

1 Title: Characterizing changes in glucocorticoid receptor internalization in the fear circuit in an animal
2 model of post traumatic stress disorder

3 Running title: stress, GR, fear conditioning

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ABSTRACT

Glucocorticoid receptors (GRs) shuttle from the cytoplasm (cy) to the nucleus (nu) when bound with glucocorticoids (i.e. GR internalization) and alter transcriptional activity. GR activation within the fear circuit has been implicated in fear memory and post traumatic stress disorder (PTSD). However, no study to date has characterized GR internalization within the fear circuit during fear memory formation or examined how traumatic stress impacts this process. To address this, we assayed cy and nu GR levels at baseline and after auditory fear conditioning (FC) in the single prolonged stress (SPS) model of PTSD. Cy and nu GRs within the medial prefrontal cortex (mPFC), dorsal hippocampus (dHipp), ventral hippocampus (vHipp), and amygdala (AMY) were assayed using western blot. The distribution of GR in the cy and nu (at baseline and after FC) was varied across individual nodes of the fear circuit. At baseline, SPS enhanced cyGRs in the dHipp, but decreased cyGRs in the AMY. FC only enhanced GR internalization in the AMY and this effect was attenuated by SPS exposure. SPS also decreased cyGRs in the dHipp after FC. The results of this study suggests that GR internalization is varied across the fear circuit, which in turn suggests GR activation is selectively regulated within individual nodes of the fear circuit. The findings also suggest that changes in GR dynamics in the dHipp and AMY modulate the enhancing effect SPS has on fear memory persistence.

51 **INTRODUCTION**

52 Glucocorticoid receptors (GRs) are ligand-gated transcription factors. Upon binding with
53 glucocorticoids they leave the cytoplasm (cy) and enter the nucleus (nu) as dimers (i.e. GR
54 internalization) where they bind to GREs to regulate transcription (1-4). GRs can also enter the nucleus as
55 monomers and interact with other transcription factors (e.g. AP-1) to indirectly regulate transcriptional
56 activity (2).

57 Glucocorticoid release during fear conditioning (FC) has been implicated in fear memory
58 consolidation (5-8) and specifically GR activation in the basolateral amygdala (BLA) and dorsal
59 hippocampus (dHipp) are critical for fear memory consolidation (9-12). Changes in GR function have
60 been consistently implicated in post traumatic stress disorder (PTSD) with an enhancement in GR levels
61 (inferred via hormonal experiments or direct measurement on lymphocytes) being reported (13-20).
62 Given the role of GRs in fear memory consolidation, it is reasonable to infer that enhanced GR expression
63 in PTSD contributes to persistent traumatic fear memory that is characteristic of PTSD (21-23). However,
64 other studies have shown that administration of glucocorticoids shortly after trauma prevent the
65 development of PTSD (24, 25) and can enhance the efficacy of exposure therapy in treating PTSD (26).
66 Thus, it is currently unclear how GRs contribute to PTSD symptoms. Indeed characterization of GR
67 internalization across the fear circuit during fear memory formation and how this process is affected by
68 traumatic stress is lacking.

69 Single prolonged stress (SPS) refers to serial exposure to restraint, forced swim, and ether and is a
70 validated animal model of PTSD (27-29). SPS exposure increases GR expression in the dHipp and mPFC
71 (30-33) and leads to the formation of fear memory that is difficult to extinguish (i.e. persistent fear
72 memory) (31, 32, 34-37). These two symptoms are characteristic of PTSD (18, 20, 38-40). Thus, SPS is
73 an appropriate animal model to examine how traumatic stress might lead to changes in GR internalization
74 in the fear circuit. In this study we used western blot to assay cy and nu GRs in the medial prefrontal
75 cortex (mPFC), amygdala (AMY), dHipp, and ventral Hipp (vHipp) at baseline and after FC (see Figure
76 1).

77 -----Figure 1 caption-----

78 Experimental design used in this study.

79 -----Figure 1 caption-----

80

81 These substrates were selected, because they are critical nodes of the fear circuit (41-45). Results
82 suggest that distribution of GRs in the cy and nu at baseline and after FC was varied across these nodes of
83 the fear circuit. The effects of SPS on cy and nu GR levels at baseline and after FC was restricted to the
84 dHipp and AMY. SPS increased cyGR levels in the dHipp at baseline, but decreased cyGR levels in the
85 dHipp after FC. SPS decreased cyGR levels in the AMY at baseline, but increased cyGR levels in the
86 AMY after FC. SPS also disrupted GR internalization in the AMY brought on by FC.

87 MATERIAL AND METHODS

88 Animals

89 Eighty-eight male Sprague-Dawley rats (~ 150 – 250 g upon arrival) obtained from Charles River
90 Inc. were used in this study. Upon arrival, rats were housed in pairs during a five day acclimation period
91 with ad libitum access to food and water. Following SPS and control procedures, rats were individually
92 housed and restricted to 23g/day of standard rat chow per the manufacturer's recommendation (LabDiet
93 St. Louis MO) with ad libitum access to water. Experiments commenced following the animals'
94 acclimation period. The rats were on a 12 hour light/dark cycle and all experimental procedures were
95 performed in the animals' light cycle between the hours of 9:00 am and 2:00pm. All experiments were
96 approved by the University of Delaware Institutional Animal Care and Use committee following
97 guidelines established by the NIH.

98 SPS and Behavioral Procedures

99 All rats were randomly assigned to the SPS or control stress group prior to SPS. SPS was
100 conducted as previously described (33, 46) and consisted of two hours of restraint, followed by 20
101 minutes of forced swim, then ether exposure until general anesthesia was induced. Control rats were
102 placed into a novel room in their home cages while SPS occurred. A post-stress incubation period of

103 seven days was allowed to elapse prior to experimental testing, because this is necessary to observe SPS
104 effects (32, 33).

