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Evaluation of N-acetylcysteine on ethanol self-administration in ethanol-dependent rats

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Running title: NAC reduces ethanol seeking and relapse in dependent rats

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52

53 **Introduction**

54

55 Alcohol consumption is one of the major avoidable risk factors for chronic disease, injury and
56 premature death, with an estimated 3.8% of all global deaths and 4.6% of global disability-
57 adjusted life-years attributable to alcohol worldwide (Rehm et al., 2009). Among alcohol-
58 induced pathologies, Alcohol Use Disorder (AUD) is a chronic disorder characterized by the
59 compulsive use of alcohol, loss of control over intake and development of a negative
60 emotional state when alcohol is unavailable (Koob, 2013, 2015). Given the high relapse rate
61 when patients attempt abstinence and that not all patients respond to a specific treatment,
62 research of new-treatments against relapse appears highly necessary especially in the context
63 of future personalized therapies.

64 N-acetylcysteine (NAC) is a cystine prodrug that activates the cystine-glutamate exchanger,
65 the system antiport x_c^- , that maintains extrasynaptic glutamate concentrations (Millea 2009;
66 Lewerenz *et al.* 2013). Numerous studies have shown that NAC decreases the craving for
67 cocaine in both rodents and humans, via its stimulating action of the x_c^- antiport system
68 (LaRowe et al., 2007, Kau et al., 2008, Amen et al., 2011, Kupchik et al., 2012, Brown et al.,
69 2013, LaRowe et al., 2013, Ducret et al., 2016).

70 This effect was not only observed for cocaine but also for other substances. In rat, NAC
71 decreases the relapse to nicotine self-administration (Ramirez-Nino et al., 2013, Moro et al.,
72 2016). In humans, NAC is also promising since it has been shown to reduce craving for
73 cannabis (Gray et al., 2012) and nicotine (Froeliger et al., 2015, Prado et al., 2015). NAC was
74 accordingly proposed as an interesting therapeutic option in the treatment of addiction
75 (Kalivas and Volkow, 2011, Asevedo et al., 2014).

76 Regarding ethanol, chronic NAC treatment inhibited ethanol intake in rats bred for their high
77 ethanol intake when NAC was administered to animals displaying chronic ethanol
78 consumption (Quintanilla et al., 2016). In a recent study (Lebourgeois et al., 2017), we
79 demonstrated that a high dose (100 mg/kg) of NAC reduced ethanol consumption, motivation,
80 seeking and relapse in rats self-administering 1.2 g/kg of 20% ethanol for 15 minutes per day
81 and during several weeks, a model referred to as binge-drinking in rats (Jeanblanc et al.,
82 2018). In human, NAC reduced the amount and frequency of alcohol use among participants
83 with cannabis use disorders (Squeglia et al., 2018), and decreased subjective craving score in
84 veterans with Posttraumatic Stress Disorders with co-occurring Substance Use Disorders
85 (Back et al., 2016).

86 Here we investigated the efficacy of NAC in ethanol-dependent rats exposed for several
87 weeks to repeated ethanol intoxication-withdrawal cycles using chronic intermittent ethanol
88 vapor inhalation (CIE) (Aufrere et al., 1997, Gilpin et al., 2008, Houchi et al., 2013, Simon-
89 O'Brien et al., 2014, Alaux-Cantin et al., 2015, Meinhardt and Sommer, 2015). This paradigm
90 is highly relevant as a model of post-dependent state because of the induction of both
91 behavioral (loss of control over intake, high motivation for intake, high level of extinction
92 responding in the absence of alcohol, anxiety-like behavior, depression, compulsive use) and
93 physical signs (tolerance, tremor, convulsive crisis, hyperactivity) (Vendruscolo and Roberts,
94 2014, Meinhardt and Sommer, 2015). In particular, acute withdrawal from CIE produces an
95 increase in negative affect symptoms, which could facilitate excessive drinking (Somkuwar et
96 al., 2017, Tunstall et al., 2017).

97 The glutamate release is enhanced in nucleus accumbens during cocaine (McFarland
98 et al., 2003) nicotine (Gipson et al., 2013), opiates (LaLumiere and Kalivas 2008) and ethanol
99 seeking (Gass et al., 2011). NAC is thought to reduce craving through the normalization of
100 glutamatergic homeostasis, by the mean of an increase of negative feedback produced by

101 extrasynaptic glutamate on perisynaptic mGluR2/3 autoreceptors (Scofield et al., 2016). So
102 we also tested the effect of NAC on the expression of xCT, the catalytic subunit of the x_c^-
103 system, and in the expression of GLT-1, the transporter that re-uptake glutamate from the
104 extracellular fluids, in dependent and in nondependent (ethanol-naïve) rats.
105 In this study, we demonstrated that a low dose of NAC significantly reduced ethanol
106 consumption, motivation to ethanol, and extinction responding (i.e. seeking) during acute
107 withdrawal (8 hours) from CIE, while dependent rats showed a reduced expression of xCT
108 (the specific subunit of x_c^- system) in the nucleus accumbens. NAC reduced the expression of
109 GLT-1 with the same efficiency in dependent and nondependent rats. Since NAC was
110 effective in dependent rats (25-50 mg/kg) at a lower dose than in binge-drinking rats (100
111 mg/kg), our results suggest that neuroadaptations underlying ethanol dependence may make
112 rats respondent to lower doses. We also demonstrated that NAC reduces reacquisition of
113 ethanol self-administration following protracted abstinence (1 month) in dependent rats.
114 Finally, NAC (100 mg/kg) reduced 1% sucrose self-administration demonstrating the NAC
115 efficacy on non-drug reward.

116

117 **Methods and Materials**

118

119 **Animals:**

120 Male Wistar rats, weighing 260-280g at the beginning of the experiment, were
121 purchased from Janvier labs (Le Genest Saint Isle, France). Rats were individually housed in
122 a controlled environment under a 12-h light/dark cycle (lights on at 8 a.m.) with food and
123 water available *ad libitum*. All experiments were performed in conformity with the European
124 Community guiding principles for the care and use of animals (2010/63/UE, CE Off. J. 20
125 October 2010), the French decree n° 2013-118 (French Republic Off. J., 2013) and approved

126 by the local ethics committee [Comité Régional d'Ethique en Matière d'Expérimentation
127 Animale de Picardie (CREMEAP), University of Picardie Jules Verne].

