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Early prenatal stress impact on coping strategies and learning performance is sex dependent

Bridget R. Mueller, Tracy L. Bale *

Department of Animal Biology, University of Pennsylvania, 201E Vet School, 3800 Spruce Street, Philadelphia, PA 19104, United States

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Abstract

Diseases involving cognitive disorders and maladaptive stress-coping behaviors including autism and schizophrenia are present in children born to mothers exposed to stress during pregnancy. To determine the gestational time window when stress exposure produces the greatest impact on cognition, dams were exposed to chronic variable stress (CVS) early, mid-, or late in gestation and offspring learning performance and navigation strategies assessed. These studies utilized a modified version of the Barnes maze to allow investigation of coping responses to stress stimuli. In our study, males exposed to early gestational stress showed significantly impaired learning performance, requiring twice as long to locate the target following training. In stark contrast, early prenatal stress enhanced female performance, where these females located the target in a quarter of the time required by controls. Differences in search strategies whether cued, random, or serial accounted for divergent performances between sex and CVS groups. While control males' behavior expectedly evolved to a cued strategy, the early stressed offspring continued to rely on serial and random searching. Surprisingly, in a long-term memory recall test 6 weeks following previous maze exposure, these early stressed offspring now located the target significantly faster than controls suggesting gestational effects of stress on memory retention that were specific to prenatal time window of stress exposure. Overall, these results provide important insight into the temporal specificity of the effects of prenatal CVS revealing a remarkable vulnerability during early development and a sexually dichotomous influence on cognitive abilities and stress-coping strategies.

Keywords: Pregnancy; Development; Barnes maze; Search Strategy

1. Introduction

Stress during pregnancy has been linked to an increased vulnerability for the development of cognitive and emotional disorders including autism and schizophrenia [1–3]. Animal studies have also determined that prenatal stress exposure produces sex-specific long-term alterations as examined in tests of spatial learning, object-recognition, delayed alteration, and passive avoidance conditioning [4–7]. However, the temporal specificity related to the timing of stress insult is not currently known. To elucidate factors that may increase susceptibility to complex diseases, our study aims to determine, in mice, the critical gestational time window when chronic variable stress (CVS) leads to sex-specific effects on coping strategies and learning performance.

The developing hippocampus and cortex are vulnerable targets for a stress-induced augmentation of the maternal hypothalamic–pituitary adrenal (HPA) stress axis [8–10]. Stressed offspring are often born with a reduced hippocampal volume and cerebral cortex asymmetry [8,10]. These morphological changes are often accompanied by disrupted long-term potentiation and long-term depression suggesting a functional long-term consequence of prenatal stress [11–14]. The hippocampus is normally enriched with glucocorticoid receptors, providing an inhibitory influence on the HPA stress axis [15–18]. Offspring born to dams restrained late in gestation have a reduction in glucocorticoid receptor expression and cortical dendritic arborization [9,19]. These structural and functional changes model those seen in brains of patients suffering from cognitive and emotional disorders and may play a role in disease development [20,21].

Animal studies have determined that aspects of such cognitive task disabilities in prenatally stressed offspring are sex-specific. While male offspring born to dams restrained late

* Corresponding author. Tel.: +1 215 898 1369; fax: +1 215 573 5187.
E-mail address: tbale@vet.upenn.edu (T.L. Bale).

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in gestation required more time to learn the location of the hidden platform in a Morris water maze and showed impairments in the radial arm maze, female performance remained unchanged or enhanced [4,20,22–24]. Detriments are not limited to hippocampal-dependent tasks. In a novel object-recognition test, male offspring exposed to unpredictable stress during development spent less time inspecting a novel object, indicating a possible learning deficit [7]. Alterations in learning performance may also involve the influence of emotional state and distinct stress-coping strategies required in these tests.

Strong connections link prenatal stress with the development of cognitive deficits, including autism and schizophrenia. Factors contributing to an increased male presentation of these diseases are not known. To determine the critical time period during development when a prenatal stress insult may produce disparate sex effects, we have compared Barnes maze performance of offspring exposed to CVS during early, mid-, and late gestation. The Barnes maze has been modified to involve the introduction of novel stressors throughout training to permit the assessment of both stress-coping abilities and learning performance.

2. Materials and methods

2.1. Animals

Mice used in these studies were on a mixed C57Bl/6:129 background. Virgin female mice ($n=18$) were mated at 6–8 weeks of age. Presence of a copulation plug denoted gestation day 1. The pregnant female was individually housed, given a cotton nestlet, and assigned to a stress treatment group (see below).

At birth, litters were culled to 8 pups and litters containing fewer than 6 pups were excluded from analysis. During postnatal week 4 (PN 25–PN 27) pups were weaned, group housed with same-sex littermates, and ear-tagged to provide a method of permanent identification. At 6 weeks of age, 1–2 males and 1–2 females per litter were trained on our modified Barnes maze. The performance of same-sex siblings was averaged to avoid litter effects (male control=5 animals/4 litters, female control=4 animals/4 litters, male early stress=8 animals/6 litters, female early stress=7 animals/6 litters, male mid-stress=6 animals/4 litters, female mid-stress=6 animals/4 litters, male late stress=7 animals/3 litters, female late stress=6 animals/4 litters). Females were left intact, and were not cycled.

All mice were housed under a 12-h light/dark cycle (lights on at 7:00 am) with ambient temperature of 22 °C, and relative humidity of 42%. Food (Purina Rodent Chow; 28.1% protein, 59.8% carbohydrate, 12.1% fat) and water was provided throughout the study ad libitum. All studies were done according to experimental protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

2.2. Prenatal stress paradigm

Administration of chronic, variable stress (CVS) was performed as previously described [25]. Briefly, dams were randomly assigned to treatment groups to receive stress during one of the three weeks, or to a control non-stressed group. Pregnant mice assigned to the stress groups experienced a different stressor on each of 7 days during early (days 1–7), mid (days 8–14), or late (days 15–21) gestation. CVS included: 36 h of constant light, 1 h of fox odor (1000–1100 h), novel object (marbles) exposure overnight, 5 min restraint stress (1000–1100 h), novel noise (White Noise/Nature Sound-Sleep Machine ®, Brookstone) overnight, multiple cage changes throughout the light cycle, and saturated bedding (700 mL, 23 °C water) overnight. These mild stressors were selected to be non-habituating and for not inducing pain or directly influencing maternal food intake or weight gain [25].

