

**Activation of Peroxisome Proliferator-Activated Receptor  $\gamma$  reduces alcohol drinking and seeking by modulating multiple mesocorticolimbic regions in rats**

**Cite this article as:** Yannick Fotio, Anna Maria Borruto, Federica Benvenuti, Gregory Demopoulos, George Gaitanaris, Marisa Roberto and Roberto Ciccocioppo, Activation of Peroxisome Proliferator-Activated Receptor  $\gamma$  reduces alcohol drinking and seeking by modulating multiple mesocorticolimbic regions in rats, *Neuropsychopharmacology* doi:10.1038/s41386-020-0754-4

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Author accepted manuscript

1 **Activation of Peroxisome Proliferator-Activated Receptor  $\gamma$  reduces alcohol drinking**  
2 **and seeking by modulating multiple mesocorticolimbic regions in rats**

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17  
18 Numbers of pages: 23

19 Number of figures: 5

20 Number of words: Abstract = 216; Introduction = 346, Discussion = 1213

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22

23 **Abstract**

24 Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is an intracellular transcription factor  
25 whose signaling activation by the selective agonist pioglitazone reduces alcohol drinking and  
26 alcohol-seeking behavior in rats. The present study utilized the two-bottle choice and operant  
27 self-administration procedures to investigate neuroanatomical substrates that mediate the  
28 effects of PPAR $\gamma$  agonism on alcohol drinking and seeking in msP rats. Bilateral infusions of  
29 pioglitazone (0, 5, and 10  $\mu\text{g}/\mu\text{l}$ ) in the rostromedial tegmental nucleus (RMTg) decreased  
30 voluntary alcohol drinking and alcohol self-administration. Microinjections of pioglitazone in  
31 the ventral tegmental area (VTA), central amygdala (CeA), and nucleus accumbens (NAc)  
32 shell had no such effect. Notably, water, food, and saccharin consumption was unaltered by  
33 either treatment. The yohimbine-induced reinstatement of alcohol seeking was prevented by  
34 infusions of pioglitazone (0, 2.5, 5, and 10  $\mu\text{g}/\mu\text{l}$ ) in the CeA, VTA, and RMTg but not in the  
35 NAc-shell. These results emphasize the involvement of mesocorticolimbic circuitries in  
36 mediating the effects of PPAR $\gamma$  agonists on alcohol drinking and seeking. These results will  
37 facilitate future studies that investigate the pathophysiological role of PPAR $\gamma$  in alcohol use  
38 disorder and help clarify the mechanisms by which the activation of this receptor decreases  
39 the motivation for drinking.

40

41 **Keywords:** Alcohol addiction, Mesolimbic System, Dopamine, Nuclear Receptors, Relapse

42

43

## 44 Introduction

45 Alcohol use disorder (AUD) is a chronic brain disease that is characterized by  
46 compulsive alcohol drinking and withdrawal symptoms when access to alcohol is prevented,  
47 thus heightening the risk of relapse to pathological drinking [1]. Alcohol use disorder is  
48 considered the fifth highest risk factor for premature death and disability worldwide. In 2016  
49 alone, more than 3 million deaths and 132.6 million disability-adjusted life years at the global  
50 level were attributable to AUD (World Health Organization, 2018). The neurobiological  
51 mechanisms that underlie AUD are still only partially understood but are thought to be  
52 associated with profound counteradaptive alterations of reward and stress neurocircuitries  
53 [2,3]. Untangling these neuroadaptations is complex but essential to develop more efficacious  
54 therapies.

55 Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is a ligand-activated  
56 transcription factor that belongs to a large group of nuclear receptors. Upon activation,  
57 PPAR $\gamma$  regulates gene expression by translocating to the nucleus and binding to a selective  
58 DNA sequence called PPAR response element [4]. Although PPAR $\gamma$  is mainly expressed in  
59 adipose tissue and macrophages where it controls metabolism and the immune response [5,6],  
60 recent studies showed that this nuclear factor is also densely expressed in the central nervous  
61 system. PPAR $\gamma$  is highly expressed in neurons and glial cells where it is involved in  
62 neuroprotection, cell repair, and antiinflammatory responses [7-10]. Earlier studies showed  
63 that PPAR $\gamma$  is expressed on dopaminergic cells in the ventral tegmental area (VTA),  
64 suggesting that this receptor could be involved in modulating the reinforcing effects of drugs  
65 of abuse [10]. Consistent with this hypothesis, research in our laboratory showed that the  
66 systemic administration of two selective PPAR $\gamma$  agonists, pioglitazone and rosiglitazone,  
67 significantly reduced alcohol drinking and seeking in alcohol-preferring rats [11,12].  
68 However, the neurocircuitries and putative mechanisms that subserve such effects are still

69 unknown. The present study investigated the neuroanatomical substrates that mediate the  
70 effects of PPAR $\gamma$  agonists on alcohol drinking and seeking to facilitate future  
71 characterizations of their molecular and cellular mechanisms.

72

### 73 **Materials and Methods**

#### 74 *Animals*

75 Ten- to 11-week-old male Marchigian Sardinian alcohol-preferring (msP) rats ( $N_{total} =$   
76 135), weighing 250-280 g, were employed in this study. They were bred and housed under a  
77 reverse 12 h/12 h light/dark cycle (light on at 8 PM) in the vivarium of the University of  
78 Camerino and controlled temperature (22°C) and humidity (55%). Food (4RF18, Mucedola,  
79 Settimo Milanese, Italy) and water were provided *ad libitum*. Before starting the experiments,  
80 the rats were pair housed in conventional clear plastic cages with standard bedding. The  
81 experiments were conducted during the dark phase of the light/dark cycle, and the procedures  
82 were conducted in accordance with directives on the care and use of laboratory animals of the  
83 European Community Council and National Institutes of Health. Formal approval was  
84 obtained from the Italian Ministry of Health and Internal Ethical Committee for Laboratory  
85 Animal Protection and Use of the University of Camerino. All efforts were made to minimize  
86 the rats' suffering and distress.

87

#### 88 *Chemicals and treatments*

89 Saccharin (Sigma, Italy) was dissolved in tap water to obtain a 0.2% (w/v) solution.  
90 Alcohol (Carsetti, Camerino, Italy) was diluted with tap water to obtain a 10% concentration.  
91 The selective PPAR $\gamma$  agonist pioglitazone ( $ED_{50} = 0.2-0.6 \mu\text{M}$  at PPAR $\gamma$  inactive at  
92 PPAR $\alpha$  and PPAR $\delta$  at  $10^{-3}$ ) [13-15] was purchased from Molcan Corporation (Richmond  
93 Hill, Ontario, Canada) and dissolved in vehicle that consisted of 10% dimethylsulfoxide, 3%

94 Tween 80, and 87% distilled water. To evaluate the effects of intracranial pioglitazone  
95 administration on alcohol drinking and seeking, the rats were treated twice with the  
96 compound: at the onset of the light cycle (8:00 PM) and 15 min before the dark cycle began,  
97 when alcohol was made available. The pioglitazone administration schedule was based on  
98 previous studies [11,12]. Yohimbine (Sigma, Milano, Italy) was dissolved in saline and was  
99 used to evoke the reinstatement of alcohol seeking [16]. It was administered intraperitoneally  
100 (i.p.) at a dose of 1.25 mg/kg, 15 min after the second injection of pioglitazone and  
101 corresponding to the beginning of the dark phase (8:00 AM). Reinstatement testing was  
102 performed 30 min after the yohimbine injection. To minimize the diffusion of pioglitazone  
103 from the injection site, it was administered in a volume of 0.3  $\mu$ l per site in the rostromedial  
104 tegmental nucleus (RMTg) and VTA. In the nucleus accumbens (NAc) shell and central  
105 amygdala (CeA), the injection volume was 0.5  $\mu$ l per site. All of the treatments were  
106 administered in a counterbalanced Latin-square design to limit the number of rats used.

107

### 108 *Intracranial surgery*

109 The rats were anesthetized by an intramuscular injection (100-150  $\mu$ l) of a solution  
110 that contained tiletamine (58.17 mg/ml) and zolazepam (7.5 mg/ml). Bilateral guide cannulas  
111 (0.65 mm outer diameter) that were aimed at the CeA, VTA, RMTg, and NAc shell were  
112 implanted and cemented to the skull. We used the following stereotaxic coordinates (from  
113 bregma) according to previous reports [17,18]: CeA (anterior/posterior, -1.8 mm;  
114 dorsal/lateral,  $\pm$ 4.3 mm; medial/ventral, -7.0 mm), VTA (anterior/posterior, -5.8 mm;  
115 dorsal/lateral,  $\pm$ 2.2 mm; medial/lateral, -7.4 mm; 12° angle), RMTg (anterior/posterior, -6.7  
116 mm; dorsal/lateral,  $\pm$ 2.2 mm; medial/ventral, -7.4 mm; 12° angle), NAc shell  
117 (anterior/posterior, +1.4 mm; dorsal/lateral,  $\pm$ 0.9 mm; medial/ventral, -6.1 mm). After  
118 surgery, the rats received a single subcutaneous injection of ketoprofen (2.5 mg/kg) and

119 allowed to recover for 1 week in their home cage. During this period, the rats were handled  
120 daily and habituated to the injection procedure, consisting of inserting a stainless-steel  
121 injector into the guide cannulas, for at least 3 days before the tests began. The injector was  
122 1.5 mm longer than the guide cannula and left in place for an additional 20 s after the  
123 injection to allow diffusion of the solution. Upon completion of the experiments, the rats  
124 were anesthetized with isoflurane, and black India ink (0.5  $\mu$ l per site) was injected into the  
125 studied brain areas. The rats were then immediately euthanized to remove the brain and  
126 histologically analyze the cannula placements.