128

129 **Drugs:**

130 For self-administration experiments, ethanol (VWR, Strasbourg, France) was diluted
131 in tap water at the final concentration of 10 or 20% (v/v) and sucrose (VWR, Strasbourg,
132 France) was diluted in tap water at the final concentration of 1% (w/v). For intraperitoneal
133 (i.p.) injections, N-acetylcysteine (Sigma Aldrich, Saint Quentin Fallavier, France) was
134 dissolved in sterile saline (0.9 % NaCl) and administered at the doses of 25, 50 and 100
135 mg/kg chosen accordingly to the literature, after pH adjustment to pH 7.4 (Kau et al., 2008,
136 Reichel et al., 2011, Lebourgeois et al., 2017), 60 min before the start of self-administration
137 sessions.

138

139 **Behavioral acquisition of the self-administration task**

140 The self-administration behavior was induced by a three-step paradigm described in details
141 in Supplementary Materials and Methods. First, rats were exposed to intermittent access to
142 20% ethanol for 2 weeks to facilitate the acquisition of a high level of ethanol intake (Wise,
143 1973). Thereafter, rats were trained to self-administer ethanol during 30-min access operant
144 sessions. Once rats reached a stable level of intake, they were placed in inhalation chambers
145 to induce dependence. The effect of NAC was recorded during acute withdrawal (8-hour
146 period after inhalation chambers were turned off). This timing was chosen according to a
147 previous report showing that repeated withdrawal produced an increase in extracellular
148 glutamate in hippocampus after 6-7 hours withdrawal, i.e. 6-7 hours after inhalation chambers
149 were turned off (Dahchour and De Witte, 2003).

150

151 **Behavioral testing: Effect of NAC on ethanol self-administration:**

152 The timeline of the experimental procedures is given in figure 1 (Fig. 1).

153 *Effect of NAC on ethanol self-administration during acute withdrawal:*

154 Once rats reached a stable ethanol self-administration level, the effect of an acute
155 injection of NAC (0, 25, 50 or 100 mg/kg; i.p., 60 min before the test) was tested on the level
156 of ethanol 20% self-administration during a 30-min session, 8 hours after retrieval from the
157 inhalation chambers. The doses and timing of injection doses were chosen depending on
158 previous work (Baker et al., 2003, Moussawi et al., 2009, Amen et al., 2011, Reichel et al.,
159 2011). Injections were performed according to a Latin square counterbalanced design with 1
160 day of wash-out between each injection.

161

162 *Effect of NAC on the reinforcing properties of ethanol during acute withdrawal:*

163 After a 2 week wash-out period were rats were submitted to five-day-a-week sessions
164 of ethanol self-administration at 8-hours withdrawal, a progressive ratio (PR) schedule test
165 was performed to evaluate the effect of NAC (0 or 25mg/kg) on the motivation to consume
166 ethanol (i.e. its reinforcing properties). As the 25 mg/kg dose was the effective dose in the
167 previous test, we choose this dose in the PR schedule test. In this test, the effort necessary to
168 obtain one reward (i.e. the number of presses on the active lever) was continuously increased
169 after each reward delivery (3, 4, 5, 7, 9, 12, 15, 18, 20, 23, 25, 28, 30, 33, 35). During the 30-
170 min session, the maximum effort (breakpoint) produced to receive a single reward of ethanol
171 was measured and reflected an index of motivation.

172

173 *Effect of NAC on ethanol seeking: test of extinction responding*

174 In order to evaluate the effect of NAC on ethanol-seeking behavior, we performed a
175 test in a single session of extinction. During this test, NAC was injected i.p. 60 min before a

176 30-min session test during which the light cue remained off and no alcohol was available. The
177 persistence of drug-seeking behavior in the absence of the drug (i.e., craving) was assessed by
178 recording the number of active lever presses. After a 2 weeks wash-out period where the rats
179 were submitted to five-day-a-week sessions of ethanol self-administration, we performed a
180 first test with the 25 mg/kg dose of NAC, followed by a second 2 weeks-wash out period and
181 a second test at the 50 mg/kg dose.

182

183 *Effect of NAC on reacquisition after protracted abstinence:*

184 After a 2 weeks-wash out period, rats were exposed to one month of abstinence,
185 during which rats were housed in the home cage and had no access to ethanol vapor chambers
186 and self-administration boxes. After this abstinence period, we used the reacquisition model
187 of relapse (Alaux-Cantin et al., 2013, Simon-O'Brien et al., 2014), which consists of the re-
188 introduction of ethanol in the operant self-administration chambers. Briefly, a priming
189 delivery of ethanol (0.1 ml of a 20% ethanol solution) was given, non-contingently, at the
190 beginning of the re-acquisition session to provide an olfactory and gustatory cue. Ethanol was
191 then available on an FR3 schedule for the duration of the 30-min session, and NAC or saline
192 was injected i.p. 60 min before the beginning of the behavioral test. The experiment was
193 performed twice, first with the NAC 25 mg/kg and then with the 50 mg/kg dose.

194

195 **Behavioral testing: Effect of NAC on 1% sucrose self-administration in nondependent**
196 **rats**

197 In a separate cohort of 14 Long Evans rats, the effect of NAC (100 mg/kg) was tested on 1%
198 sucrose self-administration, after eight weeks of 20% ethanol self-administration during 15-
199 min session (as described in Lebourgeois et al., 2017). It is noteworthy that rats were not
200 dependent, since they were not exposed to the CIE.

201

202 **Western-blotting assays**

203 The effect of NAC on xCT and GLT1 proteins expression was evaluated in dependent rats
204 exposed to 10 weeks of CIE following an 8-hour withdrawal period and in rats kept in the
205 same conditions, but naïve to ethanol. NAC was administered i.p. one hour before euthanasia
206 (n=6 rats per groups, 4 groups). After anaesthesia and decapitation, the nucleus accumbens
207 was dissected in 4°C PBS. The details for western blot procedures are provided in
208 Supplementary Materials and Methods.