2.3. Modified Barnes circular maze

The Barnes maze has been previously validated as a useful test to examine learning behaviors and strategies [26]. The maze consisted of a black circular disc (90 cm in diameter) with 24 holes (5 cm in diameter) around the perimeter. An escape box (15 cm × 8 cm × 7 cm) was located under one of the holes. The location of the escape box remained constant throughout training and testing. A ramp was positioned at 45°, to permit a more gradual descent into the escape hole. The disc was elevated 70 cm above the floor and situated in a room with white walls. Visual cues on each of 3 separate walls a black and white checkerboard, two red circles, and blue and white stripes. All cues were (55.8 × 71.1 cm). Each cue was positioned 80 cm above the floor and 15 cm from perimeter of maze.

2.3.1. Training

All mice were trained 2 trials/day for 3 days. Trials within each day were separated by 4 h. A video camera was used to record the

Table 1
Effect of prenatal CVS, sex, trial on performance during training

Parameter	Prenatal CVS	Offspring sex	Sex × CVS	Trial	Trial × CVS	Trial × sex	Trial × sex × CVS
Latency to 1st hole	$F_{(3,48)}=1.2$	$F_{(1,48)}=0.6$	$F_{(3,46)}=0.5$	$F_{(5,240)}=2.5^*$	$F_{(14,249)}=1.4$	$F_{(4,240)}=0.4$	$F_{(12,240)}=0.8$
ΔDT	$F_{(3,46)}=3.4^*$	$F_{(1,46)}=2.6$	$F_{(3,46)}=4.0^*$	$F_{(5,184)}=0.8$	$F_{(14,184)}=1.1$	$F_{(3,184)}=0.3$	$F_{(12,188)}=0.9$
Error (before target)	$F_{(3,41)}=4.1^*$	$F_{(1,48)}=0.1$	$F_{(3,48)}=0.5$	$F_{(4,240)}=2.6^*$	$F_{(14,164)}=0.2$	$F_{(4,164)}=0.9$	$F_{(12,164)}=1.1$
Latency to target	$F_{(3,30)}=0.8$	$F_{(1,78)}=1.1$	$F_{(3,80)}=1.3$	$F_{(4,110)}=6.9^*$	$F_{(14,249)}=2.5^*$	$F_{(4,249)}=0.1$	$F_{(14,249)}=1.3$
Error (post-target)	$F_{(3,23)}=1.4$	$F_{(1,23)}=0.1$	$F_{(3,46)}=3.7^*$	$F_{(4,69)}=0.4$	$F_{(14,69)}=2.5^*$	$F_{(4,69)}=0.4$	$F_{(12,69)}=1.2$
ΔTC	$F_{(3,80)}=0.8$	$F_{(1,78)}=1.1$	$F_{(3,80)}=1.3$	$F_{(4,72)}=0.9$	$F_{(14,72)}=0.8$	$F_{(4,72)}=0.1$	$F_{(12,72)}=1.1$
Success	$F_{(3,55)}=0.1$	$F_{(1,55)}=0.1$	$F_{(3,46)}=2.6$	$F_{(5,240)}=9.9^*$	$F_{(14,249)}=1.4$	$F_{(4,240)}=0.4$	$F_{(15,275)}=1.7^*$

*$P<0.05$.

behavior of mice during all trials and tests. To address the complex intersection between learning and motivation that occurs in any repeated task, a mild novel stimuli was introduced daily during training. During trials 1 and 2, a bright light (400 lx) was placed above the maze (positioned 35 cm above maze). During trials 3 and 4, an 8-inch Holmes® fan on the medium setting was added (positioned 35 cm above maze). During trials 5 and 6, a novel noise (100 dB positioned 35 cm above center of maze) was added. To each trial, the mouse was placed under a glass beaker in the center of the maze for 15 s prior to trial start.

Parameters scored for each trial and test included: latency to the first hole, distance of first searched hole from target escape box (ΔDT), errors obtained and time spent locating the target, errors obtained after the target was located, time interval between finding the target hole and climbing into the box (ΔTC), search strategy, and success, defined as entering the target box by the end of the trial. An error was defined as an incorrect nose-poke. ΔDT was calculated by counting the number of holes between the first hole searched and the target escape box.