127

#### 128 *Two-bottle choice procedure*

129 The two-bottle choice (2-BC) procedure (free choice between water and 10% alcohol)  
130 was used to measure voluntary alcohol drinking and preference [19]. The rats were single  
131 housed in experimental chambers (30 cm length  $\times$  30 cm width  $\times$  30 cm height) for 1  
132 week of habituation before beginning the two-bottle choice test. They were given free access  
133 to water and 10% alcohol (v/v) for the next 15 days to establish a stable baseline and  
134 preference for alcohol. Preference was defined as 80-90% preference for alcohol *vs.* water.  
135 The fluids were offered through graduated drinking tubes that were equipped with metal  
136 spouts. Fluid intake was measured by reading the volume that was consumed at specific time  
137 points (2, 8, and 24 h) following initiation of the active (dark) phase of the light/dark cycle.  
138 The drinking tubes were switched daily to avoid the development of side preference. The rats  
139 also had free access to food. Food consumption was measured by weighing the food  
140 container while considering the spillage weight. Alcohol, water, and food intakes were  
141 calculated as absolute values of consumption at each time point and are expressed as g/kg  
142 body weight [20].

143

144 *Operant alcohol and saccharin self-administration*

145 Operant chambers were used in daily 30-min sessions to establish alcohol and  
146 saccharin self-administration under fixed-ratio 1 (FR1) schedule of reinforcement [21,22].  
147 Each chamber was equipped with an active lever and an inactive lever that were  
148 symmetrically centered on the side panel. Responding at the active lever activated the  
149 infusion pump and released 0.1 ml of 10% alcohol (v/v) or 0.2% saccharin (w/v) in a liquid  
150 receptacle that was located between the two levers. Presses at the inactive lever were  
151 recorded but did not activate the infusion pump. During the infusion, a stimulus light that was  
152 located above the active lever was turned on for a 5 s timeout period. Lever pressing during  
153 the timeout period was recorded but did not lead to further infusions. When the rats achieved  
154 a stable baseline of self-administration for both alcohol and saccharin over the last 3 days of  
155 training, we evaluated the effects of microinfusions of pioglitazone in the RMTg every 4 days  
156 using a counterbalance Latin-square design.

157

158 *Yohimbine-induced reinstatement of alcohol seeking*

159 The reinstatement experiments consisted of three phases: training for alcohol self-  
160 administration, extinction (during which alcohol was no longer available), and reinstatement  
161 tests.

162 In the training phase, alcohol self-administration was performed as described  
163 previously (see *Operant alcohol self-administration* section above). Lever responding under  
164 the FR1 schedule was maintained for 10 days (sessions) before and after surgery to  
165 reestablish baseline alcohol self-administration.

166 In the extinction phase, after the last alcohol self-administration session, the rats  
167 underwent 15 days of extinction sessions, during which they were placed under  
168 environmental conditions that were similar to the alcohol training phase, with the exception



169 that responding at the active lever did not result in alcohol deliveries. During the last 3 days  
170 of extinction, the rats were habituated to the intracranial treatment procedures.

171 In the reinstatement phase, the experimental conditions were identical to the  
172 extinction phase, but the rats were subjected to a reinstatement test. In separate experiments,  
173 pioglitazone (2.5, 5, and 10  $\mu\text{g}/\mu\text{l}$ ) or its vehicle was injected in the CeA, VTA, RMTg, and  
174 NAc shell. The experiment was conducted in a counterbalanced Latin-square design, with a  
175 4-day interval between test sessions. During this interval, the rats were subjected to extinction  
176 sessions. The dose of yohimbine and experimental design were based on previous studies  
177 [11,23,24].

178

#### 179 *Statistical analyses*

180 The data were analyzed using analysis of variance (ANOVA) followed by the  
181 Newman-Keuls multiple-comparison *post hoc* test when appropriate. The effects of  
182 intracranial injections of pioglitazone in the CeA, VTA, RMTg, and NAc shell on alcohol,  
183 water, and food intake were analyzed using two-way repeated-measures ANOVA, with time  
184 and treatment as within-subjects factors. The effects of microinfusions of pioglitazone in the  
185 RMTg on alcohol and saccharin self-administration were analyzed using one-way repeated-  
186 measures ANOVA, with treatment as the within-subjects factor. The effects of  
187 microinfusions of pioglitazone in the CeA, VTA, RMTg, and NAc shell on the yohimbine-  
188 induced reinstatement of alcohol seeking were analyzed using one-way repeated-measures  
189 ANOVA, with treatment as the within-subjects factor. For the reinstatement experiments,  
190 differences between lever responding during the extinction and reinstatement sessions were  
191 analyzed using paired Student's *t*-test. The two-bottle choice data are expressed as the mean  
192 ( $\pm$  SEM) of intake (g/kg of body weight). For operant self-administration, the data are  
193 expressed as the mean ( $\pm$  SEM) of the number of responses at the active and inactive levers.

194 Only data from rats with correct cannula placements were included in the statistical analyses.  
195 The following numbers of rats were included in the statistical analyses: voluntary alcohol  
196 drinking (CeA,  $n = 9$ ; VTA,  $n = 11$ ; RMTg,  $n = 11$ ; NAc shell,  $n = 10$ ), alcohol self-  
197 administration (RMTg,  $n = 8$ ), saccharin self-administration (RMTg,  $n = 16$ ), reinstatement  
198 of alcohol seeking (CeA,  $n = 12$ ; VTA,  $n = 10$ , RMTg,  $n = 15$ ; NAc shell,  $n = 13$ ). The  
199 statistical analyses were performed using Prism 8.0 software (GraphPad, La Jolla, CA, USA).  
200 Values of  $p < 0.05$  vs. the vehicle control were considered statistically significant.

201

## 202 **Results**

### 203 *Effect of intra-CeA activation of PPAR $\gamma$ on voluntary 2-BC alcohol drinking*

204 Pioglitazone (5 and 10  $\mu\text{g}/\mu\text{l}$ ) was microinfused in the CeA in msP rats in a  
205 counterbalanced Latin-square design ( $n = 9$ ). As shown in **Fig. 1A**, voluntary alcohol  
206 drinking was monitored at 2, 8, and 24 h. The overall ANOVA revealed no difference in the  
207 amount of alcohol consumption between the pioglitazone- and vehicle-treated groups at any  
208 time-point (time:  $F_{2,16} = 33.91$ ,  $p < 0.0001$ ; treatment:  $F_{2,16} = 2.492$ ,  $p = 0.344$ ; time  $\times$   
209 treatment interaction:  $F_{4,32} = 0.7949$ ,  $p = 0.5373$ ). Similarly, no difference in the amount of  
210 water (time:  $F_{2,16} = 8.685$ ,  $p = 0.0028$ ; treatment:  $F_{2,16} = 1.311$ ,  $p = 0.2970$ ; time  $\times$  treatment  
211 interaction:  $F_{4,32} = 8834$ ,  $p = 0.4849$ ; **Table S1**) or food (time:  $F_{2,16} = 64.11$ ,  $p < 0.0001$ ;  
212 treatment:  $F_{2,16} = 6025$ ,  $p = 0.5594$ ; time  $\times$  treatment interaction:  $F_{4,32} = 2.674$ ,  $p = 0.0946$ ;  
213 **Fig. 1B**) consumption was found between the pioglitazone- and vehicle-treated groups.

214

### 215 *Effect of intra-NAc-shell activation of PPAR $\gamma$ on voluntary 2-BC alcohol drinking*

216 The ANOVA revealed that alcohol consumption was detectable 2 h after treatment  
217 and progressively increased in the following hours (time:  $F_{2,18} = 78.76$ ,  $p < 0.0001$ ; **Fig. 1D**).  
218 The ANOVA also revealed that intake were unaffected by treatment, although a slight

219 reduction was observed at 24 h ( $F_{2,18} = 0.2135$ ,  $p = 0.8098$ ). No time  $\times$  treatment interaction  
220 was detected ( $F_{4,36} = 2.067$ ,  $p = 0.1055$ ). Intra-NAc shell pioglitazone administration did not  
221 alter the consumption of water (time:  $F_{2,18} = 11.89$ ,  $p < 0.001$ ; treatment:  $F_{2,18} = 0.073$ ,  $p =$   
222  $0.9298$ ; time  $\times$  treatment interaction:  $F_{4,36} = 0.3109$ ,  $p = 0.8688$ ; **Table S1**) or food (time:  
223  $F_{2,18} = 54.39$ ,  $p < 0.0001$ ; treatment:  $F_{2,18} = 0.9515$ ,  $p = 0.4048$ ; time  $\times$  treatment interaction:  
224  $F_{4,36} = 0.5584$ ,  $p = 0.6942$ ; **Fig. 1E**).

