Research Article

Blockade of ghrelin receptor signaling enhances conditioned passive avoidance and context-associated neural circuit activation in fasted male rats

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Short Title: Ghrelin influences learned passive avoidance and neural activation

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Abstract

Interoceptive feedback to the brain regarding the body's physiological state plays an important role in guiding motivated behaviors. For example, a state of negative energy balance tends to increase exploratory/food-seeking behaviors while reducing avoidance behaviors. We recently reported that overnight food deprivation reduces conditioned passive avoidance behavior in male (but not female) rats. Since fasting increases circulating levels of ghrelin, we hypothesized that ghrelin signaling contributes to the ability of fasting to reduce conditioned avoidance. To test this, *ad libitum*-fed male rats were trained in a passive avoidance procedure using mild footshock. Later, following overnight food deprivation, the same rats were pretreated with ghrelin receptor antagonist (GRA) or saline vehicle 30 min before avoidance testing. GRA restored passive avoidance in fasted rats as measured both by latency to enter and time spent in the shock-paired context. In addition, compared to vehicle-injected fasted rats, fasted rats that received GRA before re-exposure to the shock-paired context displayed more activation of prolactin-releasing peptide (PrRP)-positive noradrenergic neurons in the caudal nucleus of the solitary tract, accompanied by more activation of downstream neurons in the bed nucleus of the stria terminalis and paraventricular nucleus of the hypothalamus. These results support the view that ghrelin signaling contributes to the inhibitory effect of fasting on learned passive avoidance behavior, perhaps by suppressing recruitment of PrRP-positive noradrenergic neurons and their downstream hypothalamic and limbic forebrain targets.

Introduction

Avoiding danger is critical for survival, and animals are equipped with neural circuits that enable adaptive avoidance responses to stimuli previously associated with potentially dangerous, aversive experiences. However, excessive conditioned avoidance can be maladaptive, contributing to clinical anxiety and other stress-related disorders [1–3]. Conditioned avoidance responses are based on learning and memory processes that include both conscious and subconscious components. Sensory feedback from body to brain during the learning and expression of emotional responses plays a major role in the subconscious modulation of motivated behavior [4]. For example, short-term negative energy balance generally biases animals towards approach behaviors and away from avoidance behaviors, thereby facilitating exploratory food-seeking [5,6]. In recent work, we demonstrated a reduction in learned passive avoidance behavior in male rats that were tested following an overnight fast [7]. The present study was designed to investigate potential neural circuits through which overnight food deprivation suppresses conditioned passive avoidance behavior.

Interoceptive feedback about the internal state of the body is continuously communicated to the brain through hormonal and neural pathways. The neural pathways feature vagal afferent inputs to the caudal nucleus of the solitary tract (cNTS) that can powerfully modulate motivated behavior [4,8–10]. Vagal afferents communicate a diverse array of cardiovascular, respiratory, gastrointestinal, and inflammatory signals to the cNTS in a manner that is further modulated by hormonal and metabolic state [11–15]. Within the cNTS, the A2 noradrenergic cell group receives direct synaptic input from vagal sensory afferents [16,17], and A2 neurons are activated to express the immediate-early gene product, cFos, in response to vagal sensory stimulation [17–20]. A caudal subset of A2 neurons that co-express prolactin-releasing peptide (PrRP) express cFos in rats exposed to a variety of innate and conditioned stressors that promote avoidance behavior [6,7,21,22]. Interestingly, overnight food deprivation markedly reduces the ability of these innate and conditioned stress stimuli to activate PrRP+ neurons within the cNTS [6,7], concurrent with reduced activation of neurons within hypothalamic and limbic forebrain regions that are innervated by PrRP+ neurons, including the paraventricular nucleus of the hypothalamus (PVN) [19] and the anterior ventrolateral bed nucleus of the stria terminalis (vlBNST) [6,7].

Food deprivation increases circulating levels of acyl-ghrelin [23–25], and acyl-ghrelin reduces tonic firing and stimulus-induced responsiveness of vagal sensory neurons [26,27] and postsynaptic A2 neurons in the cNTS [28]. We recently demonstrated that systemic administration of a ghrelin receptor antagonist in fasted rats partially restores the ability of vagal sensory stimulation to activate

PrRP+ A2 neurons and suppress food intake [20]. Further, caloric restriction reduces innate avoidance behavior in wildtype mice but not in mice lacking ghrelin receptors [5], which display more innate anxiety-like behavior than wildtype mice [29]. Consistent with these anxiolytic effects of ghrelin signaling, baseline acyl-ghrelin levels prior to auditory fear conditioning negatively predict conditioned freezing behavior in rats [30]. However, the potential impact of ghrelin signaling on the expression of learned avoidance has not been examined. Based on our previous research demonstrating that rats fasted overnight display less passive avoidance behavior and less fear context-induced neural activation [7], the present study was designed to test the hypothesis that increased ghrelin receptor signaling contributes to the ability of overnight food deprivation to suppress learned passive avoidance behavior, and to suppress conditioned context-induced activation of PrRP+ neurons in the cNTS and their downstream targets in the PVN and anterior vlBNST.

Materials and Methods

All experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were reviewed and approved by the Florida State University Animal Care and Use Committee.

Passive Avoidance Task

Adult male Sprague Dawley rats [Envigo; N=32 (250-300g body weight)] were pair-housed in standard tub cages in a temperature- and light-controlled housing environment (lights on from 0400 hr to 1600 hr). Only male rats were used in the current study based on evidence that food deprivation does not significantly reduce conditioned passive avoidance in female rats [7]. Rats were acclimated to handling for three days, with free access to water and rat chow (Purina 5001). Rats underwent passive avoidance training in a novel light/dark shuttle box (Colbourn Instruments, Allentown, PA), with training performed 4-6 hr after lights on. The light/dark box comprised a light (illuminated) chamber with clear plastic walls and a smooth white plastic floor, and a dark (non-illuminated) chamber with black plastic walls and a metal grid floor. Each chamber measured 25 x 25 cm, with 28 cm-high walls and a ceiling. The two chambers were separated by a metal divider wall with a guillotine door that could be opened and closed remotely. For passive avoidance training, rats were initially placed individually into the light chamber of the box and the dividing guillotine door was lifted immediately to allow access to the dark chamber. As expected, due to their innate aversion to the light and preference for the dark, rats very quickly entered the dark chamber (average latency = 22.73s). Upon entry into the dark chamber, the guillotine door was closed. After a 5s delay, rats received a single mild electric footshock (0.6mA; 1s). Rats remained in the enclosed

86 dark chamber for 30s following footshock, and then were returned to their homecage. Cagemates
87 were similarly trained on the same day.

88 Two hours before dark onset on the day before testing (i.e., 2 days after training), all rats were
89 transferred to clean cages with chow removed to initiate overnight food deprivation. On the next
90 day, 4-6 hr after lights on, rats received either an intraperitoneal (i.p.) injection of 0.15M NaCl
91 (saline) vehicle (~0.3 ml), or the same volume of vehicle containing ghrelin receptor antagonist (GRA;
92 [D-Lys3]-GHRP-6; 3.3mg/kg BW; Sigma-Aldrich G4535). This GRA dose was based on our previous
93 report demonstrating its ability to restore cholecystokinin-induced NTS neuronal activation in food
94 deprived rats [20]. Thirty minutes later, rats were tested for passive avoidance retention. For this
95 purpose, rats were placed individually into the light chamber of the light/dark box, the guillotine
96 door was opened, and the latency of rats to fully enter the dark chamber was recorded, with a pre-
97 set maximum latency of 900s (15 min). During the retention test, the guillotine door remained open
98 and no footshock was administered. Rats were allowed to freely explore both the light and dark
99 chambers during the 900s test, and total time spent within each chamber was recorded. After testing
100 was complete, rats were returned to their home cages. Chow was not returned until 2 hr prior to
101 dark onset (i.e., approximately 6-8 hr after testing).

