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Assessment of Stress Effects on Cognitive Flexibility using an Operant Strategy Shifting Paradigm

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TITLE:

Assessment of Stress Effects on Cognitive Flexibility Using an Operant Strategy Shifting Paradigm

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SUMMARY:

Stressful life events impair cognitive function, increasing the risk of psychiatric disorders. This protocol illustrates how stress affects cognitive flexibility using an automated operant strategy shifting paradigm in male and female Sprague Dawley rats. Specific brain areas underlying particular behaviors are discussed, and translational relevance of results are explored.

ABSTRACT:

Stress affects cognitive function. Whether stress enhances or impairs cognitive function depends on several factors, including the 1) type, intensity, and duration of the stressor; 2) type of cognitive function under study; and 3) timing of the stressor in relation to learning or executing the cognitive task. Furthermore, sex differences among the effects of stress on cognitive function have been widely documented. Described here is an adaptation of an automated operant strategy shifting paradigm to assess how variations in stress affect cognitive flexibility in male and female Sprague Dawley rats. Specifically, restraint stress is used before or after training in this operant-based task to examine how stress affects cognitive performance in both sexes. Particular brain areas associated with each task in this automated paradigm have been well-established (i.e., the medial prefrontal cortex and orbitofrontal cortex). This allows for targeted manipulations during the experiment or the assessment of particular genes and proteins in these regions upon completion of the paradigm. This paradigm also allows for the detection of different types of performance errors that occur after stress, each of which has defined neural substrates. Also identified are distinct sex differences in perseverative errors after a repeated restraint stress paradigm. The use of these techniques in a preclinical model may reveal how stress affects the brain and impairs cognition in psychiatric disorders, such as post-traumatic stress disorder (PTSD) and major depressive disorder (MDD), which display marked sex differences in prevalence.

INTRODUCTION:

In humans, stressful life events can impair cognitive function (i.e, cognitive flexibility[1]), which denotes the ability to adapt cognitive processing strategies to face new conditions in the environment[2]. Impairment in cognition precipitates and exacerbates many psychiatric disorders, such as Post Traumatic Stress Disorder (PTSD) and Major Depressive Disorder (MDD)[3,4]. These disorders are twice as prevalent in females[5–8], yet the biological basis for this disparity remains unknown. Aspects of executive functioning in humans can be assessed using the Wisconsin Card Sorting Task, a demonstration of cognitive flexibility[2]. Performance in this task is impaired in patients with PTSD[9] and MDD[10], but the neural basis of this change can only be examined by brain imaging[11].

Advances in understanding how stress affects the brain have been made through the use of animal models, particularly rodents. As cognitive flexibility is affected in stress-related diseases, it is an exceptionally relevant phenotype to examine in rodents. To date, most stress neurobiology literature has used an alternative cognitive flexibility paradigm (sometimes referred to as the digging task)[12–15]. While this task has been extensively vetted, it requires more time and effort by the experimenter to train rodents. Adapted and described here is a well-established automated set-shifting protocol[16] to assess cognitive flexibility in male and female Sprague Dawley rats using various stress models[17,18]. The procedure requires minimal oversight by the experimenter and allows multiple rats to be tested simultaneously. In addition, unlike other versions of this automated task[19], the adaptation of this paradigm only requires 3 days of training and includes an efficient programmed data analysis.

Whether stress enhances or impairs cognitive function depends on the type, intensity, and duration of the stressor, as well as the timing of the stressor in relation to learning or executing a cognitive task[20,21]. Thus, the protocol incorporates stress procedures both before and after the operant training. It also examines representative results from stress studies. In addition, the brain regions underlying particular aspects of set-shifting have been well-established[2,16,22]; thus, the report also describes how to target and assess particular brain regions during or after the stress and strategy shifting procedures.

There has been limited research on directly examining sex differences in cognitive flexibility[18,23]. The protocol describes how to 1) incorporate both male and female rats into the experimental paradigm, then 2) track estrous cycles before and during the procedures in freely cycling females. Prior studies have indicated that stress before operant training can lead to sex-specific deficits in cognitive flexibility in rats[17]. Particularly, female rats exhibit disruptions in cognitive flexibility after stress, whereas cognitive flexibility improves in male rats after stress[17]. Interestingly, a major hallmark of stress-related psychiatric disorders, which have a sex-biased incidence in humans, is cognitive inflexibility. These results suggest that females may be more vulnerable to this type of cognitive impairment than males. The use of these techniques in animal models will shed light on the effects of stress on the brain and how it impairs cognition in psychiatric disorders in humans.

PROTOCOL:

All procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Bryn Mawr College.

1. Animal preparation

1.1. Acquire male and female adult Sprague Dawley rats.

NOTE: The rats can be delivered before 65 days of age, but do not begin procedures until after this point to ensure that both males and females are fully mature.

1.2. Pair-house same-sex rats for as long as possible, as long-term isolation is a stressor[24]. For food restriction, singly house rats just prior to the operant strategy shifting protocol.