225

#### 226 *Effect of intra-RMTg activation of PPAR $\gamma$ on voluntary 2-BC alcohol drinking*

227 Pioglitazone (5 and 10  $\mu\text{g}/\mu\text{l}$ ) was microinfused in the RMTg in msP rats ( $n = 11$ ).  
228 The ANOVA revealed significant effects of time ( $F_{2,20} = 104.7$ ,  $p < 0.0001$ ) and treatment  
229 ( $F_{2,20} = 21.27$ ,  $p < 0.0001$ ) and a significant time  $\times$  treatment interaction ( $F_{4,40} = 8.701$ ,  $p <$   
230  $0.0001$ ). As shown in **Fig. 2A**, voluntary alcohol consumption was detectable but not  
231 significantly affected by intra-RMTg pioglitazone administration 2 h after treatment.  
232 However, at 8 and 24 h post-treatment, alcohol intake dose-dependently decreased.  
233 Interestingly, intra-RMTg pioglitazone administration did not alter water (time:  $F_{2,20} = 5.106$ ,  
234  $p = 0.0162$ ; treatment:  $F_{2,20} = 1.593$ ,  $p = 0.2280$ ; time  $\times$  treatment interaction:  $F_{4,40} = 0.2922$ ,  
235  $p = 0.8813$ ; **Table S1**) or food (time:  $F_{2,20} = 45.21$ ,  $p < 0.0001$ ; treatment:  $F_{2,20} = 0.3759$ ,  $p =$   
236  $0.6914$ ; time  $\times$  treatment interaction:  $F_{4,40} = 0.1251$ ,  $p = 0.3051$ ; **Fig. 2B**) consumption.

237

#### 238 *Effect of intra-VTA activation of PPAR $\gamma$ on voluntary 2-BC alcohol drinking*

239 Pioglitazone (5 and 10  $\mu\text{g}/\mu\text{l}$ ) was microinfused in the VTA in msP rats ( $n = 11$ ).  
240 Alcohol intake was detectable 2 h after initiation of the dark phase. The ANOVA revealed a  
241 significant effect of time on alcohol intake ( $F_{2,20} = 80.74$ ,  $p < 0.0001$ ; **Fig. 2D**) but no effect  
242 of treatment ( $F_{2,20} = 2.425$ ,  $p = 0.114$ ) and no time  $\times$  treatment interaction ( $F_{4,40} = 0.8606$ ,  $p =$   
243  $0.4959$ ). The intra-VTA administration of pioglitazone or its vehicle did not alter the absolute

244 amount of alcohol consumption at any time-point (2, 8, and 24 h). Treatment did not affect  
245 water (time:  $F_{2,20} = 6.38$ ,  $p = 0.0096$ ; treatment:  $F_{2,20} = 0.7005$ ,  $p = 0.5081$ ; time  $\times$  treatment  
246 interaction:  $F_{4,40} = 0.7241$ ,  $p = 0.5807$ ; **Table S1**) or food (time:  $F_{2,20} = 76.40$ ,  $p < 0.0001$ ;  
247 treatment:  $F_{2,20} = 2.178$ ,  $p = 0.1394$ ; time  $\times$  treatment interaction:  $F_{4,40} = 1.895$ ,  $p = 0.074$ ;  
248 **Fig. 2E**) consumption.

249

#### 250 *Effect of intra-RMTg activation of PPAR $\gamma$ on alcohol and saccharin self-administration*

251 To further investigate the role of the RMTg in modulating alcohol intake through  
252 PPAR $\gamma$ , msP rats ( $n = 8$ ) underwent operant alcohol (10%, v/v) self-administration training.  
253 When they reached a stable mean number of reinforcements earned, pioglitazone (5 and 10  
254  $\mu\text{g}/\mu\text{l}$ ) or its vehicle were administered in the RMTg, and their effects on operant responding  
255 were evaluated. As expected, the ANOVA showed that pioglitazone dose-dependently  
256 decreased the number of reinforced lever presses ( $F_{2,14} = 6.361$ ,  $p = 0.006$ ; **Fig. 3A**). The  
257 number of responses at the inactive lever was negligible and did not change throughout the  
258 experiment (**Fig. 3B**).

259 To test whether the observed effect of intra-RMTg PPAR $\gamma$  activation is selective for  
260 alcohol, rats ( $n = 16$ ) were trained to self-administer saccharin (0.2%, w/v) under an FR1  
261 schedule until they reached a stable baseline of reinforcements obtained. Pioglitazone (5 and  
262 10  $\mu\text{g}/\mu\text{l}$ ) was then microinfused in the RMTg. The ANOVA revealed that this treatment did  
263 not alter saccharin self-administration ( $F_{2,30} = 0.3996$ ,  $p = 0.6748$ ; **Fig. 3C**). Responding at  
264 the inactive lever was negligible and did not change throughout the experiment (**Fig. 3D**).

265

266 *Effect of intra-CeA activation of PPAR $\gamma$  on yohimbine-induced reinstatement of alcohol*  
267 *seeking*

268 Pioglitazone (2.5, 5, and 10  $\mu\text{g}/\mu\text{l}$ ) or its vehicle were microinfused in the CeA in msP  
269 rats ( $n = 12$ ) to evaluate its effect on the yohimbine-induced reinstatement of alcohol seeking.  
270 During the training phase, the mean number of responses at the active lever was  $68.73 \pm 5.95$ ,  
271 which sharply decreased during extinction ( $21.41 \pm 1.97$ ). Paired Student's  $t$ -test (vehicle vs.  
272 extinction) revealed that yohimbine administration (1.25 mg/kg, i.p.) significantly reinstated  
273 operant alcohol-seeking behavior ( $t_{11} = 3.8$ ,  $p = 0.0029$ ; **Fig. 4A**), which was prevented by  
274 intra-CeA infusions of pioglitazone ( $F_{3,33} = 16.12$ ,  $p < 0.0001$ ). Responding at the inactive  
275 lever was low ( $1.79 \pm 0.49$ ) and not significantly affected by the treatment (**Fig. 4B**).

276

277 *Effect of intra-NAc shell activation of PPAR $\gamma$  on yohimbine-induced reinstatement of alcohol*  
278 *seeking*

279 In msP rats ( $n = 13$ ) with cannula implants in the NAc shell during the training phase,  
280 the mean number of responses at the active lever was  $65.33 \pm 5.54$ , which significantly  
281 decreased during extinction ( $19.12 \pm 4.95$ ) and was reinstated ( $t_{12} = 5.096$ ,  $p = 0.0003$ ) by  
282 yohimbine treatment (1.25 mg/kg, i.p.). However, intra-NAc shell pioglitazone  
283 administration did not alter the yohimbine-induced reinstatement of alcohol seeking ( $F_{3,36} =$   
284  $1.838$ ,  $p = 0.1578$ ; **Fig. 4D**). Responding at the inactive lever was low and unchanged by the  
285 treatments (**Fig. 4E**).

286

287 *Effect of intra-VTA activation of PPAR $\gamma$  on yohimbine-induced reinstatement of alcohol*  
288 *seeking*

289 During the training phase in msP rats ( $n = 10$ ), the mean number of responses at the  
290 active lever was  $67.87 \pm 6.52$ . Operant responding markedly decreased during extinction  
291 ( $13.63 \pm 1.51$  lever presses). As shown in **Fig. 5A**, treatment with yohimbine (1.25 mg/kg,  
292 i.p.) significantly reinstated ( $t_9 = 6.552$ ,  $p < 0.0001$ ) operant responding for alcohol. This

293 effect was dose-dependently prevented by intra-VTA pioglitazone administration ( $F_{3,27} =$   
294 8.87,  $p = 0.0003$ ). Responding at the inactive lever was negligible ( $4.86 \pm 1.66$ ) and not  
295 significantly affected by the treatments (**Fig. 5B**).

296

297 *Effect of intra-RMTg activation of PPAR $\gamma$  on yohimbine-induced reinstatement of alcohol*  
298 *seeking*

299 In msP rats ( $n = 15$ ) with cannula implants in the RMTg, the mean number of  
300 responses at the active lever was  $72.35 \pm 4.81$  during the training phase, which rapidly  
301 decreased during extinction ( $19.22 \pm 1.7$ ). Yohimbine (1.25 mg/kg, i.p.) significantly  
302 increased the number of responses at the active lever ( $t_{14} = 4.460$ ,  $p = 0.0005$ ; **Fig. 5D**). This  
303 effect was dose-dependently decreased by intra-RMTg pioglitazone administration ( $F_{3,42} =$   
304 74.54,  $p < 0.0001$ ; **Fig. 5D**). Responding at the inactive lever (**Fig. 5E**) was negligible and  
305 unaffected by the treatments.

