixed approaches were initially more common among LPS-ORX males ($MD = .24$, $SE = .11$, $p = .041$). By the third day, LPS-OVX males used direct searches more often than their female counterparts ($MD = .24$, $SE = .11$, $p = .034$) but implemented serial searches less often than SAL-OVX males ($MD = -.23$, $SE = .10$, $p = .028$). SAL-OVX females implemented direct searches less often than control females on the final day ($MD = .28$, $SE = .10$, $p = .007$).

3.3.2. BM memory probes

During the short-term memory probe, females made more primary errors than males ($F_{(1, 75)} = 6.11$, $\eta_p^2 = .075$) and LPS-treated mice made more primary errors and target-opposite pokes than saline-treated mice ($F_{(1, 75)} = 5.69$, $\eta_p^2 = .070$ and $F_{(1, 75)} = 4.04$, $\eta_p^2 = .051$, all $p < .05$). Furthermore, LPS-SHAM females committed more primary errors than LPS-SHAM males ($MD = 3.82$, $SE = 1.56$, $p = .016$).

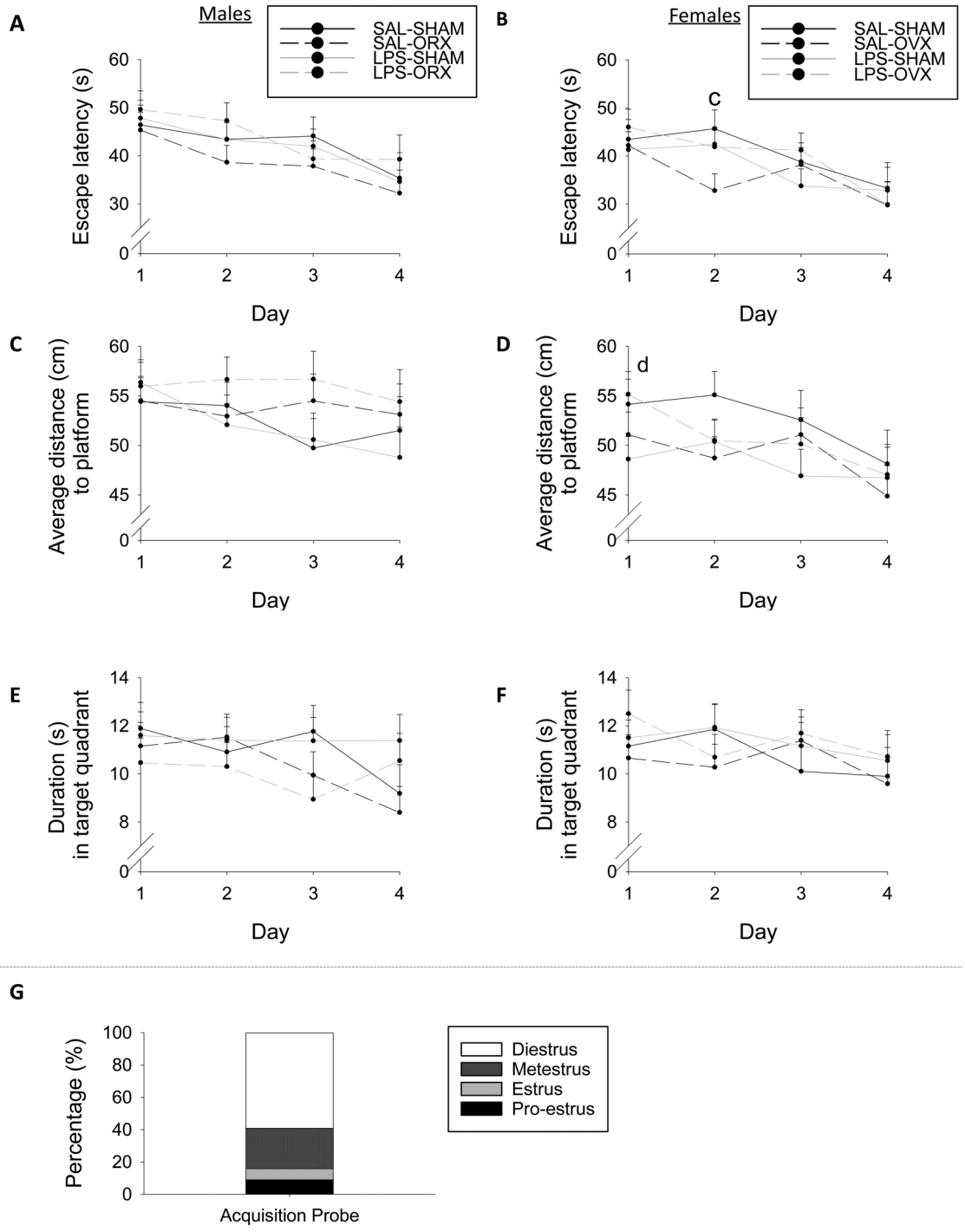

Fig. 4. Treatment and surgery differences in learning in the acquisition phase of the Morris water maze among males and females. Figures A and B refer to treatment (saline versus LPS) and surgery (intact versus gonadectomized) differences in escape latency (s) among males and females, respectively. Figures C and D refer to treatment and surgery differences in average distances (cm) to the platform among males and females, respectively. Figures E and F refer to treatment and surgery differences in duration (s) in the target quadrant (north) among males and females, respectively. Figure G depicts percentage of female mice in pro-estrus, estrus, metestrus, and diestrus during the acquisition memory probe. *Note*: SAL = saline; SHAM = sham-surgery; ORX = orchiectomy; OVX = ovariectomy; *a* = significant treatment difference ($p < .05$) among intact subjects, *b* = significant treatment difference ($p < .05$) among gonadectomized subjects, *c* = significant surgery difference ($p < .05$) among saline-treated subjects, *d* = significant surgery difference ($p < .05$) among LPS-treated subjects.

Primary latencies, target-hole pokes, and total errors did not differ significantly between groups.

During the long-term memory probe, females made more total errors than males ($F_{(1, 74)} = 6.41$, $p = .013$, $\eta_p^2 = .080$). All other parameters were similar across groups.