209

210 **Statistical analysis:**

211

212 All results are expressed as mean \pm standard error of the mean. For behavioral data, statistical
213 differences were assessed using one- or two-way ANOVAs with or without repeated
214 measures (RM). Significant main effects detected by ANOVAs were followed by a Tukey
215 *post-hoc* test. For simple comparisons, data were analyzed with a Student's *t*-test. For
216 Western blotting data, the integrated optical density for xCT and GLT-1 was divided by the
217 density of actin immunoreactivity within the same sample. This yielded a "ratio" for each rat
218 and these values were compared between groups using two-way ANOVAs. Significant main
219 effects detected by ANOVAs were followed by a Tukey *post-hoc* test. All statistical analyses
220 were performed using the SigmaPlot software (version 11; Systat software , Inc., San Jose,
221 CA USA) and a $p < 0.05$ was considered significant.

222

223 **Results:**

224

225 **N-acetylcysteine reduced ethanol self-administration:**

226 The effect of an acute injection of NAC (0, 25, 50 or 100 mg/kg; i.p.; 1h before the
227 test) on mean numbers of active lever presses in the ethanol self-administration procedure was
228 analyzed using a one way RM-ANOVA, which revealed a significant main effect of treatment
229 ($F[(3,48)=16.334, p<0.001]$). Subsequent *post-hoc* Tukey analyses showed that total active
230 lever presses were significantly lower for the NAC 25 mg/kg group compared with the saline
231 group (-58%). Similarly, total active lever presses were significantly lower for the 50 and 100
232 mg/kg doses of NAC compared with saline (respectively -48 et -95%) (Fig. 2A). Inactive
233 lever presses were not statistically different between groups: $F(3, 48)=1.153, p=0.337]$ (Fig.
234 2C). Analogous effects were observed regarding ethanol intake (Fig. 2B). Rats treated with
235 25, 50 or 100 mg/kg dose consumed significantly less ethanol than saline-treated rats
236 (respectively: 64; 49 and 95% of inhibition) (treatment main effect, $F[(3,48)=14.116,$
237 $p<0.001]$). Together, these data indicate that an acute injection of NAC at the doses of 25, 50
238 or 100 mg/kg, reduced ethanol self-administration.

239

240 **N-acetylcysteine was more effective in ethanol-dependent rats:**

241 Assuming that dependent animals have robust modifications of their glutamate
242 homeostasis, and that NAC acts by restoring glutamatergic homeostasis, we next intended to
243 test the hypothesis that NAC could be more effective in ethanol-dependent animals compared
244 with those that are not dependent. To test this hypothesis, we compared our results presented
245 here with data found in nondependent animals self-administering chronically large amount of
246 ethanol during several weeks (Lebourgeois et al., 2017). Fig. 2D represents the percentage of
247 baseline EtOH consumption in dependent and nondependent groups for 3 doses of N-
248 acetylcysteine (25, 50 or 100 mg/kg; i.p., 1h before the self-administration test). Data were
249 analyzed using a two-way ANOVA, which revealed a significant main effect of dependence
250 ($F[(1,90)=23.035, p<0.001]$) and a significant main effect of treatment ($F[(2,90)=8.049,$

251 $p<0.001$]). Subsequent pairwise analyses (Tukey test) showed that NAC produced a greater
252 relative reduction in ethanol consumption in dependent animals at all doses tested.

253

254

255 **N-acetylcysteine decreased the reinforcing efficacy of ethanol**

256 To further assess the effect of NAC (25 mg/kg) on the motivation to consume ethanol,
257 we performed a PR test. The number of active lever presses and the breakpoint values during
258 the 30-min session are depicted in Fig. 3A and 3B. Statistical analyses using Student t-tests
259 revealed that NAC-treated rats pressed significantly fewer times during the PR session (-55%;
260 $p<0.05$) and displayed a significantly lower breakpoint value compared with saline-treated
261 rats (-43%; $p<0.01$). Inactive lever presses were statistically not different between groups
262 ($p=0.591$) (Fig. 3C).

263

264 **N-acetylcysteine reduced extinction responding (ethanol seeking)**

265 Next, we assessed the effect of an acute injection of NAC (25 and 50 mg/kg; i.p., 1h
266 before the test) on ethanol seeking during extinction. In this test, rats were placed in ethanol
267 self-administration chambers and each press on the active lever was counted, but had no
268 consequences (neither ethanol nor light cue). We performed two separate experiments at 2
269 weeks interval with the 25 (Fig. 4A and 4C) and 50 mg/kg dose (Fig 4B and 4D). The total
270 active lever presses were analyzed using a Student t-test. NAC 25 mg/kg did not produce any
271 alteration of extinction responding ($p=0.59$) while the 50 mg/kg dose of NAC significantly
272 reduced by 92% the persistence of drug-seeking behavior in the absence of alcohol
273 ($p=0.0004$). However, the total number of inactive lever presses was not significantly
274 different between groups.

275

276

277 **N-acetylcysteine limited reacquisition after abstinence:**

278 Addiction is a highly relapsing disorder, so we next examined the effect of an acute
279 injection of NAC (25 and 50 mg/kg; i.p., 1h before the test) on relapse after 1 month of
280 protracted abstinence. We performed two separate experiments at 1.5 months interval with the
281 25 (Fig. 5A, C, E) and 50 mg/kg dose (Fig. 5B, D and F). NAC 25 mg/kg did not produce
282 any alteration in the total active lever presses ($p=0.35$) (Fig. 5A), while the NAC 50 mg/kg
283 decreased significantly the total active lever presses compared with saline group ($p=0.037$)
284 (i.e., a reduction of 55%) (Fig. 5B). As expected, analogous effects were observed regarding
285 ethanol intake. Rats treated with 50 mg/kg NAC consumed significantly less ethanol than
286 saline-treated rats ($p=0.027$) (55% of reduction) (Fig 5F). Inactive lever presses were
287 statistically not different between groups (Fig 5 C and D).

288

289 **Behavioral testing: Effect of NAC on 1% sucrose self-administration in nondependent**
290 **rats:**

291 We tested the effect of NAC on 1% sucrose self-administration in rats that were
292 submitted before sucrose experiment to 2 months of 5 day-a-week 20% ethanol self-
293 administration during 15 minutes sessions (Lebourgeois et al., 2017). In those rats (Fig. 6),
294 NAC 100 mg/kg significantly decreased the 1% sucrose self-administration from -83%
295 (101 ± 12.1 active lever presses for NaCl rats versus 17 ± 13.5 for NAC treated rats, paired
296 Student *t*-test $p=0.0003$).