306

## 307 **Discussion**

308 *Administration of pioglitazone in the RMTg decreased alcohol intake*

309 The mesocorticolimbic dopamine system which originates in the VTA and projects to  
310 the NAc, CeA, and prefrontal cortex. This system plays a key role in controlling the  
311 reinforcing properties of drugs of abuse, including alcohol [25-31]. The majority of afferent  
312 connections to VTA dopaminergic cells are  $\gamma$ -aminobutyric acid (GABA)ergic and inhibitory  
313 [32-34]. Emerging evidence indicates that the tail of the VTA, also known as the RMTg,  
314 provides important GABAergic inputs to VTA dopaminergic cells [32,35-37]. Therefore, the  
315 RMTg is a key structure in the development and maintenance of drug addiction. PPAR $\gamma$   
316 expression has been detected on VTA dopaminergic neurons [10] and RMTg GABAergic  
317 cells (de Guglielmo et al. 2015). Thus, we investigated whether the effect of pioglitazone on

318 alcohol drinking involves PPAR $\gamma$ -dependent signaling in these two adjacent areas. We  
319 infused pioglitazone in the VTA and RMTg and evaluated its effect in the 2-BC procedure.  
320 We found that PPAR $\gamma$  activation in the RMTg but not the VTA significantly attenuated  
321 alcohol drinking compared with vehicle-treated rats. Moreover, water and food consumption  
322 were unaltered by pioglitazone treatment, indicating that its effect in the RMTg is specific to  
323 alcohol and does not generalize to water or food. To confirm this finding, we subsequently  
324 administered pioglitazone in the RMTg in two groups of rats that were trained to self-  
325 administer alcohol or saccharin. As expected, pioglitazone significantly attenuated alcohol  
326 but not saccharin intake, suggesting that PPAR $\gamma$  activation may specifically reduce the  
327 motivation for alcohol. Notably, the VTA and RMTg are in anatomical contiguity. Hence, the  
328 fact that pioglitazone was efficacious only when injected in the RMTg demonstrated that it  
329 did not diffuse to neighboring regions at the dose and volume tested. A corollary to this  
330 finding is that the RMTg is the sole neuroanatomical substrate for the alcohol-suppressing  
331 effect of PPAR $\gamma$  agonists. This hypothesis was supported by findings that showed that  
332 pioglitazone microinfusions in other brain areas of the mesocorticolimbic system where  
333 PPAR $\gamma$  is expressed (e.g., CeA and NAc shell) did not affect alcohol drinking [8,38]. Such a  
334 specific role for PPAR $\gamma$  activation in the RMTg in controlling the reinforcing effects of drugs  
335 of abuse has also been observed in opioid self-administration studies in our laboratory [18].  
336 In this earlier study, we found that the effect of pioglitazone in the RMTg was linked to its  
337 ability to increase the inhibitory tone of RMTg GABAergic cells, thereby inhibiting  
338 dopamine neuron activation in the VTA [18]. Although more studies are needed to support  
339 this hypothesis, we speculate that a similar mechanism may be involved in the alcohol-  
340 suppressing effects of PPAR $\gamma$  agonists.

341 An interesting observation in the present study was that the effect of pioglitazone in  
342 the 2-BC test was observed at 8 and 24 h but not at 2 h. In the operant self-administration



343 experiments, this effect was observed at 30 min. Two possibilities may explain this apparent  
344 discrepancy. First, in the operant self-administration session, the rats consumed  
345 approximately 1.25 g/kg alcohol in 30 min. In the 2-BC test, the rats had to drink for more  
346 than 2 h to reach this level of consumption. This may result in different pharmacokinetics of  
347 the drug (i.e., peak levels in the brain) that in turn may influence the response to  
348 pioglitazone. Second, motivation of the animals may be more effectively captured in operant  
349 self-administration experiments than in 2-BC experiments. If pioglitazone acts by attenuating  
350 the motivation for alcohol, then a more pronounced effect may be observed under operant  
351 contingencies rather than under free-drinking conditions.

352

353 **Administration of pioglitazone in the VTA and RMTg reduced the yohimbine-induced**  
354 **reinstatement of alcohol seeking**

355 The high rate of relapse among individuals with alcohol and substance use disorders is  
356 a major clinical problem [39,40]. Studies that utilized well-validated animal models of drug  
357 reinstatement demonstrated that the  $\alpha_2$ -adrenergic receptor antagonist yohimbine increased  
358 drug craving in humans [41,42] and reinstated extinguished alcohol-seeking behavior in rats  
359 that were trained to self-administer alcohol [43-45]. Yohimbine reinstates drug seeking  
360 through complex mechanisms that partially involve activation of the stress system and the  
361 potentiation of responding to sensory cues [46-49]. Consistent with these mechanisms, earlier  
362 studies showed that the yohimbine-induced reinstatement of drug seeking was reduced by  
363 corticotropin-releasing factor-1 (CRF<sub>1</sub>) receptor antagonists and the blockade of dopamine  
364 transmission [45,47,49-52]. Previous reports from our laboratory showed that systemic  
365 PPAR $\gamma$  agonist administration prevented the yohimbine- but cue-induced reinstatement of  
366 alcohol seeking in msP rats [11,12]. Here, under identical experimental conditions, we found  
367 that PPAR $\gamma$  activation in the RMTg profoundly and dose-dependently decreased the



368 yohimbine-induced reinstatement of alcohol seeking. A similar but less marked effect was  
369 also observed following pioglitazone administration in the VTA. PPAR $\gamma$  agonists may  
370 engage intra-RMTg GABAergic signaling to reduce the firing of VTA dopaminergic neurons  
371 [18]. This hypothesis is supported by previous studies that showed that stress strongly  
372 activated VTA dopaminergic neurons to induce the reinstatement of drug seeking [53,54].  
373 This effect of stress was prevented by intra-VTA administration of the GABA<sub>B</sub> receptor  
374 agonist baclofen [55]. Moreover, yohimbine-induced reinstatement was blocked by both  
375 systemic and intra-medial prefrontal cortex (i.e., a region that receives dopaminergic afferents  
376 from the VTA) administration of dopamine receptor antagonists [50-52]. The present results  
377 demonstrate that the RMTg might play a critical role in the stress-induced reinstatement of  
378 alcohol seeking. However, because of the tight apposition of the RMTg and VTA, one  
379 possibility is that the effect of pioglitazone on yohimbine-induced alcohol seeking is at least  
380 partially attributable to spread of the drug into the nearby VTA. This possibility cannot be  
381 excluded, but appears to be unlikely because the effect of pioglitazone was much more  
382 pronounced when it was injected directly in the RMTg rather in the VTA. An opposite effect  
383 would be expected if the VTA was the main site of action of the drug.

384

385 **Administration of pioglitazone in the RMTg did not affect operant responding for**  
386 **saccharin**

387 GABAergic neurons in the RMTg are also known to strongly inhibit dopaminergic  
388 cells in the substantia nigra compacta, thereby controlling motor coordination and motor  
389 learning [56,57]. Based on evidence that RMTg GABAergic signaling is the main  
390 neurocircuitry that mediates the PPAR $\gamma$  agonist-induced reduction of alcohol intake, we  
391 considered the possibility that the effects of pioglitazone on lever pressing for alcohol may  
392 have been influenced by an influence on locomotor activity. However, when we

393 microinjected pioglitazone in the RMTg in rats that were trained to self-administer saccharin,  
394 we found that the number of reinforcements earned was unaffected by the drug. These results  
395 indicate that PPAR $\gamma$  activation in the RMTg selectively controls alcohol intake and the  
396 yohimbine-induced reinstatement of alcohol seeking by modulating the mesocorticolimbic  
397 system without altering transmission of the nigrostriatal pathway.

398

399 *Administration of pioglitazone in the CeA but not NAc shell attenuated the reinstatement of*  
400 *alcohol seeking*

401 Finally, we found that the yohimbine-induced reinstatement of alcohol seeking was  
402 attenuated by intra-CeA but not intra-NAc shell (pioglitazone administration). These results  
403 suggest that neurocircuitry in the CeA may also be recruited by PPAR $\gamma$  agonists to attenuate  
404 the reinstatement of alcohol seeking. This intra-CeA effect of pioglitazone may be secondary  
405 to anxiolytic properties of the compound [38]. In fact, it has been demonstrated that the CeA  
406 plays an important role in the expression of excessive anxiety linked to stress exposure  
407 [17,58,59]. Moreover, the pharmacological and genetic blockade of PPAR $\gamma$  signaling in the  
408 CeA exacerbated basal anxiety-like behavior and increased the vulnerability to stress [38].  
409 Therefore, a tempting speculation is that the anxiolytic properties of pioglitazone may be  
410 partially responsible for the protective effects of PPAR $\gamma$  agonists against the stress-induced  
411 reinstatement of alcohol seeking.

412 In conclusion, the present findings filled a gap in the literature by revealing brain  
413 areas that modulate the effect of PPAR $\gamma$  activation on alcohol-seeking behavior. The results  
414 also demonstrate an important role for RMTg in modulating the yohimbine stress-induced  
415 reinstatement of alcohol seeking. Pioglitazone is clinically used for the treatment of insulin  
416 resistance in patients with type 2 diabetes, and its tolerability has been largely demonstrated

417 [60-62]. Hence, the ability of pioglitazone to decrease alcohol seeking may open new  
418 avenues for further clinical investigation of its efficacy.