1.3. After 1 week of acclimation, gently begin to handle rats for 3–5 min per day. Collect the body weight of each rat. Additionally, if interested in assessing how gonadal hormones may affect the results, collect vaginal lavage for female rats (described in section 2).

1.4. Before food restriction procedures begin, obtain approval from the institutional IACUC or other regulatory body. Restrict (from food) animals that will be run in the operant strategy shifting paradigm at least 3 days before the training begins so that they successfully learn the task. Ensure that water is always freely available.

1.4.1. If employing a stress procedure for more than 3 days before training, adjust the food restriction to match the number of days of stress (e.g., 5 days of restraint plus food restriction[25]).

1.4.2. Each day, deliver 80% of the normal daily food intake (i.e., 4 g of food per 100 g of body weight)[26]. Use the daily weight collection for the rat to calculate how much food to give each day.

1.4.3. Continue the food restriction through the training and testing days. However, do not place food in the home cage until after the rat has completed training or testing for the day, or else they will not be motivated to perform the tasks for a food pellet reward. Ensure that the timing of food delivery to rats upon completion of the task is fairly unpredictable since this helps to avoid reduced motivation to perform in the operant chamber (in favor of simply waiting for food in the home cage afterwards).

NOTE: Animals undergoing the restraint stress paradigm do not exhibit significantly greater weight loss than control, unstressed subjects. However, various stress procedures may themselves induce weight loss, resulting in rats receiving less food than unstressed counterparts during body weight-based food restriction. This may present an additional,

confounding stressor. If this appears to be the case, alternatively use a fixed amount of food given to each subject, regardless of weight[27].

2. Vaginal lavage

NOTE: Gonadal hormones (i.e., estrogen and progesterone) are known to affect the stress response and cognition[28–30]. These hormones fluctuate over the estrous cycle of female rats[31]. If interested in tracking the estrous cycle of freely cycling female rodents to correlate with stress or cognitive flexibility data, collect vaginal lavage as described below. Representative data considering estrous cycle stage are not provided.

2.1. To obtain vaginal lavage samples from females, gather warm water in a clean beaker, a glass eyedropper, a "lavage" slide (microscope slide with acrylic paint circles to hold the lavage sample), and one empty beaker.

2.2. Fill the eyedropper with a small amount of warm water (~0.5 mL), then insert the tip into the vagina of the female rat (by lifting by its tail). Expel the sterile water 2x–3x and expel the collected fluid onto a microscopic slide. Do not overflow the lavage slide circle.

2.3. Expel any excess liquid into the empty beaker. Label the lavage slide with rat numbers and put the samples from each rat in that order so it is clear which sample belongs to each rat.

2.4. Thoroughly rinse the eyedropper by pipetting clean warm water and dispensing it into the "excess" beaker several times before filling the eyedropper to sample the next rat.

2.5. Carefully carry the lavage slide to a brightfield microscope to image the lavage sample and classify the day within the estrous cycle as described in Becker et al[31].

NOTE: Ideally, lavaging should be done for a few weeks to properly track a female's cycle and should be performed at a very similar time each day to control for circadian rhythms. Preferably, this procedure should be performed before stress and operant strategy shifting procedures. Data for female rats can be analyzed post-hoc according to estrous cycle day (consider days of cycle when stress is performed and/or day of cycle when testing occurs).

3. Equipment and software

3.1. Use operant chambers for behavioral training and testing.

3.1.1. Ensure that the chambers contain at least two retractable levers with two stimulus lights above, a house light, and a dispenser for reinforcement for these tasks.

3.1.2. Check that the levers are on the either side of the central reinforcement delivery area with one stimulus light above each lever.

3.1.3. Use the house light to illuminate the chamber without interfering with detection of the light stimulus (it is best if the house light is on the back wall of the chamber, opposite to the levers and stimulus lights).

3.2. Use dustless food pellets (here, 45 mg pellets are used: 18.7% protein, 5.6% fat, and 4.7% fiber) for reinforcement in food-restricted rats. Do not use pellets high in sucrose or fat (unless there is interest in how stress affects palatable food intake).

3.3. Control the presentation of stimuli, lever operation, and data collection from a computer with software capable of operating the chamber (**Table of Materials**).

NOTE: For information related to coding of programs using this software, contact the authors. MED-PC scripts are included as supplemental files. This software collects information about the animal's responses for each trial (which lever is pressed, whether it is correct/incorrect/no response, and latency to make the choice). From this information, users can calculate various measures in the behavioral paradigm, as described in the behavioral analysis section.

3.4. Perform training/testing at the same time each day to control for circadian rhythms in stress hormones[32] (and other relevant measures).

3.5. Fill the bottom tray of each operant box with fresh bedding to collect feces/waste. Following each session, dump each tray, clean trays with alcohol wipes, and replace with fresh bedding before placing a new animal in the chamber.

4. Stress procedures

4.1. Decide whether the stress procedure should be performed before, during, and/or after training on the operant strategy shifting paradigm (e.g., 5 days of restraint stress prior to 3 days of operant training *vs.* 3 days of operant training followed by a single restraint and testing).