297

298 **xCT protein levels were decreased in nucleus accumbens of dependent rats:**

299 Fig. 7A shows the expression of xCT measured using western blot in ethanol-
300 dependent and ethanol-naïve rats following treatment with NAC (100 mg/kg; i.p.,

301 administered 1h before euthanasia). The expression of Xc- system was investigated through
302 the expression of its xCT subunit. As indicated by the Abcam 175186 manufacturer, the
303 expected molecular weight for xCT protein was 55 kDa. Data were analyzed using a two-way
304 ANOVA, which revealed a significant main effect of dependence ($F(1,21)=7.697, p=0.012$),
305 no significant main effect of NAC treatment ($F(1,21)=0.0997, p=0.756$) and no significant
306 interaction ($F(1,19)=0.149, p=0.704$). Dependent rats showed a -18% decrease in xCT
307 expression compared with ethanol-naïve rats, irrespective of NAC treatment ($p<0.05$).

308 Fig. 7B shows the expression of GLT-1 measured using western blot in ethanol-
309 dependent and ethanol-naïve rats following treatment with NAC (100 mg/kg; i.p.,
310 administered 1h before euthanasia). As indicated by the Abcam ab 41621 manufacturer the
311 expected molecular weight for GLT-1 protein was 63 kDa. Data were analyzed using a two-
312 way ANOVA, which revealed no significant main effect of dependence ($F(1,21)=0.474$;
313 $p=0.499$), a significant main effect of NAC treatment ($F(1,21)=4.456, p=0.048$) and no
314 significant interaction ($F(1,19)=0.114; p=0.739$). NAC treated rats showed a -28% decrease in
315 GLT-1 expression compared with saline group, in dependent rats as well as in ethanol naive
316 rats.

317

318

319 **Discussion:**

320 We have previously demonstrated that a high dose (100mg/kg) of NAC reduced
321 excessive ethanol intake, motivation, seeking and relapse in rats displaying a binge drinking-
322 like behavior for several weeks (Lebourgeois et al., 2017). In the present study we
323 investigated the effect of NAC in rats displaying ethanol dependence following CIE to
324 ethanol vapor. One of the primary findings of the present study is that a low dose (25mg/kg)
325 of NAC reduced ethanol self-administration and motivation to consume ethanol and that a

326 higher dose (50mg/kg) reduced both extinction responding and reacquisition after abstinence.
327 We also showed that NAC (100 mg/kg) reduced 1% sucrose self-administration in non-
328 dependent rats, demonstrating the NAC efficacy on non-drug reward. When examining
329 glutamate-related neuroadaptations in this addiction model, we found a 18% decrease in the
330 xCT protein levels in the nucleus accumbens of ethanol-dependent rats compared with those
331 of nondependent rats, and we found that NAC induced -28% decrease in GLT-1 expression in
332 both groups, dependent or not.

333 *NAC efficacy on ethanol self-administration and motivation: relevance of long-term ethanol*
334 *exposure*

335 NAC was tested in rats exposed to ethanol for several weeks via the CIE paradigm, i.e. in
336 ethanol dependent animals. The dose of 25mg/kg decreased both ethanol intake and
337 motivation (evaluated in the progressive ratio paradigm) to consume ethanol, assessed after 8-
338 h of withdrawal. This finding complements our prior results in rats engaging in binge-like
339 drinking consumption for several weeks. A main finding of the present study is that NAC is
340 much more effective in ethanol-dependent animals compared with animals displaying binge-
341 like drinking behavior. While the NAC dose of 25 mg/kg significantly reduced intake and
342 motivation in ethanol-dependent rats, a higher dose of 100 mg/kg was necessary to reduce
343 them in rats displaying binge-drinking behavior (Lebourgeois et al., 2017).

344 It appears that the extended exposure to ethanol is important for NAC efficacy. In rats bred
345 for their high ethanol intake and given free access to ethanol beverage for three months, NAC
346 decreased ethanol intake. However, in rats that drank for only 5 days, NAC did not reduce
347 ethanol intake (Quintanilla et al., 2016). These results and ours suggest that NAC acts by
348 counteracting some of the chronic ethanol-induced neuroadaptations that are responsible for
349 the transition to ethanol addiction.

350 The NAC dose-response data highlight ethanol-dependence-induced neuroadaptations of
351 glutamatergic transmission. We hypothesized that NAC would be more effective in dependent
352 rats because the hyperglutamatergic state would be higher than in rats self-administrating
353 large quantities of ethanol, but not exposed to the ethanol vapor regimen. In the ethanol
354 withdrawal condition (i.e. the condition in which we tested NAC efficacy here), there are
355 alcohol-induced alterations of the glutamate homeostasis: Chronic alcohol exposure leads to
356 increased extracellular concentrations of glutamate in the nucleus accumbens during
357 withdrawal (Dahchour and De Witte, 2003, Melendez et al., 2005, Griffin et al., 2015),
358 revealing an hyperglutamatergic state in the mesocorticolimbic system following withdrawal
359 (Ding et al., 2013, Rao et al., 2015a). The CIE of ethanol vapor has been described as a model
360 inducing a post-dependent state (Heilig and Koob, 2007, Meinhardt and Sommer, 2015),
361 resulting in long-term changes toward compulsive alcohol intake and hypersensitivity to
362 stress. The use of this model allows the evaluation of NAC impact on the chronic negative
363 affective state experienced by these rats during acute withdrawal (Meinhardt and Sommer,
364 2015). Herman et al. demonstrated that the hyperglutamatergic state observed during acute
365 withdrawal in these rats parallels the same hyperglutamatergic state observed in alcoholic
366 patients (Hermann et al., 2012). This observation lends support for the CIE model to test
367 glutamate-targeting substances, as we did for NAC here.

368 *Potential role of NAC to reduce craving*

369 The 50mg/kg dose of NAC completely abolished the extinction responding in ethanol-
370 dependent rats. This result indicates that NAC is effective not only when ethanol is available,
371 but also during periods of drug seeking (a behavioral correlate of craving) when ethanol is not
372 available. This finding also suggests that the NAC effect on negative-reinforcement-induced

373 extinction responding during acute withdrawal we observed in ethanol dependent rats could
374 also be obtained in abstinent patients treated with NAC.