419

#### 420 **Funding and Disclosure**

421 *Funding:* This work was supported by National Institute on Alcohol Abuse and Alcoholism  
422 grant AA017447 (to MR and RC) and by grant (2017SXEXT5) PRIN 2017 (to RC).

423 *Disclosure:* G.D. is the Chairman and CEO of Omeros Corporation (Omeros). G.G. is the  
424 Chief Scientific Officer of Omeros. R.C. is the inventor on several patent applications that are  
425 related to the therapeutic use of PPAR $\gamma$  agonists for the treatment of addiction. Omeros,  
426 through agreements with the University of Camerino and R.C., exclusively controls the  
427 intellectual property rights that are directed to R.C.'s inventions related to the use of PPAR $\gamma$   
428 agonists for the treatment of addiction and addictive behaviors. Under these agreements, R.C.  
429 may be entitled to receive payments and royalties from Omeros. The other authors have no  
430 conflict of interest.

431

432

#### 433 **Acknowledgements**

434 We are thankful to Amina Aboufaires El Alaoui for her help during the experiments,  
435 Rina Righi, Alfredo Fiorelli, and Agostino Marchi for animal care and technical support, and  
436 Michael Arends for editing the manuscript.

437

#### 438 **Authors contributions**

439 Y.F., R.C., and M.R. designed the project. Y.F. designed and performed the  
440 experiments, analyzed the data, and wrote the manuscript. A.M.B. and F.B. performed the  
441 experiments and analyzed the data. R.C. supervised the project and contributed to writing the

442 manuscript. M.R., G.D, and G.G. provided critical comments, helped interpret the data, and  
443 contributed to writing the manuscript. All of the authors reviewed the manuscript.

444

445

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- 624

625 **Figure Legends**

626

627 **Figure 1. Effect of intra-CeA and intra-NAc shell pioglitazone administration on alcohol**  
628 **and food intake in msP rats.** (A, D) Time-course of alcohol drinking following pioglitazone  
629 administration in the CeA and NAc shell, respectively. (B, E) Changes in food intake  
630 following treatment. (C, F) Schematic illustration of vehicle and pioglitazone injection sites  
631 (dots) in the CeA (C) and NAc shell (F). The data are expressed as mean ( $\pm$ SEM) intake.  $n =$   
632 9 for CeA.  $n = 11$  for NAc shell.

633

634 **Figure 2. Effect of intra-RMTg and intra-VTA pioglitazone administration on alcohol,**  
635 **water, and food intake in msP rats.** (A, D) Time-course of alcohol drinking following  
636 pioglitazone administration in the RMTg and VTA, respectively. (B, E) Changes in food  
637 intake following treatment. (C, F) Schematic illustration of vehicle and pioglitazone injection  
638 sites (dots) in the RMTg (C) and VTA (F). The data are expressed as mean ( $\pm$ SEM) intake.  $n$   
639 = 11 for RMTg.  $n = 11$  for VTA.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ , vs. vehicle-treated  
640 control.

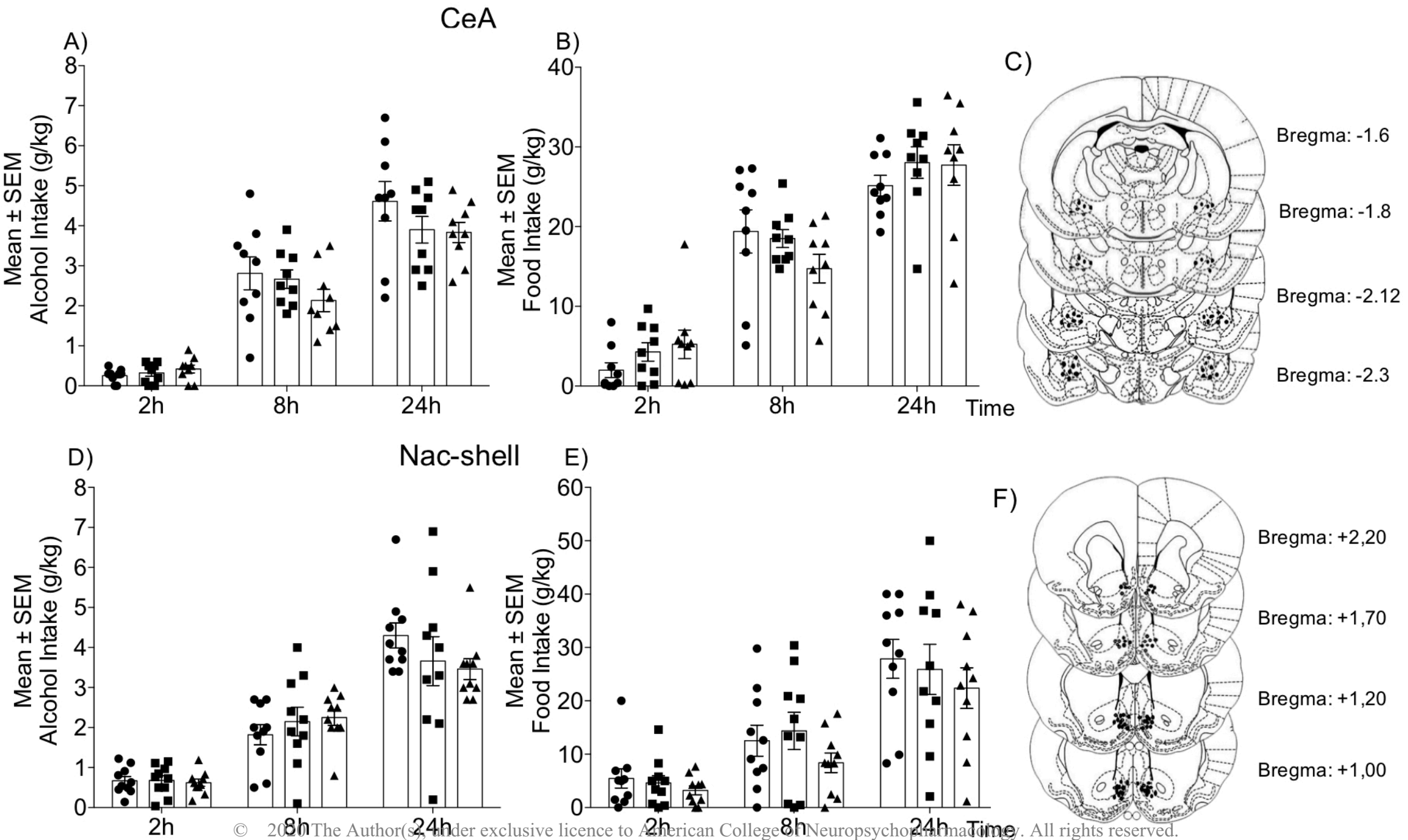
641

642 **Figure 3. Effect of intra-RMTg pioglitazone administration on operant alcohol and**  
643 **saccharin self-administration.** (A, C) Number of alcohol and saccharin reinforcements  
644 earned following pioglitazone administration in the RMTg. (B, D) Number of responses at  
645 the inactive lever. (E) Schematic illustration of vehicle and pioglitazone injection sites (dots)  
646 in the RMTg. The data are expressed as the mean  $\pm$  SEM.  $n = 8$  for alcohol.  $n = 16$  for  
647 saccharin.  $*p < 0.05$ ,  $**p < 0.01$ , vs. vehicle-treated control.