4.2. Execute the stress procedure at the same time daily with respect to operant training. (e.g., 30 min of restraint stress starting at 9 A.M., followed by placement in the operant chamber).

4.3. Perform the stress procedures in a separate room from both the colony room and strategy shifting paradigm rooms (to ensure there are no cofounding factors associated with witness stress)[33]. Briefly, place the rat in a Broome-style transparent restraint tube and seal the opening, taking care not to pinch the limbs or tail.

NOTE: Estimate how long the first group of rats will spend in the operant chambers. This will vary depending on training *vs.* test day; however, after running several cohorts, an average time to complete each task to estimate future tasks can be calculated.

4.4. Depending on how many operant chambers are available, stagger the stress procedure for subjects. For example, four rats undergo restraint stress and are placed in four operant

chambers. One hour later, four more animals undergo stress procedures to be followed by the operant chamber.

5. Training

NOTE: This paradigm is modified from the operant set-shifting procedure developed by Floresco et al. such that it can be completed in 3 days[19]. Training procedures for rats require 3 days (1 day to learn each task as described below). It is rare that a rat does not learn these tasks. If a rat fails to learn each task, it should be excluded from the final study. See **Figure 1A** for a visual depiction of the training paradigm described below.

5.1. Before placing the rat in the chamber, ensure that there are enough food pellets in the dispenser and that the operant boxes are properly functioning. To accomplish this, load and initiate a training or test day program in an empty chamber, manually testing that the correct lever appropriately delivers one reward per lever press.

5.2. Training the rat to press each lever

5.2.1. Before placing the rat in the box for the first day of training, manually set one food pellet reward on the correct lever, as designated upon loading the training procedure within each chamber.

5.2.2. Train the rat using a fixed ratio (FR-1) schedule, such that each correct lever press is rewarded with one reinforcement. Counterbalance the correct lever per day across subjects and/or experimental conditions (shaping only one lever at a time) by designating the correct lever upon loading the training procedure on the computer operating the chambers.

5.2.3. Allow the rat to press the lever until it reaches the criterion by pressing the correct lever 50x, usually completing the task between 30–45 min.

5.2.4. The following day force the rat to perform this task on the opposite lever using the same program as the first day of training, but designate the opposite lever as the correct one. There is no need to "shape" the lever with a food pellet on this day of training. Typically, this criterion is quickly acquired after rats have learned to press the first lever.

5.3. Training the rat to respond to the light cue

5.3.1. On the third day of training, illuminate the light above both levers for 15 s trials, during which the rat may press one of lever to potentially receive a food pellet reward. During the light discrimination task, this program will randomly select which lever is correct on a trial-by-trial basis.

5.3.2. If the rat presses the correct lever, ensure that the lights remain illuminated for 3 s and the reward is delivered, followed by a 5 s period, during which the lights are shut off preceding

the next trial. If the rat presses the incorrect lever, ensure that no reward is delivered and that lights are shut off for 10 s preceding the next trial.

5.3.3. Following this last day of training, calculate "side bias" to determine if the rat has a preference for the left or right lever by dividing the number of presses of one lever divided by the total number of lever presses. On the test day, the rat will start on its least preferred side to ensure that it is learning the specific response-reward contingency, rather than responding to a preferred lever.

6. Testing

NOTE: See **Figure 1B** for a visual depiction of the testing paradigm described below.

6.1. On day 4 (test day), place the rat in the operant chamber following stress procedures and test them in side discrimination, side reversal, and light discrimination tasks serially. Ensure that the light discrimination task only illuminates the light above the "correct" lever. In each task, rats must consecutively achieve eight correct trials to complete each discrimination without pressing the unrewarded, incorrect lever. An incorrect lever press will reset this chain of trials.

6.1.1. Test rats using the side discrimination task. Using the side discrimination program, reward the rat for pressing the lever on its least preferred side as determined from the third day of training, regardless of the light cue. The task ends upon pressing the correct lever 8x consecutively (excluding omissions).

6.1.2. Perform the side reversal test by running rats using the side discrimination program again, but this time designating the lever opposite to the correct one from the side discrimination task as correct. Ensure that the rat is rewarded for pressing this lever, regardless of the light cue. The task ends upon pressing the correct lever 8x consecutively (excluding omissions).

6.1.3. Perform the light discrimination task, which rewards the rat for pressing the lever with the light illuminated above. Each operant testing is complete upon pressing the correct lever 8x consecutively (excluding omissions).

NOTE: Based on previous studies, these tasks encode a minimum of 30 trials, regardless of consecutive presses, to ensure that rats have sufficient time to learn the rules of each task[18]. Thus, if the rat consecutively achieves eight correct trials before 30 trials have occurred, the task will remain engaged until 30 trials are completed.

7. Behavioral analysis

NOTE: The data acquired for each animal on the test day are automatically recorded and saved by the computer, as long as a MED-PC script for each task been initiated and allowed to complete (see supplementary materials for MED-PC scripts).