375 *Potential efficacy of NAC to reduce relapse*

376 Furthermore, the 50mg/kg dose of NAC is effective in reducing reacquisition of ethanol self-
377 administration (induced by both ethanol and visual cues) after long-term abstinence. Taken
378 together, our results demonstrate the efficacy of NAC both during acute withdrawal and after
379 long-term abstinence and these findings are consistent with the fact that enduring changes in
380 glutamatergic transmissions underlie long-term vulnerability to relapse to drug use and drug-
381 associated memories (Kalivas, 2009).

382 *NAC effect on the reinforcing properties of a non-drug reward*

383 In another experiment, we investigated whether the effect of NAC on operant self-
384 administration was specific to ethanol by measuring its effects on the reinforcing properties of
385 sucrose, a non-drug appetitive reward. The 1% sucrose concentration was chosen to achieve
386 the same level of response as that observed in rats self-administering ethanol (about 100
387 deliveries). Our data show that NAC 100 mg/kg is very effective in reducing lever pressing
388 for sucrose. These data suggest that NAC affects general rewarding and motivational
389 mechanisms. Other molecules that are effective in reducing ethanol self-administration have
390 also been shown to have an effect on self-administration of natural reinforcers such as the
391 serotonergic and noradrenergic transporter inhibitor milnacipran (Simon O'Brien et al., 2011),
392 the CB1R antagonist SR-141716A (Economidou et al., 2006), the A2R agonist CGS21680
393 (Houchi et al., 2013) and even substances used to treat AUD patients such as naltrexone
394 (Stromberg et al., 2002) and the γ -aminobutyric (GABA) B receptors agonist baclofen
395 (Anstrom et al., 2003).

396

397 *CIE-induced decrease in the x_c^- antiport system*

398 The x_c^- system is expressed on glial cells and its role is to export glutamate in the
399 extrasynaptic space at the presynaptic level. As the glial x_c^- system plays a crucial role in
400 maintaining glutamate homeostasis, its expression has previously been monitored in the
401 context of substance abuse: rats withdrawn from chronically self-administering nicotine
402 showed decreased xCT expression in the nucleus accumbens, 12 hours after the last nicotine
403 exposure (Knackstedt et al., 2009).

404 There is a debate over the correct western band that has to be chosen for xCT Western
405 analysis (Van Liefferinge et al., 2016). Using the 55 kDa band in our study, as mentioned by
406 the manufacturer for Acam 175186, we found a 18 % decrease in xCT expression in the
407 nucleus accumbens of ethanol-dependent animals compared with ethanol-naïve animals, at
408 the 8h-withdrawal period. Interestingly, the increase in XCT expression has been related to a
409 five-fold decreased ethanol consumption in alcohol-preferring rats (Rao et al., 2015b). A
410 dynamic regulation of the xCT transporter in the nucleus accumbens along ethanol chronic
411 exposure or withdrawal has been already described. The xCT transporter is strongly over-
412 expressed in ethanol-dependent rat along exposure, and strongly down-regulated during
413 withdrawal (Peana et al., 2014). In the nucleus accumbens, around 60% of the basal
414 extracellular glutamate is derived from the activity of the x_c^- antiport system (Baker et al.,
415 2003). So the withdrawal-induced decrease in x_c^- could have an impact on glutamate
416 homeostasis, leading to an ethanol withdrawal-induced decrease in extracellular glutamate
417 level. However, this hypothesis is difficult to reconcile with the observation that chronic
418 alcohol exposure leads to increased extracellular concentrations of glutamate in the nucleus
419 accumbens that persists for 24h hours after withdrawal (Melendez et al., 2005) or 7 days after
420 withdrawal (Griffin et al., 2014).

421 The NAC treatment administered 1 hour before western blot sampling was unable to modify
422 the xCT expression in the nucleus accumbens. This is concordant with the proposal that the
423 acute NAC effect would not be due to a direct, rapid increase in the x_c^- system expression, but
424 rather, increased activity of the x_c^- system.

425 However, another actor plays a major role in drug-induced alteration of glutamate
426 homeostasis: the glial glutamate transporter GLT1, that regulates glutamatergic signaling by
427 removing excess glutamate from the extrasynaptic space (Roberts-Wolfe and Kalivas, 2015).
428 For example chronic alcohol intake for five weeks in P (alcohol preferring) rats resulted in
429 downregulation of GLT1 expression in the nucleus accumbens as compared to ethanol-naive
430 animals (Sari and Sreemantula, 2012), thus demonstrating a link between ethanol intake and
431 GLT-1 expression. Besides, it has been shown that the ceftriaxone-induced reduction in
432 ethanol intake by P rats was associated with significantly enhanced expression of GLT1, in
433 the nucleus accumbens (Rao et al., 2015b). Altogether these data obtained in P rats suggest an
434 inverse correlation between GLT-1 expression and ethanol intake.

435 However here we did not observe any difference in GLT- expression during acute
436 withdrawal dependent rats compared with non dependent animals. In summary, our results
437 suggest that the higher efficacy of NAC observed in dependent rats would be related to the
438 decreased expression of the xCT transporter and not to a modification of GLT-1. This
439 assumption is based on the fact that we found only a decrease in xCT but not GLT-1 in the
440 dependent rats and no difference between our two groups of rats regarding the effects of
441 NAC.

442

443 *NAC properties on modulation of oxidative stress and inflammatory system*

444 In addition to its action on the glutamatergic system, NAC is also able to modulate the
445 oxidative stress and the inflammatory system. NAC administration leads to an increase in
446 intracellular antioxidant glutathione (GSH) and reduces oxidative stress. Indeed, NAC has

447 been found to restore a cocaine-induced decrease of glutathion levels in glial cells (Badisa et
448 al., 2015), while ethanol abstinence and NAC intake interact synergistically, improving serum
449 lipids and hepatic antioxidant defenses (Ferreira Seiva et al., 2009). The expression of the
450 glutathione-S-transferases, the enzyme that catalyzes the conjugation of the reduced form of
451 GSH to ROS for the purpose of detoxification, is altered in ethanol-naive ethanol-preferring
452 rats compared with non-preferring ones (Liang et al., 2004, Bjork et al., 2006). Moreover, the
453 activity of blood glutathione-S-transferase is reduced in alcohol-dependent male patients
454 (Peter et al., 2013), revealing the importance of the redox balance in ethanol addiction (for a
455 review see (Uys et al., 2014). Moreover, an anti-oxidative effect could be linked to an anti-
456 addictive effect. For example, it has been demonstrated that oxidative stress plays a role in the
457 reinforcing properties of substances of abuse as enhancement of ROS in the nucleus
458 accumbens contributes to the reinforcing effect of cocaine (Jang et al., 2015) and
459 methamphetamine (Jang et al., 2016).