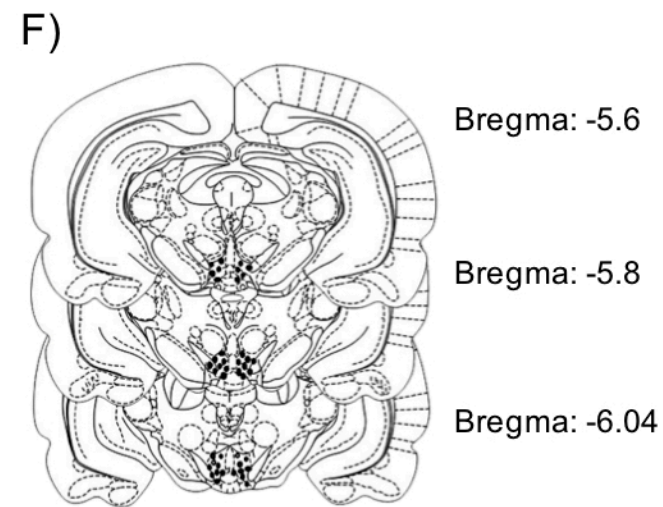
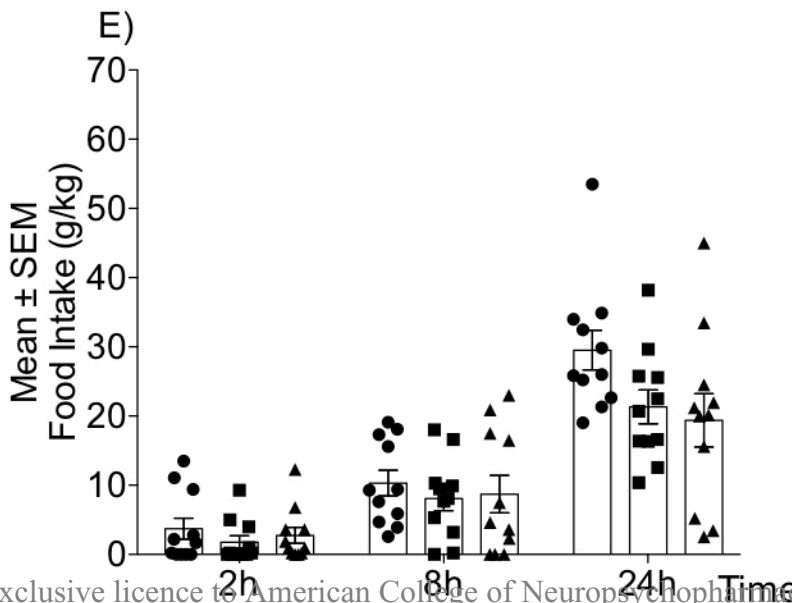
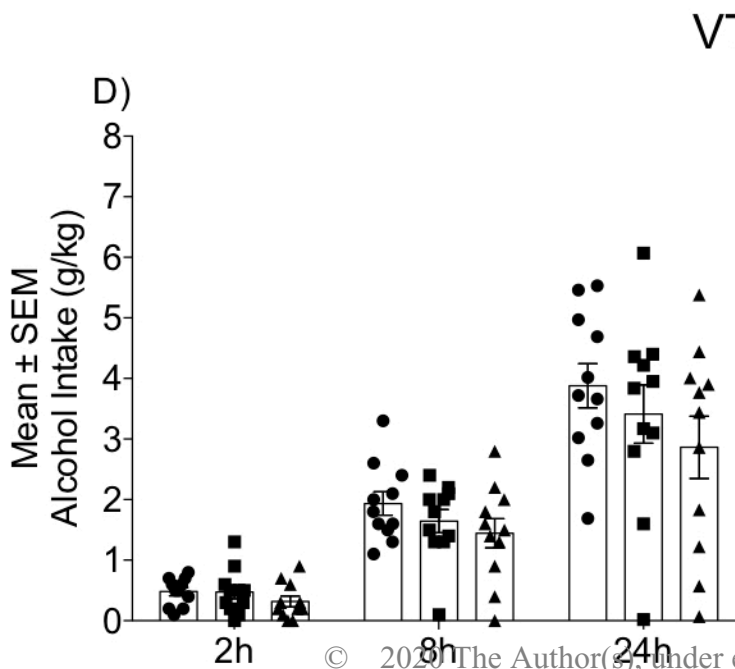
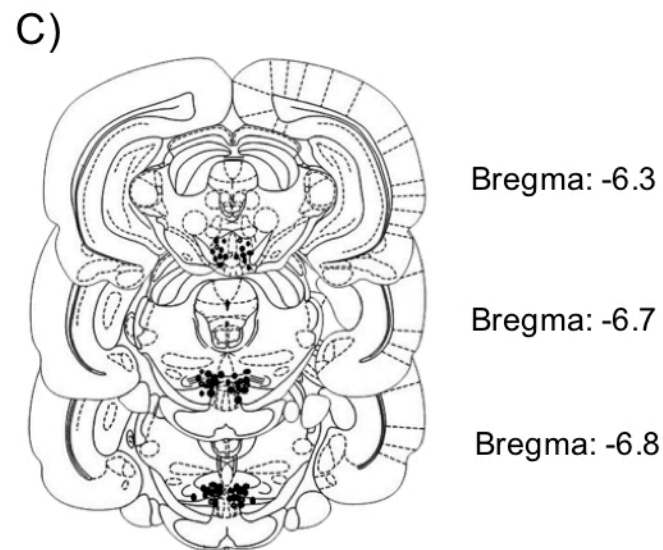
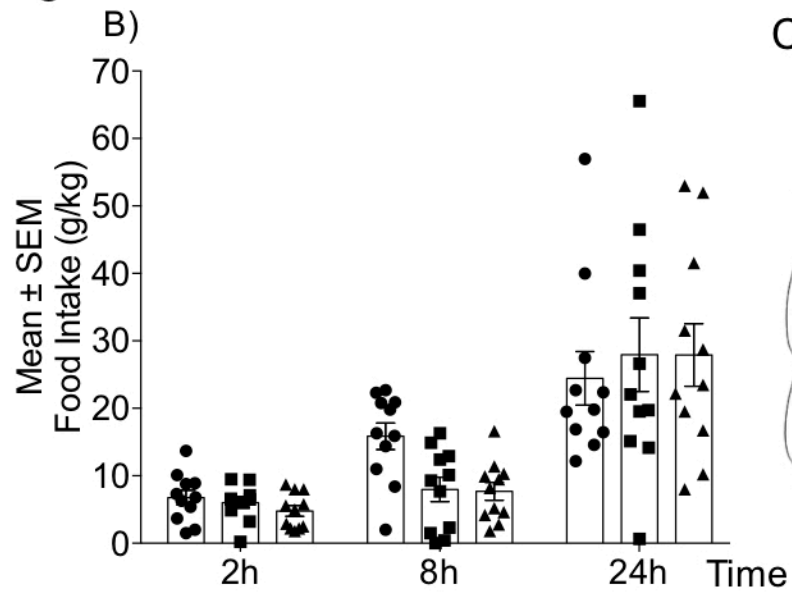
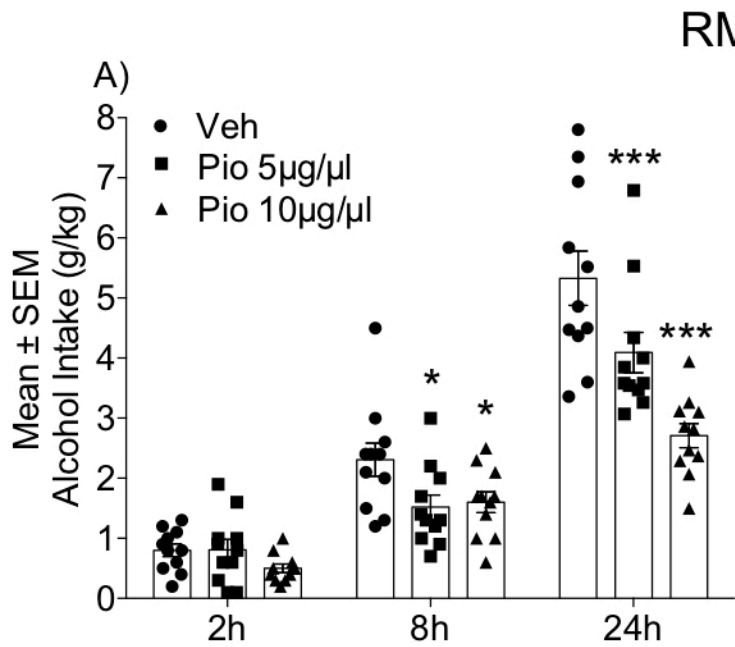
648

649 **Figure 4. Effect of intra-CeA and intra-NAc shell pioglitazone administration on the**  
650 **yohimbine-induced reinstatement of alcohol seeking. (A, D)** Number of responses at the  
651 active lever following pioglitazone administration in the CeA and NAc shell, respectively.  
652 **(B, E)** Number of responses at the inactive lever following treatment. **(C, F)** Schematic  
653 illustration of vehicle and pioglitazone injection sites (dots) in the CeA **(C)** and NAc shell  
654 **(F)**. The data are expressed as mean ( $\pm$ SEM) intake.  $n = 12$  for CeA.  $n = 13$  for NAc shell.  
655  $^{##}p < 0.01$ , vehicle vs. extinction;  $^{*}p < 0.05$ ,  $^{**}p < 0.01$ , vehicle- vs. pioglitazone-treated rats.  
656

657 **Figure 5. Effect of intra-RMTg and intra-VTA pioglitazone administration on the**  
658 **yohimbine-induced reinstatement of alcohol seeking. (A, D)** Number of responses at the  
659 active lever following pioglitazone administration in the VTA and RMTg, respectively. **(B,**  
660 **E)** Number of responses at the inactive lever following treatment. **(C, F)** Schematic  
661 illustration of vehicle and pioglitazone injection sites (dots) in the VTA **(C)** and RMTg **(F)**.  
662 The data are expressed as mean ( $\pm$ SEM) intake.  $n = 10$  for VTA.  $n = 10$  for RMTg.  $^{###}p <$   
663  $0.001$ , vehicle vs. extinction;  $^{**}p < 0.01$ ,  $^{***}p < 0.001$ , vehicle- vs. pioglitazone-treated  
664 animals.