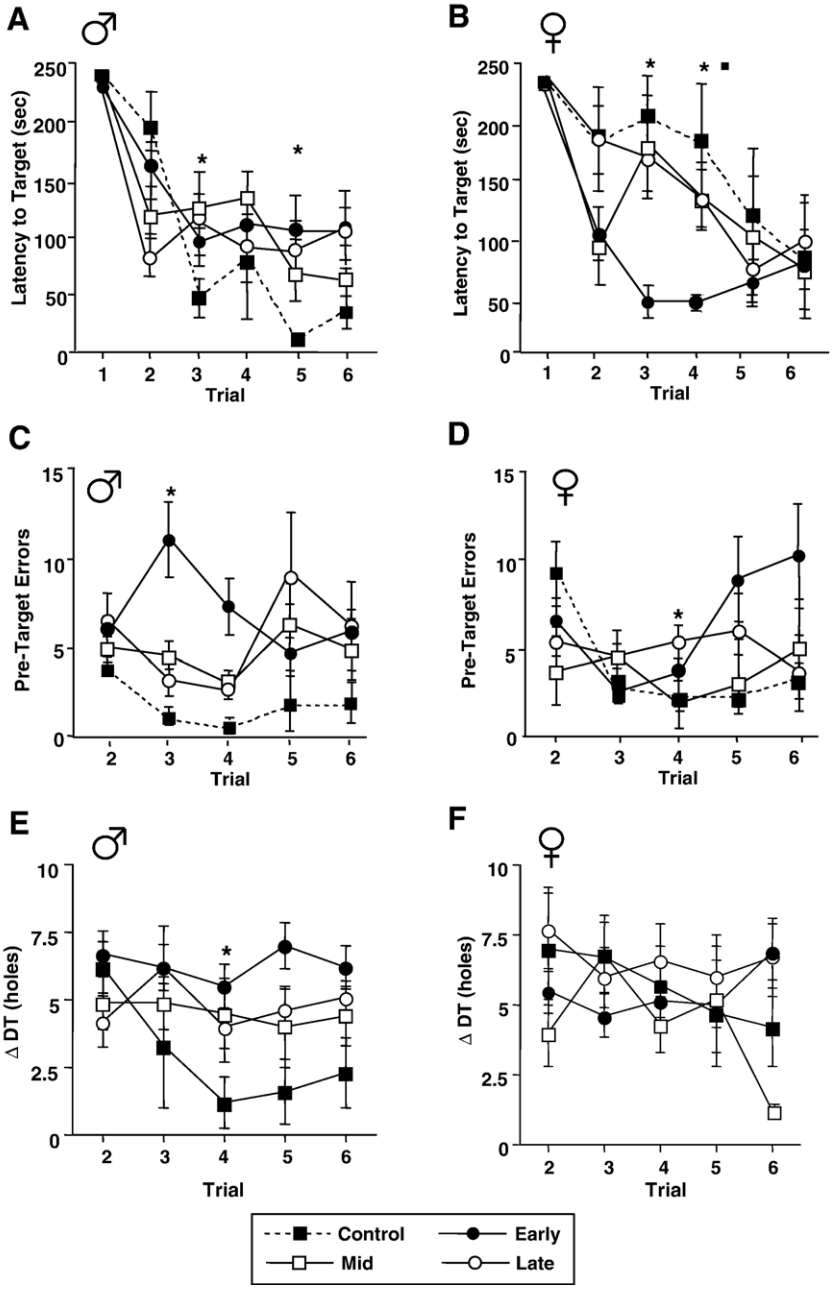


Fig. 1. Prenatal CVS produces sex-dependent and temporal specific effects on Barnes maze performance. Latency to locate target escape box (A, B), errors obtained prior to target box location (C, D), and the number of holes between the first searched hole and target escape (ΔDT) (E, F) for male (A, C, E) and female (B, D, F) offspring exposed to prenatal CVS early, mid, or late in gestation. Male offspring stressed early in gestation took longer to learn target location, obtained significantly more pre-target errors, and began searching at a greater distance from the target escape box when compared to control males *, $P<0.01$). In contrast, females stressed early in gestation learned the task faster than controls with a reduced latency (*, $P<0.01$). Data are mean±SEM.

Table 2A
Percentage of male CVS group successful in climbing into target box

CVS group	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Test trial 7	Stress test	Recall test
Control	0	40	100	75	100	100	100	100	50
Early	0	29	29	86	42	57	71	71	57
Mid-	0	29	29	86	86	100	86	86	71
Late	0	33	50	50	60	83	83	83	60

As previously described, search strategies were classified as random, serial, or cued [27]. Briefly, the random search strategy was defined as a search separated by maze center crosses and >1 alterations in direction. The serial search strategy was defined as a systematic search of consecutive holes in a clockwise or counterclockwise direction with ≤1 alteration in direction. The cued search strategy was defined as navigating directly to the target escape with error and distance scores ≤2. The trial terminated when the mouse entered the target escape box or after 4 min had elapsed. If the mouse did not successfully locate the target box, the investigator guided the mouse to the target location. The stress stimuli were deactivated when the mouse entered the target box. The mouse was allowed to remain in the escape box for 30 s prior to transfer back to the home cage. The entire apparatus was thoroughly cleaned with water and dried between each mouse.

2.3.2. Test trial 7

As a final assessment of task acquisition, offspring were examined on the Barnes maze 24 h following trial 6 (1400–1600 h). Conditions remained identical to those of the final training trial 6 and parameters described above for training were measured during testing.

2.3.3. Stress test

Twenty-four hours following trial 7, we assessed how prenatal CVS affected maze performance immediately following a novel acute stress exposure. Mice were placed in a cage containing a cotton swab of predator odor (diluted 1:10,000, Arco Organics) for 5 min. To prevent contact with chemical, the cotton swab was taped to the interior of the cage top. Following a 5 min recovery in a new clean cage, the mouse was tested on the maze as described.

2.3.4. Locomotion

During test trial 7 and the stress test, the distance traveled and speed of each mouse was recorded using the ANY-maze™ automated tracking system (Stoelting Co, Wood Dale, Illinois, USA).

2.3.5. Recall test

To examine prenatal stress effects on long-term memory performance, 6 weeks following the last exposure to the maze, mice were again tested. Conditions were identical to those of acquisition test exposure (including bright light, fan, and noise, 1400–1600 h).

2.4. Data analysis

For each parameter, data were analyzed using a two-way ANOVA for sex and prenatal CVS, with trial as a repeated-measures factor. Significance was set at $P \leq 0.05$. Bonferroni multiple comparisons test determined post hoc significance when appropriate. A nominal logistic followed by a Pearson's Chi-square test determined significant differences in search strategy. A Fisher's r to z was used to determine whether a correlation coefficient was statistically different from zero. All statistical analysis was performed using StatView SE+(Abacus Concepts, Berkeley, CA).

3. Results

3.1. Training

The effect of trial on measures of maze learning was assessed to verify that training led to improved performance (Table 1). There was a significant effect of trial on time needed to locate the target box (Fig. 1A and B, Table 1), pre-target errors obtained (Fig. 1C and D, Table 1), and success (Tables 1, 2A and 2B). Distance of first searched hole from the target (ΔDT, Fig. 1E and F) latency to locate target, errors, and success remained correlated throughout training and testing ($0.23 \leq r \geq 0.67$, $P < 0.05$). Prenatal chronic variable stress (CVS) or sex did not affect latency to search first hole (Table 1).

To assess the effect of prenatal stress on task acquisition, time required to locate target, errors, and success were compared across prenatal CVS groups. A significant interaction between trial and prenatal CVS affected time required to locate the target (Fig. 1A and B, Table 1). An interaction between trial, prenatal CVS, and sex affected success (Tables 1, 2A and 2B). In addition, there was a main effect of prenatal CVS on errors obtained before locating the target (Fig. 1C and D, Table 1).

When compared to control males, males exposed to prenatal CVS showed an impaired performance, as measured by latency to target, pre-target errors, and success. The impairment in performance was most pronounced in males stressed early in gestation. During trial 3, early stressed males required nearly twice as long to locate the target, and made significantly more pre-target errors than control males ($P < 0.01$, Fig. 1A and C). At the end of training, the latency to locate the target differed three-fold between these groups ($P < 0.01$, Fig. 1A). Early stressed males also exhibited diminished success during training (Tables 1 and 2A).