102 To confirm that GRA treatment does not alter conditioned avoidance behavior in non-fasted rats, a
103 separate cohort of adult male rats (n=4) was trained in the passive avoidance task as described
104 above. Two days later, rats were tested in an *ad libitum* fed state, 30 min after receiving i.p. injection
105 of GRA.

106 **Novel Object Recognition Task**

107 To examine the impact of GRA on general memory function, rats (N=32) were tested for novel object
108 recognition at least 5 days after completing passive avoidance testing. For this, rats underwent 3
109 days of acclimation (10 min/day) to a previously novel tub cage that had a thin layer of bedding on
110 the floor, with the tub enclosed in an illuminated sound-attenuating chamber. On the 4th day, rats
111 underwent object training 4-6 hr after lights on, during which two similarly sized but geometrically
112 distinct ceramic objects with rounded contours (i.e., a white pyramidal "Eiffel tower" and a blue
113 spherical "rocket ship") were placed at opposite ends of the tub cage. Rats were allowed to freely
114 explore both objects for 15 minutes while their behavior was recorded and later analyzed using
115 behavior analysis software (ANY-maze, Stoelting Co.). During training, there was no significant
116 difference between time spent with either object, defined as the rat being in contact with or actively
117 examining the object.

Two hours before dark onset on the day before testing (i.e., 2 days after object training), all rats were transferred to clean cages with chow removed. On the subsequent day, fasted rats received either an i.p. injection of saline vehicle (~0.3 ml), or the same volume of vehicle containing GRA (3.3mg/kg BW). Thirty minutes later, rats were tested for novel object recognition. For this, the previously explored "Eiffel tower" object was replaced with a novel object (i.e., a grey cube-shaped "robot"). Rats were allowed to freely explore the novel and familiar objects for 15 minutes. The time each rat spent exploring each object was recorded, and the discrimination index was calculated as the difference between time spent with each object divided by the total time spent with either object:

$$Discrimination\ Index = \frac{(Time_{Novel\ Object} - Time_{Familiar\ Object})}{(Time_{Novel\ Object} + Time_{Familiar\ Object})}$$

In the discrimination index, a value of 0 indicates no discrimination between the novel and familiar objects. A positive value indicates more time spent with the novel object, whereas a negative value indicates more time spent with the familiar object. One rat from each injection group was excluded from analysis after one of the objects was knocked over during the novel object recognition test.

Open Field Test

To examine the impact of GRA on general locomotion and anxiety-like behavior, the same rats (N=32) were tested in the open field after overnight food deprivation. On the testing day (4-6 hr after lights on), fasted rats received either an i.p. injection of saline vehicle (~0.3 ml), or the same volume of vehicle containing GRA (3.3mg/kg BW). Thirty minutes later, rats were placed into the center of a novel 95 x 95cm open arena, and then left to freely explore for 10 min. Behavior in the open field was recorded using a Logitech webcam and was analyzed using automated behavior analysis software (ANY-maze, Stoelting Co.). Locomotion was defined as the total distance traveled during the test. Anxiety-like behaviors were interpreted based on time spent in the center zone of the field (~30 x 30 cm) and latency to first enter the center zone. Data from one cohort of rats (n=4/injection group) were excluded due to lighting issues in the testing room.

Conditioned Context Re-exposure and Perfusion

To examine neural activation in response to the conditioned context associated with footshock, a subset of rats used in the behavioral studies described above (N=24) were retrained in the passive avoidance task, in which they were placed again into the light side of the light/dark box. When rats entered the dark chamber (average latency to enter = 134s, reflecting their earlier shock conditioning session), the guillotine door closed behind them. Shock-retrained rats immediately received a mild electric footshock, as they did during their initial conditioned avoidance training session. Additional

148 shock-naive control rats (N = 12) were placed into the light side of the light/dark box but did not
149 receive any footshock upon entry into the dark chamber (average latency to enter = 19s, similar to
150 that in our previous report [7]). Rats in both the shock retrained (i.e., shocked) and non-shocked
151 groups were kept in the dark chamber for 30s before being returned to their home cages. Two days
152 later, 2 hr before dark onset, all rats were transferred to clean cages with chow removed to initiate
153 overnight food deprivation. On the next day, 4-6 hr after lights on, fasted rats in both groups
154 received either an i.p. injection of 0.15M NaCl (saline) vehicle (~0.3 ml), or the same volume of
155 vehicle containing GRA (3.3mg/kg BW). Thirty minutes later, rats were placed directly into the dark
156 chamber, which was an aversively conditioned stimulus only in the shock-retrained group. Rats
157 remained in the dark chamber for 10 min with the guillotine door closed, and were then returned to
158 their home cages. Sixty minutes later, rats were deeply anesthetized with pentobarbital sodium (39
159 mg/ml i.p., Fatal Plus Solution; Butler Schein) and transcardially perfused with saline (100mL)
160 followed by 4% paraformaldehyde (500mL).

161 **Histology**

162 Fixed brains were removed from the skull, post-fixed overnight at 4˚C, then cryoprotected in 20%
163 sucrose. Brains were blocked and sectioned coronally (35 µm) using a Leica freezing-stage sliding
164 microscope. Tissue sections were collected in six serial sets and stored at -20˚C in cryopreservant
165 solution [31] until immunohistochemical processing. Primary and secondary antisera were diluted in
166 0.1M phosphate buffer containing 0.3% Triton X-100 and 1% normal donkey serum. One set of tissue
167 sections from each rat (with each set containing a complete rostrocaudal series of sections spaced by
168 210 µm) was incubated in a rabbit polyclonal antiserum against cFos (1:10,000; Cell Signaling, 2250;
169 AB_2247211), followed by biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch).
170 Sections were then treated with Elite Vectastain ABC reagents (Vector) and reacted with diamino-
171 benzidine (DAB) intensified with nickel sulfate to produce a blue-black nuclear cFos reaction product.

172 To visualize cFos within hindbrain PrRP neurons, one set of cFos-labeled tissue sections from each rat
173 was subsequently incubated in a rabbit polyclonal antiserum against PrRP (1:10,000; Phoenix
174 Pharmaceuticals, H-008-52), followed by biotinylated donkey anti-rabbit IgG (1:500; Jackson
175 ImmunoResearch) and Elite Vectastain ABC reagents (Vector), and finally reacted with plain DAB to
176 produce a brown cytoplasmic reaction product.

177 To visualize cFos within all noradrenergic neurons, a second set of tissue from each rat was incubated
178 in a cocktail of rabbit anti-cFos (1:1000) and a mouse monoclonal antiserum against dopamine beta-
179 hydroxylase (DbH; 1:5000; Millipore, MAB308; AB_2245740), followed by a cocktail of Cy3-

180 conjugated donkey anti-rabbit IgG (red) and Alexa Fluor 488-conjugated donkey anti-mouse IgG
181 (green).

182 **Quantification of Neural Activation**

183 Sections through the cNTS (~15.46-13.15 mm caudal to bregma) were imaged at 20x using a Keyence
184 microscope (BZ-X700). Using separate sets of dual-labeled tissue sections from each rat, the total
185 numbers of DbH+ and PrRP+ cNTS neurons were counted, and the percentage of each chemically
186 identified neural population co-expressing cFos was determined. A neuron was considered to be
187 cFos-positive if its nucleus contained any visible cFos immunolabeling.

188 Sections through the anterior vlBNST (~0.26-0.40mm caudal to bregma) were imaged at 10x. Images
189 from two rats were excluded from further analysis due to poor tissue quality. Using ImageJ, a circular
190 region of interest (ROI) of 0.10mm^2 diameter was centered on the area containing the highest
191 density of DbH+ fibers and terminals, corresponding to the fusiform subnucleus of the anterior
192 vlBNST [6,32,33]. The number of cFos-positive neurons within each 0.10mm^2 ROI was determined
193 bilaterally in 2-3 sections per brain and then averaged across assessed ROIs in each animal.