# RMTg

## Alcohol

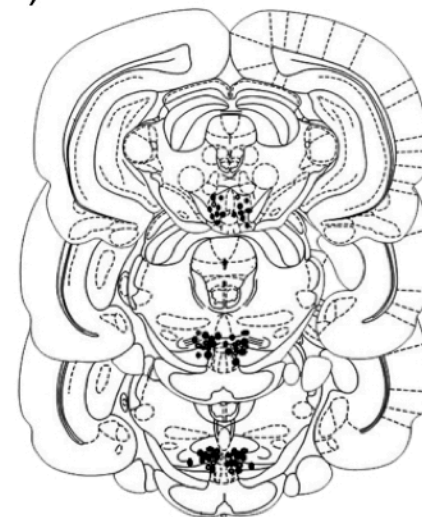
## Saccharin

E)

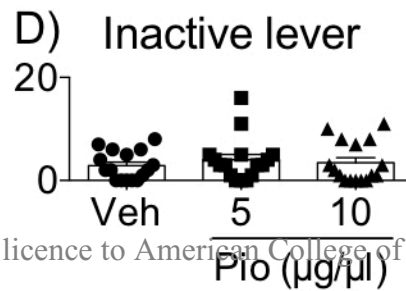
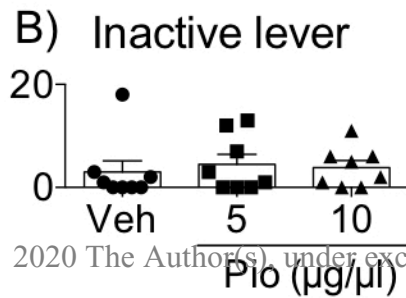
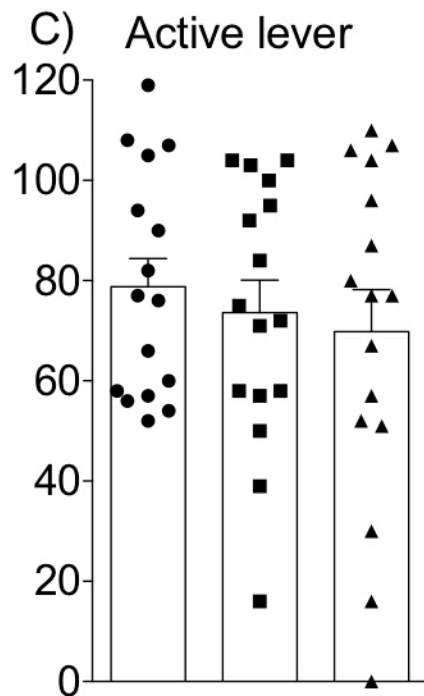
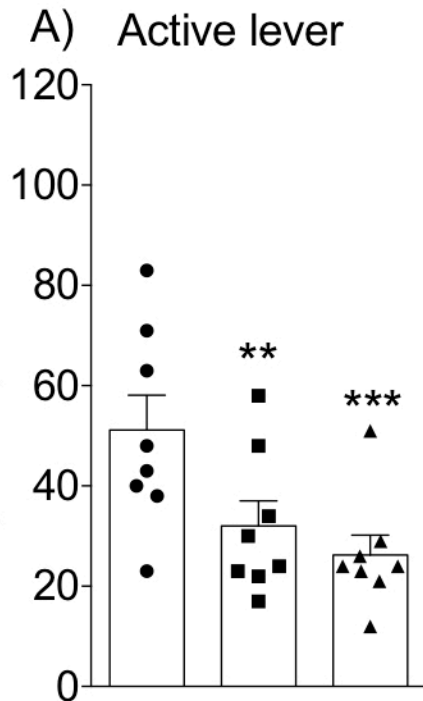
Bregma: -6.3

Bregma: -6.7

Bregma: -6.8



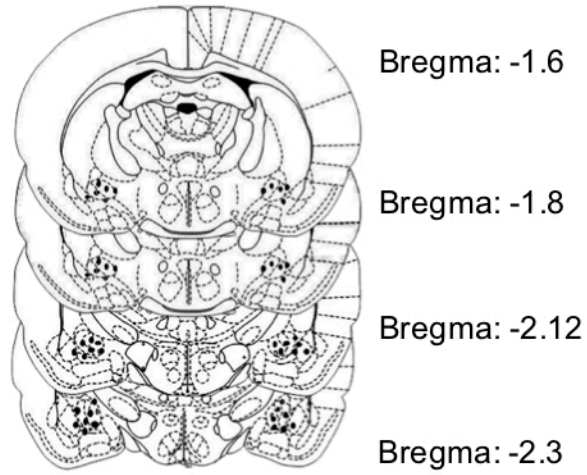
Mean  $\pm$  SEM Number of Responses  
(30-min)



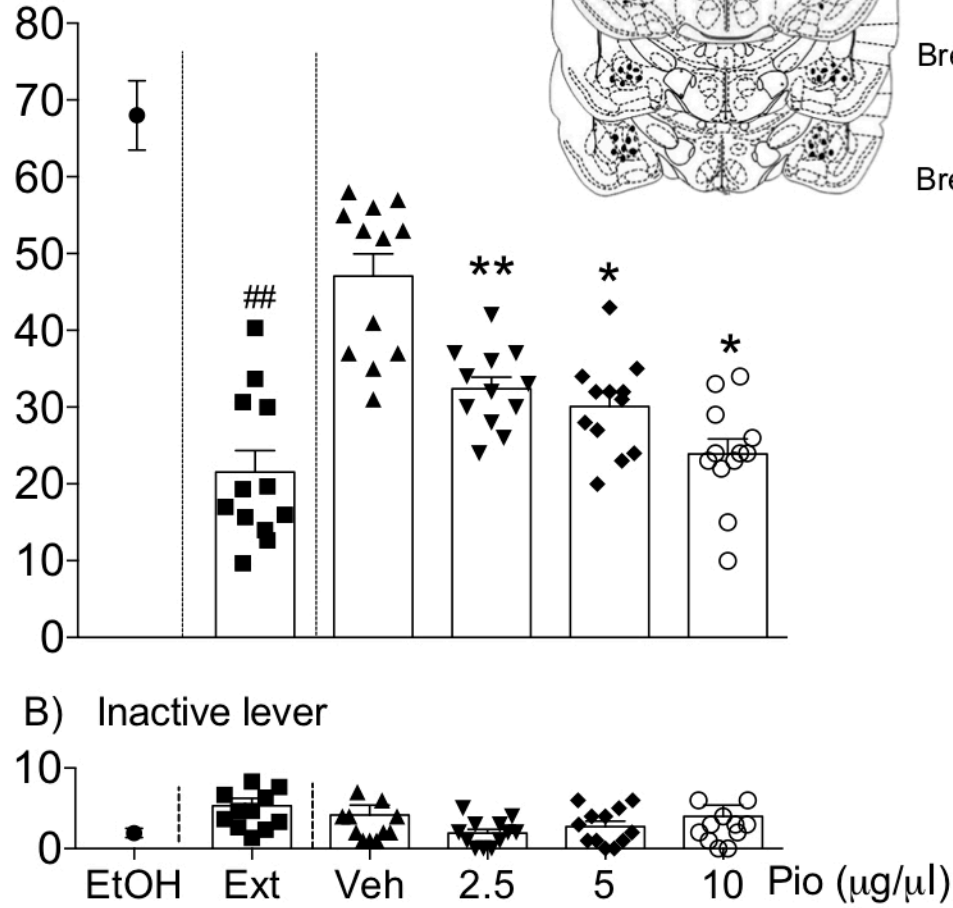


# CeA

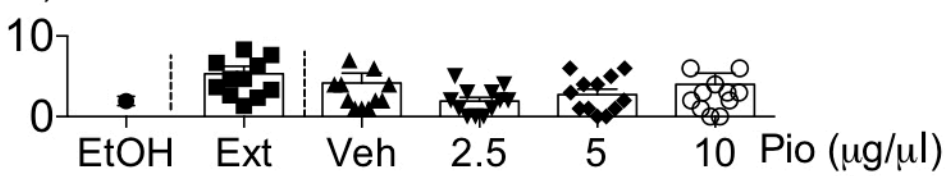
C)