3.3.3. MWM acquisition

The analyses suggest that all mice learned the platform location (north). Accordingly, the analyses revealed significant main effects of Time for escape latency ($F_{(3, 225)} = 19.33$, $\eta_p^2 = .205$), path length ($F_{(2.73, 204.58)} = 21.28$, $\eta_p^2 = .221$), target-quadrant duration ($F_{(3, 225)} = 2.95$, $\eta_p^2 = .038$), average distance to the platform ($F_{(3, 225)} = 9.19$, $\eta_p^2 = .109$), and opposite-quadrant duration ($F_{(3,}$

$_{225)}$ = 13.94, η_p^2 = .157) (all p < .05). Overall, females swam closer to the platform than males ($F_{(1, 75)}$ = 4.93, p = .029, η_p^2 = .062) (Fig. 4C and D).

LPS-SHAM females initially swam closer to the platform than their male and OVX counterparts (*MD* = -7.75, *SE* = 3.26 and *MD* = -6.55, *SE* = 3.26, respectively) and took shorter paths to locate the platform than LPS-SHAM males (*MD* = -288.68, *SE* = 121.82) (all p < .05). By the second day, control females explored the south longer and swam further to find the platform than SAL-OVX females (*MD* = 4.64, *SE* = 2.23 and *MD* = 273.99, *SE* = 106.91, respectively), whereas LPS-ORX males spent more time in the south than SAL-ORX males (*MD* = 5.11, *SE* = 2.16) (all p < .05).

3.3.4. MWM acquisition probe

All groups spent similar amounts of time in the target quadrant (north). However, gonadectomized mice travelled more in the north and spent less time in the south than intact mice ($F_{(1, 75)}$ = 4.18, η_p^2 = .053 and $F_{(1, 75)}$ = 4.17, η_p^2 = .053, all p < .05). The ANOVA also identified significant interactions of Sex x Treatment for path length ($F_{(1, 75)}$ = 4.15, p = .045, η_p^2 = .042) and Sex x Treatment x Surgery for target proximity ($F_{(1, 75)}$ = 5.58, p = .021, η_p^2 = .069). SAL-OVX females swam closer to the platform than intact counterparts (*MD* = 10.10, *SE* = 4.56), as did SAL-OVX and LPS-SHAM females relative to their male counterparts (*MD* = -9.86, *SE* = 4.32 and *MD* = -8.80, *SE* = 4.32, respectively) (all p < .05). LPS-ORX males swam shorter distances than LPS-OVX females (*MD* = -263.56, *SE* = 95.71, p = .007) and LPS-SHAM males (*MD* = -215.74, *SE* = 95.71, p = .027).

