Chronic Unpredictable Intermittent Restraint Stress

Disrupts Hippocampal-dependent Spatial Memory in Male, but not Female Rats

by

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ABSTRACT

The present series of studies examined whether a novel implementation of an intermittent restraint (IR) chronic stress paradigm could be used to investigate hippocampal-dependent spatial ability in both sexes. In experiments 1 and 2, Sprague-Dawley male rats were used to identify the optimal IR parameters to assess spatial ability. For IR, rats were restrained for 2 or 6hrs/day (IR2, IR6, respectively) for five days and then given two days off, a process that was repeated for three weeks and compared to rats restrained for 6hrs/d for each day (DR6) and non-stressed controls (CON). Spatial memory was tested on the radial arm water maze (RAWM), object placement (OP), novel object recognition (NOR) and Y-maze. The results for the first two experiments revealed that IR6, but not IR2, was effective in impairing spatial memory in male rats and that task order impacted performance. In experiment 3, an extended IR paradigm for six weeks was implemented before spatial memory testing commenced in male and female rats (IR-M, IR-F). Unexpectedly, an extended IR paradigm failed to impair spatial memory in either males or females, suggesting that when extended, the IR paradigm may have become predictable. In experiment 4, an unpredictable IR (UIR) paradigm was implemented, in which restraint duration (30 or 60-min) combined with orbital shaking, time of day, and the days off from UIR were varied. UIR impaired spatial memory in males, but not females. Together with other reports, these findings support the interpretation that chronic stress negatively impairs hippocampal-dependent function in males, but not females, and that females appear to be resilient to spatial memory deficits in the face of chronic stress.

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Major Depressive Disorder (MDD) affects more than 300 million people worldwide and is the leading cause of global disability, making MDD a common and serious psychiatric condition (World Health Organization, WHO, 2017). MDD symptoms include, markedly diminished interest or pleasure, significant weight loss/gain, insomnia/hypersomnia, psychomotor agitation/retardation, feelings of worthlessness or excessive guilt, diminished ability to focus, and recurrent thoughts of death or suicide. To be diagnosed, an individual must have one of the hallmark symptoms, 1) depressed mood or 2) loss of interest or pleasure in combination with other symptoms mentioned at a given time for at least two weeks and the symptoms must cause clinically significant distress to the individual in important areas of functioning (American Psychiatric Association, 2013). Many therapies are available to treat MDD, but despite the wide variety of treatments, approximately a third of those treated fail to improve (Keller, 2005; Souery et al., 1999), emphasizing the need to identify novel mechanisms and potentially new therapeutic targets.

Animal models can be indispensable when identifying novel neural underpinnings of MDD. While no one animal model can produce all symptoms of a neuropsychiatric condition, animal models can be useful to understand subsets of symptoms (Lapiz-Bluhm et al., 2008). Applying chronic stress to rodents is commonly used to study depressive-like symptoms because they produce anhedonia, altered weight gain, and disrupted sleep and circadian rhythms, to name a few parallels (Nestler & Hyman, 2010; Willner & Mitchell, 2002; Willner, 1991). In addition, MDD leads to hypothalamic-pituitary-

adrenal (HPA) axis dysregulation, cognitive deficits and corresponding reductions in hippocampal volumes (Dolan, 2002; Hickie et al., 2005; Kuzis, Sabe, Tiberti, Leiguarda, & Starkstein, 1997; Ravnkilde et al., 2002; Uekermann et al., 2003). Similarly, chronic stress in rodents leads to HPA axis dysregulation and reduced hippocampal plasticity and function. Thus, chronic stress in rodents can be helpful in understanding components of MDD neural underpinnings.

While many types of chronic stress are in use to produce depressive-like symptoms, chronic restraint is very common. While the chronic daily restraint paradigm has many benefits, there are some caveats. An important strength is that restraint is relatively cost-effective, requiring minimal materials that are readily available. Restraint is also relatively straightforward to implement, with usually one or two sessions to train the experimenter. Another benefit is that restraint has fairly consistent outcomes across animals, which is not always the case for paradigms that require two animals to engage and yet, may fail to behave as intended (such as a resident failing to defeat a test animal). Some caveats include the concern that restraint stress is not ecologically relevant; however, this is less of an issue in these experiments because the goal is to induce neurobiological changes in certain limbic structures, such as the hippocampus, before initiating behavioral assessments. Some also argue that chronic restraint is a homotypic (i.e., a repeat of the same type of) stressor, leading to HPA axis habituation in which corticosterone levels in the blood become less pronounced than compared to the first restraint exposure. Again, this is less of a concern because the muted corticosterone response is in alignment with HPA axis dysregulation found in patients with MDD (Grissom & Bhatnagar, 2009; Grissom, Iyer, Vining, & Bhatnagar, 2007; Jean Kant et al.,

1985; Marti & Armario, 1997; Pitman, Ottenweller, & Natelson, 1988; Stamp & Herbert, 1999). Despite the limitations of chronic restraint, many of the outcomes align with MDD in the human condition, highlighting its usefulness to study mechanisms underlying MDD.

One of the most puzzling outcomes following chronic daily restraint is the sex differences. Following chronic restraint stress, the hippocampus in male rodents is significantly compromised, leading to impaired hippocampal-dependent spatial learning and memory (Conrad, 2010; Conrad, Galea, Kuroda, & McEwen, 1996; Wright & Conrad, 2005, 2008). Specifically, common outcomes of chronic stress include reduction in hippocampal volume (Lee, Jarome, Li, Kim, & Helmstetter, 2009), reduced synaptic plasticity (Bodnoff et al., 1995) and the retraction of hippocampal CA3 apical dendritic arbors (Conrad, LeDoux, Magariños, & McEwen, 1999). Chronic stress also has implications in hippocampal neurogenesis as it reduces proliferation, differentiation, maturation and survival of new granule cells (Cameron & Schoenfeld, 2018; Dagyte et al., 2009; Pham, Nacher, Hof, & McEwen, 2003; Schoenfeld, McCausland, Morris, Padmanaban, & Cameron, 2017; Snyder, Glover, Sanzone, Kamhi, & Cameron, 2009; Snyder, Soumier, Brewer, Pickel, & Cameron, 2011). These hippocampal morphological deficits correspond to impaired hippocampal function, such as spatial ability (Hoffman et al., 2011; McLaughlin, Gomez, Baran, & Conrad, 2007). Consequently, male rodents show robust dendritic retraction and spatial memory deficits following chronic stress. In contrast, female rodents fail to show cognitive and morphological deficits in the hippocampus following chronic stress (Conrad, Grote, Hobbs, & Ferayorni, 2003; Galea et al., 1997; Kitraki, Kremmyda, Youlatos, Alexis, & Kittas, 2004; Luine, Gomez, Beck,

& Bowman, 2017). Instead, female rodents almost seem to be resilient in the face of chronic restraint, showing no morphological impairments in the hippocampus (McLaughlin et al., 2010) and may even show improved spatial ability in the Morris Water Maze, Y-maze and Radial arm water maze (RAWM, Beck & Luine, 2002; Bowman, Zrull, & Luine, 2001; Conrad et al., 2003; Conrad, McLaughlin, Huynh, El-Ashmawy, & Sparks, 2012; McFadden et al., 2011a; Ortiz et al., 2015). The concern is that in humans, women are nearly twice as likely as men to be diagnosed with MDD, even after considering willingness to seek out help (Heller, 1993; Weissman et al., 1993). Consequently, identifying an animal model of MDD that corroborates the sex differences observed in humans is important.

When characterizing the behavioral phenotype in animal models, it is helpful to obtain several behavior measures. For that reason, behavioral batteries using multiple tests can be advantageous in order to examine different aspects of the spatial memory domain and cognitive abilities. We would like the opportunity to measure cognition over multiple days, but the timeline of our daily restraint paradigm is restrictive because spatial memory deficits begin to improve in the days and weeks after chronic stress ends (Hoffman et al., 2011; Luine, Villegas, Martinez, & McEwen, 1994; Sousa, Lukoyanov, Madeira, Almeida, & Paula-Barbosa, 2000). Given the limited window of time to capture cognitive deficits following chronic restraint, identifying a paradigm that allows for multiple cognitive assessments during this window may be of great benefit.

Recently, researchers investigated a restraint paradigm that aligned with the present-day psychosocial workload that people in developed countries typically encounter. Zhang and colleagues (2014) found that a modified restraint model using a

work-week design, produced stress and anxiety responses that were greater than observed with chronic daily restraint for the same duration. Specifically, in male Sprague-Dawley rats, restraint for 20 minutes/day in a hemi-cylinder for 5 days, followed by two days off, and then with restraint for two more days produced robust effects than compared to restraint daily for the same period. Stress responses as measured by corticosterone and anxiety profile on the elevated plus maze taken the next day were greatly enhanced in the interrupted restraint rodents than the daily restraint cohort. The outcome of this study suggested that perhaps the robust nature of this interrupted restraint stress paradigm may be useful in producing substantial effects on the hippocampus and on spatial memory in both male and female rats.

While this interrupted restraint paradigm has the potential to be a more robust stressor than daily restraint, many questions remain as it pertains to the way it is used in our paradigm. The goal of the current series of experiments is to use a modified version of the interrupted restraint paradigm described by Zhang et al., (2014), which we term intermittent restraint (IR), on hippocampal function and dendritic morphology in both male and female rodents. Before using both sexes, we first wanted to determine whether an extended duration of three weeks (instead of nine days as used by Zhang et al., 2014) would have similar potentiating effects on impairing hippocampal function in males when compared to the daily restraint paradigm. Moreover, Zhang and colleagues (2014) used plastic hemi-cylinders to restrain rats for just 20 min each day. The concern is that in our prior work, daily restraint in wire mesh for 2 hours/day for three weeks failed to impair spatial memory and alter hippocampal CA3 apical dendrites (McLaughlin, Gomez, Baran, & Conrad, 2007). Consequently, we wanted to determine whether an IR

paradigm of 2 hours/day in restraint would even be effective in altering spatial memory. In the first experiment, IR paradigms for two-hours (IR2) and six-hours (IR6) were used and compared to the traditional daily restraint paradigm for six hours/day (DR6) for outcomes on hippocampal function and dendritic morphology in male rats (please note that for the master's thesis, just the behavior is reported). In addition, a behavioral battery was incorporated because if IR would be more robust than daily restraint on spatial memory impairment, then there may be more opportunity to perform multiple behavioral assessments. We found that IR6 showed the worst performance, albeit non-significant, in part, due to many rats failing to investigate spatial tasks that followed the RAWM. The order of behavioral tasks is important to consider as effects can be obscured when a more aversive task precedes one that is less aversive (Blokland et al., 2012; McIlwain, Merriweather, Yuva-Paylor, & Paylor, 2001; Sousa et al., 2000). IR rats exhibited a tendency for spatial memory deficits during RAWM, though the need for further investigation was clear given the lack of exploration by rats during the tasks that followed.

For the second experiment, tasks were ordered from the least to most aversive and compared IR6 with DR6 in male rats. We found that IR6 resulted in spatial memory deficits as robust as DR6 in the Y-maze when tested soon after restraint ending. As behavioral testing progressed over the next days, performance became similar and all groups showed similar spatial memory by the time of RAWM testing on days 6 and 7.

Given that IR6 failed to produce long-lasting spatial memory deficits beyond what would be expected from the DR6 group, we next considered whether an extended IR6 paradigm would produce robust spatial deficits. Our prior work showed that five

weeks of daily restraint results in more robust deficits in spatial ability when compared to three weeks of restraint (Hutchinson et al., 2012). Moreover, IR6 produced spatial memory deficits in male rats in Experiment 2 and so Experiment 3 included both male and female rats. Consequently, we tested whether an extended IR6 paradigm of six weeks would lead to spatial memory deficits in male and female rats. We found that after six weeks, both male and female IR6 groups exhibited normal spatial ability on the Y-Maze and other behavioral tasks, despite the extended period of restraint. This suggests that the IR paradigm may become predictable over time.