A) Active lever



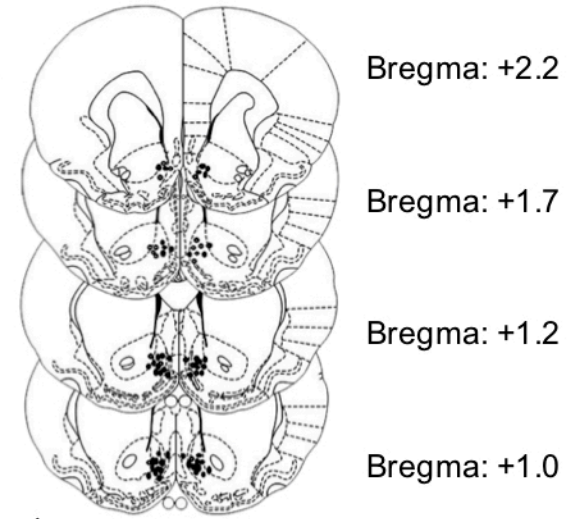
B) Inactive lever



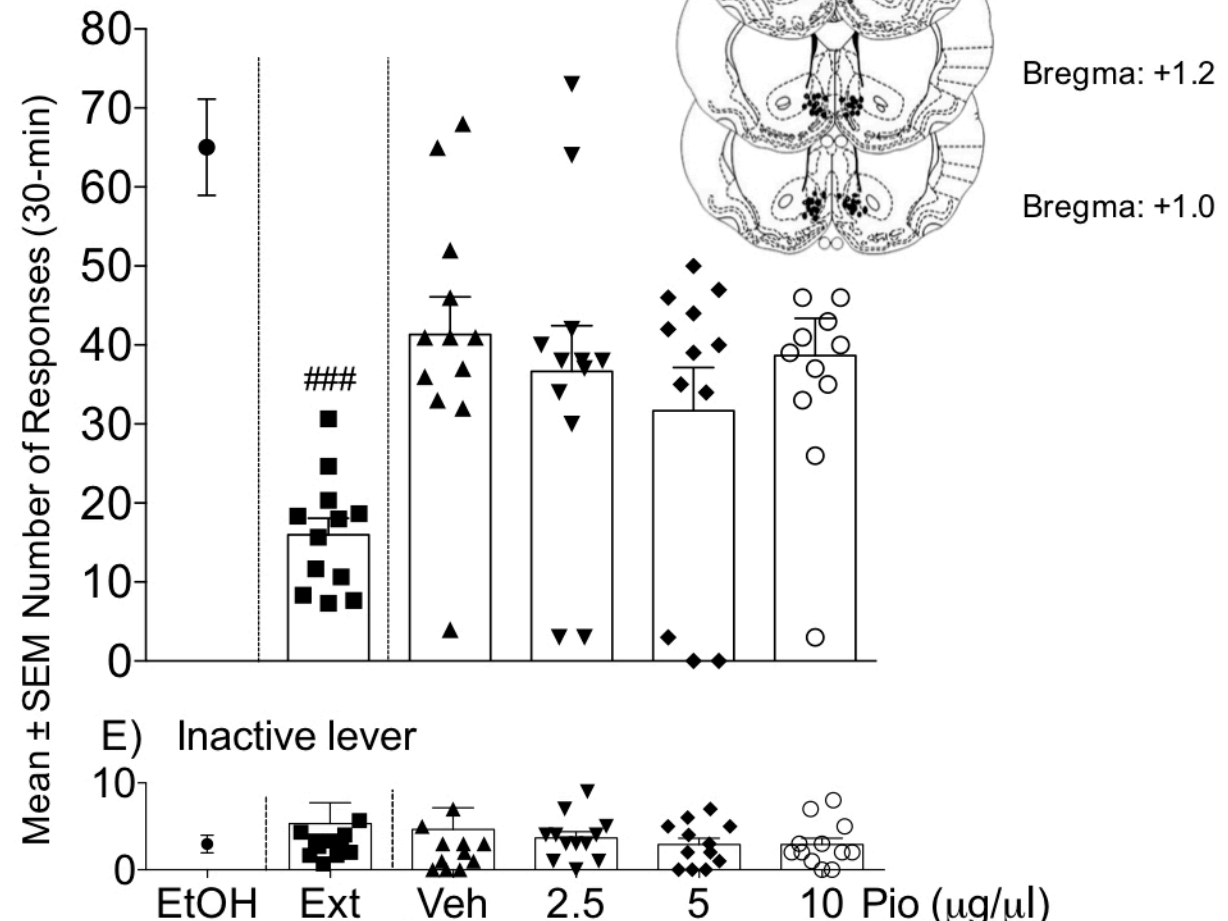
Yohimbine (1.25 mg/kg)

# Nac-shell

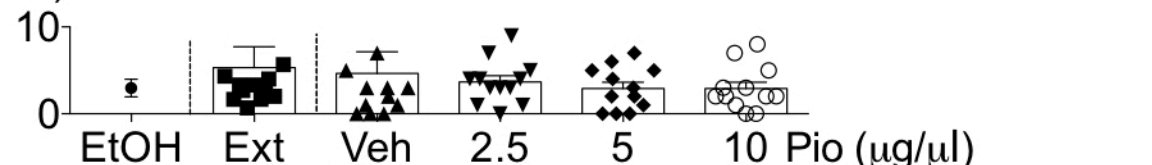
F)



D) Active lever

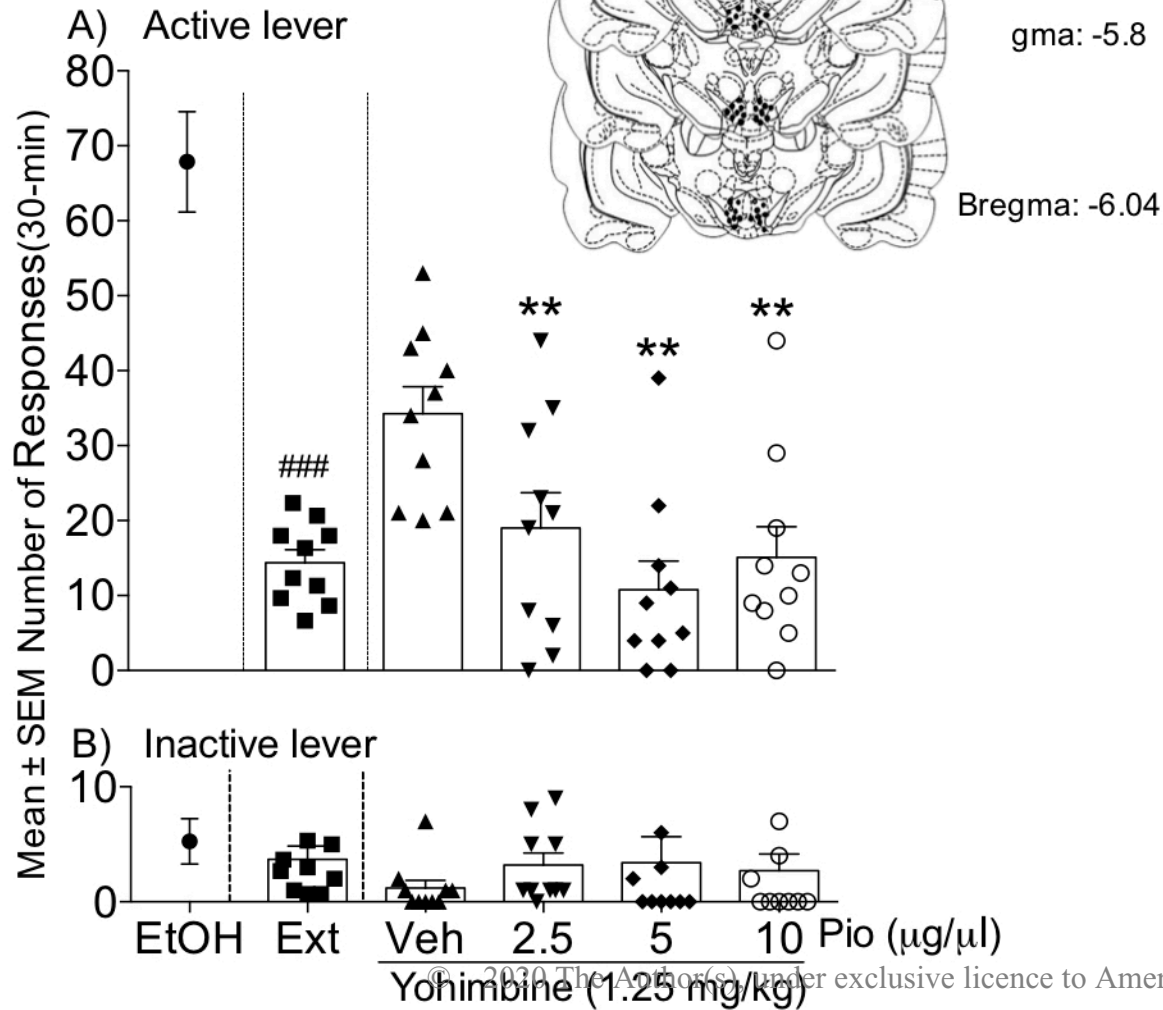
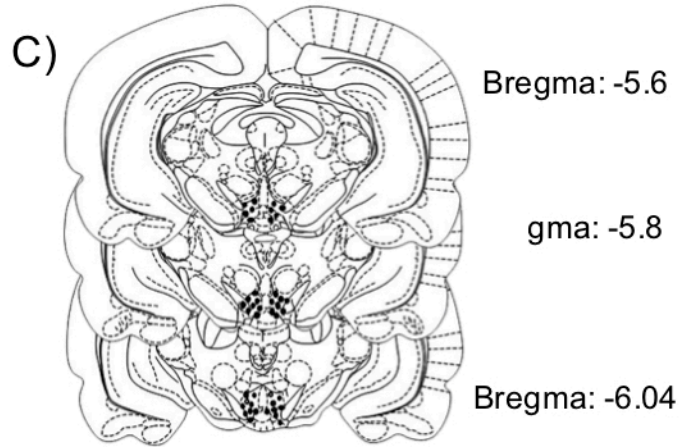


E) Inactive lever



Yohimbine (1.25 mg/kg)

# VTA



# RMTg