460 Regarding ethanol-induced neuroinflammation, elevation of TNF α and IL-6 are correlated
461 with high levels of craving in humans (Leclercq et al., 2012) and studies have shown that
462 chronic administration of NAC during abstinence helps to prevent increasing
463 proinflammatory cytokines (Schneider et al., 2017). Therefore, NAC could limit relapse and
464 somatic disorders by acting simultaneously on these 3 systems: glutamate homeostasis,
465 oxidative stress and inflammation.

466 Two other recent preclinical studies underscore the relevance of NAC in the context of
467 ethanol addiction. First, NAC treatment blocks the development of ethanol-induced
468 behavioral sensitization (Morais-Silva et al., 2016). Second, abstinence from ethanol induces
469 an increase in leptin and corticosterone levels in rats consuming alcohol by gavage for 30
470 days (2g/kg; twice a day), while NAC treatment prevents these biochemical changes
471 (Schneider et al., 2015). Thus, given that increased corticosterone concentration is a stress

472 indicator and that elevated levels of leptin are correlated with a high level of craving in
473 patients (Kiefer et al., 2001), this suggests that NAC could act to reduce stress and craving
474 observed during alcohol abstinence.

475 *Conclusion*

476 Overall, the current findings demonstrate that NAC reduces ethanol self-administration,
477 motivation, craving and reacquisition after abstinence in relevant preclinical model of alcohol
478 addiction. NAC was also effective in reducing sucrose self-administration thus indicating that
479 it may also have a more general effect on the reward system. The present data confirm and
480 extend previous studies showing that NAC is a promising pharmacological therapy for
481 addiction in general, but especially for ethanol addiction, which is characterized by enduring
482 changes in glutamatergic transmission. Our results strongly support clinical development of
483 NAC for alcohol use disorders and this is further supported by recent findings indicating that
484 NAC may be effective at reducing consumption of alcohol by ~30% among treatment-seeking
485 adults with cannabis use disorder (Squeglia et al., 2018).

486

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490 **Author's contribution:**

491 SL, CV, MCGM and MN were responsible for the study concept and design. SL,
492 MCGM and CV contributed to the acquisition of animal data. SL, MCGM and CV assisted
493 with data analysis and interpretation of findings. SL and CV drafted the manuscript. MN
494 provided critical revision of the manuscript for important intellectual content. All authors
495 critically reviewed content and approved final version for publication.

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698
699

700 Legends

701 **Figure 1: Timeline of experimental procedures on the effect of NAC on ethanol self-**
702 **administration:** The procedure was performed in dependent rat following CIE procedure for
703 10 weeks. NAC or saline was always injected i.p. one hour before the 30-min session ethanol
704 20% self-administrations, 8 hours after retrieval from the inhalation chambers. All along the
705 experiments, during the two weeks recovery and during NAC testing, rats were maintained
706 under the CIE procedure and were submitted to sessions of ethanol self-administration five-
707 days a week, at 8h withdrawal. The sole periods without the CIE procedure was during the
708 two one-month abstinence period. PR: Progressive Ratio

709
710 **Figure 2: N-acetylcysteine reduced ethanol self-administration:** Effect of an i.p. injection
711 of NAC (0, 25, 50 or 100 mg/kg; 1h before the test; n=17/group) on 20% ethanol self-
712 administration. **A.** Total active lever presses were significantly lower for the NAC 25, 50 and
713 100 mg/kg group compared with saline group, and there was no effect on inactive lever
714 presses. **B.** Rats treated with NAC 25, 50 and 100 mg/kg consumed significantly less ethanol
715 than saline-treated rats. ***: $p < 0.001$, **: $p < 0.01$. c; **C.** Total inactive lever presses were not
716 different for the NAC 25, 50 and 100 mg/kg group compared with saline group **D.** N-
717 acetylcysteine was more effective in dependent rats than in binge-drinking rats: Efficacy of an
718 acute injection of NAC (25, 50 or 100 mg/kg; i.p., 1h before the test) on ethanol self-
719 administration in dependent (n=17) vs binge-drinking rats (n=15). For each dose tested, the
720 percent of baseline EtOH consumption was significantly lower in dependent compared with
721 binge-drinking rats. In binge-drinking rats, an acute injection of 100 mg/kg NAC was
722 necessary to observe a reduction in EtOH consumption whereas in dependent rats, an
723 injection of 25 mg/kg was sufficient. **: $p < 0.01$, *: $p < 0.05$ compared with binge-drinking
724 group.

725
726 **Figure 3: N-acetylcysteine decreased the reinforcing efficacy of ethanol:** A. NAC 25
727 mg/kg-treated rats (n=8) pressed significantly fewer on the active lever during the PR session
728 (-55%) compared to saline-treated rats (n=9), while there was no effect on inactive lever
729 presses. B. NAC 25 mg/kg-treated rats displayed a significantly lower breaking point value
730 compared to saline-treated rats (-43%) **: $p < 0.01$, *: $p < 0.05$. **C.** Total inactive lever presses
731 were not different between groups.

732
733 **Figure 4: N-acetylcysteine 50 mg/kg decreased extinction responding:** A. NAC 25 mg/kg
734 (n=9) treated rats showed no alteration in extinction responding compared with saline (n=9).
735 B. NAC 50 mg/kg-treated rats (n=8) pressed significantly fewer on the active lever compared
736 to saline-treated rats C. There was no effect of NAC 25 mg/kg on inactive lever presses D.
737 There was no effect of NAC 50 mg/kg on inactive lever presses. ***: $p < 0.001$

738
739
740 **Figure 5: N-acetylcysteine 50 mg/kg reduced reacquisition after abstinence:** A. During
741 the reacquisition procedure, NAC 25 mg/kg (n=9) treated rats showed no alteration of
742 reacquisition of operant self-administration behavior compared with saline. B. NAC 50mg/kg
743 (n=11) treated rats showed a reduced reacquisition of operant self-administration behavior

744 compared with saline (- 57%). C. There was no effect of NAC 25 mg/kg on inactive lever
745 presses D. There was no effect of NAC 50 mg/kg on inactive lever presses. E: NAC 25 mg/kg
746 treated rats showed no alteration in ethanol intake during the reacquisition session compared
747 with Saline Group. F. NAC 50 mg/kg treated rats showed a decrease in ethanol intake during
748 the reacquisition session (-59%). *: $p < 0.05$ compared with Saline Group.