Experiment 4 was performed to increase the unpredictability of the IR model, as well as to enhance the stressful nature of the restraint. An unpredictable intermittent restraint (UIR) paradigm was designed to incorporate aspects of the IR paradigm in which days without restraint were intermixed with IR, the time of day varied, duration of restraint was reduced to 30 or 60 min, and IR occurred simultaneously with gentle shaking. Our hypothesis was that UIR will lead to spatial memory deficits in both male and female rats and these impairments will occur over multiple behavioral assessments obtained on the off days from UIR.

Methods

Subjects

Male and Female Sprague-Dawley (Charles River Laboratories, Hollister, CA, USA) rats weighing approximately 200-225 grams upon arrival were pair housed in standard laboratory cages (21-22 °C, corncob bedding). Except where noted below, animals were allowed food and water *ad libitum*. Animals were housed on a reverse 12:12 light cycle; lights off at 07:00. All procedures occurred during the dark phase of the light cycle and were performed in accordance with the Guide for the Care and Use of Laboratory Animals and the approval of the Arizona State University Institutional Animal Care and Use Committee.

Chronic Stress Procedure

Rats were chronically stressed by wire mesh restraint. Restrainers were constructed from wire mesh (19 cm diameter × 26.5 cm long for males, 16.5 cm diameter x 26.5 cm long for females, aluminum screen wire Model #3001120, Lowes) with the cut edges and ends sealed with Plasti Dip (Performix #075815116024, Amazon.com). Once rats were placed in the restraint, the ends were secured with black binder clips (Staples Inc., Framingham, MA, USA). Animals were upgraded to larger wire-mesh restrainers as they grew (21.5 cm diameter x 29 cm long for males, 19 cm circumference x 26.5 cm long for females). Control rats (CON) were always housed in a chamber separate than the stressed rats in order to reduce the likelihood of communication through odor, sounds and sight. To maintain similar handling procedures and access to food and water across groups, CON rats were handled daily, and food restricted for the same duration as the restrained rats. Body weights were measured weekly for all groups and CON rats were

weighed first in order to reduce the likelihood of exposure to sounds and odors from the stressed animals. For experiments 1-3, restraint occurred between the hours of 09:00 and 15:00 of the dark phase of the light cycle. In experiment 4, restraint occurred between the hours of 07:00 and 21:00. One to three days following the last behavioral testing day in Experiments 1 and 4, rats were euthanized using isoflurane and rapidly decapitated. Adrenal glands, thymus and uterus were excised and weighed for a secondary measure of stressor effectiveness. Brains were removed as well to be processed for Golgi staining.

Chronic Stress Paradigm for Each Experiment

Experiment 1: Male Sprague Dawley rats were used (n= 12 for all groups). One stress group was restrained for 6h/day for 21 consecutive days (daily restraint, 6-hours/d, DR6), another was restrained for 6h/day in an interrupted pattern: 5 days restrained then two days without restraint over a period of 23 days (Intermittent restraint, 6-hours/d, IR6) and the third was restrained for 2h/day over a period of 23 days (IR2). The sum total of restraint days for DR and IR was 21 and 15 days, respectively.

Experiment 2: Male Sprague Dawley rats were used (n= 14 for CON and IR6, n= 12 for DR6). Two restraint groups were used: DR6 and IR6, using the same parameters as described in experiment 1.

Experiment 3: Male and Female Sprague Dawley rats were used (n= 12 for all groups). IR6 was used on half the male and female rats and was performed for 6 weeks before the first spatial task was implemented.

Experiment 4: Male Sprague Dawley rats were used (n= 12 for all groups). The stressor was changed to be unpredictable and robust, termed Unpredictable Intermittent Restraint (UIR). Restraint occurred for 30 or 60 min while on an orbital shaker (120 rpm)

and occurred at different times of day (between 7:00 and 21:00) and for different consecutive day lengths (2-6 days) before a day or two off without a stressor.

Experimental Procedures

All investigators working with the rats contributed worn/unlaundered tee-shirts to ensure that the rats were familiar with the investigators' body odor (Sorge et al., 2014). The shirts were located in the testing room out of visual site of the rats when the rats were in the mazes. Curtains were used to ensure that testing environments and cues remained different across tasks.

Y-Maze: The Y-maze is validated as a task that requires hippocampal function and spatial memory to navigate (Conrad et al., 1996; Wright & Conrad, 2005). Y-Maze testing occurred over two days to accommodate the large number of rats (20-24 rats tested on each day, counterbalanced for treatment).

Apparatus. Two Y-Maze apparatuses were located in the same room and were constructed of black Plexiglas. Three identical and symmetrical arms (58.4 cm long × 20.3 cm wide x 38.1 cm height) radiated from the center. The sides were tall enough that the rats could not jump out of the maze. Outside the maze, large explicit cues (painted shapes, shelving, bins and curtains) were located on the walls and around the room in order to encourage the use of extra-maze cues. The light intensity at the floor of the maze was 80-90 lux for the duration of testing. No explicit cues were present inside of the maze. Two fans were placed in the room to provide white noise and to disperse odors. Corncob rat bedding covered the maze floor to about an inch thick. On the ceiling was attached two cameras (GoPro Hero3) that was connected to an iPad (Apple) or a

monitor. The investigator remained in the room during testing and was out of sight of the rats, behind a curtain watching the rats on an iPad or the monitor.

Procedure. Rats were carted to the testing room with their cage mate in their home cage, three cages at a time. Cage mates were tested simultaneously, in side-by-side Y-mazes. For trial 1, a rat was placed in one arm, which was then designated the "start" arm for that rat. Another arm was blocked with black Plexiglas, so the rat was able to explore the start and the open arm, called the "other" arm. Rats were given 15 min to explore the maze and two accessible arms before they were removed, returned to their home cage and brought back to the animal colony. After each Y-maze exposure, the bedding in the maze was mixed around to dissipate the odors before the next set of rats was tested. At the end of trial 1 and before the start of trial 2, the two Y-mazes were swapped so that rats would be tested in a new Y-maze, but in the exact same position as before to further reinforce that rats were relying upon extra-maze cues. Tape on the floor denoted the Y-maze position to ensure it was configured as in trial 1.

Trial 2 began after a 4-hr inter-trial-interval (ITI). Rats were brought back to the testing room and now the previously blocked arm was open to investigation and was called the "Novel" arm. Rats started in the same arm as in trial 1 and were given 5 minutes to explore. The Start, Other and Novel arms were counter-balanced across groups but held constant for a given rat.

Quantification: Behavior was quantified at a later date by an investigator who was blind to the treatments and novel/other arm identities. The dependent variables measured in trial 2 were the number of entries made (entry) and time spent in each arm (dwell) during each minute. An entry was defined as the forelimbs crossing from the

middle of the maze into an arm entrance. The first two minutes of exploration during testing were used for analysis because rats habituate quickly to the Y-maze (Dellu, Mayo, Cherkaoui, Le Moal, & Simon, 1992). The start arm was not included in the analysis because the rats were placed there at the beginning of the trial, causing an inherent bias compared to the Novel and Other arms. Entry and dwell data were converted into percentages for all three arms (Novel, Start, and Other), with chance being equal to 33.3%. Discrimination performance for the Novel arm compared to the Other arm was calculated by subtracting the percentage of entries into the Other arm from the percentage of entries into the Novel arm. Dwell was calculated similarly for percentage of time spent in the arms. For simplicity, entry data are shown only. Chance for the discrimination index would be 50% and with a preference for the Novel arm being a value greater than 50%.

Open Field (OF): OF acclimates rats to the environment in which they are to be tested on subsequent days. Moreover, OF can serve as a measure of locomotor activity and anxiety-like behavior (Prut & Belzung, 2003; Seibenhener & Wooten, 2015).

Apparatus. The OF apparatus consisted of two side-by-side black square fields (96.5 cm x 96.5 cm) with high walls (38.1 cm height) to prevent escape and yet permitting the rats to see the extra-maze cues around the walls and room (painted geometric shapes, shelving). The light intensity at the floor of the field was 150-160 lux for the duration of OF testing and during following testing days. Two fans in the room provided white noise and helped disperse odors. On the ceiling was attached a camera (GoPro Hero3) that was connected to an iPad (Apple) and a monitor. The investigator remained behind a curtain during trials, out of sight of the rats.

Procedure. An investigator carted three pairs of rats from the home colony room and placed in an adjacent room until OF testing. In the OF test, rats were allowed to explore for 10 min and then removed and returned to the animal colony. An investigator removed any fecal boli and wiped the arena with paper towels and Lime/Sea Salt Scented, Method All-Purpose cleaner (Target ®) after each exposure.

Quantification: Behavior was quantified at a later date by an investigator who was unaware of the treatment identities. The 10-min open field trial was quantified in two, 5-min blocks, utilizing a 4 x 4 grid. The first 5-min block was utilized for analyses because rats readily habituate to OF testing (Brenes, Padilla, & Fornaguera, 2009; Walsh & Cummins, 1976). Peripheral crossings were quantified as the front two paws crossing a line on the periphery of the grid. Central crossings were quantified as the front two paws crossing a center gridline. An anxiety index was calculated as used in our past work (Nishimura, Ortiz, & Conrad, 2017):

$$1 - \frac{(\text{Center crossings} / \text{Total crossings}) + (\text{Time in center}/300)}{2}$$

Total locomotor activity was scored as the number of line crossings (front two paws crossing any line).

Novel Object Recognition (NOR) and Object Placement (OP): NOR can assess a form of memory that does not necessarily require the hippocampus (Balderas et al., 2008; Barker & Warburton, 2011; Mumby, 2002) and was used to provide minimal cognitive challenge with just 1 minute inter-trial-interval (ITI). Experiment 4 also implemented a 1 hour NOR test as an added measure of cognitive ability. OP testing assesses hippocampal-mediated spatial memory in which rats use the spatial context to detect

familiar objects in new locations (Ennaceur, Neave, & Aggleton, 1997; Mumby, 2002; Nishimura et al., 2017; Spanswick & Sutherland, 2010).

Apparatus. NOR and OP occurred in the same OF arena in which rats were previously acclimated and in a testing room under similar conditions. The same room was used, but vinyl curtains were used to obscure/change the cues in the room. Two fans in the room provided white noise and helped disperse odors. On the ceiling was attached a camera (GoPro Hero3) that was connected to an iPad (Apple) and a monitor.

The objects were all large enough so that rats could not climb or topple them and made of ceramic, metal, or glass for easy cleaning with at least four duplicates of each type. For the NOR, a plastic red opaque rectangular object (23.6 cm height x 10.2 cm x 7.6 cm) was filled with sand to keep it steady. The other object was a tall, slender opaque green glass bottle (35.6 cm height) with a heavy aluminum disk (12.7 cm Diameter, 1.3 cm height) secured to its bottom to keep it steady. In the OP, objects were a tall, slender gold candlestick (22.9 cm height) attached to a heavy rectangular base for stability (10.2 cm x 11.4 cm x 1.3 cm height) and a tall, slender opaque green glass bottle (35.6 cm height) with a heavy aluminum disk (12.7 cm Diameter, 1.3 cm height) secured to its bottom to keep it steady. The objects and arena were cleaned with Method All-Purpose Cleaner (Target ®) before each trial. Different scented cleaners were used for OP and NOR but was the same scent within a session.

Procedure. All rats were tested on the same day with pairs of cage mates being tested simultaneously in adjacent fields. Three home cages of pair-housed rats were carted from the animal colony and placed in an adjacent room until it was time for testing. An investigator retrieved the home-cage from the adjacent room and brought it to

the testing room. During trial 1, pairs of rats were placed in two separate OF arenas and each OF contained 2 identical objects. Rats were always placed in a corner of the arena, away from the objects. Rats were allowed to explore for 3 min. After completing trial 1, both objects were replaced. In the NOR, one object was identical to the object used in trial 1 and the other was exchanged for an object that was unique and these objects were placed in the same location as was used in trial 1. In the OP, the objects were replaced with new, but identical objects to those used in trial 1; however, one object was moved to a novel location and the other remained in the same location as in trial 1. In trial 2, rats were returned to the arena after a 1-min or 1-hr ITI for the NOR or a 1-hr ITI for the OP (a timeframe that is sufficient for hippocampal deficits to be observed, de Bruin et al., 2011; Pitsikas et al., 2007). Rats were allowed to explore the objects in for 3 min in trial 2. The start locations of the rats and the object locations were counter-balanced across groups but held constant for a given rat. For the 1-hr ITI, three pairs of rats were carted to the colony room after trial 1 and carted back prior to the start of trial 2.