The timing of prenatal CVS also influenced the performance of female offspring. As seen in males, CVS early in gestation

Table 2B
Percentage of female CVS group successful in climbing into target box

CVS Group	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Test trial 7	Stress test	Recall test
Control	0	25	25	75	25	50	75	100	25
Early	0	57	86	86	86	86	71	100	83
Mid-	0	33	50	67	33	83	67	67	50
Late	0	33	33	83	100	67	83	83	0

produced the greatest impact on female maze performance. However, early stressed females were faster than control females (Fig. 1B). Specifically, during trials 3 and 4, early stressed females located the target in 1/4 the time control females required ($P<0.01$, Fig. 1B). The number of pre-target errors obtained by females stressed early and late in gestation was significantly higher during trial 4 ($P<0.01$, Fig. 1D).

3.1.1. Distance of first investigated hole from the target box

The distance of the first hole investigated by the mouse from the target escape box (ΔDT) was compared between sexes and CVS groups over the course of training to assess initial accuracy in locating the target. There was an effect of prenatal CVS ($F_{(3,46)}=3.4$, $P<0.05$) and an interaction between sex and prenatal CVS ($F_{(3,46)}=4.0$, $P<0.05$) on initial distance from the target. During trial 4, the first hole investigated by male controls was significantly closer to the target escape hole than males stressed early in gestation and female controls ($P<0.01$, Fig. 1E and F).

3.1.2. Time to climb into the target hole

The difference between time to locate the target and time to climb into the target box (ΔTC) was compared between sexes and CVS groups over the course of training. There was no effect of trial, prenatal CVS, or sex on ΔTC (Fig. 2A and B, Table 1).

3.1.3. Post-target errors

Additional errors obtained following locating the target were also measured. There was an interaction between sex and prenatal CVS on post-target errors ($F_{(3,46)}=3.7$, $P<0.05$) with males making more errors than females (Fig. 2C and D). Specifically, males stressed early in gestation obtained significantly more post-target errors than control males and females of the same stress treatment ($P<0.01$).

3.1.4. Test trial 7

Prenatal CVS and sex affected latency to target ($F_{(3/50)}=3.18$, $P<0.05$, Fig. 3A and B) and pre-target errors ($F_{(3/55)}=3.3$,

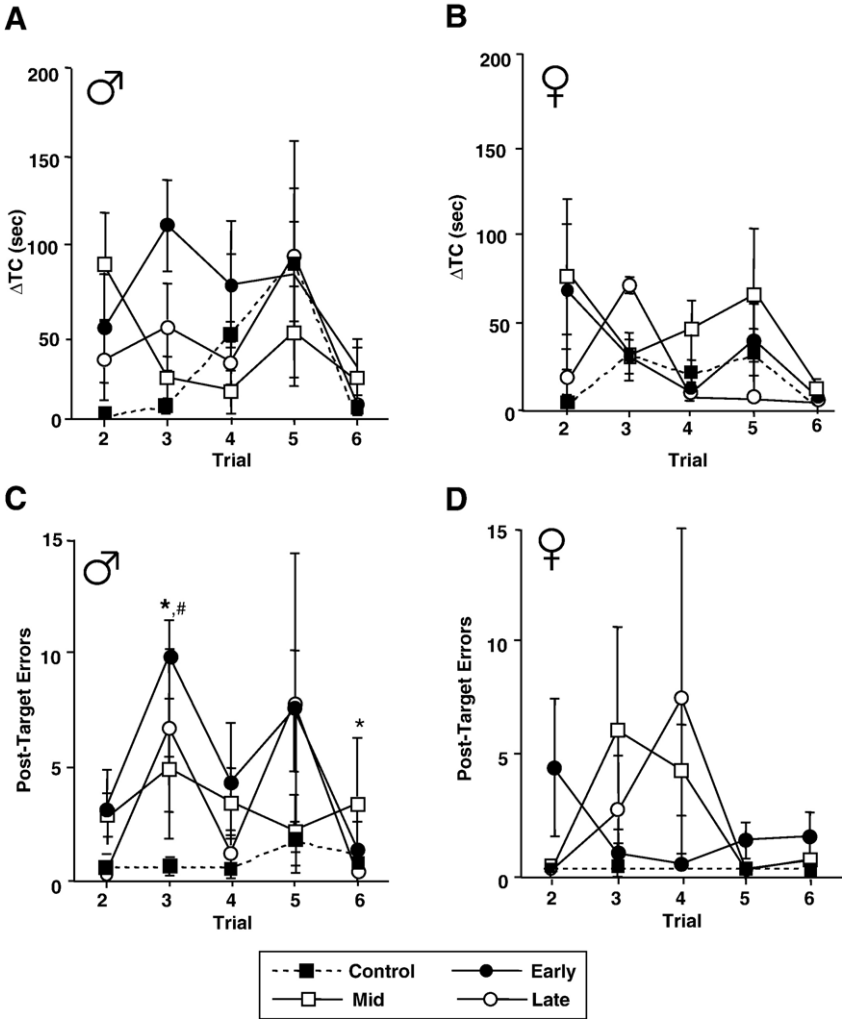


Fig. 2. Prenatal CVS produces sex-dependent and temporal specific effects on post-target behavior. The time interval between locating the target box and climbing into the target box (ΔTC) for (A) male and (B) female offspring exposed to prenatal CVS early, mid, or late in gestation. ΔTC was not affected by prenatal CVS treatment or sex. Post-target errors obtained by males (C) and females (D) were significantly elevated for males stressed early in gestation (*, $P<0.01$ significantly different from control males). Data are mean±SEM.