7.1 Open the data for each test day task (side discrimination, side reversal, and light discrimination) using the computer program. The main measures recorded by the program are trials to criterion, errors in criterion, and time to criterion. These measures are described in detail below.

NOTE: The authors have generated a MATLAB script that allows for automation of the analysis process as well as analysis of perseverative vs. regressive errors (contact authors for code information to streamline data analysis).

7.1.1. Use trials to criterion (which refers to the total number of trials [not including omissions] necessary for the rat to consecutively complete eight correct trials, including those eight trials) as the main indicator of accuracy. This data is located in the first column in array B in a data file generated by the MED-PC script for any of the tasks on test day.

7.1.2. Examine the total errors made during each task. This data is located in the third column of array B in a data file generated by the MED-PC script for any of the tasks on test day. These errors are also categorized into perseverative or regressive errors. Perseverative errors are committed when the rat continues to follow the earlier rule from the previous task. Regressive errors are committed after it has disengaged from the previous rule but continues to try to acquire the new rule (for more details on how these types of errors are calculated, refer to the published method[18]).

7.1.3. If the rat did not respond to a light cue within 15 s, the trial is categorized as an omission, not counting it towards the total number of trials to criterion. Calculate this by first adding together the number of correct responses (located in the second column of array B in data file) and number of errors (located in the third column of array B in data file). Next, subtract this number from the total number of trials to criterion (this is the last number in the first column of array B in a data file, different from the trials to criterion).

7.1.4. Use start and finish times recorded by the program (located at the top of a data file generated by the MED-PC script for any of the tasks on test day) to calculate time to criterion. Latency to the first lever press can also be calculated from the data file by subtracting the variable K (elapsed time in seconds from the first lever press) from the time to criterion.

7.1.5. Average the data for each behavioral measure for rats within the same treatment group. Perform appropriate statistical analyses (depending on how many variables are being examined).

8. Brain substrates

8.1. Determine an interested brain area and/or aspect of cognitive flexibility. For example, if stress increases perseverative errors in the side reversal task, the orbitofrontal cortex (OFC) may be of particular interest, as previous lesion studies have indicated this brain region plays a

role in many forms of reversal learning (i.e., spatial reversal tested in the side reversal task)[34–36]. In this example, sacrifice rats after the strategy shifting paradigm is completed and examine c-fos (measure of neural activation[37]) in the OFC using described immunohistochemical methods[25] and described briefly here.

8.1.1. First, extract brains from animals and cut into 40 µm slices.

8.1.2. Wash the tissue in phosphate-buffered saline (PBS) 4x for 5 min each, then incubate in 0.3% hydrogen peroxide for 10 min to quench endogenous peroxidases.

8.1.3. Wash tissue in PBS 2x for 5 min each, then incubate in mouse anti-c-fos primary antibody (1:500), 3% normal donkey serum (NDS), and 0.3% Triton X overnight.

8.1.4. The next day, wash tissue in PBS 3x for 5 min each, then incubate in biotin-SP-conjugated donkey anti-mouse sary antibody (1:500) for 2 h.

8.1.5. Wash tissue in PBS 3x for 5 min each, then incubate in avidin-streptavidin AB complex for 1 h.

8.1.6. Wash tissue in PBS 3x for 5 min each, then incubate in DAB solution for up to 10 min as tissue undergoes an oxidation chromogenic reaction.

8.1.7. Wash tissue in PBS 3x for 5 min each, then mount the brain slices on glass microscope slides.

8.1.8. Coverslip the tissue using toluene based mounting medium and image using a brightfield microscope.

NOTE: Here, as reflected in the representative results, rats are sacrificed 30 min after the strategy shifting paradigm ends, roughly 60–90 min after the reversal task has been completed (depending on each rat's performance in the light task). This should represent optimal timing for c-fos expression[38], reflecting performance in the reversal task.

8.2. Alternatively, cannulate a specific brain area for drug injection or viral injection prior to the execution of stress or the operant strategy shifting paradigm.

NOTE: Researchers may want to examine how manipulating neural substrates alters the effects of stress on cognitive flexibility. For example, researchers can block a particular neurotransmitter receptor in the prefrontal cortex prior to testing.

REPRESENTATIVE RESULTS:

The adapted automated operant strategy shifting paradigm outlined above was used to determine if repeated restraint stress affects cognition in male and female Sprague Dawley

rats. Representative behavioral data are described in **Figure 2** below. In short, control and repeatedly restrained rats performed this operant strategy shifting test, which consisted of a series of tasks: side discrimination, side reversal, and light discrimination.

Trials to criterion for each task are depicted in **Figure 2A**. Typically, better performance on each task was represented by a reduced number of trials to criterion. These data indicate that, following acute restraint, males completed the side reversal task in significantly fewer trials than unstressed, control males. Conversely, stressed females required a significantly greater number of trials to complete the side reversal task. These results suggest that males exhibited improved performance following stress, whereas females exhibited impaired performance. In the light discrimination task, stress increased the number of trials to criterion compared to control females, thereby impairing performance in females but not males in this task.

