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

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1 **TITLE:**
2 Assessment of Stress Effects on Cognitive Flexibility Using an Operant Strategy Shifting
3 Paradigm
4

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17 **KEYWORDS:**
18 sex differences, cognitive flexibility, stress, prefrontal cortex, attention, perseverative errors
19

20 **SUMMARY:**
21 Stressful life events impair cognitive function, increasing the risk of psychiatric disorders. This
22 protocol illustrates how stress affects cognitive flexibility using an automated operant strategy
23 shifting paradigm in male and female Sprague Dawley rats. Specific brain areas underlying
24 particular behaviors are discussed, and translational relevance of results are explored.
25

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

45

46 **INTRODUCTION:**

47

48 In humans, stressful life events can impair cognitive function (i.e, cognitive flexibility¹), which
49 denotes the ability to adapt cognitive processing strategies to face new conditions in the
50 environment². Impairment in cognition precipitates and exacerbates many psychiatric
51 disorders, such as Post Traumatic Stress Disorder (PTSD) and Major Depressive Disorder
52 (MDD)^{3,4}. These disorders are twice as prevalent in females⁵⁻⁸, yet the biological basis for this
53 disparity remains unknown. Aspects of executive functioning in humans can be assessed using
54 the Wisconsin Card Sorting Task, a demonstration of cognitive flexibility². Performance in this
55 task is impaired in patients with PTSD⁹ and MDD¹⁰, but the neural basis of this change can only
56 be examined by brain imaging¹¹.

57

58 Advances in understanding how stress affects the brain have been made through the use of
59 animal models, particularly rodents. As cognitive flexibility is affected in stress-related diseases,
60 it is an exceptionally relevant phenotype to examine in rodents. To date, most stress
61 neurobiology literature has used an alternative cognitive flexibility paradigm (sometimes
62 referred to as the digging task)¹²⁻¹⁵. While this task has been extensively vetted, it requires
63 more time and effort by the experimenter to train rodents. Adapted and described here is a
64 well-established automated set-shifting protocol¹⁶ to assess cognitive flexibility in male and
65 female Sprague Dawley rats using various stress models^{17,18}. The procedure requires minimal
66 oversight by the experimenter and allows multiple rats to be tested simultaneously. In addition,
67 unlike other versions of this automated task¹⁹, the adaptation of this paradigm only requires 3
68 days of training and includes an efficient programmed data analysis.

69

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

77

78 There has been limited research on directly examining sex differences in cognitive flexibility^{18,23}.
79 The protocol describes how to 1) incorporate both male and female rats into the experimental
80 paradigm, then 2) track estrous cycles before and during the procedures in freely cycling
81 females. Prior studies have indicated that stress before operant training can lead to sex-specific
82 deficits in cognitive flexibility in rats¹⁷. Particularly, female rats exhibit disruptions in cognitive
83 flexibility after stress, whereas cognitive flexibility improves in male rats after stress¹⁷.
84 Interestingly, a major hallmark of stress-related psychiatric disorders, which have a sex-biased
85 incidence in humans, is cognitive inflexibility. These results suggest that females may be more
86 vulnerable to this type of cognitive impairment than males. The use of these techniques in
87 animal models will shed light on the effects of stress on the brain and how it impairs cognition
88 in psychiatric disorders in humans.

89

90 **PROTOCOL:**

91

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

94

95 **1. Animal preparation**

96

97 1.1. Acquire male and female adult Sprague Dawley rats.

98

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

101

102 1.2. Pair-house same-sex rats for as long as possible, as long-term isolation is a stressor²⁴. For
103 food restriction, singly house rats just prior to the operant strategy shifting protocol.

104

105 1.3. After 1 week of acclimation, gently begin to handle rats for 3–5 min per day. Collect the
106 body weight of each rat. Additionally, if interested in assessing how gonadal hormones may
107 affect the results, collect vaginal lavage for female rats (described in section 2).