3.3.5. MWM spatial reversal

All groups appeared to learn the new platform location (south) given the significant main effects of Time for escape latency ($F_{(2.60, 195.15)}$ = 26.44, η_p^2 = .261), path length ($F_{(2.56, 191.92)}$ = 24.57, η_p^2 = .247), duration in the target and opposite quadrants ($F_{(2.57, 193.06)}$ = 7.67, η_p^2 = .093 and $F_{(2.28, 171.20)}$ = 24.59, η_p^2 = .247, respectively), and average distance to the platform ($F_{(2.51, 188.39)}$ = 17.97, η_p^2 = .193) (all p < .001). Overall, gonadectomized mice took longer to escape and spent more time exploring the south than intact mice ($F_{(1, 75)}$ = 5.78, η_p^2 = .072 and $F_{(1, 75)}$ = 6.09, η_p^2 = .075, respectively, all p < .05) (Fig. 5A – B and E–F).

We also observed significant Time x Sex x Treatment x Surgery interactions for target-quadrant duration, escape latency, and path length ($F_{(2.57, 193.06)}$ = 4.15, η_p^2 = .052; $F_{(2.60, 195.15)}$ = 3.94, η_p^2 = .050; and, $F_{(2.56, 191.92)}$ = 3.47, η_p^2 = .044, respectively) (all p < .05). LPS-OVX females initially spent less time in the south than SAL-OVX females (Fig. 5D) and LPS-ORX males (*MD* = 4.08, *SE* = 1.60) and swam further away from the platform than LPS-ORX males (*MD* = 9.64, *SE* = 3.96) and their intact counterparts (*MD* = 11.10, *SE* = 5.17) (all p < .05). On the second day, SAL-ORX males took longer to escape than their female counterparts (*MD* = 12.34, *SE* = 5.72). Furthermore, compared to their saline-treated and sham-operated counterparts, LPS-OVX females took longer to escape and explore the north (Fig. 5B and D), swam further away from the platform (*MD* = 14.58, *SE* = 5.04 and *MD* = 12.19, *SE* = 5.04, respectively), and took longer paths to reach the platform (*MD* = 376.10, *SE* = 127.58 and *MD* = 465.15, *SE* = 127.58, respectively) (all p < .05). Longer durations in the north, path lengths, and target proximities were also seen among LPS-OVX females relative to LPS-ORX males on the second day (*MD* = 4.43, *SE* = 1.52; *MD* = 340.18, *SE* = 130.73; and, *MD* = 4.43, *SE* = 1.52, respectively, all p < .05). On the third day, control males took longer swim paths than LPS-SHAM males (*MD* = 265.30, *SE* = 129.08, p = .043), were quicker to escape than LPS-SHAM males (Fig. 5A) and their female counterparts (*MD* = 10.25, *SE* = 5.04, p = .046), and spent more time in the south than their LPS-treated counterparts (Fig. 5C). On the last day, LPS-OVX females were again slower to escape and spent more time in the north than SAL-OVX females (Fig. 5E and F) and LPS-ORX males (*MD* = 9.27, *SE* = 3.72 and, *MD* = 3.56, *SE* = 1.18, respectively) (all p < .05). LPS-ORX males also took shorter paths to reach the platform than LPS-OVX females (*MD* = -252.03, *SE* = 103.47) and intact counterparts (*MD* = -231.62, *SE* = 103.47) (all p < .05).

3.3.6. MWM spatial reversal probe

Gonadectomized mice travelled less in the target south quadrant and had shorter path lengths than intact mice ($F_{(1, 75)}$ = 4.76, η_p^2 = .060 and $F_{(1, 75)}$ = 5.65, η_p^2 = .070, respectively, all p < .05). Compared to males, females spent more time in the north, had longer swim paths, and swam further away from the platform ($F_{(1, 75)}$ = 8.50, η_p^2 = .102; $F_{(1, 75)}$ = 6.35, η_p^2 = .078; and, $F_{(1, 75)}$ = 4.75, η_p^2 = .060, respectively, all p < .05). Control males and females travelled more than their gonadectomized counterparts (*MD* = 203.85, *SE* = 97.55 and *MD* = 197.43, *SE* = 97.55, respectively, all p < .05).