The total number of errors made for each attention task is depicted in **Figure 2B.** Consistent with the number of trials to criterion, stressed males made significantly fewer errors than control males, whereas stressed females made more errors in the side reversal task. Furthermore, in the light discrimination task, females also made significantly more errors. In sum, these data suggest that repeated stress improves cognitive performance in males but impairs cognitive performance in females.

Total errors were further categorized into perseverative or regressive errors in **Figure 2C** (for a distinction between these two types of errors, refer to section 7 of the protocol). Interestingly, stressed males made fewer perseverative errors in the side reversal task than control males. On the other hand, in both the side reversal and light discrimination tasks, stressed females made a greater number of perseverative errors than control females. There were no differences between the treatment groups in the number of regressive errors made during either task.

Omissions in each trial and time to reach criterion are shown in **Figure 2D** (for more information on how these were calculated, refer to section 7 of the protocol)**.** These measures were evaluated in the side reversal task only, as this task exhibited the largest sex differences. Stressed females made a higher percentage of omissions compared to all other treatment groups. In addition, while stress appeared to decrease the time to complete the side reversal task in males, stress prolonged completion of the task in females. In sum, repeated stress impaired cognitive flexibility in females but not males.

Brain substrates underlying cognitive flexibility are depicted in **Figure 3**. As stark sex differences were observed in the side reversal task, the brain areas underlying this task were examined to determine whether they displayed similar sex differences in neural activity. As previously discussed, lesion studies have indicated that the orbitofrontal cortex (OFC) mediates the side reversal task[34]. Thus, c-fos, a measure of neural activation[37], was labeled in the OFC at 30 min after the completion of strategy shifting, which should have reflected performance in the side reversal task[38]. However, it is possible that OFC may also play a role in the extradimensional strategy shifting component of this task[39]. Thus, it is important to perform the sacrifice at the appropriate time to reflect brain activity during a particular task within the operant strategy

shifting paradigm. Here, stress induced a significant increase in neuronal activation in the OFC of males compared to controls**.** However, stress induced a significant decrease in neuronal activation in the OFC of females compared to controls. Furthermore, in males, OFC activation and trials to criterion were negatively correlated; specifically, higher OFC activation was associated with fewer trials to criterion. In contrast, there was no correlation between OFC activation and performance in females, suggesting that the OFC was disengaged during these performances.

FIGURE LEGENDS:

Figure 1: Schematic of the operant strategy shifting paradigm during training and test days.

Figure 2: Representative behavioral data from operant strategy shifting paradigm. (A) Trials to criterion for each task on test day. In the side reversal task, stress improved performance in males but impaired performance in females. In the light discrimination task, stress weakened performance in females, while it did not affect males. **(B)** Number of errors for each task on test day. Stress reduced the number of errors made in males but increased errors in females in both side reversal and light discrimination tasks. **(C)** Perseverative and regressive error categorization. Stress decreased perseverative errors made in males but increased perseverative errors made in females in both side reversal and light discrimination tasks. **(D)** Percent trials omitted and time to criterion in the side reversal task. Stress increased the percent omissions in female rats. Stress decreased the time required by males but increased the time required by females to complete the task. Statistics were calculated using two-way ANOVA followed by Tukey's t-test (n = 12 rats per group; error bars represent SEM; #$p \leq 0.10$, *$p < 0.05$). This figure has been modified from a previous publication[17].

Figure 3: Representative neural activation after operant strategy shifting paradigm. (A) OFC activation after strategy shifting task. Representative images of immunohistochemical 3,3'-diaminobenzidine (DAB) staining using an antibody against c-fos in the OFC visualized using brightfield microscopy, then quantified. Stress significantly increased activation (demonstrated by the number of c-fos-expressing cells) in the OFC of males, while it decreased activation in females. Scale bar in bottom-right image panel represents 200 µm. Statistics were calculated using two-way ANOVA followed by Tukey's t-test (n = 12 rats per group, 6–8 sections of OFC analyzed per rat; error bars represent SEM; *$p < 0.05$). **(B)** Trials to criterion in the side reversal task correlated with OFC activation. Males displayed a significant negative correlation, whereas females did not.

DISCUSSION:

The protocol demonstrates how to measure the effects of stress on cognitive function. Specifically, a modified operant strategy shifting paradigm is used in rodents, which measures cognitive flexibility (analogous to the Wisconsin Card Sorting Task in humans)[1]. Cognitive flexibility denotes the ability to adapt cognitive processing strategies to face new conditions in the environment, and it is crucial for normal daily functioning[2]. As human studies on cognitive

flexibility are mostly limited to brain imaging[11], the use of this paradigm in animals will greatly advance the understanding of effects of stress on the brain and cognition.

Stress can impair cognitive function[40]. In fact, this is one of the most common phenotypes in stress-related illnesses such as PTSD and MDD[3,41]. Moreover, there are stark sex differences in the occurrence of stress-related psychiatric illnesses[5–7], yet there is little understanding of the neurobiology behind these biased incidences. Thus, use of this operant strategy shifting paradigm in animals of both sexes may help advance the current understanding of sex differences in psychiatry.