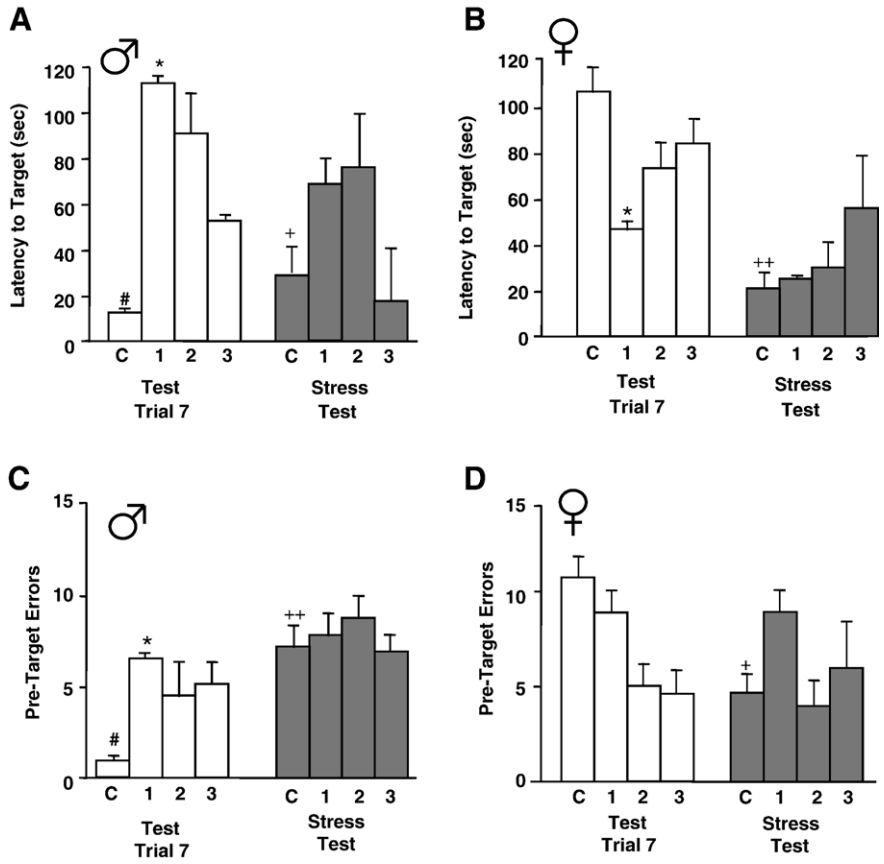


Fig. 3. Acute stress exposure prior to testing exerts sex and temporal specific influence on task performance. Latency to target (A, B) and pre-target errors (C, D) for male (A, C) and female (B, D) CVS offspring following an acute stress exposure of predator odor (stress test) compared to the last training trial (test trial 7). Offspring born to unstressed dams (control, C), or dams exposed to prenatal CVS during early (1st week, 1), mid (2nd week, 2), or late (3rd week, 3) gestation were compared to assess acquisition (test trial 7) with performance following an acute predator odor exposure (stress test). For males, latency to target and pre-target errors were significantly elevated for early stressed offspring (*, $P<0.01$). Male controls required significantly more time to locate the target ($^{+}$, $P<0.05$) and obtained significantly more errors ($^{++}$, $P<0.01$) when compared to test trial 7 performance. In addition, a sex difference in latency to target and pre-target errors was found on test trial 7 ($^{\#}$, $P<0.01$). Early stressed females required less time to locate the target on test trial 7 (*, $P<0.05$). Female controls required significantly less time to locate the target ($^{++}$, $P<0.01$) and obtained significantly fewer errors ($^{+}$, $P<0.05$) when compared to test trial 7 performance. Following predator odor stressor, CVS treatment or sex did not affect latency to target. Data are mean±SEM.

$P<0.05$, Fig. 3C and D). Males stressed early in gestation required significantly more time to locate the target than control males ($P<0.01$, Fig. 3A), while females stressed early in gestation found the target in significantly less time than control females ($P<0.01$, Fig. 3B). Males stressed early in gestation also made significantly more pre-target errors than control males ($P<0.01$, Fig. 3C) before locating the target. Although prenatal CVS did not significantly impact female errors ($P>0.05$, Fig. 3D), control females took significantly longer to locate the target and obtained more errors compared to control males ($P<0.01$, Fig. 3).

3.1.5. Stress test

As prenatal stress has been shown to alter stress-related coping behaviors [1], we exposed offspring to predator odor. Following 5 min of recovery, performance on the Barnes maze was assessed. There was no effect of prenatal CVS or sex on the latency to locate target ($F_{(3,51,\ \text{treatment})}=0.8$, $P>0.05$; $F_{(1,\ 51,\ \text{sex})}=0.3$, $P>0.05$) or pre-target errors ($F_{(3,51,\ \text{treatment})}=0.7$, $P>0.05$; $F_{(1,\ 51,\ \text{sex})}=0.5$, $P>0.05$, Fig. 3) during the stress test.

3.1.6. Test trial 7 v. stress test

A comparison between test trial 7 and the stress test revealed a significant interaction between test day and offspring sex on latency to target ($F_{(3,101)}=4.1$, $P<0.05$) and pre-target errors ($F_{(3,102)}=3.9$, $P<0.05$, Fig. 3). In addition, prenatal CVS affected latency to target and pre-target errors ($F_{(3,101)}=4.8$, $P<0.05$, Fig. 3). Compared to test trial 7, control males required more time to locate the target and obtained more errors following acute predator odor exposure ($P<0.01$, Fig. 3A and C). This decrease in control performance eliminated the disparity between prenatal CVS groups found during training and test trial 7. For female controls, following acute predator odor exposure, performance improved. Control females required less time to locate the target during the stress test when compared to test trial 7, eliminating the disparity in

performance between prenatal CVS groups present during training and test trial 7 ($P<0.01$, Fig. 3B and D).

3.1.7. Locomotion

As performance on the Barnes maze is dependent on locomotor ability, we measured total distance traveled and speed prior to reaching the target during test trial 7 and the stress test. Prenatal CVS did not significantly impact distance traveled ($F_{(3,12,\ \text{test trial 7})}=1.2$, $P>0.05$; $F_{(3,12,\ \text{stress test})}=1.3$, $P>0.05$, Fig. 4A and B), or speed ($F_{(3,12,\ \text{test trial 7})}=1.9$, $P>0.05$; $F_{(3,12,\ \text{stress test})}=1.1$, $P>0.05$, Fig. 4C and D) during the stress test. Sex affected distance traveled during test trial 7, with females traveling a longer distance than males before reaching the target ($F_{(1,12)}=7.2$, $P<0.05$). There was no effect of sex on distance traveled in the stress test ($F_{(3,12,\ \text{stress test})}=1.8$, $P>0.05$, Fig. 4A and B). Further, speed did not vary between sexes during test trial 7 ($F_{(1,12)}=0.9$, $P>0.05$) or the stress test ($F_{(1,12)}=0.6$, $P>0.05$, Fig. 4C and D).

3.2. Recall test

To examine the effect of prenatal stress on long-term memory retention and performance, offspring were examined on the maze 6 weeks following the last test. The gestational timing of prenatal CVS affected latency to target ($F_{(3/33)}=2.9$, $P<0.05$, Fig. 5A). Early stress groups of both sexes required less time to locate the target ($P<0.01$, Fig. 5A). Although there was not a significant effect of prenatal CVS on errors obtained in this test, males stressed early in gestation appeared to make fewer errors than control males ($P=0.07$, Fig 5B).