194 Three sections through the PVN (~1.5-1.9mm caudal to bregma) in each animal were imaged at 10x.
195 For this, a region of interest (ROI) encompassing each PVN (bilaterally) was identified by the presence
196 of dense DbH+ fibers and terminals, and an outline was drawn around each region using ImageJ. The
197 number of cFos-positive neurons within each ROI was determined, divided by the ROI area, and
198 averaged in each animal to calculate the number of cFos-positive neurons per mm^2.

199 **Ghrelin ELISA**

200 After finding that GRA treatment significantly increased conditioned avoidance behavior and cFos
201 activation in fasted rats (see *Results*), we sought to confirm our assumption that plasma acyl-ghrelin
202 levels increase in rats exposed to either a single or repeated sessions of overnight food deprivation.
203 For this, terminal blood samples were collected from a separate cohort of adult male rats that were
204 either *ad lib* fed with no fasting experience (n=3), fasted once overnight before morning blood
205 collection (n=3), or fasted overnight on 3 separate occasions (with at least two days between each
206 episode) with the 3rd fast occurring the night before morning blood collection (n=4). Just before
207 blood collection, rats were injected with pentobarbital sodium (39 mg/ml i.p., Fatal Plus Solution;
208 Butler Schein), and blood samples were collected within 5 min from the right atrium of the heart
209 using a 3mL syringe pre-rinsed with 1% EDTA. Blood samples were transferred to a K2E EDTA
210 vacuette tube (BD Ref 367841), and aprotinin (50µl/mL) was added to each sample. Samples were
211 centrifuged at 3500 rpm for 10 min at 4°C to separate the plasma. Hydrochloride was added to the

212 plasma supernatant (10µl/100mL), and then samples were centrifuged again at 3500 rpm for 5 min
213 at 4°C. Plasma samples were stored at -80°C until acyl-ghrelin was measured using an ELISA kit
214 (BioVendor, R&D; RA394062400R), following the manufacturer's instructions.

215 **Statistics**

216 Data were analyzed using GraphPad Prism. Passive avoidance behaviors (latency to enter and total
217 time spent in the dark chamber) during the retention test were analyzed using a Welch's t-test [34].
218 Time spent exploring novel vs. familiar objects in the novel object recognition task was analyzed with
219 a mixed 2x2 ANOVA with i.p. injection as a between-subjects independent variable and Object (novel,
220 familiar) as a within-subjects independent variable. When ANOVA F values indicated significant main
221 effects and/or interactions, post-hoc comparisons were made using Šídák's multiple comparisons
222 tests. Discrimination indices for novel object recognition by each i.p. injection group of rats were
223 analyzed using Welch's t-test, and by individual one-subject t-tests with a hypothetical value of 0
224 (i.e., no discrimination). Each open field behavioral measure (distance traveled, time in center, and
225 latency to first enter the center) was analyzed separately with a Welch's t-test to compare i.p.
226 injection groups. Cell count data (cFos activation of PrRP and DbH neurons, cFos activation within the
227 anterior vlBNST and PVN) were analyzed using between-subjects 2x2 ANOVA with previous shock-
228 pairing and i.p. treatment (i.e., GRA vs. vehicle) as independent variables. In addition, Pearson's r
229 correlations were run between the percent of DbH+/PrRP+ cNTS neurons expressing cFos and cFos
230 counts within vlBNST/PVN in the same rat. Plasma acyl-ghrelin data were analyzed using a one-way
231 ANOVA with food deprivation history as the independent variable. An alpha level of 0.05 ($p \leq 0.05$)
232 was used as the criterion for considering group differences to be statistically significant. Estimation
233 statistics [35,36] were used to report effect sizes for both passive avoidance behavior and cell count
234 data.

235 ## Results

236 **Passive Avoidance Task**

237 Welch's t-test indicated that fasted rats injected with GRA demonstrated more passive avoidance
238 behavior than fasted rats injected with saline. Compared to saline-treated rats, GRA-treated rats took
239 longer to enter the shock-paired dark chamber [$t(26.1) = 1.728$; $p = 0.0479$] and spent less time in the
240 dark chamber [$t(18.67) = 2.162$; $p = 0.0219$] during the 900s testing period (Fig. 1). Estimation
241 statistics indicated that compared to fasted saline-treated control rats, fasted GRA-treated rats took
242 an average of 195s (3.25min) longer to enter the shock-paired dark chamber [95%CI: -11.4s, 419s],

243 and spent an average of 178s (2.97min) less time in the shock-paired dark chamber during the 900s
244 retention test [95%CI: 47.2s, 369s], evidence for increased passive avoidance after GRA treatment.

245 A separate group of avoidance-trained, non-fasted rats that received GRA treatment before the
246 passive avoidance test (n=4) displayed a typically long average latency to enter [895.75s ± 4.25] and
247 minimal time spent within the shock-paired dark chamber [1s ± 1], quite similar to the behavior of
248 fasted GRA-treated rats (see Fig. 1). The similarly robust conditioned avoidance behavior displayed
249 by GRA-treated rats tested in the fasted and fed states also was similar to published data from our
250 laboratory obtained from avoidance-trained, non-fasted male rats with no i.p. treatment prior to
251 testing [7]. These observations support the view that GRA treatment does not alter conditioned
252 avoidance behavior in *ad libitum*-fed rats, in which plasma acyl-ghrelin levels are not elevated.

253 **Novel Object Recognition Task**

254 GRA treatment did not alter the behavior of fasted rats in the novel object recognition task (Fig. 2).
255 Two-way ANOVA indicated a significant main effect of Object [$F(1,28) = 15.59$; $p = 0.0005$] but no
256 main effect of i.p. treatment and no interaction. Post-hoc Šídák's multiple comparisons tests
257 indicated that fasted rats spent significantly more time exploring the novel object compared to the
258 familiar object in both the saline- ($p = 0.0093$) and GRA-injected ($p = 0.0361$) groups. Individual one-
259 sample t-tests indicated that the discrimination indices for both the saline- [$t(14) = 3.575$, $p = 0.0030$]
260 and GRA-injected groups [$t(14) = 2.294$, $p = 0.0378$] were significantly higher than 0, evidence that
261 both groups were similarly able to discriminate between the familiar and novel objects.

262 **Open Field Test**

263 Pre-treatment of fasted rats with GRA did not alter assessed behaviors in the 10-min open field test
264 (Fig. 3). There were no significant differences in the total distance traveled by saline-injected (mean =
265 17.9m) vs. GRA-injected rats (mean = 17.3m; Fig. 3A). Additionally, there were no significant group
266 differences in anxiety-like behaviors as measured by either time spend in the central zone (Fig. 3B) or
267 latency to first enter the central zone of the open field (Fig. 3C).

268 **Neural Activation after Dark Chamber Re-exposure**

269 Two-way ANOVA revealed a significant main effect of shock retraining [$F(1,30 = 14.02$; $p < 0.001$] on
270 PrRP+ neuron activation in fasted rats that were re-exposed to the dark (i.e., shock-paired) chamber,
271 as well as a trending main effect of GRA treatment prior to dark chamber re-exposure [$F(1,30) = 3.14$;
272 $p = 0.087$] and a trending interaction between shock retraining and GRA treatment [$F(1,30) = 3.33$; p
273 $= 0.078$] (Fig. 4A). Post-hoc Sidak's multiple comparisons tests indicated that significantly more PrRP+
274 neurons were activated in shock-retrained fasted rats treated with GRA vs. rats treated with saline

before dark chamber re-exposure ($p = 0.010$). Conversely, there was no GRA-related effect on the very low levels of PrRP+ neural activation in fasted rats that were not shocked retrained before dark chamber re-exposure. Estimation statistics indicated that 12.1% more PrRP+ neurons were activated in shock-retrained, fasted rats pretreated with GRA before dark chamber re-exposure compared to PrRP+ neural activation in similarly shock-retrained, fasted rats treated with saline before dark chamber re-exposure [95%CI: 2.39%, 21.9%].