Quantification. Behavior was quantified at a later date by an investigator who was unaware of the treatment identities. Exploration was defined as the rat facing the object within 3 cm and attentively interacting with the object. Rats were excluded from analyses if they failed to explore both objects or explored the objects for less than 10 second total. An object exploration index was calculated as reported elsewhere (Nishimura et al., 2017):

$$\frac{\text{time spent exploring novel object (NOR) or location (OP) in trial 2}}{\text{total amount of time spent exploring both objects in trial 2}}$$

Radial Arm Water Maze (RAWM): RAWM testing was conducted because of its well-documented use in measuring spatial ability in rodents (Diamond, Park, Heman, & Rose, 1999; Hoffman et al., 2011; Ortiz et al., 2014).

Apparatus. The RAWM was constructed of black polypropylene, with eight symmetrical arms (27.9 cm long × 12.7 cm wide) originating from a circular center (48 cm diameter). The maze was filled with water and allowed to equilibrate to room temperature ranging from 20 to 22 °C. Black powder tempera paint was added to the water until its opacity was sufficient to conceal a black rubber platform placed in one of the eight arms. Two similar testing rooms provided several prominent extra-maze cues including the door to the room, shelves, heat lamps, and cues made of black and white construction paper located on the walls.

Procedure. Groups were counterbalanced between the two similar testing rooms and were tested by two different experimenters. Rats were tested in squads of 6-8 (e.g. two rats from each experimental group). Once one rat was tested, it wasn't tested again until the other rats in the squad completed the trial. Testing occurred over three days with 8 trials occurring on each of the first 2 days and a single retention trial occurring on the third day (17 total trials). Each trial consisted of releasing the rat into an arm (start arm) that did not contain the platform, the start arm was also never directly across from the platform arm to increase the navigational demand of the task. The start arm was varied across trials for each rat and the platform arm remained the same. Once the rat reached the platform, it remained there for 15 s to localize the platform spatially before being returned to its testing cage in the same room, under a heat lamp. If a rat failed to find the platform within 3 min, experimenters used a pole to guide the rat to the platform. A net

was used to stir the water and collect debris to reduce the likelihood of rats using non-spatial cues.

Quantification. Arm entrances were recorded by the investigator on the day of testing and quantified at a later date. An entrance was recorded when the tip of the rat's nose crossed a mark on the outside of the maze (about 22 cm into the arm). Reference memory errors were considered the number of first-time entries into arms that did not contain the platform within a given trial (first entries into the start arm were also quantified as reference memory errors). Working memory errors were considered the number of repeat entries into an arm that did not contain the platform within a given trial (i.e. repeat entries into an arm where a reference memory error was previously committed in the same trial).

Novelty Suppressed Feeding (NSF): Anxiety-like behavior was assessed using a test to examine how readily rats will eat food in a novel environment (Gould et al., 2015; Snyder et al., 2011).

Apparatus. NSF occurred in a novel testing room but in the same dual-field apparatus as the OF, OP and NOR. The OF arena was brightly-lit (170-180 lux) and located in a novel testing room without obvious spatial cues. Two fans in the room provided white noise and helped disperse odors. Before each rat was tested, the arena was cleaned with 70% isopropyl alcohol. On the ceiling was attached a camera (GoPro Hero3) that was connected to an iPad (Apple). The investigator and the home cage remained behind a curtain during trials, out of sight of the rats.

Procedure. Twenty-four hours prior to NSF, rats were food deprived, but with unlimited access to drinking water. On the day of testing, rats were carted, two pairs at a

time in their home cages, to a holding room prior to testing. When it was time, an investigator retrieved the cage and brought it to the NSF testing arena. In the center of the arena was located a pile of standard rodent chow. The rat was placed in one corner of the field, and the amount of time it took the rat to approach the food was recorded. If the rat did not approach the food after 8 min, the testing was terminated, and the animal was given a score of 480 sec. Slow latency to approach the food and begin eating is indicative of high anxiety. When completed, the animals were returned to the animal colony and placed individually in their cages for 10-minutes. In each cage was pre-weighed rat chow and water. The amount of food consumed during the home-cage feeding was measured in order to assess the motivation of the rats to eat in a familiar environment. Littermates were re-united with each other after the end of the home-cage eating assessment.

Elevated Platform Stressor (EP): When implemented seven days following chronic stress, novel acute stressors may increase activation in corticolimbic structures in female rats (Moench, Breach, & Wellman, 2019). The impact of this novel stressor was assessed on spatial ability at the end of the fourth experiment. Seven days following the last UIR day, rats that previously underwent UIR were exposed to this EP stressor and then spatial memory was tested one day after on the Y-maze.

Apparatus. An elevated platform (12 cm x 12 cm, elevated 90 cm from ground) was used as a unique stressor. EP occurred in a novel testing room. Two fans in the room provided white noise and helped disperse odors. A camera (GoPro Hero3), connected to an iPad (Apple), was attached to the ceiling of the room. The investigator and the home cage remained behind a curtain during trials, out of sight of the rats.

Procedure. The UIR rats were transported in pairs in their home cages and the rat pairs were tested simultaneously in two separate rooms. Rats were placed on the top of the EP for 30 minutes. In the event that a rat fell, an investigator surveilled the rat and returned it to the platform. The EP was performed seven days after the end of UIR, based upon a study that found effects using this acute novel stressor in female rats seven days after the end of restraint (Moench et al., 2019). One day after the EP stressor, all rats (UIR and CON), were tested in the Y-maze.

Statistical Analysis

Analysis of variance (ANOVA) was used to analyze parametric data. Body weights were analyzed as a change in body weight from the start of the study to the end of the study, as the experimental timelines varied. Fisher's LSD post hoc tests were used when ANOVA reached significance. In some cases, planned comparisons were performed. Parametric data were represented as means ± S.E.M. For object tests and the Y-Maze, Wilcoxon Signed Rank tests were used on nonparametric data and represented as medians and quartiles. Statistical significance was defined when p-values were equal to or less than .05.

Results

Experiment 1: Effects of IR2 and IR6 over three weeks on various measures of spatial memory.

In this study, we compared two IR paradigms of 2 and 6 hours of restraint with the commonly used 6 hours of daily restraint on several cognitive tests (Fig. 2A). Rats were tested first on the RAWM because of consistent chronic stress effects from past work (Hoffman et al., 2011; Ortiz et al., 2015) Also, the most robust chronic stress effects occurred using the RM assessment (Hoffman et al., 2012; Ortiz et al., 2015) and so RM was measured in these studies.

As expected, all groups acquired the task rapidly, as shown by decreased errors as trials progressed (Day 1, $F_{7,301} = 13.515$, $p < .05$; Day 2 $F_{7,301} = 8.224$, $p < .05$), with no significant main effect of group or interaction (Fig. 2B). Surprisingly, on Day 3 when a single retention trial was given, no significant effects were observed (Fig. 2C). However, the data showed high variability and so a subsequent analysis was performed comparing IR6 with CON, as we expected IR6 to be most impaired (McLaughlin et al., 2007). Also, errors from training day 1 and 2 were used as a covariate to reduce the variance and revealed a significant effect with IR6 making more errors than did CON ($p < 0.05$ for group, using T1 and T17 as the within-subjects variable).

The next tests performed used an appetitive incentive in the OP, NOR and Y-maze. Unexpectedly, many of the rats failed to sufficiently explore OP and NOR, despite acclimation to the OF under similar parameters used with success in past studies (Ortiz et al., 2018). In the OP, 50% of the rats failed to meet criteria and this was distributed similarly across the experimental groups (n=6/all groups). Distributions of the OP index

from individual rats are plotted (Fig. 2D). While the subject number is low and with insufficient power, it is notable that the group with an OP index distributed around chance levels is IR6. In the NOR with a 1-min ITI and minimal cognitive load, the subject number ranged from six (CON), eight (IR6, IR2) and nine (DR6). Wilcoxon paired analysis revealed that CON spent more time with the novel object than they did with the familiar object ($p < 0.05$, Fig. 2E). The NOR index from the other groups did not reach statistical significance, even though they had more rats than did the CON ($p > 0.1$ for DR6, IR6, IR2). On the Y-maze, nearly all rats explored the arms in trial 2 with just one rat in each of CON and DR6 failing to leave the start arm. Wilcoxon paired tests showed that rats entered and spent more time in the novel arm compared to the other arm over the first two minutes for CON ($p < 0.05$), DR6 ($p < 0.05$), and IR6 ($p < 0.05$). IR2 failed to show a significant preference for the novel arm and performed at chance levels (Fig. 2F).

To determine whether anxiety or motor ability may have impacted performance, additional assessments were performed on the OF, OP and Y-maze. An anxiety index was calculated to determine whether the groups differed in anxiety profile regardless of locomotor activity. In the OF, the anxiety index was high and similar for all groups (greater than 90%). Consequently, anxiety profile was unlikely to explain differences in performance among groups. However, the groups demonstrated heightened anxiety overall, perhaps from the prior day exposures on the RAWM. This may also explain the lack of investigation for many of the rats on the OP, which requires motivation to explore. For the the total time spent exploring objects during trial 2 in OP, an ANOVA revealed significant differences between groups on Trial 2 ($F_{1,3} = 3.56$, $p < 0.05$). LSD

post-hoc tests showed that IR2 spent more time exploring both objects than the rest of the groups during OP ($p < 0.05$ compared to CON, DR6, IR6, Fig. 2H, please note, no group differences were found on NOR for trial 2). No other statistical differences were found. On the Y-maze, all groups entered a similar number of arms over the first two minutes, ranging from 6.3 ± 0.6 for CON to 7.8 ± 0.4 for IR6 (Fig. 2I). These OP data suggest that motor or motivation may have contributed to the IR2 group's spatial profile on OP, but a lack of an effect on the total entries of the Y-maze suggest that motor/motivation was unlikely to contribute to spatial ability in the Y-maze. Importantly, CON and IR6 showed similar motor/motivational ability and suggests that they are similarly motivated.

In summary, patterns were observed to suggest that IR6 may have exhibited impaired spatial memory on the RAWM compared to CON, but that performance on the OP and NOR may have been obscured by high anxiety. In addition, spatial memory was displayed on the Y-maze by days 6 and 7 from the CON, DR6 and IR6. These findings suggest that IR6 may have compromised spatial ability, but that this effect was not long lasting.

Experiment 2: Effects of IR6 Over Three Weeks on Various Measures of Spatial Memory with the most Aversive RAWM Last

In this study, we compared the effects of the 6-hr IR paradigm with the commonly used 6-hr of DR on a behavioral battery when the testing order was reversed with the least aversive tasks first (Y-maze) and ending with the most aversive task (RAWM, Fig. 3A).

On the first two days after restraint ended, rats were tested on the Y-maze. CON rats demonstrated spatial memory, whereas DR6 and IR6 did not (Fig. 3B). For the CON

rats, Wilcoxon Signed Rank Tests indicated a significantly greater number of entries in the Novel arm than in the Other arm ($p < .05$) as well as significantly more time spent in the novel arm ($p < .05$). For the DR6 and IR6 rats, the Wilcoxon analyses failed to reveal a significant difference for entries made or time spent in the Novel and Other arms.

The rats were tested in the NOR on the third day after the end of restraint. Since rats had just 1-min ITI, all groups were expected to recognize and spend more time with the novel object compared to the familiar object. A Wilcoxon Signed Rank Test showed that CON and IR6 rats explored the novel object significantly more than the familiar object ($p < .05$), an effect that was not found with the DR6 rats (Fig. 3D).