749

750

751 **Figure 6: N-acetylcysteine 100 mg/kg reduced 1% sucrose self-administration.** In 14 rats
752 that self-administered 20% ethanol for eight weeks during 15-min daily sessions (as described
753 in Lebourgeois et al., 2017), NAC (100 mg/kg) was tested on 1% sucrose self-administration.
754 NAC treatment was very effective in reducing the number of active lever presses for 1%
755 sucrose, *** $p < 0.001$.

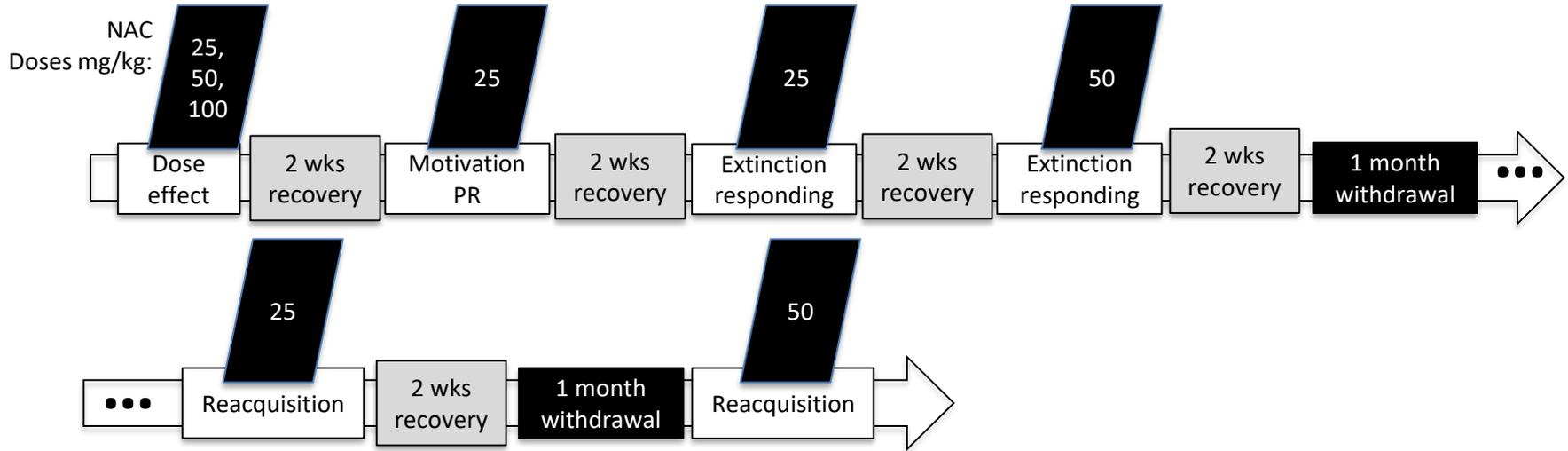
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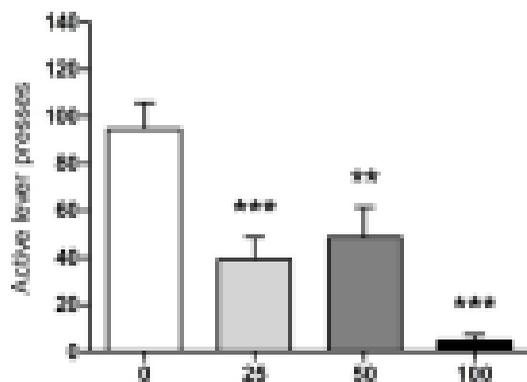
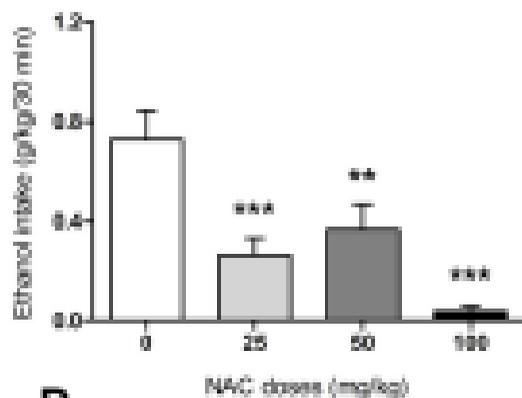
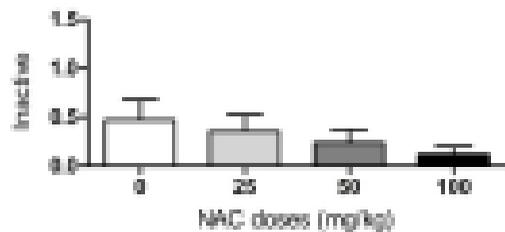
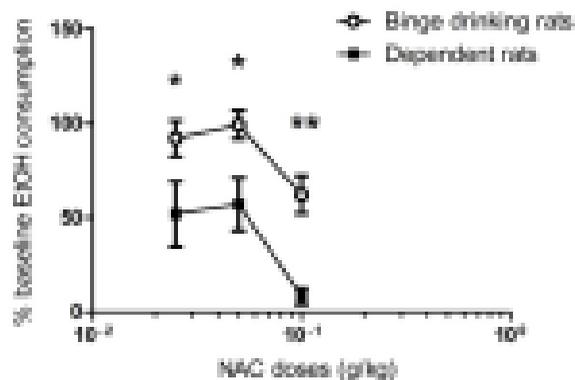
757 **Figure 7: Western blot of xCT and GLT-1 expression in nucleus accumbens of**
758 **dependent versus naive rats, following N-acetylcysteine 100 mg/kg treatment.** A.
759 Dependent rats showed a -18% decrease of xCT expression compared with nondependent rats
760 that have never been exposed to ethanol. There was no effect of NAC treatment. $n = 6$ rats
761 per groups. *: $p < 0.05$: Main “Dependence” effect, dependent versus non dependent rats.
762 Immunoblot for xCT protein yielded a band of approximately 56 kDa. Below are
763 representative xCT and actin blots. B. NAC-treated rats showed a -28% decrease of GLT-1T
764 expression compared with non-treated rats, whatever they were dependent or not. $n = 6$ rats
765 per groups. *: $p < 0.05$: Main “NAC” effects, NAC-treated rats versus non-treated rats.
766 Immunoblot for xCT protein yielded a band of approximately 63 kDa. Below are
767 representative GLT-1 and actin blots.