105 SPS and control rats were randomly assigned to one of four groups: baseline, FC0, FC30, or
106 FC60. Rats in the baseline treatment were removed from the housing colony and immediately euthanized
107 in order to determine baseline GR levels. All other rats were removed from the housing colony and
108 subjected to FC. FC sessions were conducted as previously described (32, 34) using six MedAssociates
109 (Fairfax VT) operant boxes. Briefly, FC consisted of five presentations of a 10 second auditory
110 conditioned stimulus (CS, 2 kHz, 80dB) that co-terminated with a 1 second, 1mA footshock
111 unconditioned stimulus (UCS). All FC sessions began with a 210s baseline period and had inter-stimulus
112 intervals (ISIs) of 60s. All rats subjected to FC were removed from the operant boxes and euthanized
113 either immediately (FC0), 30 minutes (FC30), or 60 minutes (FC60) following the cessation of FC. These
114 time points were selected, because previous studies have shown that corticosterone levels are elevated
115 immediately after FC, sustained for approximately 30 minutes, but after this time point begins to decrease
116 (47-49). As a result, these time points are appropriate for examining changes in GR internalization
117 induced by enhanced adrenal corticosterone release.

118 All animals were euthanized via rapid decapitation and their brains were immediately extracted
119 and flash frozen in isopentane chilled on dry ice. Brains were then stored in a -80°C freezer until further
120 processing. To dissect brain regions, brains were thawed to -13°C cryostat (Leica CM1350) and 300 µm
121 coronal sections through the mPFC, AMY, dHipp, and vHipp were taken and these brain regions
122 dissected out and placed into 1.5 mL microtubes. Dissected brain regions in microtubes were then stored
123 in a -80°C freezer.

124 **Western Blot**

125 All brain sections were treated to separate cy and nu fractions using a method described by
126 Spencer et al., (50). We empirically tested this protocol to ensure it was successful at separating cy and nu
127 fraction (see Appendix). Dissected brain regions were homogenized in 250 µL of buffer (50mM Tris

128 buffer, 10% sucrose, 1mM EDTA, 0.5mM DTT, 1mM benzamidine, 0.3mM PMS Fl) by a motor-driven
129 homogenizer (Fisher Scientific, PowerGen125). The homogenate was then then centrifuged (2,000 x g)
130 for five minutes at 4°C to obtain a supernatant and rough pellet. The supernatant was centrifuged at
131 14,800 x g for 45 minutes at 4°C and the supernatant treated as the cy fraction of brain tissue.

132 The rough pellet from the initial centrifuge treatment was used to obtain the nu fraction from
133 dissected brain regions. The pellet was washed twice in 400 μ L of buffer and then resuspended in 150 μ L
134 of buffer that had a high concentration of NaCl (50mM Tris buffer, 0.5M NaCl, 10% sucrose, 1mM
135 EDTA, 0.5mM DTT, 1mM benzamidine, 0.3mM PMS Fl). Samples were placed on a fixed speed vortex
136 mixer and the suspension was incubated in ice for 1 hour with frequent shaking. Following incubation,
137 samples were centrifuged at 8,000 x g for 15 minutes at 4°C. The supernatants from these samples were
138 treated as the nu fraction of brain tissue.

139 The protein concentration of cy and nu fractions were increased using protein concentrator
140 columns (GE Healthcare, Vivaspin 500). Protein assay was then performed on each sample per
141 manufacturer's directions (Pierce BCA Protein Assay Kit). 0.5X Laemmli sample buffer was mixed with
142 approximately 15 μ g of protein from each sample. These samples were stored in a -80°C until western
143 blot. Protein samples were heated at 70°C for 7 minutes before being loaded into 10% Tris-HCl
144 polyacrylamide gels and separated by SDS polyacrylamide gel electrophoresis. Separated proteins were
145 electrophoretically transferred from gels to nitrocellulose membranes. The membranes were subsequently
146 left to dry for 30 minutes at 37°C followed by rehydration washes in 0.5 M Tris-buffered saline (TBS).
147 Blots were blocked for 1 hour at room temperature in TBS containing 5% non-fat milk. Nitrocellulose
148 membranes were probed for GR and β actin (reference protein) by incubating overnight at room
149 temperature with a polyclonal rabbit GR antibody (1:50, Santa Cruz Biotechnology, M-20) and a mouse
150 β -actin antibody (1:2000, Cell Signaling Inc. 8H10D10) in TBS. After 18-20 hours, the membranes were
151 subjected to several washes in 0.5 M TBS with 0.1% Tween-20 then a 3 hour incubation at room
152 temperature with polyclonal goat anti-rabbit (800CW) (1:500, Li-COR) and anti-mouse IgG (680RD)

153 (1:5000, Li-COR) secondary antibodies in 0.5M TBS containing 0.1% Tween and 5% non-fat milk.

154 Nitrocellulose membranes were then washed in TBS and scanned in the Li-cor Odyssey Clx scanner

155 under the following settings: resolution – 169 μ m, quality – lowest, focus offset – 0.0 mm.

156 **Data and Statistical Analysis**

157 Freezing behavior was analyzed using ANY-maze (Stoelting Inc.) as previously described (32).

158 Fear-conditioned freezing was averaged in trials that consisted of a CS and respective ISI (e.g. CS1 and

159 IS1) and analyzed using a stress (SPS vs. control) \times trial (baseline, trials 1–5) factor design, with trial

160 being a repeated measure. The condition factor was pooled because rats in the FC0, FC30, and FC60

161 levels were treated in an identical manner during FC.

162 In order to reduce variability in western blot data, representative rats from each independent

163 factor (i.e. stress and condition) were always included in each protein assay and western blot. The

164 integrated intensity (I.I.) of GR and β -actin protein bands were scored using ImageStudio. The profile

165 curves of all bands were inspected to ensure that there was significant I.I. signal above the background

166 within each lane in every western. For all statistical analyses cyGR and nuGR were expressed relative to

167 cy β -actin and nu β -actin to yield relative GR levels. We also divided relative nuGR levels (nuGR/nuAct)

168 into relative cyGR levels (cyGR/cyAct) to yield a single measure of GR internalization (i.e. nGR/cGR) in

169 all brain regions.