OP occurred on the fourth day after the end of restraint. This task requires a functional and intact hippocampus in order for rats to recognize the moved object (Ennaceur et al., 1997; Mumby, 2002; Nishimura et al., 2017; Spanswick & Sutherland, 2010). Wilcoxon Signed Rank Tests were performed to determine whether each group explored the object in the novel location more than the object in the same location (Fig. 3D). The CON rats spent significantly more time with the object in the novel location than the object in the same location ($p < .05$). DR6 rats performed at chance by exploring both objects similarly. Interestingly, IR6 rats explored the object in the same location more than the new location ($p < .05$). Additional analysis was performed to compare across groups using a 1-way ANOVA for the OP discrimination index, revealing a significant effect ($F_{2,31} = 6.200$, $p < .05$, Power = .860, Fig. 3D). LSD post-hoc analyses found a significant difference between the OP discrimination index for CON and IR6 rats, with CON rats having a greater OP discrimination index than did IR6 ($p < .05$, Fig. 3D).

RAWM testing began on the fifth day following the end of restraint and occurred over three days. RAWM testing has typically revealed differences in performance between chronically stress male rats and non-stressed controls (Ortiz et al., 2015, 2018). During acquisition on days 1 and 2, all three groups made fewer first time entry errors as trials proceeded (Fig. 3E). A repeated measures ANOVA for groups across the 8 trials on day 1 showed a significant effect of trial on first time entry errors ($F_{7,259} = 8.838$, $p < .05$). By day 2, a repeated measures ANOVA for groups across the 8 trials did not show a significant effect of trial on first time entry errors to suggest that the groups reached a plateau. However, when these trials were analyzed in bins of 2 trials (e.g., a repeated measure of four bins), a significant effect of bin was observed with rats making fewest errors during the last bin compared to the first ($F_{3,111} = 3.537$, $p < .05$). There were no other significant effects on either day 1 or 2. On the third day, a one-way ANOVA for first time entry errors was not significant to reveal that rats were making similar number of first-time entry errors (Fig. 3F).

To determine whether anxiety or motor ability may have impacted performance, additional assessments were performed on the OF, OP and Y-maze. A one-way ANOVA performed on anxiety index in the OF revealed significant differences ($F_{2,33} = 6.644$, $p < 0.05$, Power = .980, Fig. 3G). LSD post-hoc analyses showed that DR6 and IR6 rats expressed a higher anxiety profile than did CON ($p < .05$). To determine whether locomotor activity or motivation to explore the Y-maze differed across groups, total entries (sum of entries into Novel, Start and Other arms over minutes 1 and 2) were analyzed using a one-way ANOVA. No significant differences were detected (Fig. 3H). The total number of entries averaged 8.1± 0.6 for CON, 7.5 ± 0.6 for DR6 and 7.5 ± 0.5

for IR6. Therefore, differences in spatial memory in the Y-maze were unlikely due to motivation to explore. For the OP, the total time spent exploring the objects was compared with a 1-way ANOVA and revealed no significant effects. The total time exploring objects (in seconds) averaged 31.7 ± 3.6 for CON, 28.1 ± 3.6 for DR6 and 28.8 ± 3.2 for IR6 (Fig. 3I).

In summary, both IR6 and DR6 showed impaired spatial memory on the first assessment using the Y-maze at a time when the CON rats exhibited spatial memory by entering the Novel arm more than they did the Other arm. As testing continued in different mazes over days, IR6 and DR6 began to show the potential to demonstrate spatial ability. In the next spatial task, CON showed a better OP discrimination Index than did IR6, but IR6 may have avoided the moved object. On the NOR when cognitive load was minimal, CON and IR6 preferred the novel object over the familiar one. By the time they were tested on the RAWM, the last task of the session, all rats acquired it and performed similarly. Motor abilities are unlikely to explain the spatial memory differences observed in the beginning on the Y-maze and OP. We conclude that a 6-hour IR paradigm may lead to impaired hippocampal-dependent spatial ability with comparably robust deficits as found with DR6. A caveat is that a narrow window of time exists to assess behavior from the end of restraint, as stress-induced cognitive deficits improve within four to seven days after restraint has ended. The spatial memory deficits that follow daily restraint are potentiated with a longer period of restraint (Hutchinson et al., 2012). Therefore, we aim to extend the duration of IR prior to the onset of behavioral testing in order to investigate the potentially more robust deficits in spatial ability and utilize the days without restraint to capture cognitive assessments.

Experiment 3: Effects of an extended Intermittent restraint timeline on spatial memory in male and female rats

In this study, the effects of an extended 6-hr IR paradigm were explored in both male and female rats by increasing the stress period to 6-weeks before behavioral testing as longer periods of restraint have been show result in more robust cognitive deficits (Hutchinson et al., 2012). After the 6-week IR6 period, behavioral testing began on days without restraint, with restraint continuing in a similar pattern. After the first behavioral assessment, there was an additional 3-week IR6 period prior to the next assay, with the following assessments occurring weekly. A timeline of the experiment is shown in figure 4A.

The Y-maze (4hr ITI) was utilized for the first two behavioral assays. In the first Y-Maze (6-wks of restraint), all groups (CON-M, IR6-M, CON-F, IR6-F) entered or spent more time in the novel arm than the other arm to reflect intact spatial ability (Wilcoxon Signed Rank Tests, Fig. 4B). CON-M rats entered and spent more time in the novel arm than the other arm ($p < .05$). IR6-M entered the novel arm more than the other arm ($p < 0.05$). The CON-F and IR6-F entered the novel arm more than the other arm ($p < 0.05$) and had a tendency to spend more time in the novel arm compared to the other arm ($p < .10$). A one-way ANOVA did not show a significant effect.

The rats were given another three weeks of IR and then tested again in the Y-maze in a different room. After 9-wks of restraint, the rats were still showing spatial ability. Wilcoxon Signed Rank Tests indicated a significantly greater number of entries in the novel arm than in the other arm for CON-M ($p < 0.05$) and IR6-M ($p < .05$). CON-F entered the novel arm more than the other arm ($p < 0.05$) and also had a tendency to

spend more time in the novel arm compared to the other arm ($p < .10$). IR6-F showed a tendency to make more entries into the novel arm ($p = .10$). A two-way ANOVA revealed no significant stress effects on %Entry Index across groups.

After another week of IR, the rats were tested on the OP, which occurred during the 10th week of restraint. Wilcoxon Signed Rank Tests were performed to determine whether each group explored the object in the novel location more than the object in the same location. No significant differences were detected. CON-M, IR6-M, CON-F and IR6-F rats explored the objects in the novel and same location similarly. Two-way ANOVA also found no significant differences across groups for OP discrimination.

After another week of IRS, the rats were tested in the NOR during the 11th week of restraint. Since rats had just 1-min ITI, all groups were expected to recognize and spend more time with the novel object compared to the familiar object. Wilcoxon Signed Rank Tests showed that CON-M, IR6-M, CON-F and IR6-F rats explored the novel object significantly more than the familiar object ($p < .05$, Fig. 4E). There were no significant differences across groups in NOR discrimination.

To determine whether anxiety or motor ability may have impacted performance, additional assessments were performed on the OF, OP and Y-maze. A two-way ANOVA performed on anxiety index in the OF revealed a significant interaction of stress and sex ($F_{1,43} = 3.827$, $p = 0.05$, Power = .481, Fig. 4F). LSD post-hoc analyses showed that IR6-F rats expressed a reduced anxiety profile compared to CON-F ($p < .05$), CON-M ($p < .05$), and IR6-M ($p < .05$). To determine whether locomotor activity or motivation to explore the Y-maze differed across groups, total entries (sum of entries into Novel, Start and Other arms over minutes 1 and 2) were analyzed using two-way ANOVAs. No

significant differences were detected in the first Y-maze after 6-weeks of IR6. The total number of entries averaged 7.8± 0.8 for CON-M, 8.2 ± 0.7 for IR6-M, 9.8 ± 0.6 for CON-F and 8.9 ± 0.7 for IR6-F (data not shown). Therefore, groups were similarly motivated. For the Y-maze after 9-weeks of IR6, a two-way ANOVA revealed a significant effect of sex with female rats making more total entries than male rats ($F_{1,42}$ = 10.205, $p < 0.05$, Power = .877, Fig. 4G). There were no other significant effects. The total number of entries averaged 7.5± 0.8 for CON-M, 7.7 ± 1.0 for IR6-M, 10.4 ± 0.8 for CON-F and 9.9 ± 0.6 for IR6-F. In OP, the total time spent exploring the objects was compared with a two-way ANOVA and revealed a significant effect of sex with male rats spending more time exploring objects compared to female rats ($F_{1,39}$ = 4.228, $p < 0.05$, Power = .518, Fig. 4H). There were no other significant effects. The total time exploring objects (in seconds) averaged 48.0 ± 6.5 for CON-M, 56.7 ± 4.2 for IR6-M, 48.4 ± 6.5 for CON-F and 54.3 ± 6.1 for IR6-F.

In summary, both IR6-M and IR6-F showed signs of spatial memory on the first two assessments using the Y-maze by entering the Novel arm more than they did the Other arm, despite exposure to an extended stressor paradigm. OP behavior was less clear as all groups, including controls, failed to discriminate and spent similar amounts of time exploring both objects. On the NOR when cognitive load was minimal, all groups discriminated and preferred the novel object over the familiar one. We conclude that a 3-week 6-hour IR paradigm can lead to impaired hippocampal-dependent spatial ability in males, but these deficits fail to present when this stressor is extended to a longer, 6-week timeline. Therefore, we aim to use an IR paradigm that is less predictable and potentially more robust in order to assess cognitive and anxiety profile repeatedly.

Experiment 4: Effects of unpredictable intermittent restraint on spatial memory in male and female rats

In this study, the effects of an unpredictable intermittent restraint (UIR) paradigm were explored in both male and female rats. Chronic unpredictable restraint by changing contexts and given daily resulted in spatial memory deficits in male, but not female rats (Ortiz et al., 2015). The UIR paradigm in this experiment was designed to incorporate the intermittent pattern of the IR paradigm, as well being unpredictable by having the time and duration of restraint vary. The UIR paradigm involved varying the time of day which restraint occurred as well as the duration of restraint (either 30 min. or 1 hr.), with restraint repeating once a day for a period of 2 to 6 consecutive days before a 1- or 2-day restraint hiatus. Moreover, restraint occurred on an orbital shaker to increase the robustness of the restraint with a shorter duration. After a 26-day UIR period, behavioral testing began and occurred weekly on days without restraint with UIR continuing the day after testing. At the end of the study, a robust heterotypic stressor was performed because it produces sex differences in set-shifting ability, but its effect on spatial ability is unknown (Moench et al., 2019). A timeline of the experiment is shown in figure 5A.

The Y-maze (4hr ITI) was utilized for the first behavioral assessment. In the Y-Maze, sex differences were observed in spatial memory (Fig. 5B). In the males, Wilcoxon Signed Rank Tests indicated a significantly greater number of entries and time spent in the Novel arm than in the Other arm for CON-M ($p < .05$), but not in UIR-M. In the females, a tendency to enter and spend more time in the novel arm more than the other arm was found in UIR-F ($p < .10$, Fig. 5B), but not in CON-F. A two-way ANOVA on the %Entry Index did not reveal any significant effects.

OP occurred twice in this experiment, during the 2nd and 5th weeks of behavioral testing. In the first OP assessment (1 hr. ITI), none of the groups showed a significant preference for one object over the other and explored both objects similarly (Wilcoxon Signed Rank, Fig. 5E). Moreover, a two-way ANOVA for OP index did not reveal any significant effects. The second OP assessment (1 hr. ITI) replicated the first, with rats exploring both objects similarly and with no statistical differences across groups for the OP index (data not shown).