3.3. Search strategy

To investigate if differences in navigation strategy accounted for performance disparities, we analyzed strategy utilized to locate the hidden target. Overall, search strategy was significantly correlated with latency to locate the target, errors obtained prior to reaching the target, and success ($0.32 \leq r \geq 0.87$, $P<0.01$). Further, a significant interaction between training and prenatal CVS ($\chi^2=12.6$, $P<0.05$, Fig. 6) and training and sex ($\chi^2=119.5$, $P<0.05$, Fig. 6) affected strategy used.

At the start of training, prenatal CVS (Day 1 $\chi^2=9.5$, $P>0.5$) and sex (Day 1 $\chi^2=9.5$, $P>0.5$) did not affect search strategy. However, on trials 3–7, early stressed males employed random and serial searching while controls used a cued strategy

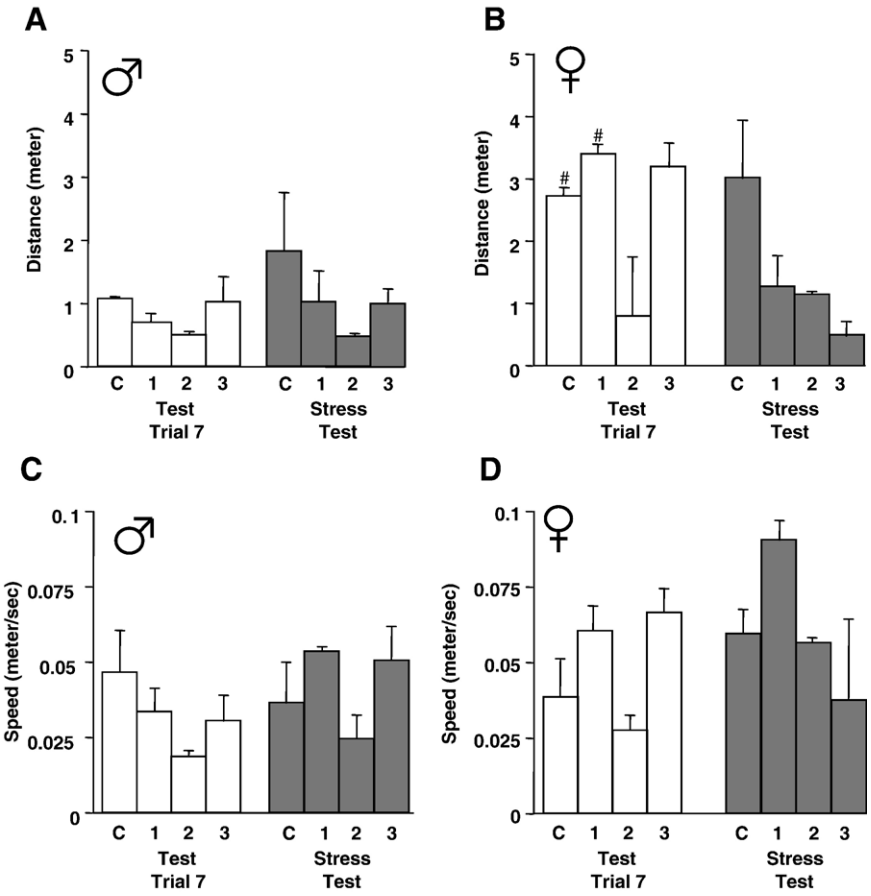


Fig. 4. Prenatal CVS produces sex-dependent and temporal specific effects on location of first searched hole. Distance traveled (A, B) and speed (C, D) for male (A, C) and female (B, D) CVS offspring during the last training trial (test trial 7) and following an acute stress exposure of predator odor (stress test). Ambulation of offspring born to unstressed dams (control, C), or dams exposed to prenatal CVS during early (1st week, 1), mid (2nd week, 2), or late (3rd week, 3) gestation was compared to assess potential effects of prenatal CVS on locomotion. For females, distance traveled was significantly elevated for early and mid stressed offspring compared to male siblings ($^{\#}$, $P<0.01$). CVS treatment or sex did not affect speed. Data are mean ± SEM.

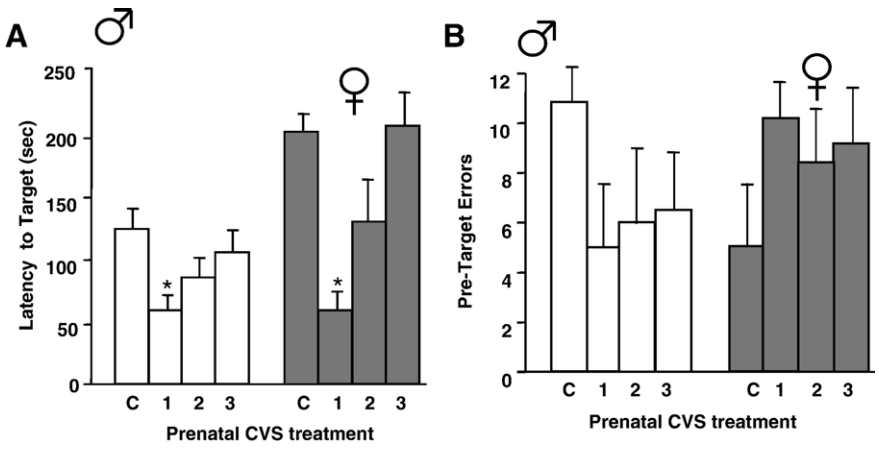


Fig. 5. Prenatal CVS influence on long-term memory recall is dependent on gestational timing of insult. Latency to target (A) and pre-target errors (B) were compared across prenatal CVS groups 6 weeks following last maze exposure to assess long-term recall. Latency to the target box was significantly reduced in offspring stressed early in gestation (*, $P<0.05$). Compared to previous maze performance, early stressed males and females showed an enhanced long-term memory. There were no significant effects of prenatal CVS ($p>0.05$) or sex ($p>0.05$) on pre-target errors during the long-term recall. Data are mean±SEM.