3.4. Cellular expression

The significant Treatment x Surgery interaction ($F_{(1, 56)}$ = 5.11, p = .028, η_p^2 = .084) showed significantly higher Ki67$^+$ cells in the CA1 of LPS-SHAM males compared to their saline-treated and castrated counterparts (Fig. 6C).

All groups showed similar expression of DCX$^+$, Ki67$^+$, and DCX$^+$/Ki67$^+$ cells in the SGZ and Ki67$^+$ cells in the CA3.

4. Discussion

As expected, LPS treatment during the stress-sensitive pubertal period impaired learning in adulthood. In the BM, LPS-treated mice used more inaccurate and inefficient searches to locate and enter the escape hole than saline-treated mice. Pubertal LPS treatment also negatively impacted short-term but not long-term retention of the target location, although females tended to show poorer spatial retention than males overall. In contrast, all groups showed similar learning and retention of the escape platform in the MWM, but females generally swam closer to the platform than males while learning its location. Intact mice travelled less in the target quadrant and spent more time in the opposite quadrant during the probe trial than gonadectomized mice. When tasked with learning a new platform location, only gonadectomized groups were significantly slower to adapt to this change relative to their intact counterparts, although LPS-treated groups showed slower learning, particularly among LPS-OVX females.

The discrepant findings between the BM and MWM can be attributed to differences in stress experienced by the mice during behavioral testing. This stressor-dependent performance is particularly relevant for sex differences because testosterone and estradiol cause opposing stress responses under certain conditions (Boivin et al., 2017; Romeo et al., 2016; ter Horst et al., 2012). The dry-land BM is a less stressful and more ethologically-relevant task than the MWM and is therefore more advantageous at elucidating learning/navigation behaviors (D'Hooge and De Deyn, 2001; Sharma et al., 2010; Whishaw and Tomie, 1996). Furthermore, the predominantly rat-based findings from water mazes can be difficult to translate to mice (Sharma et al., 2010; Whishaw and Tomie, 1996). Although both rats and mice perform similarly on dry-land mazes, mice are more sensitive to the aversive nature of the MWM and show an inverse relationship between spatial learning and corticosterone levels only in the water maze (D'Hooge and De Deyn, 2001; Harrison et al., 2009; Whishaw and Tomie, 1996).

Given these considerations in the dry-land and water mazes, pubertal LPS treatment appears to impair hippocampus-dependent task performance in adulthood during low-stress but not high-stress conditions. Additional support for this stress-dependent expression of LPS-induced cognitive deficits comes from the increase in treatment interactions seen during the reversal phase of the MWM once the mice have presumably become more familiarized and thus less stressed with the