Two-way ANOVA also revealed a significant interaction between shock training and GRA treatment [$F(1,32) = 7.29$; $p = 0.011$] on DbH+ neuron activation (Fig. 4D), but no main effects of either condition. Post-hoc Sidak's multiple comparisons tests indicated that GRA treatment significantly increased DbH+ neural activation in shock retrained rats after re-exposure to the shock-paired dark chamber ($p = 0.024$), but no effect of GRA treatment on DbH+ neural activation in non-shocked rats. Estimation statistics indicated that dark chamber re-exposure activated 11.9% more DbH+ neurons in shock-trained rats pretreated with GRA compared to rats treated with saline [95%CI: 1.59%, 20.4%].

Regarding activation of neurons within forebrain regions that receive input from hindbrain A2 neurons, two-way ANOVA indicated a significant main effect of shock training [$F(1,30) = 57.08$; $p < 0.001$] and a significant interaction between shock training and GRA treatment [$F(1,30) = 5.55$; $p = 0.025$] on dark chamber-induced cFos in the vlBNST (Fig. 5A). Post-hoc Sidak's multiple comparisons tests indicated significantly more vlBNST cFos activation in GRA- vs. saline-treated rats that had been shock retrained ($p = 0.031$), but no effect of GRA treatment on vlBNST cFos in non-shocked rats. Estimation statistics indicated that 12.9 more vlBNST neurons per ROI were activated in response to the shock-paired dark chamber in rats pretreated with GRA compared to those treated with saline [95%CI: 3.4, 25.3]. A significant main effect of shock training on PVN cFos also was observed [$F(1,32) = 19.54$; $p < 0.001$], with a marginally trending interaction between shock training and GRA treatment [$F(1,32) = 2.296$; $p = 0.14$] (Fig. 5D). Post-hoc Sidak's multiple comparisons tests indicated a trend towards more PVN cFos activation in GRA- vs. saline-treated rats that had been shock retrained before dark chamber re-exposure ($p = 0.059$), but no effect of GRA treatment on PVN cFos in non-shocked rats. Estimation statistics indicated that 74.6 more PVN neurons/mm^2 were activated in shock retrained rats pretreated with GRA compared to rats treated with saline [95%CI: 8.64, 137].

Across all shock retrained rats, Pearson's r correlations indicated strong positive within-subjects relationships between activation of DbH+ cNTS neurons and activation of neurons in the vlBNST [$r(20) = 0.5093$; $p = 0.0155$] (Fig. 6A) and PVN [$r(22) = 0.6494$; $p = 0.0006$] (Fig. 6B). Similar positive relationships were observed between activation of PrRP+ cNTS neurons and activation of neurons in the vlBNST [$r(20) = 0.5497$; $p = 0.0080$] (Fig. 6C) and PVN [$r(22) = 0.5488$; $p = 0.0055$] in shock

308 retrained rats (Fig. 6D). Conversely, there were no significant relationships between cFos expression
309 by PrRP+ or DbH+ neurons within the cNTS and cFos expression in either the PVN or vlBNST in the
310 non-shock retrained rats.

311 **Acyl-Ghrelin Levels**

312 One-way ANOVA confirmed a trending effect of food deprivation to increase plasma levels of acyl-
313 ghrelin in adult male rats [$F(2,7) = 3.851$; $p = 0.0745$]. Acyl-ghrelin levels in *ad libitum* fed rats
314 averaged 120.1±8.0 pg/ml. Average acyl-ghrelin levels rose to 192.3±24.5 pg/ml following a single
315 overnight fast, and measured 399.9±105.2 pg/ml following a third overnight fast. Thus, there was no
316 decrement of the acyl-ghrelin response to an overnight fast in rats exposed to repeated spaced
317 episodes of overnight fasting.

318 Discussion/Conclusion

319 Results from the present study build on our previous findings that overnight food deprivation
320 reduces conditioned passive avoidance behavior in adult male rats, with concurrent reduction of
321 conditioned context-induced activation of PrRP+ NA neurons in the NTS and downstream target
322 neurons in the vlBNST [7]. Here we demonstrate a role for ghrelin signaling in these fasting-induced
323 behavioral and neural effects. Specifically, we show that pharmacological blockade of fasting-related
324 ghrelin receptor signaling (via systemic administration of GRA) prior to the retention test enhanced
325 conditioned passive avoidance behavior (shown in Fig. 1) and neuronal activation by the aversively
326 conditioned context (Figs. 4, 5). These novel findings support the view that reduced avoidance
327 behavior and reduced neural activation in food-deprived rats is at least partly due to increased
328 ghrelin signaling.

329 We considered the possibility that reduced passive avoidance behavior in fasted rats with elevated
330 ghrelin is due to a general reduction in memory retrieval, such that GRA may increase passive
331 avoidance by improving memory of the shock-associated context. However, GRA treatment did not
332 improve novel object recognition memory in food deprived rats (shown in Fig. 2). A key distinction
333 between mnemonic processes underlying passive avoidance vs. novel object recognition is that the
334 former but not the latter involves contextual memory [37,38]. Previous research has shown that mice
335 with a global ghrelin receptor knockout and rats with a vagal afferent-specific ghrelin receptor
336 knockdown show impairment in a hippocampal-dependent "novel object in context" task [39,40].
337 However, GRA effects on passive avoidance memory in the present study were in the opposite
338 direction, such that GRA treatment improved avoidance memory in fasted rats. This discrepancy may
339 be related to chronic rather than acute disruption of ghrelin signaling in the knockout/knockdown

340 studies, which could interfere with other stages of memory formation. Alternatively, reduced
341 performance in the "novel object in context" task by rodents with ghrelin receptor
342 knockout/knockdown could reflect avoidance of the novel object. For example, Davis and colleagues
343 [40] reported a negative shift in discrimination index in their vagal afferent ghrelin receptor
344 knockdown model, such that knockdown rats spent less time exploring the novel object than would
345 be expected if their behavior was due only to impaired memory of the familiar object.

346 If a fasting-induced elevation in ghrelin signaling is normally anxiolytic, then GRA treatment may
347 reverse this effect to enhance general/innate anxiety-like behavior and avoidance displayed by non-
348 fasted rats. Supporting this idea, previous work demonstrated that overnight food deprivation
349 suppresses innate anxiety-like behavior in the elevated plus maze and light-enhanced acoustic startle
350 tests [6], and in the open field test [41]. In the present study, GRA treatment did not alter innate
351 anxiety-like behavior displayed by food-deprived rats in the open field test (shown in Fig. 3B, C),
352 evidence that ghrelin signaling alone cannot fully explain the anxiolytic effect of food deprivation. A
353 previous study reported that vagal afferent-specific ghrelin receptor knockdown does not alter
354 anxiety-like behavior in the elevated zero maze [40], although rats in that experiment were not food
355 deprived so endogenous ghrelin presumably would have been relatively low in both knockdown and
356 control rats.

357 Our findings demonstrate that GRA treatment enhanced the ability of re-exposure to the shock-
358 associated context to activate cFos expression in PrRP+ noradrenergic neurons in shock-retrained
359 fasted rats (shown in Fig. 4). These results are consistent with evidence that ghrelin suppresses the
360 activity of vagal sensory afferents [26,27], and suppresses activation of postsynaptic A2 neurons in
361 the cNTS [28]. Our current results also are in line with our previous report that GRA enhances the
362 ability of pharmacological vagal afferent stimulation to activate PrRP+ A2 neurons [20]. In that study
363 [20] and in the current study, GRA treatment did not completely restore A2 neural activation to
364 levels observed in *ad libitum* fed rats (i.e., see [7]). Thus, elevated ghrelin signaling after food
365 deprivation does not fully explain the A2 neural "silencing" effect of negative energy balance. Other
366 contributing factors may include altered mechanosensory signals and/or hormonal signals [6,42,43].