The rats were tested in two versions of the NOR during the 3rd week of testing. NOR testing occurred on back to back days in different testing rooms, with 1-min ITI followed by a 1-hr. ITI. Wilcoxon Signed Rank Tests showed that all groups (CON-M, UIR-M, CON-F and UIR-F) discriminated and explored the novel object significantly more than the familiar object in the 1-min ITI ($p < .05$, Fig. 5F) and the 1-hr ITI ($p < .05$, Fig. 5G). A two-way ANOVA did not show any significant differences among groups for the NOR index in either task.

To determine whether anxiety may have impacted performance, the OF and NSF were used. In the OF, a two-way ANOVA performed on the anxiety index did not reveal any significant differences among groups, although there was a tendency for females to have a higher anxiety index than males ($F_{1,44} = 3.265$, $p < 0.10$, Fig. 5H). In the NSF, there were no significant differences across groups in latency to approach food (Fig. 5I) and home cage feeding was statistically similar. Together, the OF and NSF data suggest that groups had similar overall anxiety profiles. Although females may have had a higher anxiety index, this does not explain why IR6-F may have differed from CON-F.

To determine whether motivation to explore could have impacted performance, total entries on the Y-maze and time spent with both objects in the OP and NOR were analyzed. For the Y-maze, total entries (sum of entries into Novel, Start and Other arms over minutes 1 and 2) were analyzed using two-way ANOVAs and no significant differences were detected. The total number of entries averaged 10.2 ± 0.9 for CON-M, 9.3 ± 0.8 for UIR-M, 9.3 ± 0.8 for CON-F and 9.6 ± 0.8 for UIR-F (Fig. 5C). Therefore, differences in spatial memory in the Y-maze were unlikely due to motivational differences to explore. In OP1 and OP2, the total time spent exploring the objects in trial 2 was compared with a two-way ANOVA and revealed a significant effect of sex in OP1 ($F_{1,40} = 5.338$, $p < 0.05$, Power = .616) and OP2 ($F_{1,41} = 16.641$, $p < 0.05$, Power = .978), with no other significant effects. The total time exploring objects (in seconds) averaged for OP1: 29.3 ± 2.5 for CON-M, 23.6 ± 2.6 for UIR-M, 32.5 ± 4.5 for CON-F and 38.9 ± 5.3 for UIR-F (Fig. 5J) and for OP2 (data not illustrated): 27.8 ± 5.1 for CON-M, 24.4 ± 3.8 for UIR-M, 37.8 ± 4.8 for CON-F and 43.5 ± 3.5 for UIR-F. While females spent more time with the objects than did males, all rats performed similarly and at chance on the OP.

Seven days after the last UIR session, male and female rats in the UIR condition were placed on the EP and then tested on the Y-maze the following day. In the post-EP Y-maze (4-hr ITI), all groups (CON-M, UIR-M, CON-F and UIR-F) demonstrated spatial memory (Fig. 5D). Wilcoxon Signed Rank Tests indicated that CON-M, CON-F, UIR-F significantly entered and spent more time in the novel arm than in the other arm ($p < .05$). UIR-M rats significantly entered the novel arm more than the other arm ($p < 0.05$) and

has a tendency to spend more time in the novel arm over the other arm ($p < .10$). A two-way ANOVA revealed no significant effects.

To investigate potential motivational differences in the post-EP Y-maze, a two-way ANOVA revealed a significant interaction of sex and stress ($F_{1,44} = 7.609$, $p < 0.05$, Power = .770) with no other significant effects. LSD post-hoc analyses showed that IR6-M rats made fewer total arm entries compared to CON-M ($p < .05$), CON-F ($p < .05$), and UIR-F ($p < .05$). The total number of entries averaged 8.6 ± 0.7 for CON-M, 5.8 ± 0.6 for UIR-M, 8.2 ± 0.5 for CON-F and 8.8 ± 0.8 for UIR-F.

In summary, the UIR paradigm resulted in spatial memory deficits in male rats as UIR-M showed signs of spatial memory deficits on the first assessment using the Y-maze by entering the Novel arm and Other arms similarly. In contrast to males, UIR in females did not result in spatial memory deficits and may have even been beneficial, as UIR-F rats showed improved discrimination compared to CON-F with entries and dwell measures favoring the Novel arm more than the Other arm. Motor abilities are unlikely to explain the spatial memory differences observed in the first Y-maze and lack of an effect in OP. We did not find any deficits in spatial memory in the Y-maze following the EP stressor, suggesting rats had recovered from deficits by that time point and that the EP did not interfere with this process. We conclude that a UIR paradigm may lead to impaired hippocampal-dependent spatial ability with robust deficits in male rats that fail to present in female rats.

Physiological Measures

In all four experiments, restraint attenuated body weight gain compared to control. In experiment 1, (DR6, IR6, IR2) led to attenuated weight gain compared to

CON over three weeks. A one-way ANOVA revealed significant differences among groups ($F_{3,44} = 16.357$, $p < .05$, Table 1). LSD post hoc tests revealed that DR6 gained the least body weight compared to CON, IR6 and IR2 ($p < .05$). IR6 and IR2 gained similar amounts of weight, but significantly less than CON ($p < .05$). In experiment 2, both restraint paradigms (DR6 and IR6) led to attenuated weight gain compared to CON . A one-way ANOVA for revealed significant differences among groups ($F_{3,44} = 1153.777$, $p < .05$, Table 1). LSD post hoc tests revealed that DR6 gained significantly less body weight than did IR6 and CON ($p < .05$), and that IR6 gained less body weight than did CON ($p < .05$). In experiment 3 and 4, females gained less weight than did males, as would be expected. Importantly, IR6 attenuated body weight gain over the 11-week stress period compared to their respective same-sex CON. A two-way ANOVA for stress and sex revealed a significant effect of stress ($F_{1,43} = 86.088$, $p < .05$), a significant effect of sex ($F_{1,43} = 454.343$, $p < .05$), as well as a stress x sex interaction ($F_{1,43} = 11.888$, $p < .05$, Table 2). Further analysis revealed that IR6-M gained significantly less weight than did CON-M rats ($p < .05$) and that IR6-F gained less weight than CON-F ($p < .05$). In experiment 4, UIR attenuated body weight gain compared to their respective same-sex CON. A two-way ANOVA for stress and sex revealed a significant effect of stress ($F_{1,44} = 59.523$, $p < .05$), a significant effect of sex ($F_{1,44} = 436.739$, $p < .05$), as well as a stress x sex interaction ($F_{1,44} = 6.359$, $p < .05$, Table 2). Further analysis revealed that UIR-M gained significantly less weight than did CON-M ($p < .05$) and UIR-F gained less weight than did CON-F ($p < .05$). In experiment 4, uterus, adrenal and thymus weights were also analyzed as an additional measure of stressor effectiveness. A one-way ANOVA of uterine weights revealed no significant effect of stress (Table 4). As expected, male rats

had larger adrenal glands compared to female rats. A two-way ANOVA of weights for the adrenal glands revealed no significant effect of stress, but there was a significant effect of sex ($F_{1,43} = 15.090$, $p < .05$, Table 4). An analysis of thymus weight after the end of the experiment revealed that male rats had larger thymus glands compared to female rats as well as significant group differences with stressed rats bearing smaller thymus glands compared to controls. A two-way ANOVA for thymus weight revealed a significant effect of stress ($F_{1,44} = 10.982$, $p < .05$) and a significant effect of sex ($F_{1,44} = 27.557$, $p < .05$, Table 4). Further analysis revealed that UIR-M gained significantly less weight than did CON-M ($p < .05$) and UIR-F gained less weight than did CON-F ($p < .05$).

Discussion

The current study investigated whether an IR paradigm could be extended to study chronic restraint effects on spatial memory deficits in both male and female rats. We report that IR may be useful to investigate spatial ability in male rats within a relatively brief period, such as following approximately three weeks of IR, but not after an extended IR duration of six or nine weeks. Moreover, when spatial memory deficits were detected in male rats, the effects of IR were transient because spatial memory deficits begin to improve with a few days after restraint ended. When IR continued for an extended duration for up to six weeks, IR male and female rats failed to demonstrate spatial memory impairments, suggesting that the IR paradigm may have become predictable. A modified version of IR that was made to be unpredictable (UIR) through restraining rats at different 1) numbers of consecutive days restrained (2 to 6 days), 2) times of day, 3) durations of restraint (30 or 60 min) combined with gentle shaking led to a more robust and less-predictable version of the IR paradigm. The outcome showed that UIR males were impaired on spatial ability, whereas UIR females still remained relatively unaffected on spatial navigation. These experiments demonstrate important sex differences in how chronic restraint alters hippocampal function and introduces UIR as an effective chronic stressor in producing spatial memory deficits in male rats.

A consistent theme following chronic stress is that males show spatial memory deficits, which improve in the days after the chronic restraint paradigm ends. One study found that 4 weeks of chronic restraint impaired spatial learning on the Morris Water Maze task, with these deficits improving after a month has passed from the end of the stressor (Sousa et al., 2000). In addition, our lab found that 3 weeks of chronic restraint

hindered spatial memory on the RAWM, an effect that improved with the passage of time (Hoffman et al., 2011). Consequently, the IR paradigm was predicted to produce spatial memory deficits that would persist longer than just for a few days after the restraint ended. As expected in male rats, IR6 (6hrs of intermittent restraint) produced spatial memory deficits on the RAWM in experiment 1 and the Y-maze and OP in experiment 2. These deficits occurred within the first four days after IR ended; however, as testing days progressed from the end of IR, the groups began to perform similarly by the end of the week. We also included IR2 (2hrs of intermittent restraint), because IR was predicted to be more robust than daily restraint but found that IR2 failed to impair spatial memory. Hence, IR6 is an effective and robust stressor to assess spatial memory deficits within days after restraint ends in male rats, but improvements in spatial ability occur along similar timelines as observed with daily restraint. These experiments support the concept that a short window exists after restrained ends to capture spatial memory deficits in chronically stressed male rats.

Another consideration for the relatively fast spatial memory improvement is that the rats may have benefited from the repeated behavioral assessments. For example, environmental enrichment counteracts chronic stress-induced learning and memory deficits (Cui et al., 2006; Hutchinson et al., 2012; Ilin & Richter-Levin, 2009; Wright & Conrad, 2008). Aspects of the cognitive assessments implemented in this study, such as the opportunity to explore objects and environments, could be perceived as enriching and may have similar to effects as environmental enrichment in rats. Another interpretation is that the rats were able to transfer information from one testing situation to another (Winocur & Gilbert, 1984; Winocur & Mills, 1970; Winocur & Salzen, 1968), but this

likelihood was minimized by using unique testing rooms for each cognitive task. Whether or not spatial memory deficits improved from the repeated testing conditions or from the passage of time, the outcome for using IR or UIR is similar to that found with chronic daily restraint.

We also observed that the behavioral assessment testing order impacted performance. In experiment 1, when the RAWM occurred first, followed by the OP and NOR, half of the rats failed to explore despite being presented with an OF arena for acclimation. In experiment 2, when the Y-maze occurred first, subsequent object exploration was greatly increased across all groups and ranged from 83% to 100% participation across treatment conditions. Others reports document order effects and one found that mice explored less in the open field and the Y-Maze when a behavioral battery preceded them, but how a behavioral battery impacted performance on the Morris Water Maze was less obvious (Võikar, Vasar, & Rauvala, 2004). When aversive tasks, such as the Morris Water Maze, precede comparatively less aversive tasks, such as OF, mice exhibit reduced locomotion (Blokland et al., 2012; McIlwain et al., 2001). Taken together, the current series of studies corroborate the literature that if multiple behavioral tasks are to be used to assess chronic stress effects, testing order should start from the least to the most aversive paradigm.