108

109 1.4. Before food restriction procedures begin, obtain approval from the institutional IACUC or
110 other regulatory body. Restrict (from food) animals that will be run in the operant strategy
111 shifting paradigm at least 3 days before the training begins so that they successfully learn the
112 task. Ensure that water is always freely available.

113

114 1.4.1. If employing a stress procedure for more than 3 days before training, adjust the food
115 restriction to match the number of days of stress (e.g., 5 days of restraint plus food
116 restriction²⁵).

117

118 1.4.2. Each day, deliver 80% of the normal daily food intake (i.e., 4 g of food per 100 g of body
119 weight)²⁶. Use the daily weight collection for the rat to calculate how much food to give each
120 day.

121

122 1.4.3. Continue the food restriction through the training and testing days. However, do not
123 place food in the home cage until after the rat has completed training or testing for the day, or
124 else they will not be motivated to perform the tasks for a food pellet reward. Ensure that the
125 timing of food delivery to rats upon completion of the task is fairly unpredictable since this
126 helps to avoid reduced motivation to perform in the operant chamber (in favor of simply
127 waiting for food in the home cage afterwards).

128

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

133 confounding stressor. If this appears to be the case, alternatively use a fixed amount of food
134 given to each subject, regardless of weight²⁷.

135

136 **2. Vaginal lavage**

137

138 NOTE: Gonadal hormones (i.e., estrogen and progesterone) are known to affect the stress
139 response and cognition^{28–30}. These hormones fluctuate over the estrous cycle of female rats³¹.
140 If interested in tracking the estrous cycle of freely cycling female rodents to correlate with
141 stress or cognitive flexibility data, collect vaginal lavage as described below. Representative
142 data considering estrous cycle stage are not provided.

143

144 2.1. To obtain vaginal lavage samples from females, gather warm water in a clean beaker, a
145 glass eyedropper, a “lavage” slide (microscope slide with acrylic paint circles to hold the lavage
146 sample), and one empty beaker.

147

148 2.2. Fill the eyedropper with a small amount of warm water (~0.5 mL), then insert the tip into
149 the vagina of the female rat (by lifting by its tail). Expel the sterile water 2x–3x and expel the
150 collected fluid onto a microscopic slide. Do not overflow the lavage slide circle.

151

152 2.3. Expel any excess liquid into the empty beaker. Label the lavage slide with rat numbers and
153 put the samples from each rat in that order so it is clear which sample belongs to each rat.

154

155 2.4. Thoroughly rinse the eyedropper by pipetting clean warm water and dispensing it into the
156 “excess” beaker several times before filling the eyedropper to sample the next rat.

157

158 2.5. Carefully carry the lavage slide to a brightfield microscope to image the lavage sample and
159 classify the day within the estrous cycle as described in Becker et al³¹.

160

161 NOTE: Ideally, lavaging should be done for a few weeks to properly track a female’s cycle and
162 should be performed at a very similar time each day to control for circadian rhythms.

163 Preferably, this procedure should be performed before stress and operant strategy shifting
164 procedures. Data for female rats can be analyzed post-hoc according to estrous cycle day
165 (consider days of cycle when stress is performed and/or day of cycle when testing occurs).

166

167 **3. Equipment and software**

168

169 3.1. Use operant chambers for behavioral training and testing.

170

171 3.1.1. Ensure that the chambers contain at least two retractable levers with two stimulus lights
172 above, a house light, and a dispenser for reinforcement for these tasks.

173

174 3.1.2. Check that the levers are on the either side of the central reinforcement delivery area
175 with one stimulus light above each lever.

176

177 3.1.3. Use the house light to illuminate the chamber without interfering with detection of the
178 light stimulus (it is best if the house light is on the back wall of the chamber, opposite to the
179 levers and stimulus lights).

180

181 3.2. Use dustless food pellets (here, 45 mg pellets are used: 18.7% protein, 5.6% fat, and 4.7%
182 fiber) for reinforcement in food-restricted rats. Do not use pellets high in sucrose or fat (unless
183 there is interest in how stress affects palatable food intake).