367 In addition to partial restoration of cNTS neural activation in shock retrained, GRA-treated rats after
368 re-exposure to the shock-paired context, GRA increased shock-paired context-induced activation of
369 neurons within the vlBNST and PVN, two downstream targets of PrRP+ noradrenergic cNTS neurons
370 (shown in Fig. 5). Importantly, GRA treatment prior to dark chamber re-exposure did not influence
371 cFos activation in PrRP+ noradrenergic neurons or within the vlBNST and PVN in rats that were not
372 shocked during training (Figs. 4, 5). These data support the view that ghrelin signaling in fasted rats

373 acts to limit aversive context-induced neural activation, without suppressing neural activation in
374 response to a non-aversive context. Based on significant positive relationships between activation of
375 PrRP+ and DbH+ cNTS neurons and activation of neurons within the vlBNST and PVN (shown in Fig.
376 6), we hypothesize that activation of A2 cNTS neurons in response to the shock-paired context
377 promotes activation of downstream neurons within the vlBNST and PVN. We previously
378 demonstrated that A2 noradrenergic inputs to the vlBNST and PVN are necessary for stress-induced
379 innate avoidance and behavioral suppression [44–46], and noradrenergic signaling in the BNST is
380 implicated in avoidance behavior conditioned by aversive opiate withdrawal [47,48]. The current
381 results provide new evidence that A2 noradrenergic inputs to the vlBNST and/or PVN also participate
382 in conditioned passive avoidance behavior.

383 The effect of GRA treatment on behavior and neural activation in the present study could reflect
384 interference with either central or peripheral ghrelin signaling mechanisms. Ghrelin receptor mRNA
385 is expressed by vagal sensory neurons in the nodose ganglia [26,49] and by neurons within the cNTS
386 [50,51], PVN [51], and hippocampus [51,52]. Since systemic administration of the same GRA used in
387 the present study was shown to block the effects of centrally-administered ghrelin [53], central
388 receptor blockade may have contributed to the behavioral and neural effects of GRA in our
389 experiments. Additional work will be needed to differentiate between peripheral/vagally-mediated
390 vs. centrally-mediated ghrelin signaling in conditioned avoidance behavior. Future research also
391 should explore sex differences in passive avoidance behavior and the role of ghrelin signaling (and
392 other interoceptive feedback signals) in that behavior, given our previous report that food
393 deprivation suppresses conditioned passive avoidance in male, but not female, rats [7]. In this
394 regard, recent evidence indicates that female rats have significantly higher plasma levels of acyl-
395 ghrelin compared to male rats under both fed and fasted conditions, and that females may be more
396 sensitive to the anxiolytic effects of fasting-induced ghrelin signaling [25]. We reported that
397 compared to male rats, female rats display markedly less conditioned passive avoidance behavior
398 regardless of metabolic state at testing, consistent with other reports that female rodents show less
399 passive and more active responses to threatening stimuli [7]. Thus, an active avoidance learning and
400 testing approach is likely more suitable to explore potential effects of ghrelin signaling on behavioral
401 (and neural) responses to conditioned aversive stimuli in female rodents.

402 In summary, our results demonstrate a role for endogenous ghrelin signaling in the ability of
403 negative energy balance to reduce conditioned avoidance behavior in male rats. We further
404 demonstrate that GRA treatment enhances the ability of a footshock-conditioned context to activate
405 PrRP+ A2 noradrenergic neurons, and to activate neurons in the downstream targets of these cNTS

406 neurons (i.e., vlBNST and PVN) in fasted male rats. These findings support the view that interoceptive
407 feedback from body to brain modulates learned avoidance behavior, and that a ghrelin-sensitive
408 circuit contributes to this modulatory effect.

409

Statements

Statement of Ethics

This study protocol was reviewed and approved by Florida State University Animal Care and Use Committee and was conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

CME and LR designed the experiments. CME, IEG, HZ, and TD performed the experiments and analyzed the resulting data. CME, IEG, and LR wrote the manuscript.

Data Availability Statement

Data are available on request from the corresponding author.

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Figure Legends

Fig. 1. Systemic ghrelin receptor antagonist (GRA) administration prior to the retention test increased passive avoidance behavior in food deprived male rats. (A) Schematic depicting the passive avoidance experimental timeline. (B) Rats pretreated with GRA took an average of 195s longer to enter the shock-paired dark chamber compared to rats pretreated with saline. (C) Rats pretreated with GRA spent an average of 178s less in the shock-paired dark chamber during the 900s retention test compared to rats pretreated with saline. *$p < 0.05$. Graphs display group mean +/- SEM, with individual data points plotted.

Fig. 2. Systemic ghrelin receptor antagonist (GRA) administration prior to the novel object test did not influence novel object recognition memory in food deprived male rats. (A) Schematic depicting the novel object recognition training and test. (B) Both GRA- and saline-pretreated rats spent significantly more time with the novel object compared to the familiar object. (C) The discrimination indices for GRA- and saline-pretreated rats were similar, and significantly greater than zero in both groups. Graphs display group mean +/- SEM, with individual data points plotted. *$p<0.05$ and **$p<0.01$.

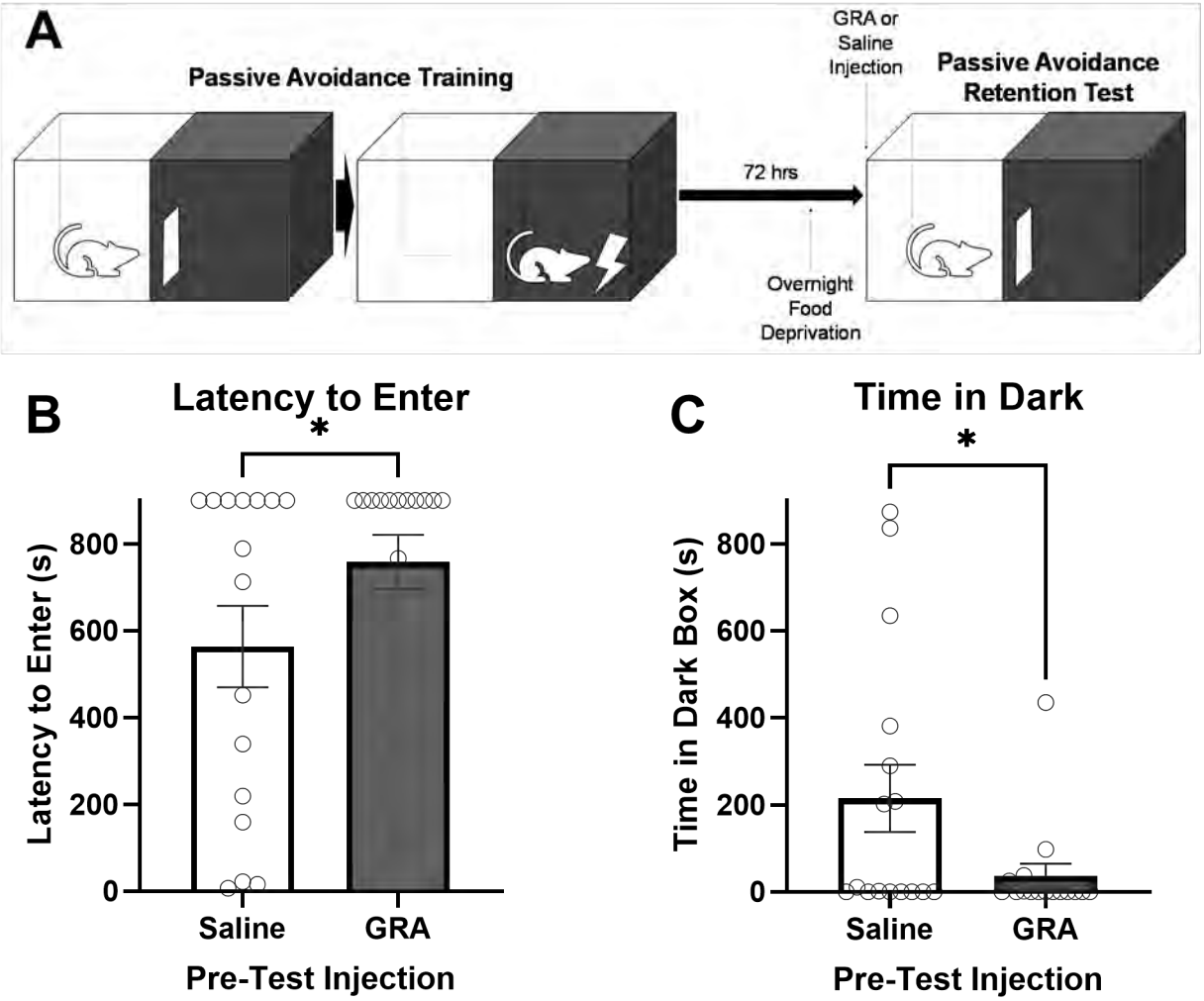
Fig. 3. Systemic ghrelin receptor antagonist (GRA) administration prior to the open field test did not influence locomotion or anxiety-like behavior in food deprived male rats. (A) GRA- and saline-pretreated rats did not differ in distance traveled during the open field test. (B) GRA- and saline-pretreated rats did not differ in time spent in the center zone of the open field. (C) GRA- and saline-pretreated rats did not differ in latency to enter the center zone of the open field during the test. Graphs display group mean +/- SEM, with individual data points plotted.