170 A stress \times fraction (cy vs. nu) factor design was used to examine the effect of SPS on relative

171 cyGR and nuGR levels at baseline, with fraction being a repeated measure. T-test (SPS vs. control) was

172 used to analyze baseline nGR/cGR ratios. A stress \times fraction \times condition (FC0, FC30, FC60) factor design

173 was used to examine changes in relative cyGR and nuGR levels after FC. A stress \times condition factor

174 design was used to examine changes in nGR/cGR ratios. To specifically examine how cyGR and nuGR

175 levels changed after FC, relative cyGR, nuGR, and nGR/cGR ratios in the condition factor were

176 expressed as a percent change from baseline. These normalized values were subjected to separate stress \times

177 condition factor designs.

178 Main and simple effects were analyzed using analysis of variance (ANOVA), while main and
179 simple comparisons were analyzed using independent, paired sample, or one sample t-test with a
180 Bonferroni correction applied where appropriate. The reference value for all one sample t-tests was set to
181 100. Statistical significance was assumed with $p < .05$ for all statistical tests.

182 RESULTS

183 Behavior

184 There was a main effect of trial [$F_{(5,125)} = 127.615, p < .001$], which suggested all rats acquired
185 fear memory. There was also a significant stress x trial interaction [$F_{(5,125)} = 2.669, p = .036$]. This was
186 driven by enhanced freezing in SPS rats during FC trial 1 of FC in comparison to control rats [$t_{(25)} =$
187 $2.268, p = .032$]. However, at the end of FC all animals had equivalent levels of freezing ($p > .05$), which
188 suggests SPS did not alter acquisition of FC; a finding consistent with previous studies (31, 32, 34). These
189 results are illustrated in Figure 2.

190

191 -----Figure 2 caption-----

192 Effect of SPS on acquisition of fear conditioning (FC). Even though SPS enhanced conditioned freezing
193 during trial 1 of FC, SPS did not affect acquisition of FC. SPS = 15, control = 12.

194 -----Figure 2 caption-----

195

196 Western Blot

197 *vHipp*

198 Sample western is shown in Figure 3A.

199

200 -----Figure 3 caption-----

201 Effect of SPS on GR internalization in the vHipp. A) Cartoon of dissected brain region and representative
202 western of cytoplasmic (cy) and nuclear (nu) vHipp samples. B) SPS had no effect on baseline GR levels
203 or nGR/cGR ratios (SPS = 12, Con = 13). C) Cy GR levels were enhanced during FC. SPS had no effects

204 on relative GR levels or nGR/cGR ratios after FC. D) SPS had no effect on normalized GR levels or
205 nGR/cGR ratios, but both cy and nu GR levels were enhanced relative to baseline levels. Also, nGR/cGR
206 levels after FC were decreased relative to baseline. + - significant one-sample t-test. SPS/0' = 12, SPS/30'
207 = 8, SPS/60' = 9; Con/0' = 11, Con/30' = 7, Con/60' = 7.

208 -----Figure 3 caption-----

209
210 Relative baseline GR levels and nGR/cGR ratios were unaffected by stress and relative baseline cyGR
211 and nuGR levels were equivalent (Figure 3B, $p > .05$). However, there was a rise in cyGR levels, relative
212 to nuGR levels, after FC. This was revealed by a main effect of fraction [$F_{(1,48)} = 11.149$, $p = .001$; Figure
213 3C]. There were no stress or condition effects on relative GR levels or nGR/cGR ratios after FC (Figure
214 3C; $p > .05$). There was no effect of stress on normalized GR levels after FC ($p > .05$). However, there
215 was an enhancement in both cy and nu GR levels brought on by FC across all time points (i.e. 0', 30',
216 60'). This was revealed by significant one-sample t-test [cyGR - $t_{(53)} = 2.831$, $p = .014$; nuGR - $t_{(53)} =$
217 2.525 , $p = .03$]. Normalized nGR/cGR ratios were unaffected by stress ($p > .05$), but were significantly
218 lower after FC in comparison to baseline [$t_{(53)} = 4.976$, $p < .001$]. This suggests GR internalization in the
219 vHipp was decreased after FC. These results are illustrated in Figure 3D.

220 *dHipp*

221 A sample western is shown in Figure 4A.

222

223 -----Figure 4 caption-----

224 Effect of SPS on GR internalization in the dHipp. A) Cartoon of dissected brain region and representative
225 western of cy and nu dHipp samples. B) At baseline, cy GR levels were enhanced relative to nu GR levels
226 and this enhancement was further increased in SPS rats. SPS had no effect on nGR/cGR ratios (SPS = 9,
227 Con = 13). C) Cy GR levels, relative to nu GR levels, were enhanced after FC. SPS had no effects on
228 relative GR levels or nGR/cGR ratios after FC. D) Normalized cy GR levels in SPS rats were enhanced
229 relative to control rats. This effect was most pronounced at the FC-60 time point. One sample t-test

230 revealed that SPS decreased normalized cy GR levels after FC. SPS had no effect on normalized nu GR
231 levels or nGR/cGR ratio. * - significant effect of stress. + - significant one-sample t-test. SPS/0' = 10,
232 SPS/30' = 8, SPS/60' = 9; Con/0' = 12, Con/30' = 9, Con/60' = 6.

233 -----Figure 4 caption-----

234
235 There was a main effect of fraction [$F_{(1,20)} = 57.529$, $p < .001$] for relative GR at baseline. This reflected
236 enhanced cyGR, relative to nuGR, in the dHipp. There was also a significant stress x fraction interaction
237 [$F_{(1,20)} = 7.345$, $p = .013$], which was driven by SPS enhancement of cyGR relative to nuGR. This
238 assertion was supported by significant t-test when comparing difference scores between relative cy and nu
239 GR levels (i.e. cyGR – nuGR) for SPS vs. control rats [$t_{(20)} = 2.71$, $p = .013$]. Independent t-test for
240 baseline nGR/cGR ratios was not significant ($ps > .05$). These results are illustrated in Figure 4B.