This operant strategy shifting task allows researchers to examine key aspects of cognition relevant to psychiatric disorders. For example, perseverative errors after experimental manipulation are calculated in this paradigm. Perseveration is observed in stress-related psychiatric disorders such as PTSD, and it impairs the ability of one to learn a new set of rules, ultimately impairing working memory[3]. Thus, the measure of perseverative errors is translationally relevant. Moreover, omissions in attention tasks have been noted in patients with PTSD, indicating slower cortical processing[3]. Accordingly, omission data from this paradigm may have clinical counterparts. In sum, cognitive flexibility measured as by this experimental paradigm models key phenotypes that are observed in psychiatric disorders.

This experimental paradigm also allows for precision in targeting neural substrates underlying cognitive flexibility. For example, the literature has indicated that the prefrontal cortex (PFC) is a crucial brain region for cognitive flexibility[3], including the medial prefrontal (mPFC) and orbitofrontal cortex (OFC). Of these subregions in the PFC, the OFC is important for performance in the side reversal task[34,35]. These brain areas are also a key targets for stress-induced functional alterations[42 43]. Interestingly, the model of stress used here does appear to play a role in the subsequent performance of rodents in tests of cognitive flexibility; thus, it should be considered in the design of future experiments. These varying responses to stress point to potentially novel mechanisms by which cognition is impacted by stress. Thus, targeting specific neurotransmitters, proteins, or activation of these brain regions may shed light on how stress affects cognition in male and female rodents. Researchers can choose to manipulate these neural substrates at different timepoints in conjunction with stress or strategy shifting, or alternatively measure neural substrates after exposure to these behavioral paradigms.

This modified operant strategy shifting task has clear advantages over other cognitive flexibility paradigms used in the stress literature (i.e., the digging task[12–15]), which require more time and effort by the experimenter to train rodents. This procedure requires minimal oversight by the experimenter and allows multiple rats to be tested simultaneously. In addition, unlike other versions of this automated task[19], the paradigm only requires 3 days of training and includes an efficient programmed data analysis.

The operant strategy shifting paradigm does have certain limitations. One limitation is that it can only test two stimulus dimensions (e.g., left or right lever vs. light cue), whereas the digging task[12–15] can test a third stimulus dimension (e.g., digging media vs. odor vs. texture). However,

the task described in this protocol still allows for testing of the rat's ability to shift to different rules, which allows testing of the cognitive flexibility constructs. In addition, it is possible to add other parameters to the operant chambers to allow for a third stimulus (e.g., an odor), but this may prolong the training required for the task.

The primary advantage of this task is its simplicity and ability to pair it with stressful or pharmacological manipulations to further understand how stress affects the brain. It should be noted that this simplicity comes with an increased difficulty that subjects face while learning to lever press, compared to the ecologically relevant digging task. While this operant task is far less labor-intensive, rodents will generally require more trials to acquire this task. However, both the digging task and this paradigm engage similar neurobiological mechanisms and thus represent valid options for the examination of cognitive flexibility[16,44]. While there have been varied results in the literature regarding the effects of stress on cognitive flexibility using the digging task and this operant procedure[23,25,27,45,46], the presented method reflects the complex effects that the type, intensity, and duration of a stressor can have on cognitive function[20,21].

Another limitation of the task is that rodents are housed in closed opaque boxes; thus, behaviors other than those that are collected via the computer interface cannot be coded. For example, a high number of omissions by a rat may be due to behavioral inhibition inflicted by stress, or because the rat is asleep. Moreover, other stereotypical behaviors, such as grooming (which is particularly relevant in studying stress), may be interesting to analyze during the task. Mounting cameras in operant chambers may allow for this type of behavioral precision.

Overall, this report details the use of stress procedures in conjunction with an operant strategy shifting paradigm to further understand how stress affects the brain. It should be noted that, in addition to stress procedures and cognitive assessment in adults, research on different developmental stages may provide crucial information about the etiology of cognitive inflexibility. In addition to studying the effects of stress on cognitive flexibility, this simple and efficient operant strategy shifting paradigm can be paired with many experimental manipulations to investigate how the brain adapts to changing environments. Moreover, alternate experimental approaches can be used to study the neural basis of cognitive flexibility, including lesions, pharmacology, gene editing, and electrophysiology. As cognitive inflexibility is one of the key phenotypes in psychiatric disease, more research must be conducted to further understand its neurobiological substrates.

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DISCLOSURES:
The authors have nothing to disclose.