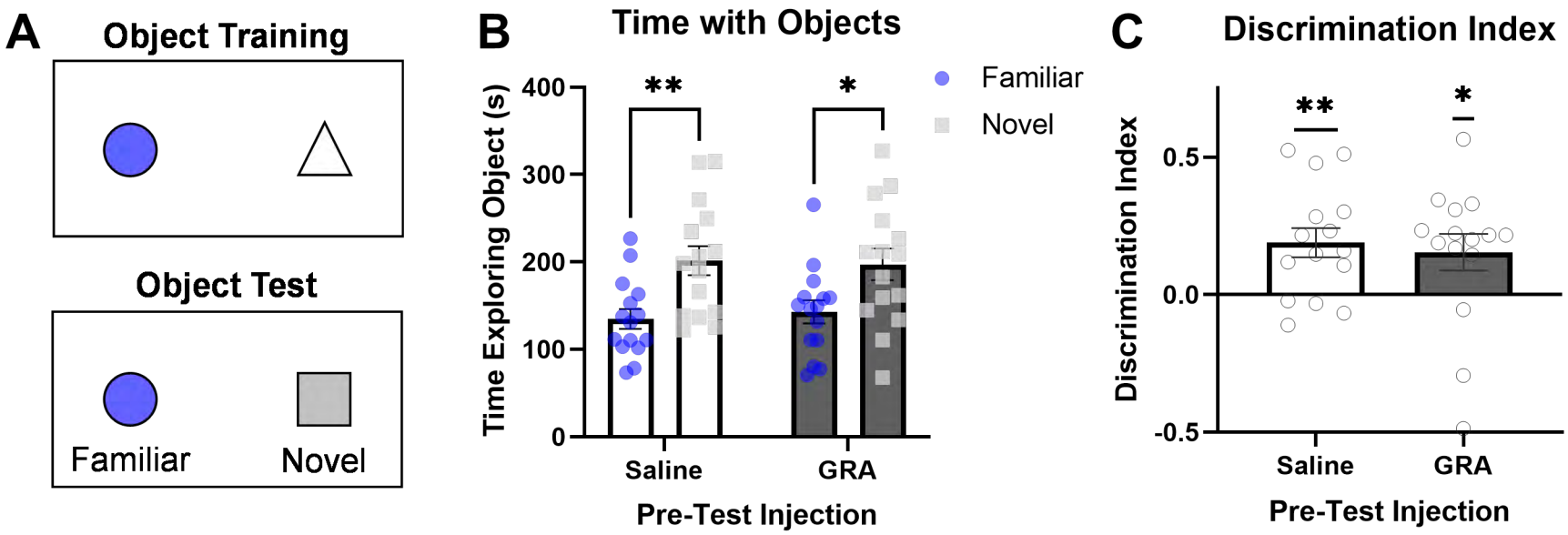
Fig. 4. In food deprived male rats, systemic ghrelin receptor antagonist (GRA) administration prior to dark chamber re-exposure increased cFos expression in noradrenergic cNTS neurons in rats that were retrained with footshock (Shock +), but not in rats that were not shock retrained (Shock -). (A) GRA pretreatment increased shock-paired dark chamber-induced activation of the PrRP+ subset of noradrenergic A2 neurons. (B, C) Representative images taken at 40x magnification of nuclear cFos (dark blue) and cytoplasmic PrRP immunolabeling (brown) in saline vehicle- (B) and GRA-treated (C) rats that were shock retrained before being re-exposed to the shock-paired dark chamber. Small black arrows indicate several double-labeled (cFos+) PrRP+ cells. (D) GRA pretreatment also

increased shock-paired dark chamber-induced activation of the larger population of DbH+ A2 neurons. (E+F) Representative images taken at 40x magnification of nuclear cFos (red) and cytoplasmic DbH immunolabeling (green) in saline vehicle- (E) and GRA-pretreated (F) rats that were shock retrained before being re-exposed to the shock-paired dark chamber. Small white arrows indicate several double-labeled (cFos+) DbH+ cells. Graphs display group mean +/- SEM, with individual data points plotted. *p<0.05.

Fig. 5. In food deprived male rats that were previously retrained with footshock exposure (Shock +), systemic ghrelin receptor antagonist (GRA) administration increased the ability of dark chamber re-exposure to activate cFos expression in the DbH+ terminal-rich regions of the vlBNST and PVN. GRA did not alter cFos activation in non-shock retrained (Shock -) rats. (A) GRA pretreatment increased shock-paired dark chamber-induced activation of neurons in the DbH+ terminal-rich region of vlBNST. (B+C) Representative images of nuclear cFos (red) and DbH+ terminal labeling (green) in saline vehicle- (B) and GRA-pretreated (C) rats that were re-exposed to the shock-paired dark chamber. ac, anterior commissure. (D) GRA pretreatment tended to increase shock-paired dark chamber-induced activation of neurons in the DbH+ terminal-rich region of the PVN. (E+F) Representative images of nuclear cFos (red) and DbH+ terminal labeling (green) in vehicle- (E) and GRA-pretreated (F) rats that were re-exposed to the shock-paired dark chamber. 3rd V., third ventricle. Graphs display group mean +/- SEM, with individual data points plotted. *p<0.05.

Fig. 6. Scatterplots depicting significant positive relationships in shock retrained rats between activation of (A) DbH+ neurons in the cNTS and neurons in the DbH terminal-rich region of the vlBNST, (B) DbH+ neurons and neurons in the DbH terminal-rich region of the PVN, (C) PrRP+ neurons in the cNTS and neurons in the vlBNST, and (D) PrRP+ neurons in the cNTS and neurons in the PVN. Conversely, there were no significant relationships in these neural activation patterns in rats that were not shock retrained before dark chamber re-exposure (Non-shocked).