A caveat of the IR paradigm is that spatial memory was unaltered in both male and female rats when IR was extended from three to six weeks. An extended stress timeline was incorporated because previous studies showed that 5 weeks of chronic daily restraint resulted in more robust spatial memory deficits than compared to 3 weeks of chronic daily restraint (Hutchinson et al., 2012). Unexpectedly, our study revealed that

after six weeks, IR males and females displayed unhindered spatial ability on the Y-maze. Consequently, the IR continued for another three weeks and again, spatial memory remained intact. This outcome was unlikely attributed to stressor effectiveness because IR led to attenuated body weight gain, a reliable measure of chronic stress in rodents (Bollinger, Bergeon Burns, & Wellman, 2016; Henckens et al., 2015; Marin, Cruz, & Planeta, 2007; Martí, Martí, & Armario, 1994; Retana-Márquez et al., 2003). Perhaps the consistent five day exposure and two days off from restraint led to attenuated HPA responses, a phenomenon documented to occur with repeated exposures to the same stressor (Viau & Sawchenko, 2002). Some argue that this attenuation or habituation reflects an adaptive process that leads to increased predictability and control over the challenging condition (Grissom & Bhatnagar, 2009). While this interpretation does not explain why daily restraint would lead to more severe spatial memory deficits when extended from three to five weeks (Hutchinson et al., 2012), this may apply to the IR paradigm. Some argue that as stressors become predictable, they become less stressful (Koolhaas et al., 2011). Together, these reports suggest that IR repetition and/or predictability could help to explain the lack of spatial memory deficits following the extended IR paradigm.

An important outcome of these experiments is that UIR impaired spatial memory in male rats without impairing spatial memory in female rats. UIR male rats failed to discriminate during the Y-maze, indicating impaired spatial ability. Whereas, UIR female rats discriminated during the Y-maze. Since stressed females performed superior to their same-sex controls, this outcome suggests that UIR enhanced the spatial ability of female rats. The results are consistent with the findings of others documenting chronic stress-

induced deficits in spatial ability in tasks such as, Morris Water Maze (Moosavi, Naghdi, Maghsoudi, & Zahedi Asl, 2007; Sandi et al., 2003), OP (Bowman, Beck, & Luine, 2003; Conrad et al., 2012; Luine, 2002; Nishimura et al., 2017), Y-maze (Conrad et al., 1996; Kleen, Sitomer, Killeen, & Conrad, 2006; Ortiz et al., 2015; Wright & Conrad, 2005) and RAWM (Hoffman et al., 2011; Luine, 2002; Ortiz et al., 2015). These findings also support an extensive literature that chronic stress enhances spatial memory of female rats (Luine et al., 2017), across a variety of tasks, such as, Morris Water Maze (Kitraki et al., 2004), OP (Beck & Luine, 2002; Bisagno et al., 2004; Luine, 2002), Y-maze (Conrad et al., 2003) and RAWM (Bowman et al., 2003, 2001; Luine, 2002). Physiological measures such as attenuated body weight gain, enlarged adrenal glands and reduced thymus gland weight are common metrics used to validate the effectiveness of chronic stress and led to similar outcomes with both male and female rats in the current study (Bhatnagar & Dallman, 1998; Conrad, Mauldin-Jourdain, & Hobbs, 2001; Conrad et al., 2012; Galea et al., 1997; McFadden et al., 2011b; McKittrick et al., 2000). UIR corroborates the literature highlighting the sex differences in chronic stress-induced spatial memory effects.

The novel UIR stress paradigm produces effects consistent with prevailing chronic stress paradigms and offers many benefits over standard daily chronic restraint. Many current chronic stress paradigms involve daily stressors leaving no opportunity for cognitive assessment during the stress period. A major advantage with the UIR model, is the opportunity to assess behavior without disrupting the pattern of restraint, by utilizing the days without restraint. This intermittent nature of UIR enables an investigator to obtain multiple cognitive assessments without allowing for improvement from chronic

stress-induced cognitive effects. UIR also reduces the burden and constraint on the investigator. The variability in time of day and days without restraint, as well as the reduced overall stress duration make the implementation of UIR a seamless and flexible process. The UIR paradigm is a practical and robust tool to study the effects of chronic stress on cognition assessment in male rats and female rats

Despite our attempts to introduce a robust and unpredictable chronic stressor, sex differences in spatial ability persisted showing deficits in males without impairing females. A possible explanation for the resilience of female rats to the spatial memory deficits is that males and females exhibit different cognitive vulnerabilities to chronic stress. While the chronic stress-induced spatial memory deficits in male rats are well documented (Bodnoff et al., 1995; Conrad et al., 1996; Luine et al., 1994; Ortiz et al., 2015), recent studies are beginning to reveal different types of vulnerabilities in females. For example, one study revealed that chronic stress impaired cognitive flexibility using a set-shifting task in female rats (Grafe, Cornfeld, Luz, Valentino, & Bhatnagar, 2017). Set shifting requires prefrontal cortex, suggesting that chronically stressed females may be vulnerable to tasks involving the prefrontal cortex. Another interpretation is that the type of stressor could lead to sex differences in cognitive vulnerability. For example, chronically stressed female rats may be unaffected on spatial ability, but they may be influenced by a heterotypic stressor, defined as a novel stressor unique from prior stressors. Moench et al., (2019) reported that chronically stressed females, but not chronically stressed males, exposed to a heterotypic stressor were impaired on a set-shifting task. Our work adds to the literature that chronic stress leads to impaired hippocampal function in males, but not females, and that heterotypic stressors did not

modify these outcomes when administered seven days following the end of chronic stress. Future studies should investigate female vulnerability to chronic stress-induced changes on prefrontal cortex mediated behaviors and how heterotypic stressors may contribute to these outcomes.

The results from the present set of experiments show important sex differences in chronic stress-produced spatial ability and introduce UIR as a useful paradigm to probe these effects. Despite introducing IR as a possible robust stressor and then modifying the IR paradigm to be unpredictable in UIR, spatial memory deficits were detected in male, but not female rats. The overwhelming evidence from the current study and others suggest that chronic stress affects male and female rats differently. Chronic stress impairs hippocampal function in male rats, as evidenced by poor spatial ability, but fails to impair spatial ability in female rats. Instead, other studies suggest chronic stress may alter the vulnerability of females to cognitive arousal and related attentional tasks (Bangasser, Wiersielis, & Khantsis, 2016). Future studies should continue to probe the types of respective cognitive vulnerabilities exhibited by males and females and make a point to assess a variety of forms of cognition. The UIR paradigm is a novel stressor which provides the ability for robust cognitive assessment in both male and female rats. This makes it a valuable tool for investigating the neurobiological mechanisms behind the sex differences in chronic stress effects on cognition.

Figures

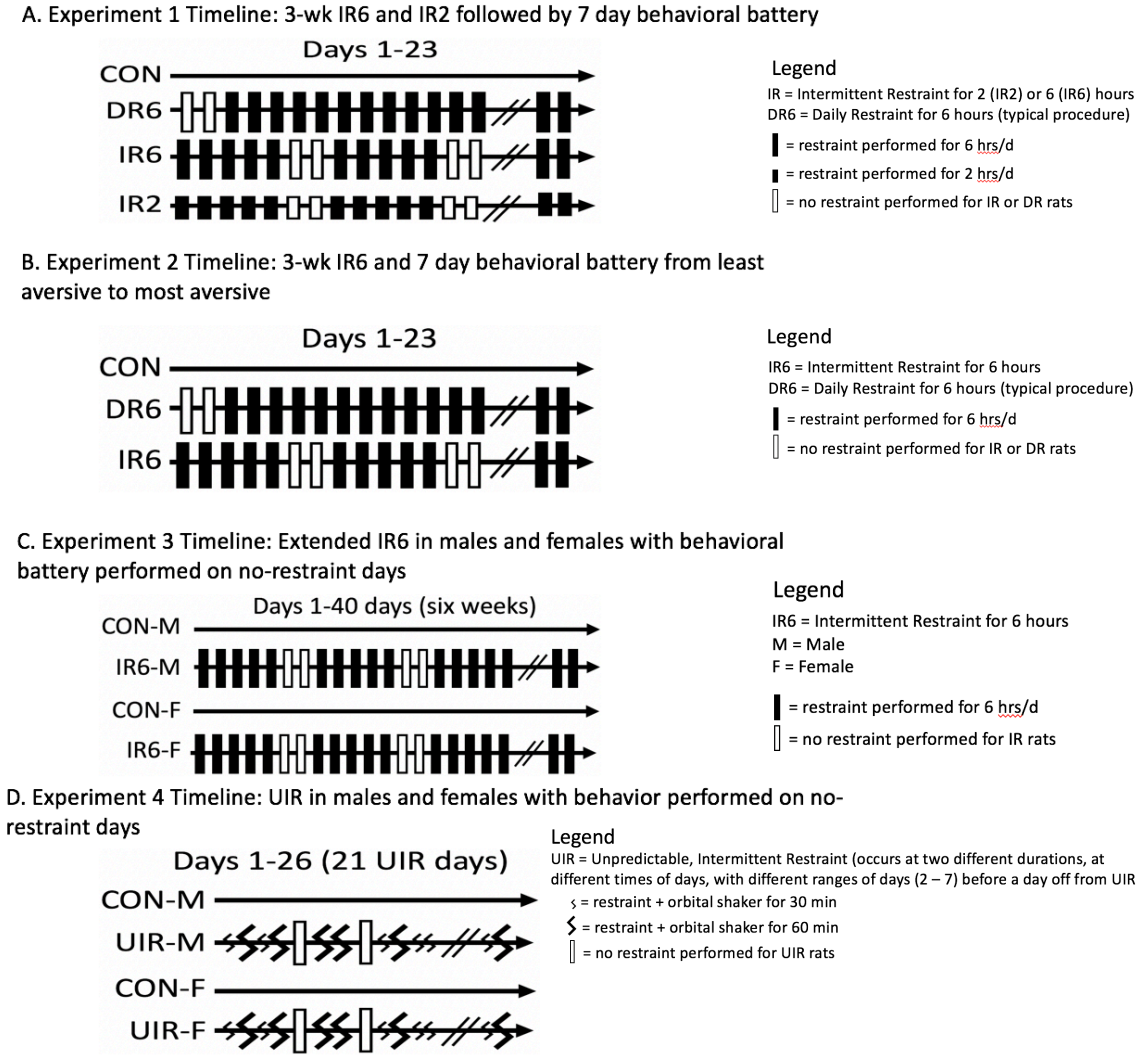

Fig. 1: Group assignments and timeline of the four experiments in this study. Restraint (6-hrs) occurred on days indicated by a solid rectangle. Restraint for 2-hrs is denoted by a shorter rectangle. In experiment 4 when restraint was combined with orbital shaking, the denotation is indicated by a zigzag. The size of the zigzag reflects the duration for 60 min (large zigzag) or 30 min (small zigzag) Days without restraint are indicated by a white rectangle. CON = control, DR6 = daily restraint for 6-hrs, IR6 = intermittent restraint for 6-hrs., IR2 = intermittent restraint for 2-hrs. CON-M = control males, IR6-M = intermittent restraint for 6-hrs in males, IR6-F =

intermittent restraint for 6-hrs in females. UIR-M = unpredictable intermittent restraint in males, UIR-F = unpredictable intermittent restraint in females.