241 Relative cyGR was higher when compared to nuGR [main effect of fraction: $F_{(1,48)} = 27.056$, $p <$
242 $.001$] after FC. There were no significant effects of stress and/or condition ($ps > .05$). There were no
243 stress and/or condition effects for nGR/cGR ratios after FC ($ps > .05$). These results are illustrated in
244 Figure 4C. There was a main effect of stress for normalized cyGR levels [$F_{(1,48)} = 5.707$, $p = .021$] that
245 was driven by lower levels of cyGR in SPS rats. This effect was pronounced at the FC60 time point.
246 Significant one sample t-test for SPS rats [$t_{(26)} = 3.624$, $p = .001$], but not control rats ($p > .05$), also
247 supported the assertion that cyGR levels were decreased in SPS rats after FC. There were no significant
248 stress and/or condition effects on relative nuGR levels or nGR/cGR ratios after FC ($ps > .05$). These
249 results are illustrated in Figure 4D.

250 *AMY*

251 A sample western is shown in Figure 5A.

252
253 -----Figure 5 caption-----

254 Effect of SPS on GR internalization in the AMY. A) Cartoon of dissected brain region and representative
255 western of cy and nu AMY samples. B) Relative cy GR levels were enhanced in comparison to nu GR

256 levels. This effect was decreased in SPS rats. This effect approached statistical significance ($p = .057$).
257 SPS had no effect on nGR/cGR ratios at baseline (SPS = 6, control = 10). C) Cy GR levels were enhanced
258 during FC. SPS had no effects on relative GR levels or nGR/cGR ratios after FC. D) SPS enhanced
259 normalized cy GR levels. This effect approached statistical significance ($p = .064$). Enhanced nGR/cGR
260 ratios after FC were observed in control rats, which suggests enhanced GR internalization. This effect was
261 not observed in SPS rats. + - significant one-sample t-test. * significant effect of stress. SPS/0' = 7,
262 SPS/30' = 5, SPS/60' = 6; Con/0' = 8, Con/30' = 7, Con/60' = 4.

263

264 -----Figure 5 caption-----

265 Effect of SPS on GR internalization in the AMY. A) Cartoon of dissected brain region and representative
266 western of cy and nu AMY samples. B) Relative cy GR levels were enhanced in comparison to nu GR
267 levels. This effect was decreased in SPS rats. This effect approached statistical significance ($p = .057$).
268 SPS had no effect on nGR/cGR ratios at baseline (SPS = 6, control = 10). C) Cy GR levels were enhanced
269 during FC. SPS had no effects on relative GR levels or nGR/cGR ratios after FC. D) SPS enhanced
270 normalized cy GR levels. This effect approached statistical significance ($p = .064$). Enhanced nGR/cGR
271 ratios after FC were observed in control rats, which suggests enhanced GR internalization. This effect was
272 not observed in SPS rats. + - significant one-sample t-test. * significant effect of stress. SPS/0' = 7,
273 SPS/30' = 5, SPS/60' = 6; Con/0' = 8, Con/30' = 7, Con/60' = 4.

274 -----Figure 5 caption-----

275

276 There was a significant main effect of fraction [$F_{(1,14)} = 28.224, p < .001$] for baseline relative GR,
277 which reflected enhanced cyGR levels relative to nuGR. This effect was attenuated in SPS rats, which
278 was suggested by a stress x fraction interaction that approached significance [$F_{(1,14)} = 4.307, p = .057$].
279 There was no effect of stress on baseline nGR/cGR ratios ($p > .05$). These results are illustrated in Figure
280 5B.

281 CyGR levels were enhanced, relative to nuGR, after FC in all rats. This revealed by a significant
282 main effect of fraction [$F_{(1,31)} = 48.734, p < .001$]. There were no effects of stress and/or condition on
283 relative GRs or nGR/cGR ratios after FC ($ps > .05$). Relative to baseline, cyGR increased after FC in SPS
284 rats, but not control rats. This was suggested by a stress effect that approached statistical significance
285 [$F_{(1,32)} = 3.672, p = .064$]. These results are illustrated in Figure 5C. There were no effects of stress and/or
286 condition on normalized cy and nu GR levels ($ps > .05$). There was a main effect of stress on normalized
287 nGR/cGR ratios [$F_{(1,31)} = 5.607, p = .024$], which was driven by a failure to enhance nGR/cGR ratios after
288 FC in SPS rats. This interpretation was supported by one sample t-test that was significant for control rats
289 [$t_{(18)} = 2.637, p = .034$], but not SPS rats ($p > .05$). These findings suggests that FC enhanced GR
290 internalization in the AMY and this effect was attenuated by SPS (see Figure 5D).

291 *mPFC*

292 A sample western is shown in Figure 6A.

293

294 -----Figure 6 caption-----

295 Effect of SPS on GR internalization in the mPFC. A) Cartoon of dissected brain region and representative
296 western of cy and nu mPFC samples. B) SPS had no effect on baseline GR levels or nGR/cGR ratios, but
297 cy GR levels were enhanced relative to nu GR levels (SPS = 7, control = 9). C) Relative cy GR levels
298 were enhanced during FC. SPS had no effects on relative GR levels or nGR/cGR ratios after FC. D) SPS
299 had no effect on normalized GR levels or nGR/cGR ratios. * significant effect of stress. SPS/0' = 8,
300 SPS/30' = 4, SPS/60' = 5; Con/0' = 6, Con/30' = 5, Con/60' = 4.

301 -----Figure 6 caption-----

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303 There was a main effect of fraction [$F_{(1,14)} = 10.147, p = .007$] for relative baseline GR, which reflected
304 enhanced cyGR, relative to nuGR, in the mPFC. There was no significant stress effect for relative
305 baseline GR levels or nGR/cGR ratios (Figure 6B; $ps > .05$). Relative cyGR levels after FC was enhanced
306 in comparison to nuGR levels. This was revealed by a main effect of fraction [$F_{(1,26)} = 4.367, p = .047$].