184

185 3.3. Control the presentation of stimuli, lever operation, and data collection from a computer
186 with software capable of operating the chamber (**Table of Materials**).

187

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

193

194 3.4. Perform training/testing at the same time each day to control for circadian rhythms in
195 stress hormones³² (and other relevant measures).

196

197 3.5. Fill the bottom tray of each operant box with fresh bedding to collect feces/waste.
198 Following each session, dump each tray, clean trays with alcohol wipes, and replace with fresh
199 bedding before placing a new animal in the chamber.

200

201 **4. Stress procedures**

202

203 4.1. Decide whether the stress procedure should be performed before, during, and/or after
204 training on the operant strategy shifting paradigm (e.g., 5 days of restraint stress prior to 3 days
205 of operant training vs. 3 days of operant training followed by a single restraint and testing).

206

207 4.2. Execute the stress procedure at the same time daily with respect to operant training. (e.g.,
208 30 min of restraint stress starting at 9 A.M., followed by placement in the operant chamber).

209

210 4.3. Perform the stress procedures in a separate room from both the colony room and strategy
211 shifting paradigm rooms (to ensure there are no confounding factors associated with witness
212 stress)³³. Briefly, place the rat in a Broome-style transparent restraint tube and seal the
213 opening, taking care not to pinch the limbs or tail.

214

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

218

219 4.4. Depending on how many operant chambers are available, stagger the stress procedure for
220 subjects. For example, four rats undergo restraint stress and are placed in four operant

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

223

224 **5. Training**

225

226 NOTE: This paradigm is modified from the operant set-shifting procedure developed by
227 Floresco et al. such that it can be completed in 3 days¹⁹. Training procedures for rats require 3
228 days (1 day to learn each task as described below). It is rare that a rat does not learn these
229 tasks. If a rat fails to learn each task, it should be excluded from the final study. See **Figure 1A**
230 for a visual depiction of the training paradigm described below.

231

232 5.1. Before placing the rat in the chamber, ensure that there are enough food pellets in the
233 dispenser and that the operant boxes are properly functioning. To accomplish this, load and
234 initiate a training or test day program in an empty chamber, manually testing that the correct
235 lever appropriately delivers one reward per lever press.

236

237 5.2. Training the rat to press each lever

238

239 5.2.1. Before placing the rat in the box for the first day of training, manually set one food pellet
240 reward on the correct lever, as designated upon loading the training procedure within each
241 chamber.

242

243 5.2.2. Train the rat using a fixed ratio (FR-1) schedule, such that each correct lever press is
244 rewarded with one reinforcement. Counterbalance the correct lever per day across subjects
245 and/or experimental conditions (shaping only one lever at a time) by designating the correct
246 lever upon loading the training procedure on the computer operating the chambers.

247

248 5.2.3. Allow the rat to press the lever until it reaches the criterion by pressing the correct lever
249 50x, usually completing the task between 30–45 min.

250

251 5.2.4. The following day force the rat to perform this task on the opposite lever using the same
252 program as the first day of training, but designate the opposite lever as the correct one. There
253 is no need to “shape” the lever with a food pellet on this day of training. Typically, this criterion
254 is quickly acquired after rats have learned to press the first lever.

255

256 5.3. Training the rat to respond to the light cue

257

258 5.3.1. On the third day of training, illuminate the light above both levers for 15 s trials, during
259 which the rat may press one of lever to potentially receive a food pellet reward. During the light
260 discrimination task, this program will randomly select which lever is correct on a trial-by-trial
261 basis.

262

263 5.3.2. If the rat presses the correct lever, ensure that the lights remain illuminated for 3 s and
264 the reward is delivered, followed by a 5 s period, during which the lights are shut off preceding

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

267
268 5.3.3. Following this last day of training, calculate “side bias” to determine if the rat has a
269 preference for the left or right lever by dividing the number of presses of one lever divided by
270 the total number of lever presses. On the test day, the rat will start on its least preferred side to
271 ensure that it is learning the specific response-reward contingency, rather than responding to a
272 preferred lever.