A
Passive Avoidance Training
GRA or
Saline
Injection
Passive Avoidance
Retention Test
72 hrs
Overnight
Food
Deprivation
B
Latency to Enter
*
Latency to Enter (s)
800
600
400
200
0
Saline
GRA
Pre-Test Injection
C
Time in Dark
*
Time in Dark Box (s)
800
600
400
200
0
Saline
GRA
Pre-Test Injection


A
Object Training
Object Test
Familiar
Novel
B
Time with Objects
Time Exploring Object (s)
400
300
200
100
0
**
*
Familiar
Novel
Saline
GRA
Pre-Test Injection
C
Discrimination Index
Discrimination Index
0.5
0.0
-0.5
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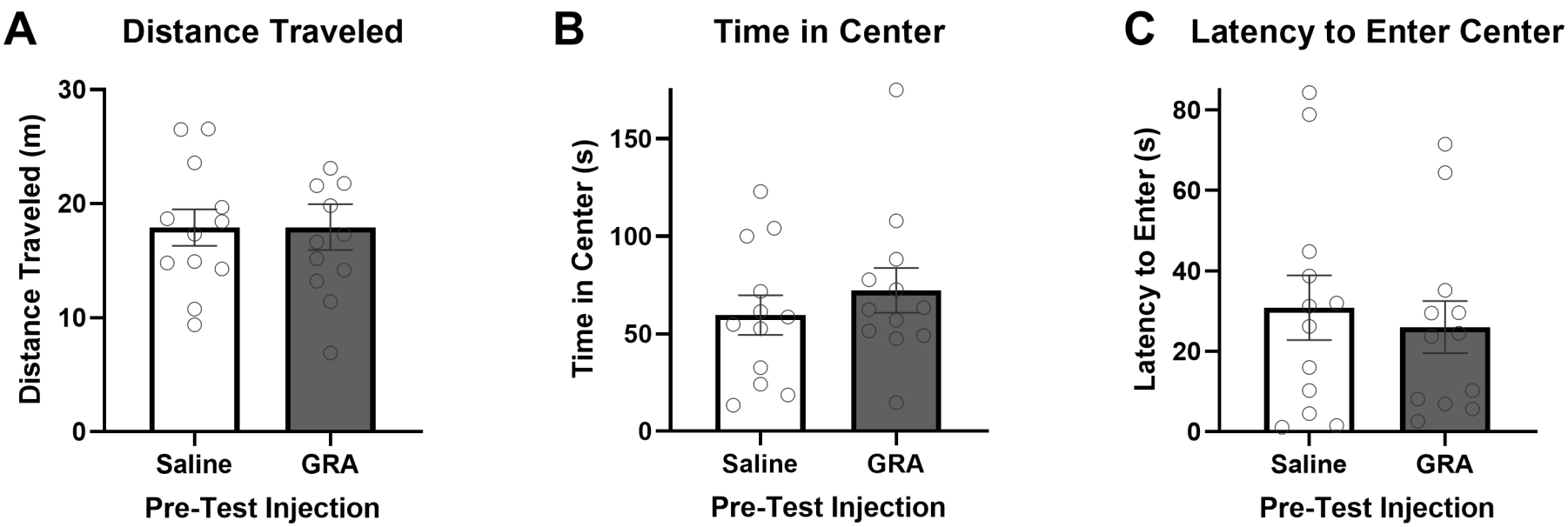
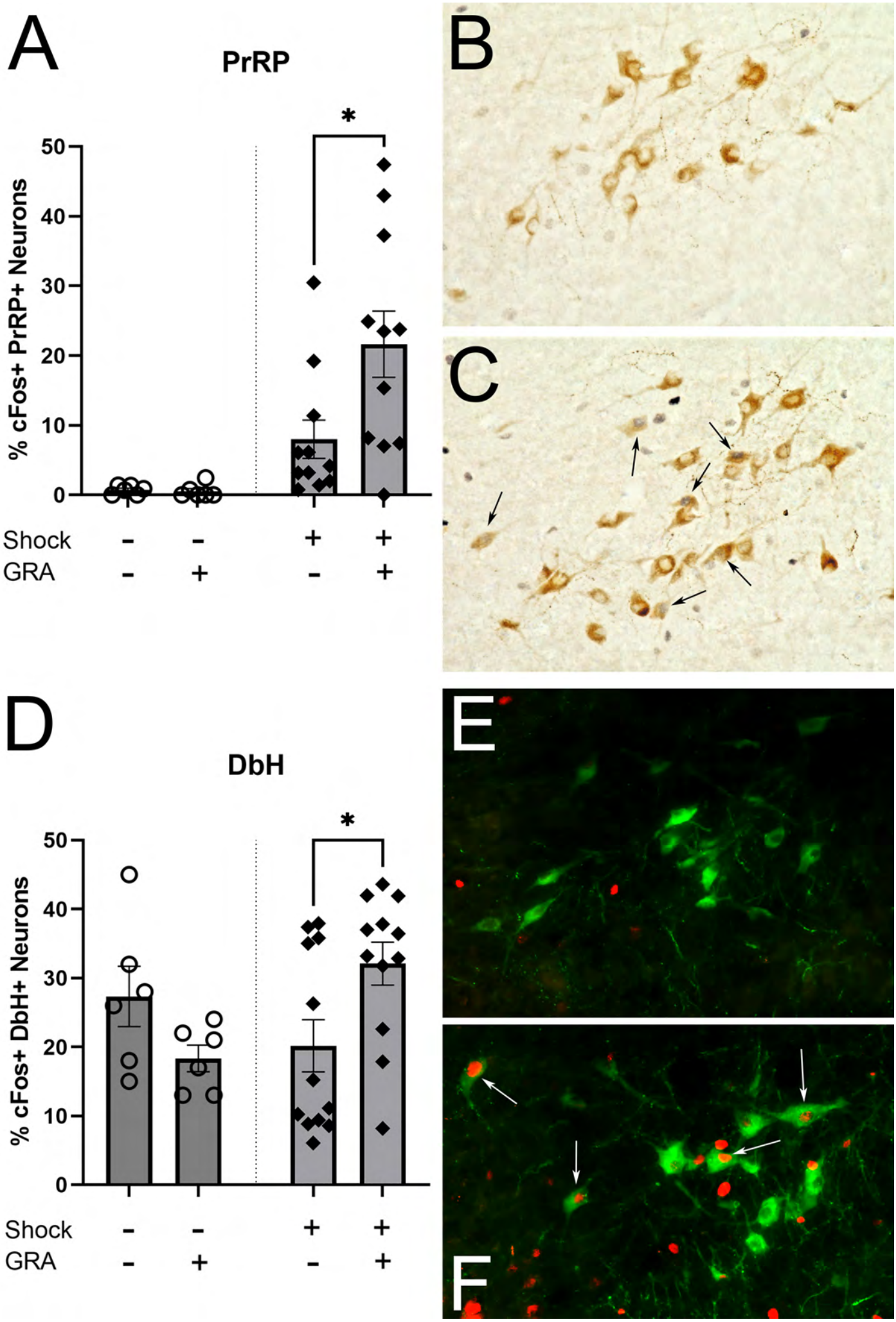
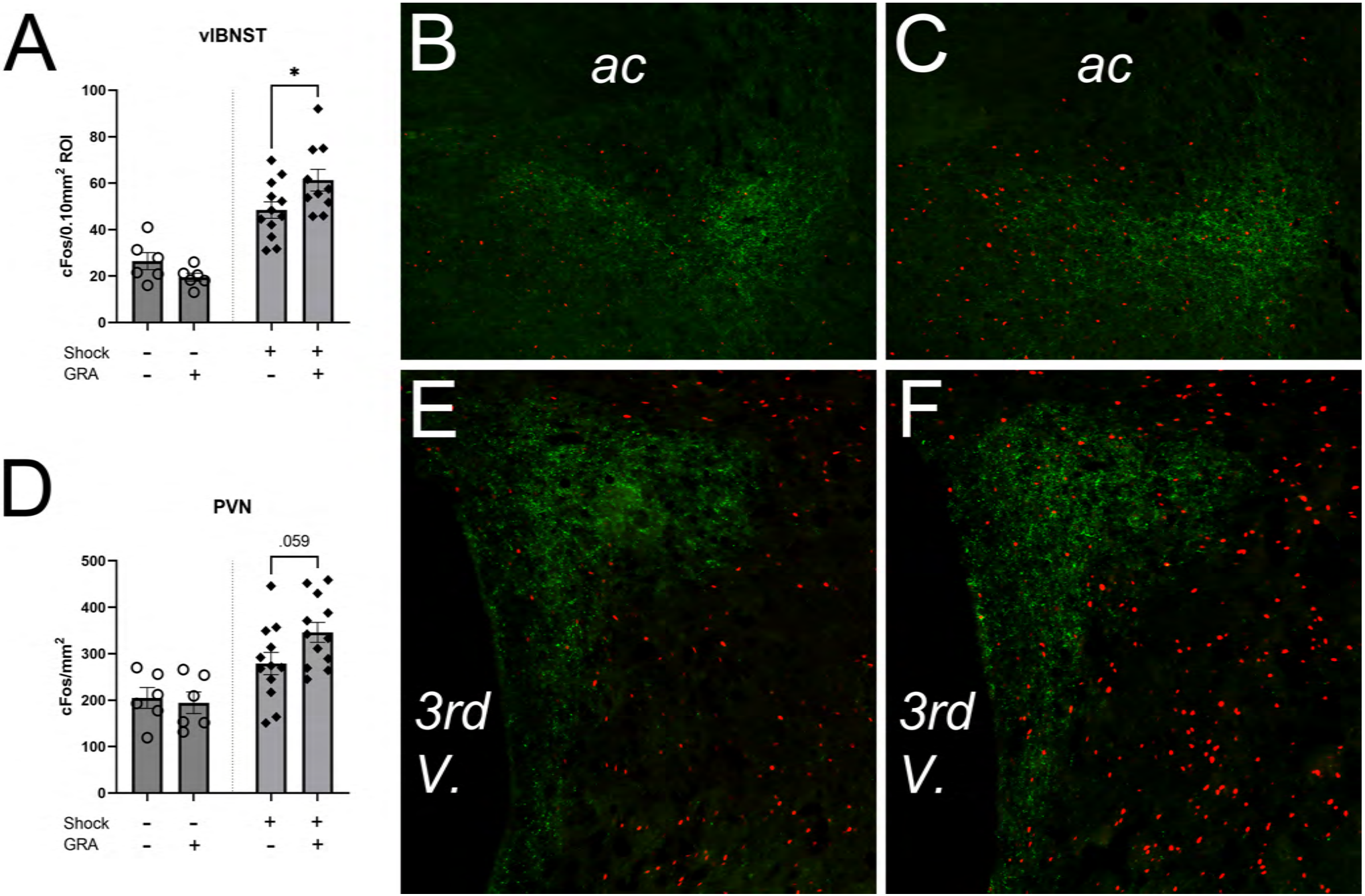
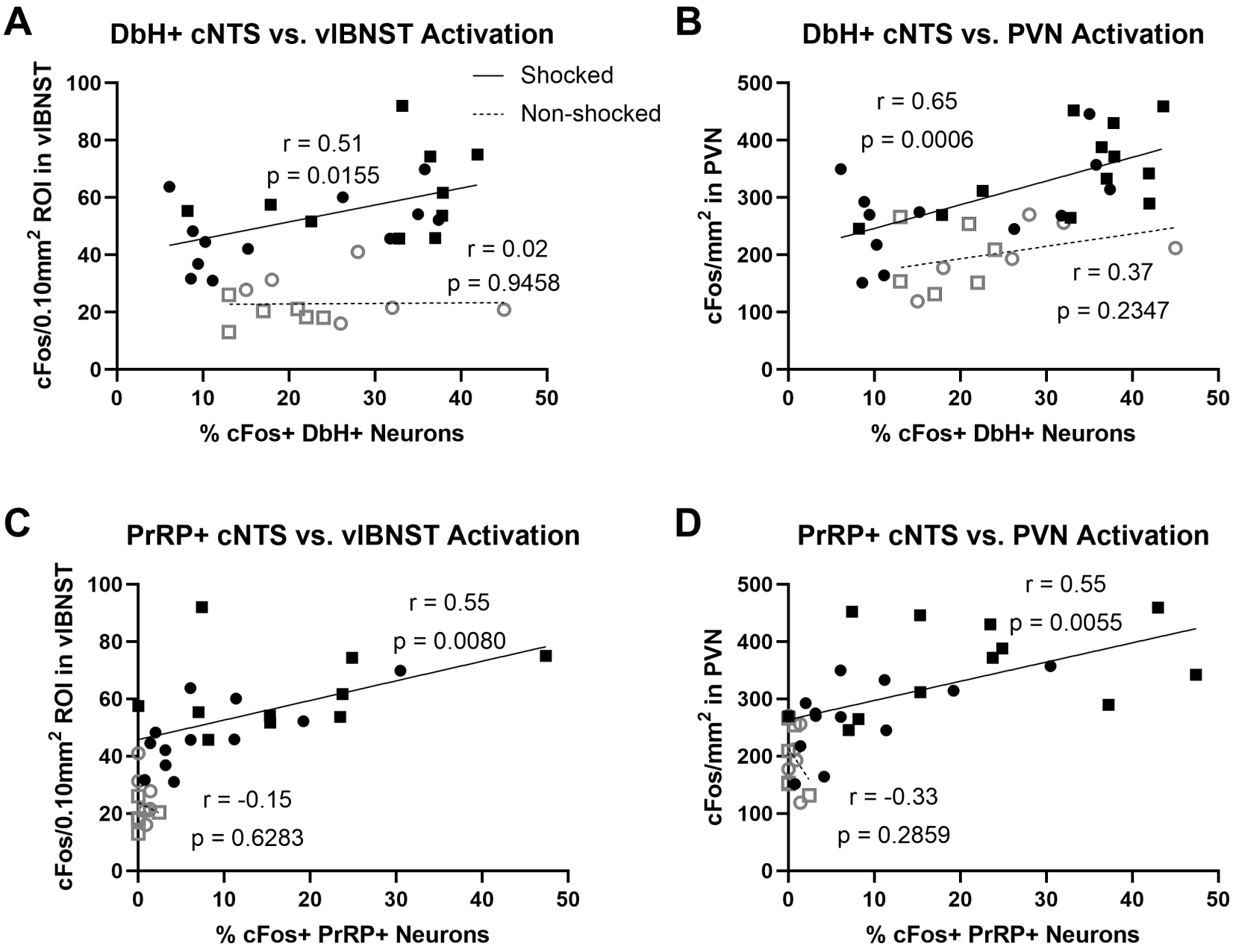
*
Saline
GRA
Pre-Test Injection