307 There were no stress and/or condition effects on relative GR levels or nGR/cGR ratios after FC (Figure
308 6C; $ps > .05$). There was no effect of stress and/or condition on normalized GR levels or nGR/cGR ratios
309 after FC (Figure 6D; $ps > .05$).

310 DISCUSSION

311 By examining changes in GR levels in the cy and nu at baseline and after FC in different neural
312 substrates that comprise the fear circuit we were able to examine how the distribution of GRs in the cy
313 and nu (i.e. GR dynamics) changes with SPS exposure and after FC. The results suggest there is selective
314 regulation of GR dynamics within individual neural substrates of the fear circuit at baseline and with FC.
315 Baseline cyGR was enhanced relative to nuGR in all brain regions except for the vHipp. FC had no effect
316 on GR dynamics in the mPFC and dHipp, but increased cy and nu GR levels in the vHipp. In spite of this
317 there was an overall decrease in vHipp GR internalization after FC. Enhanced GR internalization after FC
318 was only observed in the AMY. Thus, glucocorticoid release (whether at baseline or stress-induced) does
319 not uniformly determine GR trafficking between the cy and nu within the fear circuit.

320 SPS disrupted GR dynamics in the dHipp and AMY at baseline and after FC, with cyGRs being
321 sensitive to SPS in both brain regions. SPS enhanced cyGRs in the dHipp, but decreased cyGRs in the
322 AMY at baseline. These effects were inverted after FC with lower cyGRs in the dHipp of SPS rats, but
323 enhanced cyGRs in the AMY. The enhancement in GR internalization in the AMY observed in control
324 rats was disrupted by SPS. A previous study has observed that systematically inhibiting GR activation
325 during FC exacerbates persistent fear memory induced by SPS exposure without having any effect on
326 non-stressed rats (35). Inhibiting GR activation during FC may further inhibit GR internalization in AMY
327 cells and decrease cyGR activation in dHipp cells. Via these processes, inhibiting GR activation during
328 FC may enhance persistent fear memory in the SPS model. In turn, this suggests that the changes in GR
329 function brought on by SPS can be adaptive, where GR activation during FC inhibits the development of
330 fear memory persistence in the SPS model.

331 Previous studies have also shown that GR activation in the dHipp and AMY enhance memory
332 consolidation in non-stressed rats (see Introduction), which at first appears contrary to the hypothesis that

333 GR activation during FC inhibits persistent fear memory in the SPS model. One explanation of this
334 apparent discrepancy is that SPS alters GR function in the dHipp and the AMY such that activation of
335 GRs induce different cellular effects in SPS rats when compared to non-stressed rats. Alternatively, a
336 decrease in GR activation tends to disrupt stress adaptation (5). By decreasing GR internalization in the
337 AMY and availability of cyGRs in the dHipp after FC, SPS may prolong the stress of FC, which renders
338 fear memory more resistant to the inhibitory effects of extinction. Indeed previous studies have observed
339 that the stress of FC inhibits the formation of extinction memory (51). Further research is needed to
340 examine these possibilities.

341 **Substrate specific regulation of GR dynamics in the fear circuit**

342 How might substrate specific regulation of GR dynamics occur in the fear circuit when the ligand
343 that activates GRs originates from a single source outside of the central nervous system (i.e. adrenal
344 cortex)? 11 β -hydroxysteroid dehydrogenase types 1 and 2 (11 β -HSD1, 11 β -HSD2) are enzymes capable
345 of either converting inert 11-keto forms of glucocorticoids (e.g. 11 dehydrocorticosterone) into active
346 glucocorticoid (11 β -HSD1) or metabolizing glucocorticoids (11 β -HSD2). Via these mechanisms substrate
347 specific levels of glucocorticoids can be achieved within the brain (52, 53). Both enzymes have selective
348 expression in the brain, with high levels of 11 β -HSD1 being restricted to the neocortex, hippocampus,
349 and hypothalamus; and moderate levels of 11 β -HSD2 being expressed in selective neurons in the nucleus
350 of the solitary tract (54-57). Interestingly, genetic deletion of 11 β -HSD1 results in stress resiliency (58).

351 GRs are phosphorylated at various sites, which alters GR function, including GR internalization
352 (1, 59, 60). Substrate specific changes in GR phosphorylation status is observed with chronic stress and
353 SPS (61, 62) and could be a mechanism whereby GR dynamics is selectively regulated within the fear
354 circuit. FKBP5 is a chaperone protein for GR that inhibits GR binding by interacting with heat shock
355 protein 90 (63-65) and has been implicated in the etiology of PTSD (47, 63, 66). These chaperone
356 proteins have the potential to regulate GR dynamics in a substrate-specific manner by selectively
357 lowering GR binding within neural substrates. In this study we observed rapid increases in cy and nu
358 vHipp GRs that occurred immediately after FC and these changes may also be somewhat independent of

359 GR internalization (see Results). Further research examining how rapid changes in GR availability might
360 be achieved is needed, as these processes could be critical for substrate-specific regulation of GR
361 activation in the fear circuit.

362 **Summary**

363 The results of this study demonstrate that GR dynamics are varied in different neural substrates
364 that comprise the fear circuit. This suggests that basal glucocorticoid release and stress-enhanced adrenal
365 glucocorticoid release can have varied effects on the fear circuit via local regulation of GR activation.
366 Furthermore, the effect of traumatic stress on GR dynamics at baseline and during fear memory formation
367 are restricted to specific nodes within the fear circuit. Previous studies have shown that glucocorticoid
368 administration shortly after trauma (24, 25) and during exposure therapy (26) can prevent and treat the
369 development of PTSD. It is very likely that these treatments do not have homogenous effects on GR
370 dynamics in the fear circuit. Characterizing how these treatments change GR dynamics at baseline and
371 during emotional memory phenomena (e.g. FC, fear extinction) in animals models of PTSD is needed to
372 better understand how they work and implement them in the treatment of PTSD.