273

274 **6. Testing**

275

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

277

278 6.1. On day 4 (test day), place the rat in the operant chamber following stress procedures and
279 test them in side discrimination, side reversal, and light discrimination tasks serially. Ensure that
280 the light discrimination task only illuminates the light above the “correct” lever. In each task,
281 rats must consecutively achieve eight correct trials to complete each discrimination without
282 pressing the unrewarded, incorrect lever. An incorrect lever press will reset this chain of trials.

283

284 6.1.1. Test rats using the side discrimination task. Using the side discrimination program,
285 reward the rat for pressing the lever on its least preferred side as determined from the third
286 day of training, regardless of the light cue. The task ends upon pressing the correct lever 8x
287 consecutively (excluding omissions).

288

289 6.1.2. Perform the side reversal test by running rats using the side discrimination program
290 again, but this time designating the lever opposite to the correct one from the side
291 discrimination task as correct. Ensure that the rat is rewarded for pressing this lever, regardless
292 of the light cue. The task ends upon pressing the correct lever 8x consecutively (excluding
293 omissions).

294

295 6.1.3. Perform the light discrimination task, which rewards the rat for pressing the lever with
296 the light illuminated above. Each operant testing is complete upon pressing the correct lever 8x
297 consecutively (excluding omissions).

298

299 NOTE: Based on previous studies, these tasks encode a minimum of 30 trials, regardless of
300 consecutive presses, to ensure that rats have sufficient time to learn the rules of each task¹⁸.
301 Thus, if the rat consecutively achieves eight correct trials before 30 trials have occurred, the
302 task will remain engaged until 30 trials are completed.

303

304 **7. Behavioral analysis**

305

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

309

310 7.1 Open the data for each test day task (side discrimination, side reversal, and light
311 discrimination) using the computer program. The main measures recorded by the program are
312 trials to criterion, errors in criterion, and time to criterion. These measures are described in
313 detail below.

314

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

318

319 7.1.1. Use trials to criterion (which refers to the total number of trials [not including omissions]
320 necessary for the rat to consecutively complete eight correct trials, including those eight trials)
321 as the main indicator of accuracy. This data is located in the first column in array B in a data file
322 generated by the MED-PC script for any of the tasks on test day.

323

324 7.1.2. Examine the total errors made during each task. This data is located in the third column
325 of array B in a data file generated by the MED-PC script for any of the tasks on test day. These
326 errors are also categorized into perseverative or regressive errors. Perseverative errors are
327 committed when the rat continues to follow the earlier rule from the previous task. Regressive
328 errors are committed after it has disengaged from the previous rule but continues to try to
329 acquire the new rule (for more details on how these types of errors are calculated, refer to the
330 published method¹⁸).

331

332 7.1.3. If the rat did not respond to a light cue within 15 s, the trial is categorized as an omission,
333 not counting it towards the total number of trials to criterion. Calculate this by first adding
334 together the number of correct responses (located in the second column of array B in data file)
335 and number of errors (located in the third column of array B in data file). Next, subtract this
336 number from the total number of trials to criterion (this is the last number in the first column of
337 array B in a data file, different from the trials to criterion).

338

339 7.1.4. Use start and finish times recorded by the program (located at the top of a data file
340 generated by the MED-PC script for any of the tasks on test day) to calculate time to criterion.
341 Latency to the first lever press can also be calculated from the data file by subtracting the
342 variable K (elapsed time in seconds from the first lever press) from the time to criterion.

343

344 7.1.5. Average the data for each behavioral measure for rats within the same treatment group.
345 Perform appropriate statistical analyses (depending on how many variables are being
346 examined).

347

348 **8. Brain substrates**

349

350 8.1. Determine an interested brain area and/or aspect of cognitive flexibility. For example, if
351 stress increases perseverative errors in the side reversal task, the orbitofrontal cortex (OFC)
352 may be of particular interest, as previous lesion studies have indicated this brain region plays a

353 role in many forms of reversal learning (i.e., spatial reversal tested in the side reversal task)^{34–36}.
354 In this example, sacrifice rats after the strategy shifting paradigm is completed and examine c-
355 fos (measure of neural activation³⁷) in the OFC using described immunohistochemical
356 methods²⁵ and described briefly here.