A
Distance Traveled
Distance Traveled (m)
30
20
10
0
Saline
GRA
Pre-Test Injection
B
Time in Center
Time in Center (s)
150
100
50
0
Saline
GRA
Pre-Test Injection
C
Latency to Enter Center
Latency to Enter (s)
80
60
40
20
0
Saline
GRA
Pre-Test Injection


A
PrRP
*
% cFos+ PrRP+ Neurons
50
40
30
20
10
0
Shock - - + +
GRA - + - +
B
C
D
DbH
*
% cFos+ DbH+ Neurons
50
40
30
20
10
0
Shock - - + +
GRA - + - +
E
F


A
vlBNST
*
cFos/0.10mm² ROI
100
80
60
40
20
0
Shock - - + +
GRA - + - +
B
ac
C
ac
D
PVN
.059
cFos/mm²
500
400
300
200
100
0
Shock - - + +
GRA - + - +
E
3rd
V.
F
3rd
V.


A
DbH+ cNTS vs. vlBNST Activation
Shocked
Non-shocked
r = 0.51
p = 0.0155
r = 0.02
p = 0.9458
cFos/0.10mm2 ROI in vlBNST
% cFos+ DbH+ Neurons
B
DbH+ cNTS vs. PVN Activation
r = 0.65
p = 0.0006
r = 0.37
p = 0.2347
cFos/mm2 in PVN
% cFos+ DbH+ Neurons
C
PrRP+ cNTS vs. vlBNST Activation
r = 0.55
p = 0.0080
r = -0.15
p = 0.6283
cFos/0.10mm2 ROI in vlBNST
% cFos+ PrRP+ Neurons
D
PrRP+ cNTS vs. PVN Activation
r = 0.55
p = 0.0055
r = -0.33
p = 0.2859
cFos/mm2 in PVN
% cFos+ PrRP+ Neurons