(Day 2 $\chi^2=6.2$, Day 3 $\chi^2=6.6$, Test Day 1 $\chi^2=10.6$, $P<0.05$, Fig. 6A and C). Stress during mid gestation did not alter search strategy (Fig. 6E). Males exposed to stress late in development required an additional day of training prior to employing a cued navigation (Day 3 $\chi^2=7.9$, $P<0.05$, Fig. 6G). Although prenatal CVS did not significantly influence female search strategy (Fig. 6B,D,F,H), a significant difference between male and female control groups was evident throughout training (Day 2 $\chi^2=6.6$, $P<0.05$ Fig. 6A and B).

4. Discussion

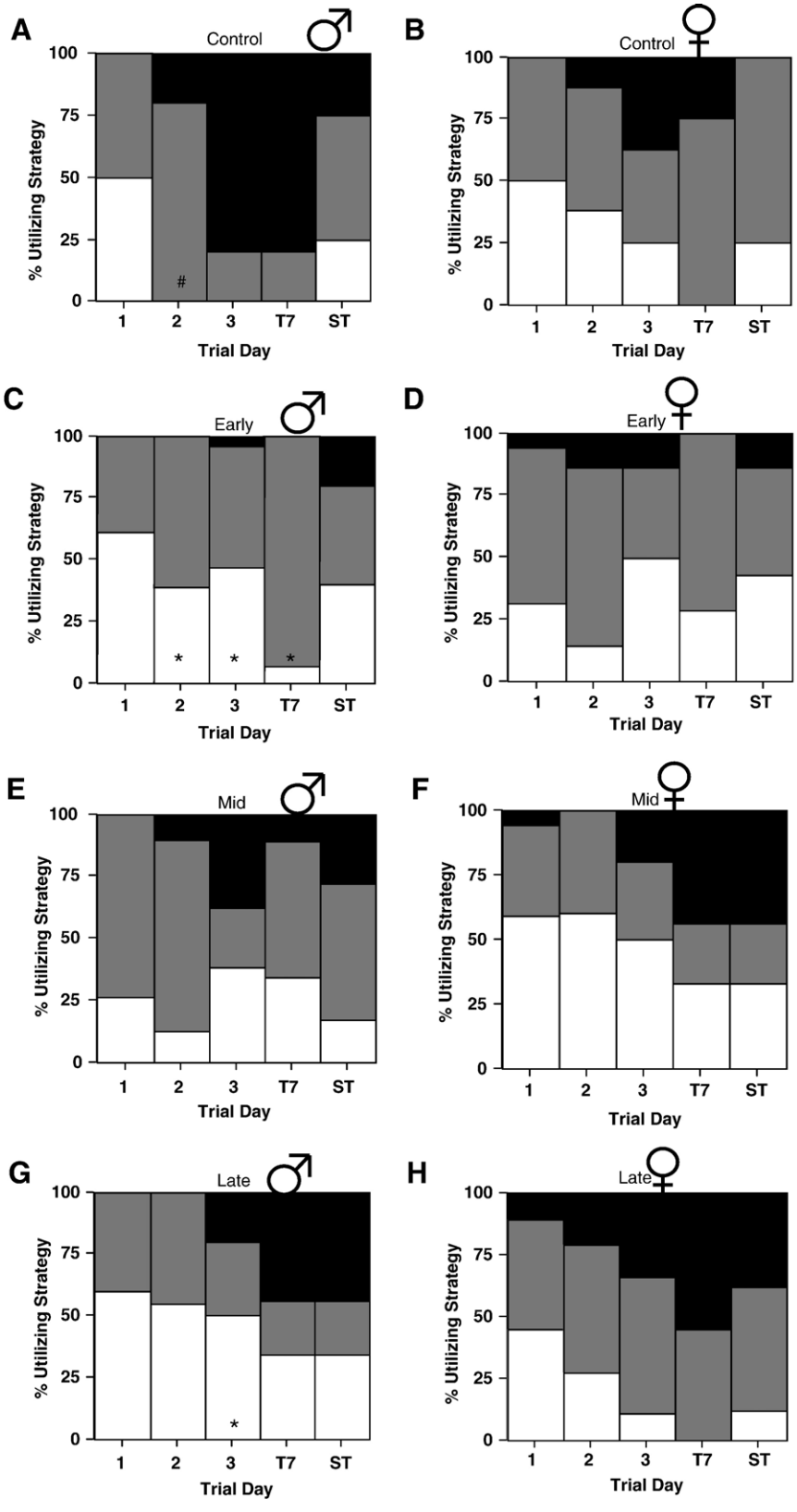
Growing evidence links perturbations of the intrauterine environment, especially stress, with the development of those diseases with affected cognitive function and stress-coping strategies including autism and schizophrenia [7,8,14,28]. The mechanism underlying the increased male presentation of these diseases is not understood. As studies that report sex-specific effects of prenatal stress have largely focused on the last week, little is known regarding the critical timing when a prenatal stress insult may produce disparate sex effects and ultimately influence disease predisposition.

In order to determine the temporal specificity of prenatal stress on cognitive abilities and coping strategies, offspring born to dams exposed to chronic variable stress (CVS) early, mid, or late in pregnancy were trained and tested on a modified Barnes maze. Our findings reveal a sexually dichotomous impact of early CVS on task acquisition, short-term memory, and long-term memory recall supporting our hypothesis that the timing of prenatal insult influences the ultimate offspring impact and may contribute to the development of sex-biased disorders.

In order to permit assessment of stress-coping behaviors as well as learning performance, the Barnes maze was modified to involve the introduction of mild novel stressors throughout training. In our examination of the temporal specificity of prenatal CVS in this test, males showed the greatest deficit when stressed early in gestation. Midway through training, control males required less than 60 s to locate the trained target, while males exposed to CVS early in gestation required nearly twice as long. Supportive of this paradigm as a learning and memory task, control males continued to improve over the course of training by reducing the time required to locate the target box. In contrast, early stressed males reached a plateau in their latency to locate the target, never reaching the efficiency of the control group. This early stressed group also obtained more errors prior to locating the target compared to control males. Specifically, there was a sharp increase in errors during trial 3 of training. This may be related to the introduction of the additional novel stimulus (fan) during this trial, and suggests that stress experienced early in gestation may elevate 'sensitivity' to novelty or change in the environment. Throughout training and testing, offspring locomotion was evaluated in each trial by comparing latency to first hole. No differences in this measure were detected between CVS treatment groups or sexes for any of the trials. In addition, we measured total distance traveled and speed prior to reaching the target during test trial 7 and the stress test. Distance traveled and speed was not affected by predator odor exposure. Prenatal CVS did not significantly impact distance traveled or speed of movement during test trial 7 and the stress test indicating that differences between CVS treatment groups in latency to locate the target escape box do not result from disparate ambulation.