357

358 8.1.1. First, extract brains from animals and cut into 40 µm slices.

359

360 8.1.2. Wash the tissue in phosphate-buffered saline (PBS) 4x for 5 min each, then incubate in
361 0.3% hydrogen peroxide for 10 min to quench endogenous peroxidases.

362

363 8.1.3. Wash tissue in PBS 2x for 5 min each, then incubate in mouse anti-c-fos primary antibody
364 (1:500), 3% normal donkey serum (NDS), and 0.3% Triton X overnight.

365

366 8.1.4. The next day, wash tissue in PBS 3x for 5 min each, then incubate in biotin-SP-conjugated
367 donkey anti-mouse sary antibody (1:500) for 2 h.

368

369 8.1.5. Wash tissue in PBS 3x for 5 min each, then incubate in avidin-streptavidin AB complex for
370 1 h.

371

372 8.1.6. Wash tissue in PBS 3x for 5 min each, then incubate in DAB solution for up to 10 min as
373 tissue undergoes an oxidation chromogenic reaction.

374

375 8.1.7. Wash tissue in PBS 3x for 5 min each, then mount the brain slices on glass microscope
376 slides.

377

378 8.1.8. Coverslip the tissue using toluene based mounting medium and image using a brightfield
379 microscope.

380

381 NOTE: Here, as reflected in the representative results, rats are sacrificed 30 min after the
382 strategy shifting paradigm ends, roughly 60–90 min after the reversal task has been completed
383 (depending on each rat’s performance in the light task). This should represent optimal timing
384 for c-fos expression³⁸, reflecting performance in the reversal task.

385

386 8.2. Alternatively, cannulate a specific brain area for drug injection or viral injection prior to the
387 execution of stress or the operant strategy shifting paradigm.

388

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

392

393 **REPRESENTATIVE RESULTS:**

394

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

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

400
401 Trials to criterion for each task are depicted in **Figure 2A**. Typically, better performance on each
402 task was represented by a reduced number of trials to criterion. These data indicate that,
403 following acute restraint, males completed the side reversal task in significantly fewer trials
404 than unstressed, control males. Conversely, stressed females required a significantly greater
405 number of trials to complete the side reversal task. These results suggest that males exhibited
406 improved performance following stress, whereas females exhibited impaired performance. In
407 the light discrimination task, stress increased the number of trials to criterion compared to
408 control females, thereby impairing performance in females but not males in this task.

409
410 The total number of errors made for each attention task is depicted in **Figure 2B**. Consistent
411 with the number of trials to criterion, stressed males made significantly fewer errors than
412 control males, whereas stressed females made more errors in the side reversal task.
413 Furthermore, in the light discrimination task, females also made significantly more errors. In
414 sum, these data suggest that repeated stress improves cognitive performance in males but
415 impairs cognitive performance in females.

416
417 Total errors were further categorized into perseverative or regressive errors in **Figure 2C** (for a
418 distinction between these two types of errors, refer to section 7 of the protocol). Interestingly,
419 stressed males made fewer perseverative errors in the side reversal task than control males. On
420 the other hand, in both the side reversal and light discrimination tasks, stressed females made a
421 greater number of perseverative errors than control females. There were no differences
422 between the treatment groups in the number of regressive errors made during either task.

423
424 Omissions in each trial and time to reach criterion are shown in **Figure 2D** (for more
425 information on how these were calculated, refer to section 7 of the protocol). These measures
426 were evaluated in the side reversal task only, as this task exhibited the largest sex differences.
427 Stressed females made a higher percentage of omissions compared to all other treatment
428 groups. In addition, while stress appeared to decrease the time to complete the side reversal
429 task in males, stress prolonged completion of the task in females. In sum, repeated stress
430 impaired cognitive flexibility in females but not males.