REFERENCES

1. Hurtubise, J. L., Howland, J. G. Effects of stress on behavioral flexibility in rodents. *Neuroscience*. **345**, 176-192 (2016).
2. Bissonette, G. B., Powell, E. M., Roesch, M. R. Neural structures underlying set-shifting: Roles of medial prefrontal cortex and anterior cingulate cortex. *Behavioural Brain Research*. **250**, 91–101 (2013).
3. Vasterling, J. J., Brailey, K., Constans, J. I., Sutker, P. B. Attention and memory dysfunction in posttraumatic stress disorder. *Neuropsychology*. **12** (1), 125–33 (1998).
4. Bangasser, D. A., Kawasumi, Y. Cognitive disruptions in stress-related psychiatric disorders: A role for corticotropin releasing factor (CRF). *Hormones and Behavior*. **76**, 125–135 (2015).
5. Nestler, E. J. et al. Neurobiology of depression. *Neuron*. **34** (1), 13–25 (2002).
6. Keane, T. M., Marshall, A. D., Taft, C. T. Posttraumatic stress disorder: etiology, epidemiology, and treatment outcome. *Annual Review of Clinical Psychology*. **2**, 161–1697 (2006).
7. Seeman, M. V. Psychopathology in women and men: focus on female hormones. *The American Journal of Psychiatry*. **154** (12), 1641–1647 (1997).
8. Hodes, G. E., Epperson, C. N. Sex Differences in Vulnerability and Resilience to Stress Across the Life Span. *Biological Psychiatry*. **86** (6), 421–432 (2019).
9. Monika, T.-B., Antoni, F., Piotr, G., Marian, M., Krzysztof, Z. Wisconsin Card Sorting Test in psychological examination of patients with psychiatric disorders. *Polski merkuriusz lekarski: organ Polskiego Towarzystwa Lekarskiego*. **25 Suppl 1**, 51–2 (2008).
10. Merriam, E. P., Thase, M. E., Haas, G. L., Keshavan, M. S., Sweeney, J.A. Prefrontal cortical dysfunction in depression determined by Wisconsin Card Sorting Test performance. *The American Journal of Psychiatry*. **156** (5), 780–782 (1999).
11. Monchi, O., Petrides, M., Petre, V., Worsley, K., Dagher, A. Wisconsin Card Sorting revisited: distinct neural circuits participating in different stages of the task identified by event-related functional magnetic resonance imaging. *The Journal of Neuroscience: the Official Journal of the Society for Neuroscience*. **21** (19), 7733–7741 (2001).
12. Bulin, S. E., Hohl, K. M., Paredes, D., Silva, J. D., Morilak, D. A. Bidirectional optogenetically-induced plasticity of evoked responses in the rat medial prefrontal cortex can impair or enhance cognitive set-shifting. *eNeuro*. **7** (1) ENEURO.0363-19 (2019).
13. Chaby, L. E., Karavidha, K., Lisieski, M. J., Perrine, S. A., Liberzon, I. Cognitive Flexibility Training Improves Extinction Retention Memory and Enhances Cortical Dopamine With and Without Traumatic Stress Exposure. *Frontiers in Behavioral Neuroscience*. **13**, 24 (2019).
14. Drozd, R., Rojek-Sito, K., Rygula, R. The trait 'pessimism' does not interact with cognitive flexibility but makes rats more vulnerable to stress-induced motivational deficits: Results from the attentional set-shifting task. *Behavioural Brain Research*. **335**, 199–207 (2017).
15. Birrell, J. M., Brown, V. J. Medial frontal cortex mediates perceptual attentional set-shifting in the rat. *The Journal of Neuroscience: the Official Journal of the Society for Neuroscience*. **20** (11), 4320–4 (2000).
16. Floresco, S. B., Block, A. E., Tse, M. T. L. Inactivation of the medial prefrontal cortex of the rat impairs strategy set-shifting, but not reversal learning, using a novel, automated