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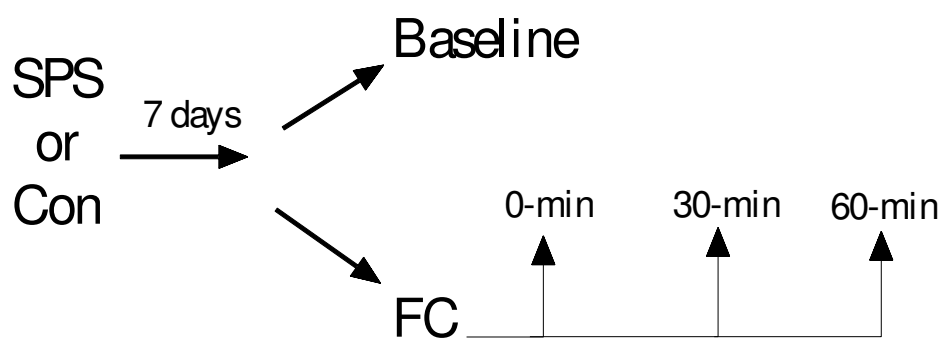
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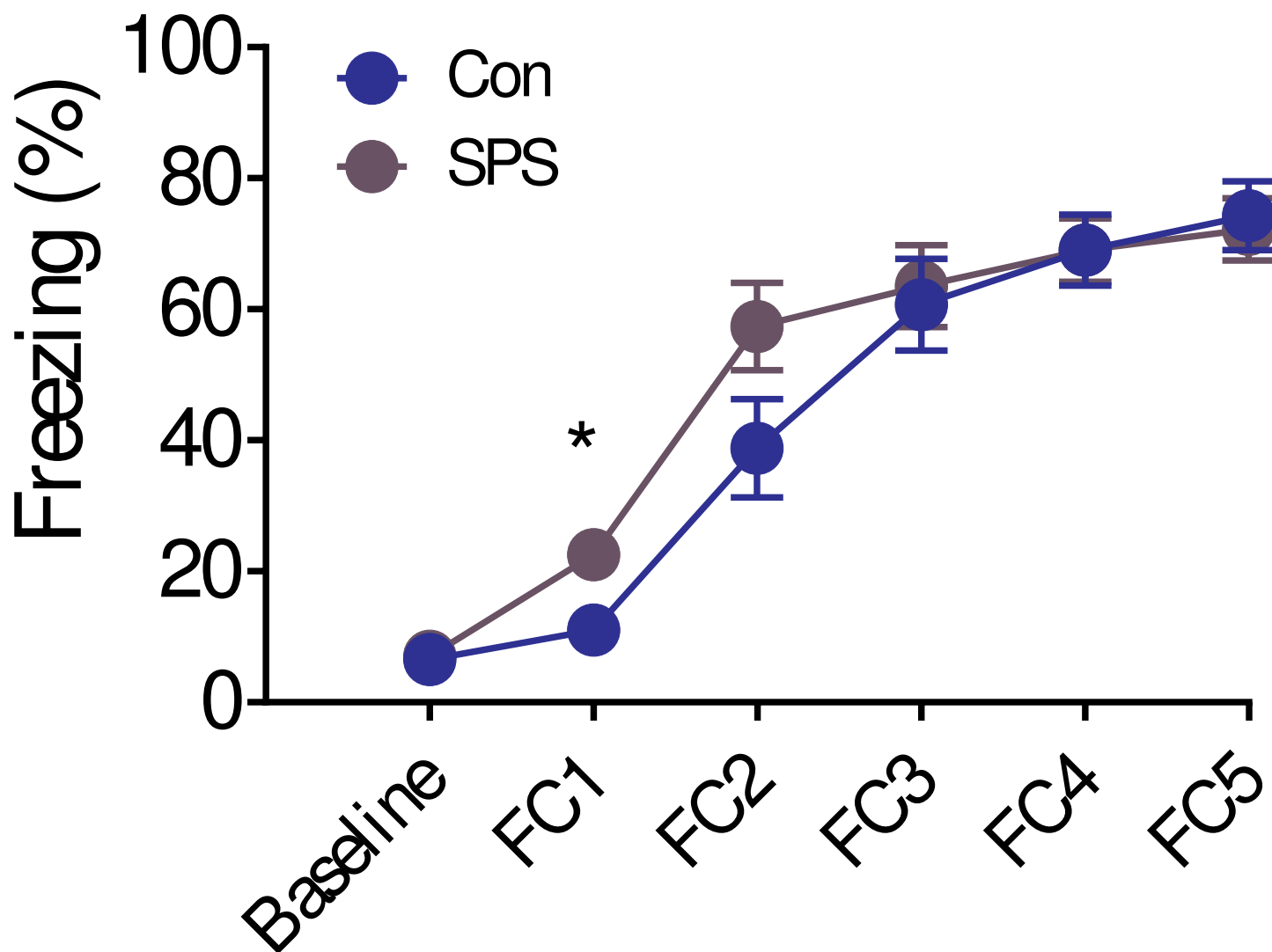
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