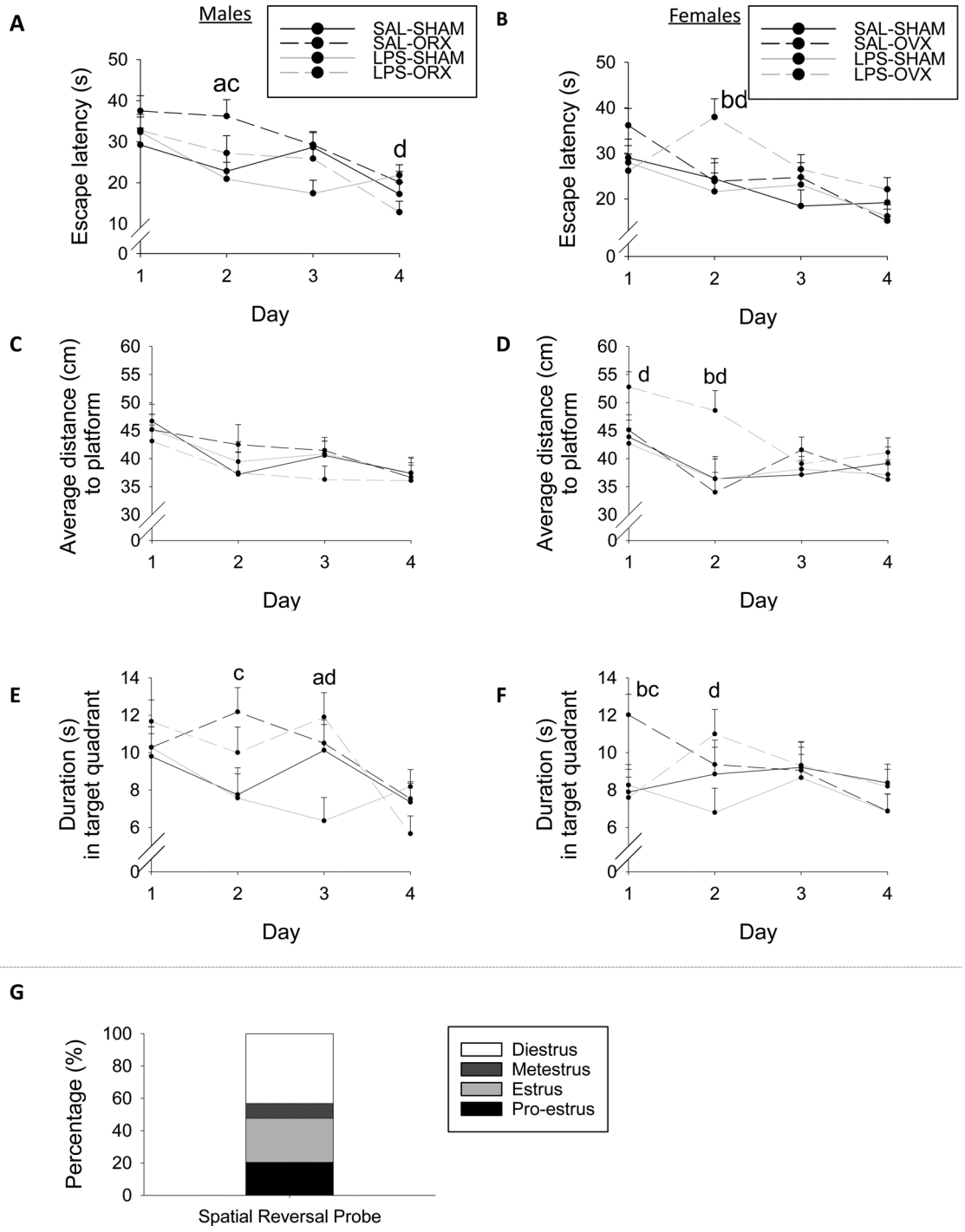

Fig. 5. Treatment and surgery differences in learning in the spatial reversal phase of the Morris water among males and females. Figures A and B refer to treatment (saline versus LPS) and surgery (intact versus gonadectomized) differences in escape latency (s) among males and females, respectively. Figures C and D refer to treatment and surgery differences in average distances (cm) to the platform among males and females, respectively. Figures E and F refer to treatment and surgery differences in duration (s) in the target quadrant (south) among males and females, respectively. Figure G depicts percentage of female mice in pro-estrus, estrus, metestrus, and diestrus during the acquisition memory probe. *Note*: SAL = saline; SHAM = sham-surgery; ORX = orchiectomy; OVX = ovariectomy; *a* = significant treatment difference ($p < .05$) among intact subjects, *b* = significant treatment difference ($p < .05$) among gonadectomized subjects, *c* = significant surgery difference ($p < .05$) among saline-treated subjects, *d* = significant surgery difference ($p < .05$) among LPS-treated subjects.

testing environment. These findings are generally consistent with the impairments seen among adult females treated with LPS during puberty in other dry-land hippocampus-dependent measures (Ismail and Blaustein, 2013). Future studies should follow up on our findings and examine whether physical markers of stress correlate with performance on hippocampus-dependent measures.

Interestingly, sex or surgery did not modulate LPS-induced learning deficits, which suggests that these LPS-induced cognitive changes occur independently of gonadal hormonal influences. This absence of sex differences in learning the BM and MWM coincide with other studies of mice at this age (e.g., Frick et al., 1999; Harrison et al., 2006; Rogers et al., 2017). Nevertheless, several noteworthy significant sex and surgery differences were observed. First, females swam significantly closer to the platform than males during the acquisition and retention