431
432 Brain substrates underlying cognitive flexibility are depicted in **Figure 3**. As stark sex differences
433 were observed in the side reversal task, the brain areas underlying this task were examined to
434 determine whether they displayed similar sex differences in neural activity. As previously
435 discussed, lesion studies have indicated that the orbitofrontal cortex (OFC) mediates the side
436 reversal task³⁴. Thus, c-fos, a measure of neural activation³⁷, was labeled in the OFC at 30 min
437 after the completion of strategy shifting, which should have reflected performance in the side
438 reversal task³⁸. However, it is possible that OFC may also play a role in the extradimensional
439 strategy shifting component of this task³⁹. Thus, it is important to perform the sacrifice at the
440 appropriate time to reflect brain activity during a particular task within the operant strategy

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

448

449 **FIGURE LEGENDS:**

450

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

452

453 **Figure 2: Representative behavioral data from operant strategy shifting paradigm. (A)** Trials to
454 criterion for each task on test day. In the side reversal task, stress improved performance in
455 males but impaired performance in females. In the light discrimination task, stress weakened
456 performance in females, while it did not affect males. **(B)** Number of errors for each task on test
457 day. Stress reduced the number of errors made in males but increased errors in females in both
458 side reversal and light discrimination tasks. **(C)** Perseverative and regressive error
459 categorization. Stress decreased perseverative errors made in males but increased
460 perseverative errors made in females in both side reversal and light discrimination tasks. **(D)**
461 Percent trials omitted and time to criterion in the side reversal task. Stress increased the
462 percent omissions in female rats. Stress decreased the time required by males but increased
463 the time required by females to complete the task. Statistics were calculated using two-way
464 ANOVA followed by Tukey's t-test ($n = 12$ rats per group; error bars represent SEM; $\#p \leq 0.10$,
465 $*p < 0.05$). This figure has been modified from a previous publication¹⁷.

466

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

477

478 **DISCUSSION:**

479

480 The protocol demonstrates how to measure the effects of stress on cognitive function.
481 Specifically, a modified operant strategy shifting paradigm is used in rodents, which measures
482 cognitive flexibility (analogous to the Wisconsin Card Sorting Task in humans)¹. Cognitive
483 flexibility denotes the ability to adapt cognitive processing strategies to face new conditions in
484 the environment, and it is crucial for normal daily functioning². As human studies on cognitive

485 flexibility are mostly limited to brain imaging¹¹, the use of this paradigm in animals will greatly
486 advance the understanding of effects of stress on the brain and cognition.

487

488 Stress can impair cognitive function⁴⁰. In fact, this is one of the most common phenotypes in
489 stress-related illnesses such as PTSD and MDD^{3,41}. Moreover, there are stark sex differences in
490 the occurrence of stress-related psychiatric illnesses⁵⁻⁷, yet there is little understanding of the
491 neurobiology behind these biased incidences. Thus, use of this operant strategy shifting
492 paradigm in animals of both sexes may help advance the current understanding of sex
493 differences in psychiatry.

494

495 This operant strategy shifting task allows researchers to examine key aspects of cognition
496 relevant to psychiatric disorders. For example, perseverative errors after experimental
497 manipulation are calculated in this paradigm. Perseveration is observed in stress-related
498 psychiatric disorders such as PTSD, and it impairs the ability of one to learn a new set of rules,
499 ultimately impairing working memory³. Thus, the measure of perseverative errors is
500 translationally relevant. Moreover, omissions in attention tasks have been noted in patients
501 with PTSD, indicating slower cortical processing³. Accordingly, omission data from this paradigm
502 may have clinical counterparts. In sum, cognitive flexibility measured as by this experimental
503 paradigm models key phenotypes that are observed in psychiatric disorders.