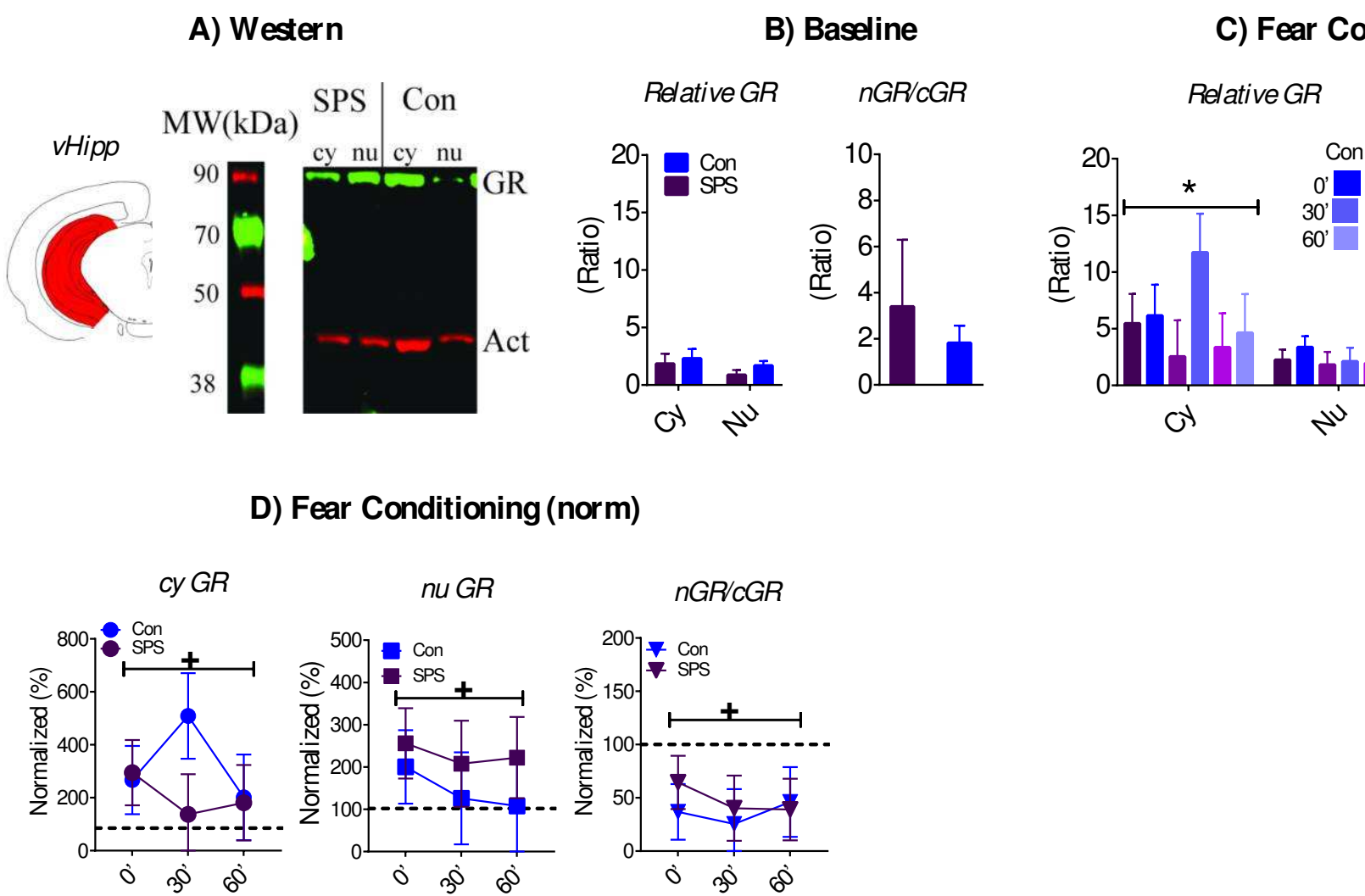
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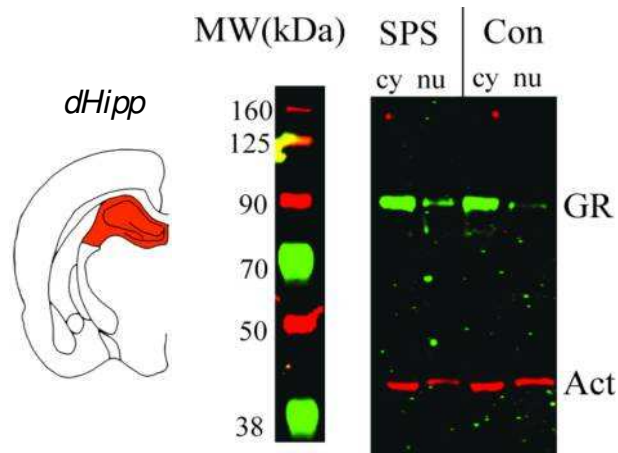
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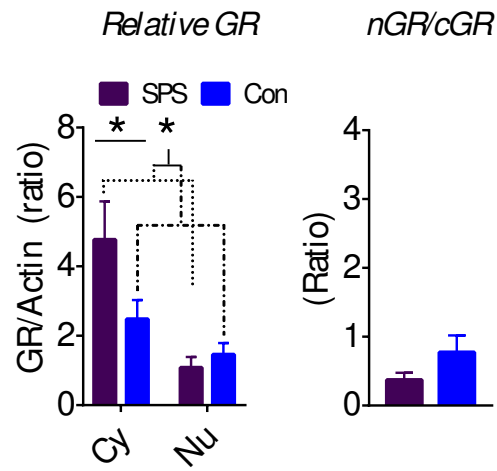