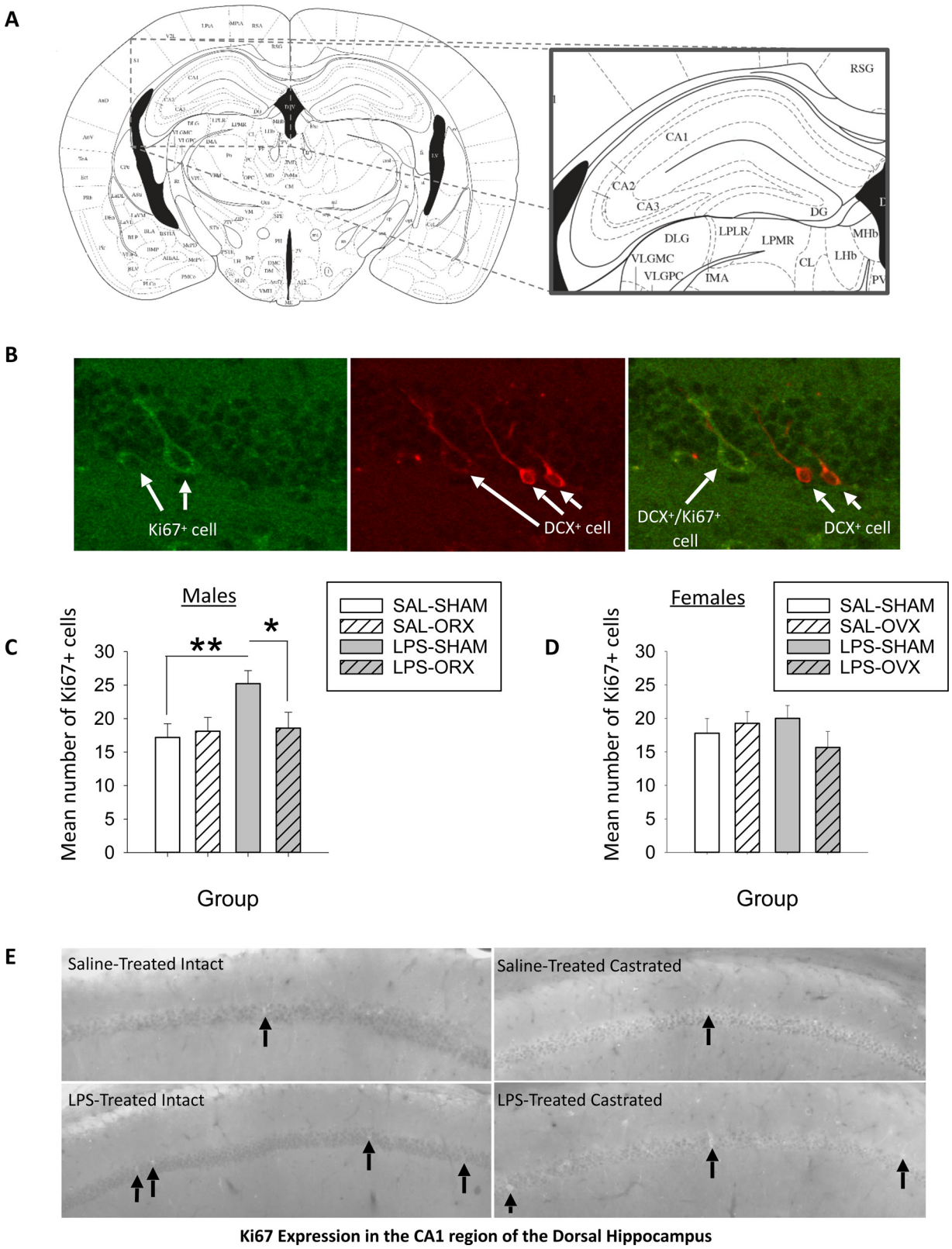

Fig. 6. Treatment and gonadal effects on DCX and Ki67 expression in the dorsal hippocampus. Figure A displays the dentate gyrus (DG), cornus ammonis (CA1) and CA3 regions of the dorsal hippocampus of the mouse brain at -2.18 mm from bregma (reproduced from Franklin and Paxinos, 1997). Figure B displays photomicrographs of DCX^+, $Ki67^+$, and double-labelled $DCX^+/Ki67^+$ cells in the subgranular zone of the dorsal hippocampus. Figures C and D show treatment (saline versus LPS) and surgery (intact versus gonadectomized) effects in mean $Ki67^+$ cell expression in the CA1 region of the dorsal hippocampus of adult (10-week-old) males and females, respectively. Figure D displays photomicrographs of treatment and surgery differences in Ki67 expression in the CA1 region of the dorsal hippocampus of adult male mice. *Note*: SAL = saline; SHAM = sham-surgery; ORX = orchiectomy; OVX = ovariectomy; * = $p < .05$, ** = $p < .01$, *** = $p < .001$.