procedure. *Behavioural Brain Research*. **190** (1), 85–96 (2008).
17. Grafe, L. A., Cornfeld, A., Luz, S., Valentino, R., Bhatnagar, S. Orexins Mediate Sex Differences in the Stress Response and in Cognitive Flexibility. *Biological Psychiatry*. **81** (8), 683–692 (2017).
18. Snyder, K. P., Barry, M., Valentino, R. J. Cognitive impact of social stress and coping strategy throughout development. *Psychopharmacology*. **232**(1),185-9 (2014).
19. Brady, A. M., Floresco, S. B. Operant procedures for assessing behavioral flexibility in rats. *Journal of Visualized Experiments*. (96), e52387 (2015).
20. Sandi, C., Pinelo-Nava, M. T. Stress and Memory: Behavioral Effects and Neurobiological Mechanisms. *Neural Plasticity*. 1–20 (2007).
21. Shansky, R. M., Lipps, J. Stress-induced cognitive dysfunction: hormone-neurotransmitter interactions in the prefrontal cortex. *Frontiers in Human Neuroscience*. **7**, 123 (2013).
22. Ragozzino, M. E., Detrick, S., Kesner, R. P. Involvement of the prelimbic-infralimbic areas of the rodent prefrontal cortex in behavioral flexibility for place and response learning. *The Journal of Neuroscience: the Official Journal of the Society for Neuroscience*. **19** (11), 4585–94 (1999).
23. Liston, C. et al. Stress-induced alterations in prefrontal cortical dendritic morphology predict selective impairments in perceptual attentional set-shifting. *The Journal of Neuroscience: the Official Journal of the Society for Neuroscience*. **26** (30), 7870–4 (2006).
24. Hatch, A., Wiberg, G. S., Balazs, T., Grice, H. C. Long-Term Isolation Stress in Rats. *Science*. **142** (3591), 507–507 (1963).
25. Grafe, L. A., Cornfeld, A., Luz, S., Valentino, R., Bhatnagar, S. Orexins Mediate Sex Differences in the Stress Response and in Cognitive Flexibility. *Biological Psychiatry*. **81** (8), 683–692 (2017).
26. Animal Care and Use Committee, T. J. H. U. Species Specific Information: Rat. at <http://web.jhu.edu/animalcare/procedures/rat.html>.
27. Lapiz-Bluhm, M. D. S. et al. Behavioural assays to model cognitive and affective dimensions of depression and anxiety in rats. *Journal of Neuroendocrinology*. **20** (10), 1115–37 (2008).
28. McEwen, B. S. Permanence of brain sex differences and structural plasticity of the adult brain. *Proceedings of the National Academy of Sciences*. **96** (13), 7128–7130 (1999).
29. Manber, R., Armitage, R. Sex, steroids, and sleep: a review. *Sleep*. **22** (5), 540–55 (1999).
30. Sherwin, B. B. Estrogen and Cognitive Functioning in Women. *Endocrine Reviews*. **24** (2), 133–151 (2003).
31. Becker, J. B. et al. Strategies and methods for research on sex differences in brain and behavior. *Endocrinology*. **146** (4), 1650–73 (2005).
32. Koch, C. E., Leinweber, B., Drengberg, B. C., Blaum, C., Oster, H. Interaction between circadian rhythms and stress. *Neurobiology of Stress*. **6**, 57–67 (2017).
33. Warren, B. L. et al. Neurobiological sequelae of witnessing stressful events in adult mice. *Biological Psychiatry*. **73** (1), 7–14 (2013).
34. McAlonan, K., Brown, V. J. Orbital prefrontal cortex mediates reversal learning and not attentional set-shifting in the rat. *Behavioural Brain Research*. **146** (1–2), 97–103 (2003).
35. Schoenbaum, G., Saddoris, M. P., Stalnaker, T. A. Reconciling the roles of orbitofrontal cortex in reversal learning and the encoding of outcome expectancies. *Annals of the New*

York Academy of Sciences. **1121** (1), 320–35 (2007).
36. Meunier, M. Effects of orbital frontal and anterior cingulate lesions on object and spatial memory in rhesus monkeys. *Neuropsychologia*. **35** (7), 999–1015 (1997).
37. Zappulla, R. A., Wang, W., Friedrich, V. L., Grabel, J., Nieves, J. CNS activation patterns underlying motor evoked potentials as demonstrated by c-fos immunoreactivity. *Electroencephalography and Clinical Neurophysiology*. **43**, 155–69 (1991).
38. Schoenenberger, P., Gerosa, D., Oertner, T. G. Temporal Control of Immediate Early Gene Induction by Light. *PLoS ONE*. **4** (12), e8185 (2009).
39. Chase, E. A., Tait, D. S., Brown, V. J. Lesions of the orbital prefrontal cortex impair the formation of attentional set in rats. *The European Journal of Neuroscience*. **36** (3), 2368–75 (2012).
40. Hancock, P. A., Warm, J. S. A dynamic model of stress and sustained attention. *Human Performance in Extreme Environments*. **7** (1), 15–28 (2003).
41. Johnson, P. L., Molosh, A., Fitz, S. D., Truitt, W. A., Shekhar, A. Orexin, stress, and anxiety/panic states. *Progress in Brain Research*. **198**, 133–61 (2012).
42. Leuner, B., Shors, T. J. Stress, anxiety, and dendritic spines: what are the connections? *Neuroscience*. **251**, 108–19 (2013).
43. Holmes, A., Wellman, C. L. Stress-induced prefrontal reorganization and executive dysfunction in rodents. *Neuroscience and Biobehavioral Reviews*. **33** (6), 773–83 (2009).
44. Placek, K., Dippel, W. C., Jones, S., Brady, A. M. Impairments in set-shifting but not reversal learning in the neonatal ventral hippocampal lesion model of schizophrenia: further evidence for medial prefrontal deficits. *Behavioural Brain Research*. **256**, 405–13 (2013).
45. Nikiforuk, A., Popik, P. Long-lasting cognitive deficit induced by stress is alleviated by acute administration of antidepressants. *Psychoneuroendocrinology*. **36** (1), 28–39 (2011).
46. Bondi, C. O., Rodriguez, G., Gould, G. G., Frazer, A., Morilak, D. A. Chronic unpredictable stress induces a cognitive deficit and anxiety-like behavior in rats that is prevented by chronic antidepressant drug treatment. *Neuropsychopharmacology*. **33** (2), 320–31 (2008).