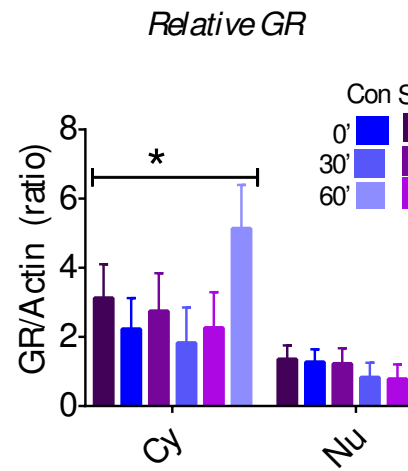
A) Western



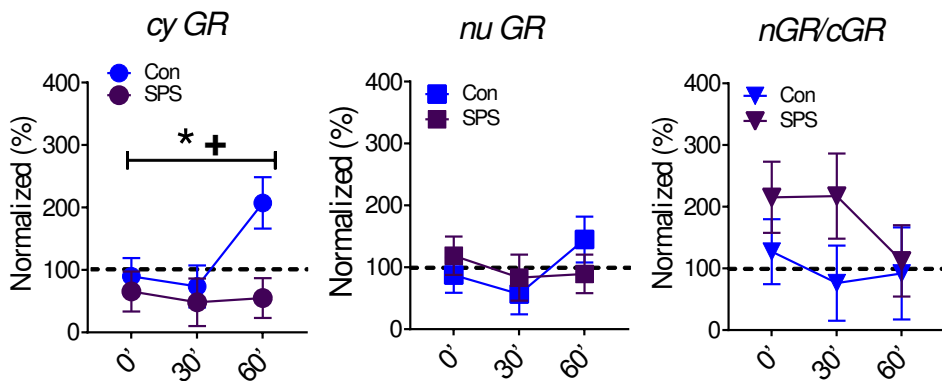
B) Baseline



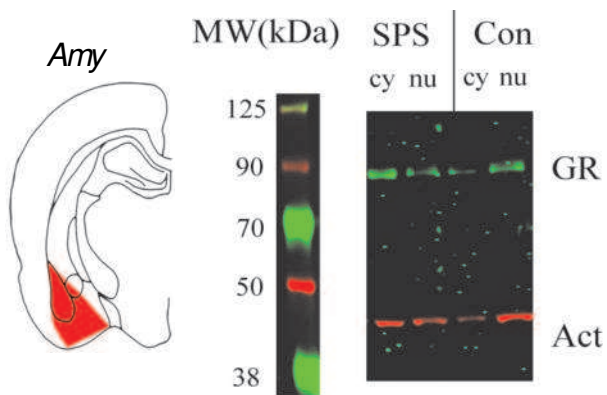
C) Fear Conditioning



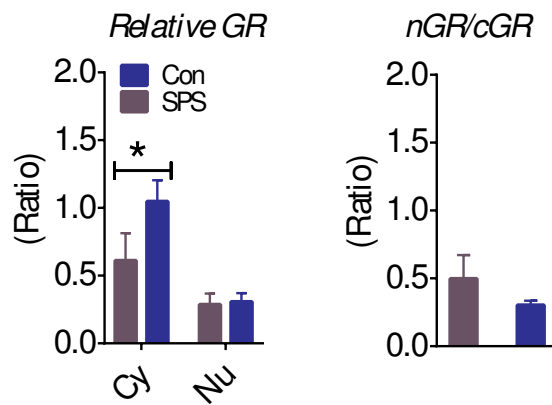
D) Fear Conditioning (norm)



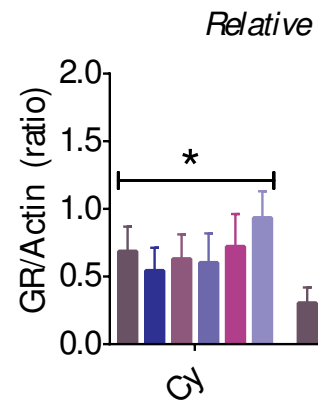
A) Western



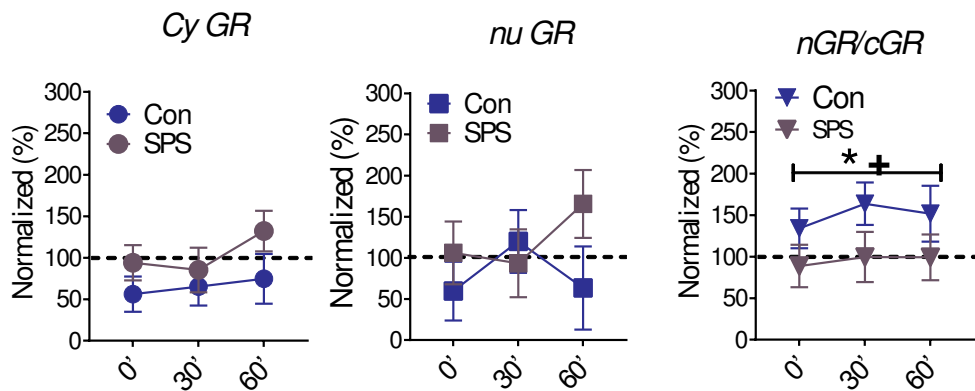
B) Baseline



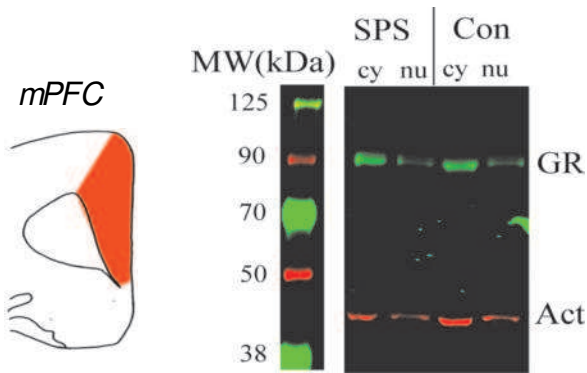
C) Fear Con



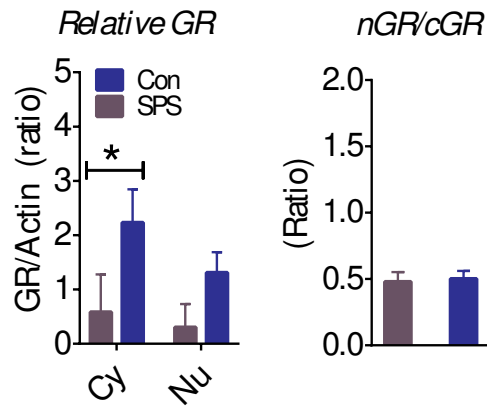
D) Fear Conditioning (norm)



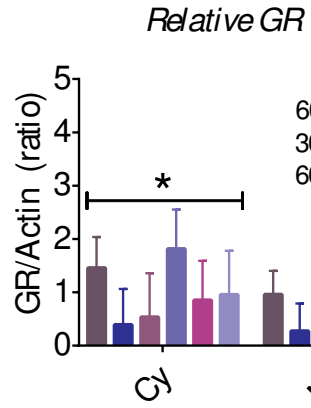
A) Western



B) Baseline



C) Fear



D) Fear Conditioning (norm)