504

505 This experimental paradigm also allows for precision in targeting neural substrates underlying
506 cognitive flexibility. For example, the literature has indicated that the prefrontal cortex (PFC) is
507 a crucial brain region for cognitive flexibility³, including the medial prefrontal (mPFC) and
508 orbitofrontal cortex (OFC). Of these subregions in the PFC, the OFC is important for
509 performance in the side reversal task^{34,35}. These brain areas are also a key targets for stress-
510 induced functional alterations^{42,43}. Interestingly, the model of stress used here does appear to
511 play a role in the subsequent performance of rodents in tests of cognitive flexibility; thus, it
512 should be considered in the design of future experiments. These varying responses to stress
513 point to potentially novel mechanisms by which cognition is impacted by stress. Thus, targeting
514 specific neurotransmitters, proteins, or activation of these brain regions may shed light on how
515 stress affects cognition in male and female rodents. Researchers can choose to manipulate
516 these neural substrates at different timepoints in conjunction with stress or strategy shifting, or
517 alternatively measure neural substrates after exposure to these behavioral paradigms.

518

519 This modified operant strategy shifting task has clear advantages over other cognitive flexibility
520 paradigms used in the stress literature (i.e., the digging task¹²⁻¹⁵), which require more time and
521 effort by the experimenter to train rodents. This procedure requires minimal oversight by the
522 experimenter and allows multiple rats to be tested simultaneously. In addition, unlike other
523 versions of this automated task¹⁹, the paradigm only requires 3 days of training and includes an
524 efficient programmed data analysis.

525

526 The operant strategy shifting paradigm does have certain limitations. One limitation is that it
527 can only test two stimulus dimensions (e.g., left or right lever vs. light cue), whereas the digging
528 task¹²⁻¹⁵ can test a third stimulus dimension (e.g., digging media vs. odor vs. texture). However,

529 the task described in this protocol still allows for testing of the rat's ability to shift to different
530 rules, which allows testing of the cognitive flexibility constructs. In addition, it is possible to add
531 other parameters to the operant chambers to allow for a third stimulus (e.g., an odor), but this
532 may prolong the training required for the task.
533

534 The primary advantage of this task is its simplicity and ability to pair it with stressful or
535 pharmacological manipulations to further understand how stress affects the brain. It should be
536 noted that this simplicity comes with an increased difficulty that subjects face while learning to
537 lever press, compared to the ecologically relevant digging task. While this operant task is far
538 less labor-intensive, rodents will generally require more trials to acquire this task. However,
539 both the digging task and this paradigm engage similar neurobiological mechanisms and thus
540 represent valid options for the examination of cognitive flexibility^{16,44}. While there have been
541 varied results in the literature regarding the effects of stress on cognitive flexibility using the
542 digging task and this operant procedure^{23,25,27,45,46}, the presented method reflects the complex
543 effects that the type, intensity, and duration of a stressor can have on cognitive function^{20,21}.
544

545 Another limitation of the task is that rodents are housed in closed opaque boxes; thus,
546 behaviors other than those that are collected via the computer interface cannot be coded. For
547 example, a high number of omissions by a rat may be due to behavioral inhibition inflicted by
548 stress, or because the rat is asleep. Moreover, other stereotypical behaviors, such as grooming
549 (which is particularly relevant in studying stress), may be interesting to analyze during the task.
550 Mounting cameras in operant chambers may allow for this type of behavioral precision.
551

552 Overall, this report details the use of stress procedures in conjunction with an operant strategy
553 shifting paradigm to further understand how stress affects the brain. It should be noted that, in
554 addition to stress procedures and cognitive assessment in adults, research on different
555 developmental stages may provide crucial information about the etiology of cognitive
556 inflexibility. In addition to studying the effects of stress on cognitive flexibility, this simple and
557 efficient operant strategy shifting paradigm can be paired with many experimental
558 manipulations to investigate how the brain adapts to changing environments. Moreover,
559 alternate experimental approaches can be used to study the neural basis of cognitive flexibility,
560 including lesions, pharmacology, gene editing, and electrophysiology. As cognitive inflexibility is
561 one of the key phenotypes in psychiatric disease, more research must be conducted to further
562 understand its neurobiological substrates.
563

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570 The authors have nothing to disclose.
571

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