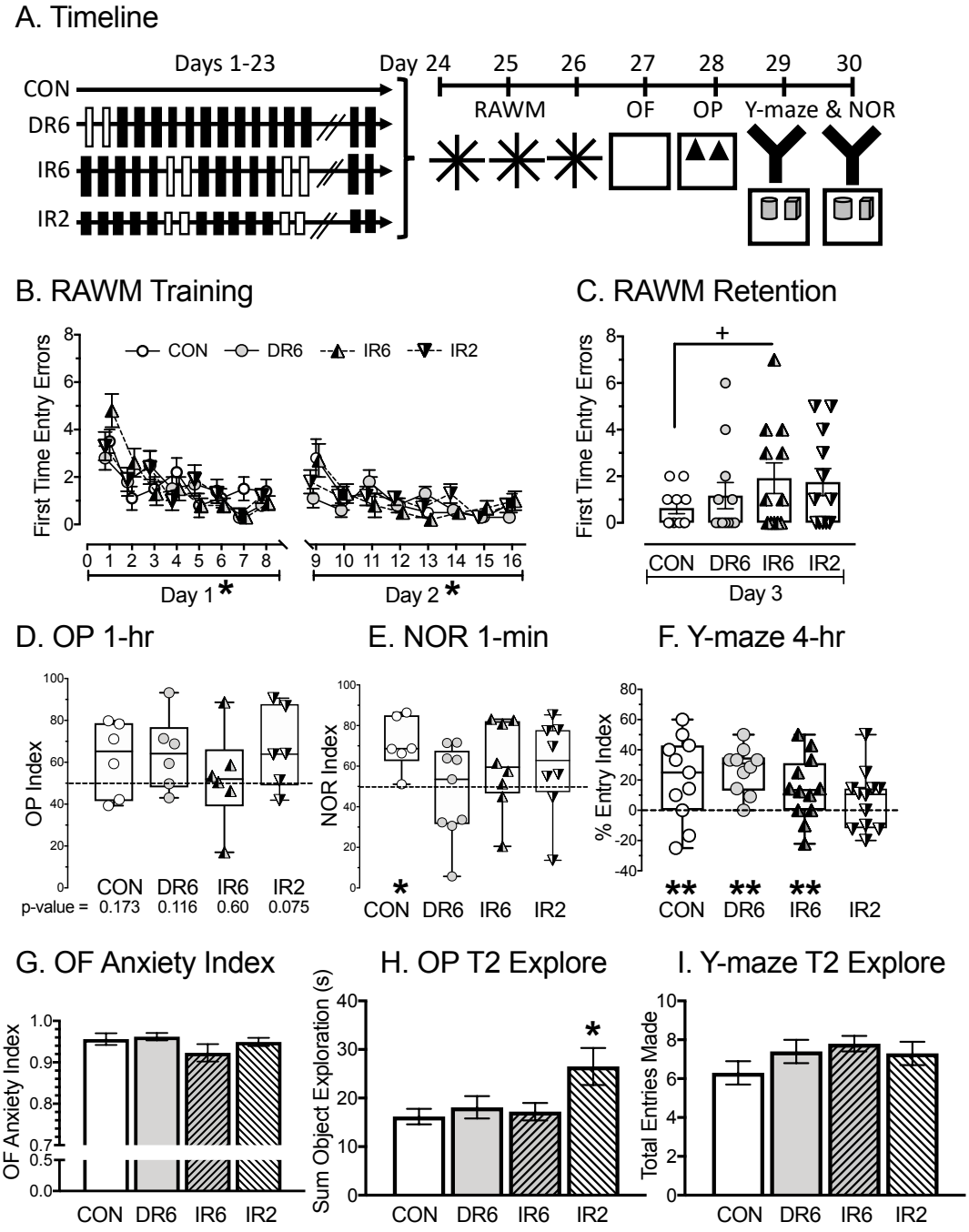

Fig. 2: Effect of two different IR durations on spatial ability and anxiety profiles in male rats. A) Timeline of manipulations. See Figure 1 for restraint legend. The day after restraint ended, rats were tested on the RAWM for 3 days, followed by the OF, OP and then the Y-maze and NOR. B) First time entry errors on the RAWM during

training on days 1 and 2. All groups acquired the task by decreasing first time entry errors over days. There were no group differences. C) Single retention trial on RAWM. IR6 made more first-time entry errors than did CON. D) OP Index from the second trial after a 1-hr ITI. Preference for the moved object will show values greater than 0.5 with the dashed horizontal line indicating chance levels. All groups performed at chance, with p-values listed below each group name; however, half the rats failed to explore, reducing the power of the analyses. E) NOR Index from the second trial with a 1-min ITI. Preference for the new object will show values greater than 0.5 with the dashed horizontal line indicating chance levels. CON spent more time with the new object than the familiar object despite a low subject number (n=6). The remaining groups performed at chance levels. F) Y-maze performance showing the %Entry Index in trial 2 after a 4-hr ITI. CON, DR6 and IR6 entered (and spent more time in) the novel arm than the other arm (dwell data are not shown). IR2 performed at chance levels, which is denoted by the dashed horizontal line. Two asterisks indicate significance in both entry and dwell measures. G) anxiety index. All groups showed statistically similar and high anxiety profiles. H) OP total object exploration time. IR2 spent more time exploring both objects in trial 2 than did CON, DR6, and IR6. There were no other group differences. I) Entries made in all three arms of the Y-maze during Trial 2. All groups made similar number of entries. *$p < 0.05$, +$p < 0.05$ with covariate, CON = control, DR6 = daily restraint for 6hrs., IR6 = intermittent restraint for 6hrs., IR2 = intermittent restraint for 2hrs. Boxes represent median and inter-quartile ranges. All other data points are mean ± S.E.M.

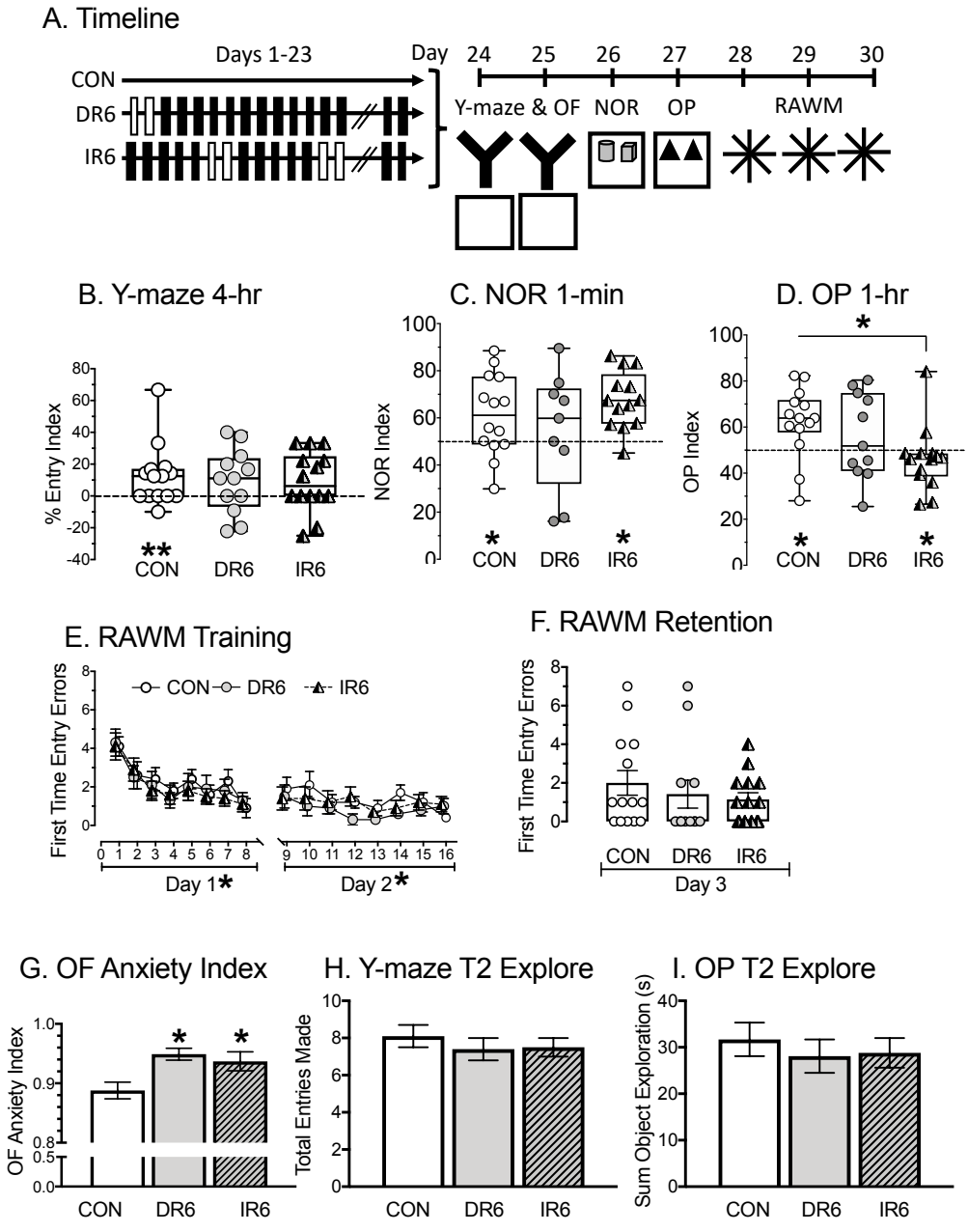

Fig. 3: Effect of 6-hr IR on spatial ability and anxiety profiles in male rats when testing begins with least aversive Y-maze and ending with the most aversive RAWM. A) Timeline of manipulations. See Figure 1 for restraint legend. The day after restraint ended, rats were tested on the Y-Maze and OF, followed by NOR, OP and then the RAWM (last 3 days). B) Y-maze performance showing the %Entry Index in trial 2 after a 4-hr ITI. CON entered (and spent more time in) the novel arm than the other

arm. DR6 and IR6 performed at chance levels, which is denoted by the dashed line. Data represent entries (dwell data are not shown). Two symbols indicate significance in both entry and dwell measures. C) NOR Index from the second trial with a 1-min ITI. Preference for the new object will show values greater than 0.5 with the dashed horizontal line indicating chance levels. CON and IR6 spent more time with the new object than the familiar object. DR6 performed at chance levels. D) OP Index from the second trial after a 1-hr ITI. Preference for the moved object will show values greater than 0.5 with the dashed horizontal line indicating chance levels. CON preferred the object in the novel location. DR6 performed at chance while IR6 preferred the object in the familiar location. IR6 significantly differed from CON in OP index. E) First time entry errors on the RAWM during training on days 1 and 2. All groups acquired the task by decreasing first time entry errors over days. There were no group differences. F) Single retention trial on RAWM. There were no group differences. G) OF anxiety index. DR6 and IR6 showed greater anxiety profiles compared to CON. H) Entries made in all three arms of the Y-maze during Trial 2. All groups made similar number of entries. I) OP total object exploration time. All groups spent similar time exploring both objects. *$p < 0.05$, CON = control, DR6 = daily restraint for 6hrs., IR6 = intermittent restraint for 6hrs.