Fig. 6. Navigation search strategy utilized during training and testing is dependent on sex and gestational timing of prenatal CVS exposure. Percentage of CVS group utilizing random, serial, or cued navigation to locate the target for male (A, C, E, G) and female (B, D, F, H) offspring over the course of training and testing (days 1, 2, 3, test trial 7, T7, and stress test, ST). A random search strategy was defined as a search separated by maze center crosses and >1 alteration in direction. The serial search strategy was defined as a search of consecutive holes with ≤1 alteration in direction. The cued search strategy was defined as navigating directly to the target escape with error and distance scores ≤2. Search strategy employed by male controls significantly differed from the strategy used by females ($^{\#}$, $P<0.05$). Search strategy employed by early stressed male offspring significantly differed from control males on training days 2 and 3 and on T7 (*, $P<0.05$). Late stressed males significantly differed from controls on training day 3 (*, $P<0.05$).

Supporting our hypothesis, these results reveal significant effects of sex on the temporal specific impact of prenatal CVS on task acquisition. As observed in males, CVS early in gestation produced the greatest impact on female learning performance. However, a divergent picture emerged in the early stressed female offspring related to the direction of effect on



learning performance. Early stressed females performed better than control females, locating the target in significantly less time during training. These results are consistent with studies showing a sexually dichotomous effect of prenatal stress on cognitive abilities, and support the standard sex difference observed in numerous spatial tasks [4,29,30]. Interestingly, the decreased latency to locate the target did not correlate with a decrease in errors during training, suggesting females may be using a distinct exploration strategy.

As a potential factor underlying the divergence in learning performance between CVS groups, the search strategy utilized throughout training was compared. Navigation strategy, whether random, serial, or cued, was predictive of latency to locate target and successful target entry. Initially, as would be predicted, all offspring utilized random or serial searching. Over the course of training, control males shifted to a cued strategy that resulted in a shorter latency to target, fewer errors, and improved success. In contrast, throughout training the early stressed males persisted with random and serial search strategies that resulted in a longer latency to locate the target, increased errors, and diminished success.

Consistent with the sex differences found in learning performance, male and female offspring also appeared to utilize distinct strategies in target location. While control males shifted to a more cued strategy, control females relied primarily on serial searching throughout training. These results are again validation of our modified maze as a learning paradigm as previous studies support these sex-specific differences in strategy [31]. While it is not currently known why male and female offspring respond so differently to the same prenatal stressors, it is possible that different rates of development may contribute to these effects [32]. Gonadal hormones may also contribute to sex-specific effects of prenatal stress. In women, higher testosterone and lower estradiol levels are associated with improved performance in male-biased spatial tasks [33]. Surprisingly, the improved performance of the early stress females did not correlate with a shift to a more male-like cued strategy. However, females stressed late in gestation did show a pattern of cued navigation, supporting previous studies where prenatal CVS late in gestation masculinized the brain, ameliorating standard neuroendocrine and behavioral–sex differences [4]. Such temporal specific effects of prenatal CVS may be related to the fact that the critical organizational influence of androgens on the brain occurs late in development [34,35].

Post-target errors and the time difference between locating the target escape box and climbing into the box (ΔTC) were measured to assess hesitancy to enter the target. While there was no effect of CVS treatment or sex on ΔTC, sex and CVS interacted to affect post-target errors. Early stressed male offspring showed a sharp increase in post-target errors when the novel stimuli were first introduced in training (trials 3 and 5), again supportive of a greater sensitivity to novelty in this CVS group. High variability in the post-target errors obtained by females during training may be related to estrous cycle variation. Future studies may further explore differences in ΔTC utilizing anxiolytic drug treatment to determine if ΔTC and post-target errors are related to altered anxiety-like behaviors.

In order to examine the interaction between prenatal CVS and acute stress recovery on maze performance, offspring were exposed to an acute novel predator odor stress immediately prior to maze testing. Surprisingly, while controls showed a predicted response to this stimulus, the exposure did not further influence the performance of prenatal CVS offspring of either sex. During this stress test, performance of controls was directionally altered to resemble that of their same-sex prenatal CVS cohorts. The acute predator odor exposure impaired male, but enhanced female performance on the Barnes maze, supporting previous reports revealing a sex-specific effect of acute stress on cognition [36,37]. Compared to test trial 7, male controls required significantly more time and obtained more errors before locating the target following the acute stress exposure. This impaired performance in males correlated with a shift from cued navigation back to serial and random searching. Although control females found the target significantly faster during this stress test, errors obtained prior to locating the target remained consistent. Differences in performance between trial 7 and stress test may be a reflection of the additional maze experience.

In addition to the impact of prenatal stress on task acquisition, there is evidence for its effect on long-term memory [38]. To evaluate the temporal specificity of prenatal CVS on long-term memory recall, offspring were tested on the maze 6 weeks following the initial training period. Surprisingly, there was a dramatic reversal in the performance of the early CVS groups. Early stressed males previously found to be impaired during training and initial testing, now located the target significantly faster than male controls. This decrease in time also correlated with a reduction in errors obtained before locating the target. Evidence suggests that aspects of spatial-related learning and memory are subserved by distinct limbic–cortical–striatal pathways, indicating enhanced long-term memory may arise as a result of the specific brain regions affected by early prenatal stress [39]. Given that the acute stress test was the last maze experience prior to recall, differences in consolidation of emotional memory may also have contributed to the enhanced long-term memory detected in early stressed males [40]. Further, as all offspring were 6 weeks older at the long-term memory recall test, we cannot rule out the influence of increased age [20]. Female performance during this long-term recall did not differ from training and short-term testing, again supporting a disparate sex effect of CVS on memory consolidation.

In summary, the present study reveals a sexually dichotomous and temporal specific effect of prenatal CVS on task acquisition and long-term memory recall. As males present with higher rates of stress-linked diseases such as autism and schizophrenia, these findings may be relevant to understanding disease etiology [41–43]. While previous work has demonstrated sex-specific effects of prenatal stress on cognition, our results reveal important new insight into early gestation as a critical time period when offspring may be vulnerable to intrauterine perturbations such as stress.

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