trials of the MWM which suggests a more accurate search strategy among females. This finding reflects Jonasson's (2005) report of better performance of females compared to males in water maze tasks in mice but not rats. Furthermore, females made more spatial retention errors in both probe trials of the BM and showed poorer memory for the new platform location in the reversal probe of the MWM. Similar male advantages in reference learning are attributed to age and sex differences in motivational tendencies, task-dependent displays of anxiety, and spatial processing (e.g., search strategies and utilization of different extra-maze and intra-maze cues on different tasks) (Grissom et al., 2013; LaBuda et al., 2002; Lamberty and Gower, 1988; Mishima et al., 1986). Some of these sex-specific results on hippocampus-dependent tasks may be due to age-related sex differences in the organizational and activational effects of gonadal steroid hormones on hippocampal physiology and function (for review see Koss and Frick, 2016).

Contrary to our hypotheses, gonadal steroid hormones did not impact performance on both spatial tasks or regulate the cognitive effects of pubertal LPS exposure. Instead, gonadectomy during early adulthood impacted cognitive flexibility in the MWM later in adulthood. This task-specific finding may be due to the timing of the behavioral test. Several studies have demonstrated that the extent of OVX-induced impairments in adult rodents differ between hippocampus-dependent tasks (for review see Tuscher et al., 2015). The cognitive effects of castration among adult male rodents are more ambiguous but generally point towards task-dependent impairments in spatial working but not reference memory paradigms (for review see Celec et al., 2015). Gonadectomy-induced impairments in spatial learning during the reversed MWM paradigm were more prominent among female mice compared to male counterparts, which is in line with the aforementioned task-dependent cognitive deficits in castrated males. Therefore, spatial reference memory in females may be more reliant on circulating gonadal steroid hormone levels than in males.

Given the association between adult hippocampus-dependent memory processes and hippocampal neurogenesis, we also examined whether group differences in learning behaviors translated to expression of DCX^+ and $Ki67^+$ cells in the dorsal hippocampus. Neither pubertal immune challenge nor gonadectomy during early adulthood permanently affected basal expression and co-labelling of these markers in the SGZ in males and females. Although the literature has generally shown decreases in DCX^+ and $Ki67^+$ cells in the SGZ following gonadectomy, our findings may be explained by species differences and the timing of our surgery. For example, Lagace et al. (2007) found similar proliferation and neurogenesis in adult C57BL/6 mice, regardless of OVX and estrous stage, whereas others have found that significant sex differences in meadow voles are impacted by breeding season (Galea and McEwen, 1999). The lack of treatment effect in SGZ cellular proliferation also suggests that these treatment-induced effects may have recovered by the time of euthanasia. In fact, the current literature on stress-related changes in hippocampal neurogenesis points towards greater vulnerabilities and longer recoveries following chronic versus acute stressors in older ages compared to younger groups (for reviews see Hueston et al., 2017; Loi et al., 2014; Lucassen et al., 2015).

In contrast, our experimental manipulations induced sex-specific changes in cellular proliferation in the CA1, a region involved in detecting spatial novelty and episodic-like memory (Barbosa et al., 2012; Drieskens et al., 2017). Intact males exposed to LPS during puberty expressed more $Ki67^+$ cells in the CA1 than their saline-treated and castrated counterparts, whereas females were not significantly impacted. Although we did not examine the functional significance of these cellular changes, similar stress-induced neurogenic changes have been implicated in the expression of depressive-like and anxiety-like behaviours (for reviews see Loi et al., 2014; Lucassen et al., 2015). Future studies should also explore whether pubertal LPS treatment similarly impacts other aspects of hippocampal neurogenesis (e.g., integration, activation).

4.1. Conclusions

We conclude that pubertal immune challenge elicits similar learning deficits in adult males and females on low-stress hippocampus-dependent spatial memory tasks but impairs cognitive flexibility more in females. High-stress tasks, however, mask the cognitive effects of pubertal LPS exposure. These enduring cognitive deficits do not appear to be modulated by gonad-derived hormones or changes in basal neurogenesis. Pubertal males, nevertheless, are more sensitive than age-matched females to LPS-induced priming of basal CA1 cellular proliferation, whereas both sexes show resiliency towards LPS-induced changes in basal hippocampal neurogenesis. These novel findings provide invaluable insights into our growing understanding of the stress-sensitive pubertal period and is the first to examine sex differences in the enduring cognitive effects of pubertal immune challenge.

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Conflicts of interest

None.

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