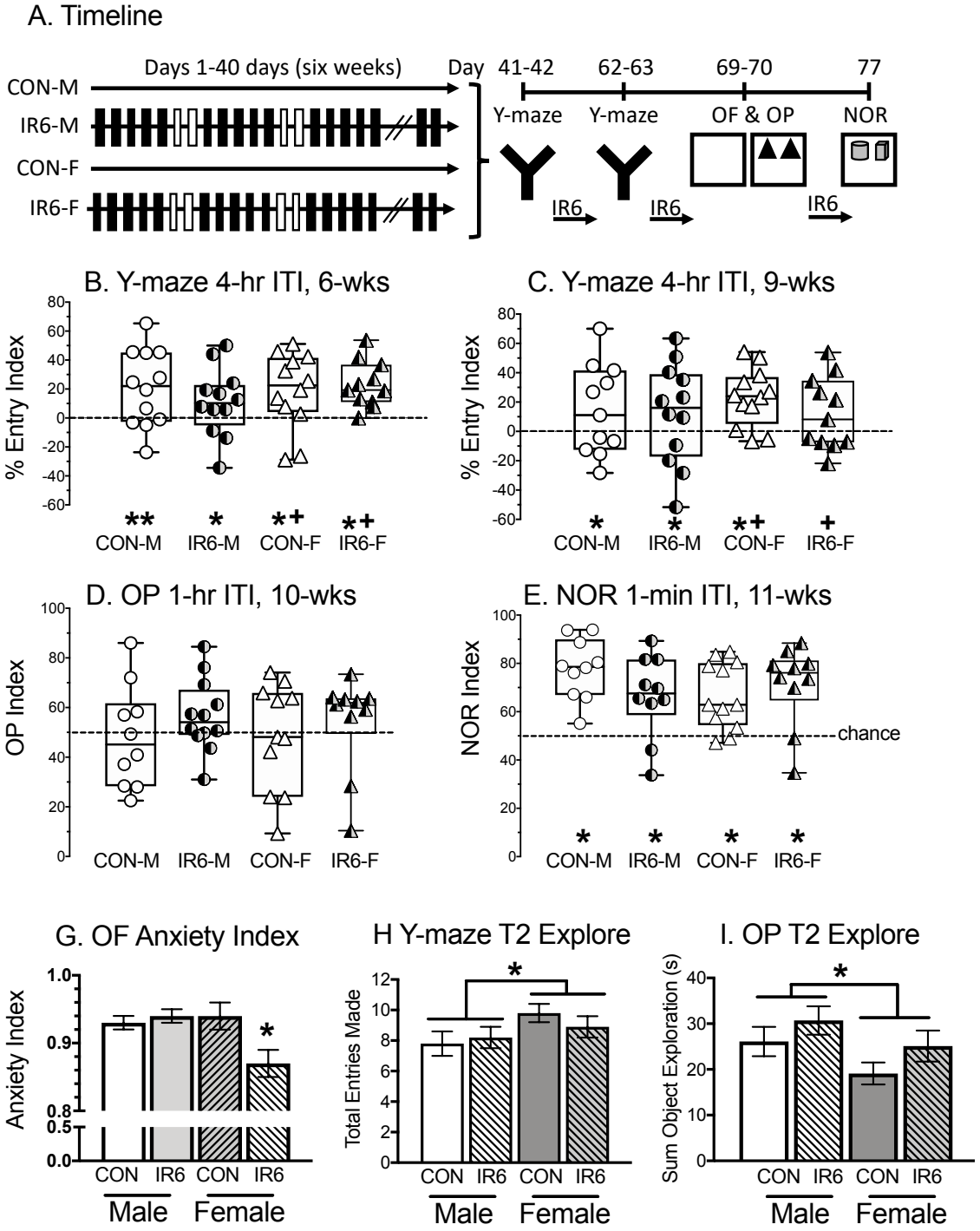

Fig. 4: Effect of an extended IR on spatial ability and anxiety profiles in both male and female rats. A) Timeline of manipulations. See Figure 1 for restraint legend. After 6-weeks of restraint, rats were tested on the Y-maze and then returned to the restraint paradigm for an additional 3-weeks. Behavioral testing then occurred weekly on

days without restraint, starting with the Y-maze, followed by OF/OP (consecutive days) and ending with NOR. B) Y-maze (6-weeks of IR) performance showing the %Entry Index in trial 2 after a 4-hr ITI. CON-M entered (and spent more time in) the novel arm than the other arm. IR6-M, CON-F and IR6-F entered the novel arm more than the other arm. Chance is denoted by the dashed horizontal line. Data represent entries (dwell time is not shown). Two symbols indicate significance or tendency toward significance in both entry and dwell measures with entry measure listed first, followed by dwell. C) Y-maze (9-weeks of IR) performance showing the %Entry Index in trial 2 after a 4-hr ITI. CON-M, IR6-M and CON-F entered the novel arm more than the other arm. IR6-F showed a tendency to enter the novel arm more than the other arm. Chance is denoted by the dashed horizontal line. Data represent entries (dwell time is not shown). Two symbols indicate significance or a tendency toward significance in both entry and dwell measures with entry measure listed first, followed by dwell. D) OP Index from the second trial after a 1-hr ITI. Preference for the moved object will show values greater than 0.5 with the dashed horizontal line indicating chance levels. All groups performed at chance. E) NOR Index from the second trial with a 1-min ITI. Preference for the new object will show values greater than 0.5 with the dashed line indicating chance levels. All groups spent more time with the new object than the familiar object. G) OF anxiety index. IR6-F had a significantly lower anxiety profile compared to CON-F, CON-M, and IR6-M. H) Entries made in all three arms of the Y-maze (9-wks of IR) during Trial 2. Female rats made more total entries compared to male rats. There were no other group differences. I) OP (trial 2) total object exploration time. Male rats spent

significantly more time exploring both objects compared to female rats. There were no other group differences. *$p < 0.05$, + $p < 0.1$, CON-M = control males, IR6-M = intermittent restraint for 6hrs. males, CON-F = control females, IR6-F = intermittent restraint for 6hrs females.

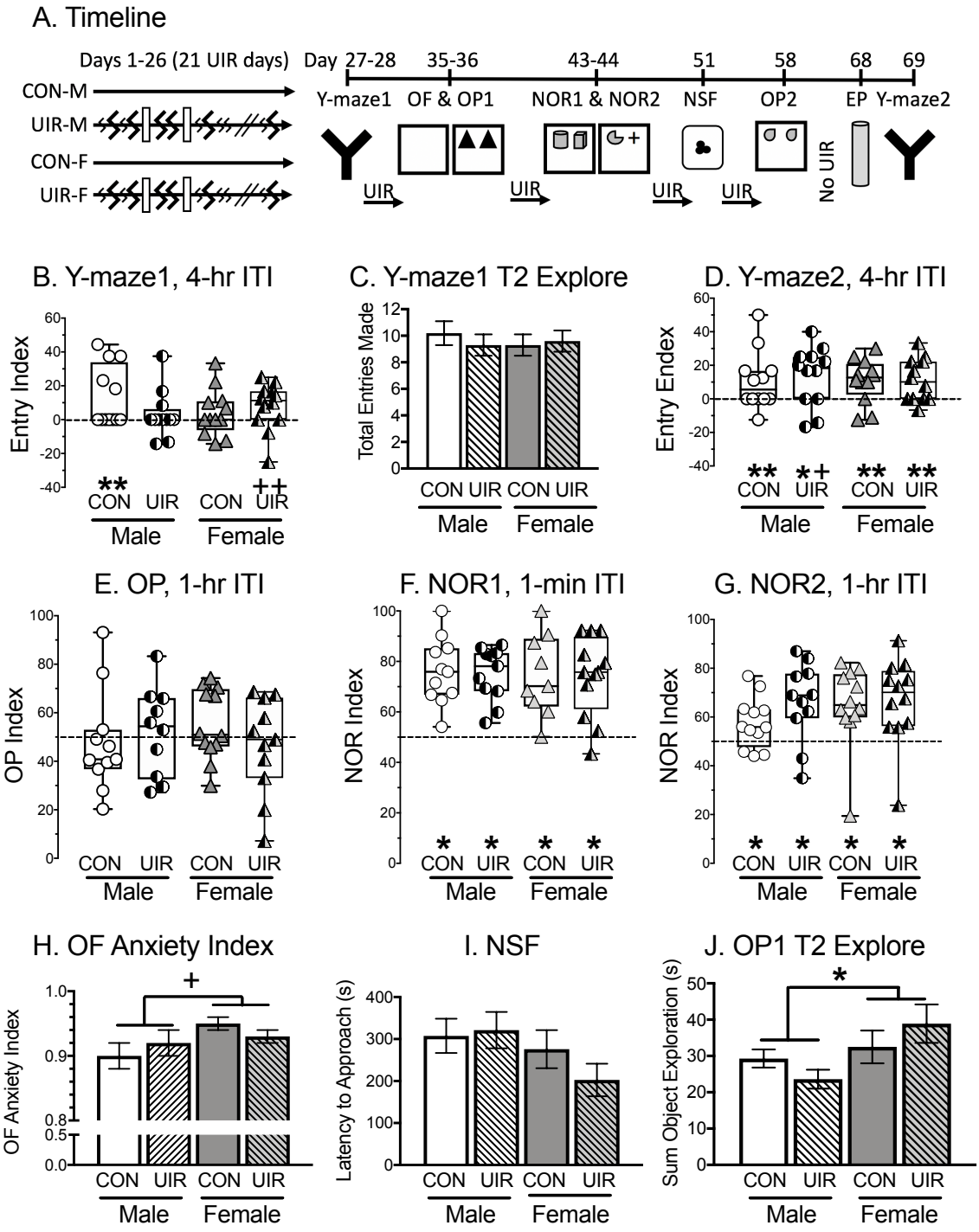

Fig. 5: Effect of a novel UIR paradigm on spatial ability and anxiety profiles in both male and female rats. A) Timeline of manipulations. See Figure 1 for restraint legend. After 21-days of UIR, behavioral testing began. The first test was the Y-maze, and then the UIR resumed in-between bouts of tests, represented by the arrow

and UIR denotation between behavioral tests. The Y-maze task was then followed by the OF and OP (on consecutive days), and then after more UIR the NOR 1-min ITI and NOR 1-hr ITI (consecutive days), and so on to the NSF. A second OP test was given at the end of UIR. Seven days after the last day of UIR, stressed rats were exposed to an elevated platform stressor for 30 minutes and then tested on a Y-maze the following day. B) Y-maze1 performance showing the %Entry Index in trial 2 after a 4-hr ITI. CON entered (and spent more time in-data not shown) the novel arm than the other arm. UIR-F tended to enter (and spend more time in-date not shown) the novel arm than the other arm. UIR-M and CON-F performed at chance levels, which is denoted by the dashed horizontal line. Two symbols indicate significance or tendency toward significance in both entry and dwell measures. C) Entries made in all three arms of the Y-maze1 during Trial 2. The groups made a similar number of total entries during trial 2 of the Y-maze. D) Y-maze2 (Post-EP) performance showing the %Entry Index in trial 2 after a 4-hr ITI. CON-M, UIR-M, CON-F and UIR-F entered (and spent more time in-data not shown) the novel arm more than the other arm. Chance is denoted by the dashed horizontal line. Two symbols indicate significance (or tendency toward significance) in both entry and dwell measures. E) OP1 Index from the second trial after a 1-hr ITI. Preference for the moved object will show values greater than 0.5 with the dashed horizontal line indicating chance levels. All groups performed at chance. F) NOR Index from the second trial with a 1-min ITI. Preference for the new object will show values greater than 0.5 with the dashed horizontal line indicating chance levels. All groups spent more time with the new object than the familiar object. G) NOR Index from the

second trial with a 1-hr ITI. Preference for the new object will show values greater than 0.5 with the dashed horizontal line indicating chance levels. All groups spent more time with the new object than the familiar object. H) OF anxiety index. Female rats tended to have an elevated anxiety profile compared to male rats. I) NSF latency to feed. All groups approached chow in similar durations. J) OP1 total object exploration time during trial 2. Female rats spent significantly more time exploring both objects compared to male rats. There were no other group differences. *$p < 0.05$, +$p < 0.1$, CON-M = control males, UIR-M = unpredictable intermittent restraint males, CON-F = control females, UIR-F = unpredictable intermittent restraint females.

Tables

Table 1: Experiments 1 and 2 body weight change (g) ± SEM over 3 weeks of intermittent restraint.

Experiment	**CON**	**DR6**	**IR6**	**IR2**
1	123.8 ± 9.8	51.8 ± 6.3	73.9 ± 5.4	93.4 ± 8.0
2	134.0 ± 5.1	77.2 ± 4.9	99.6 ± 4.5	—

Table 2: Experiment 3 body weight change (g) ± SEM over 11 weeks of intermittent restraint.

Experiment	**CON-M**	**IR-M**	**CON-F**	**IR-F**
3	254.4 ± 9.2	174.5 ± 5.1	98.8 ± 5.5	62.2 ± 3.4

Table 3: Experiment 4 body weight change (g) ± SEM over 9 weeks of unpredictable intermittent restraint.

Experiment	**CON-M**	**UIR-M**	**CON-F**	**UIR-F**
4	238.2 ± 8.5	169.8 ± 7.7	81.8 ± 6.0	47.2 ± 3.4

Table 4: Experiment 4 post-mortem physiological measures (mg) ± SEM.

Organ	**CON-M**	**UIR-M**	**CON-F**	**UIR-F**
Adrenal	62.5 ± 3.7	64.3 ± 3.0	81.6 ± 4.3	74.9 ± 4.1
Thymus	419.7 ± 26.1	325.3 ± 22.0	286.0 ± 13.2	245.8 ± 17.6
Uterus	—	—	602.7 ± 37.5	574.4 ± 38.4

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