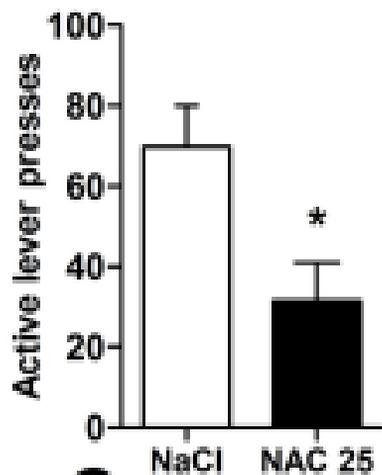
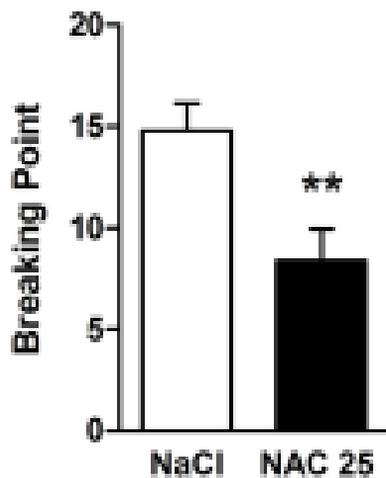
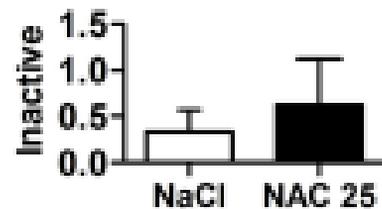
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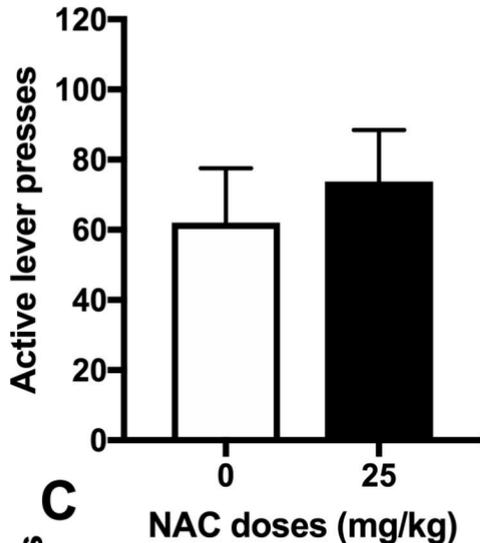
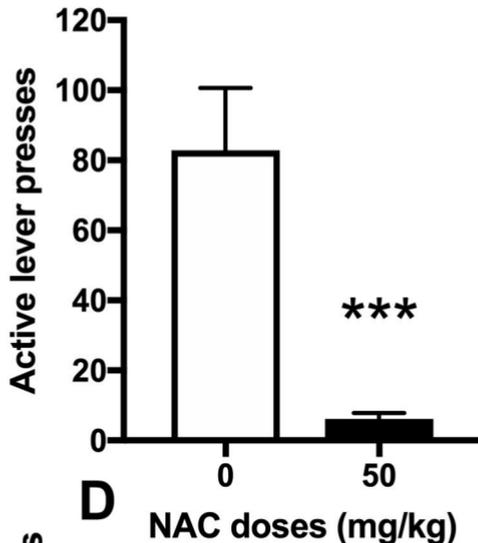
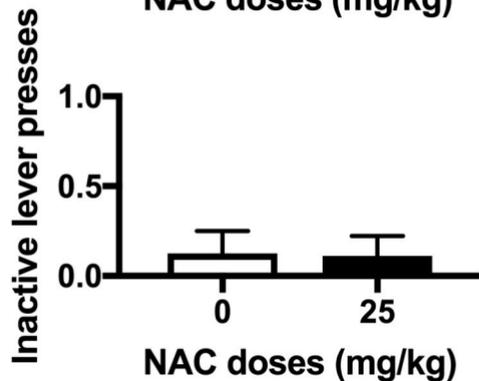
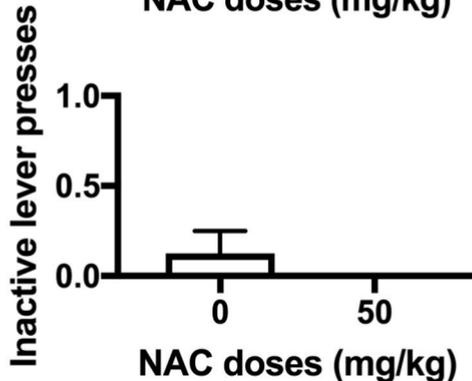
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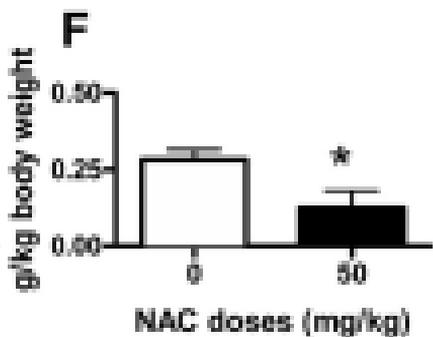
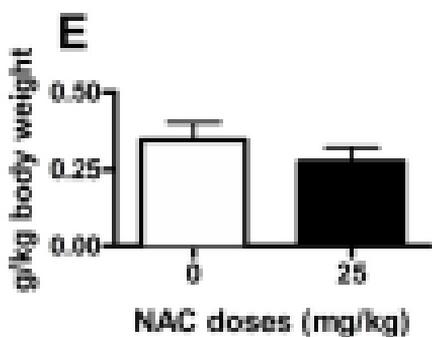
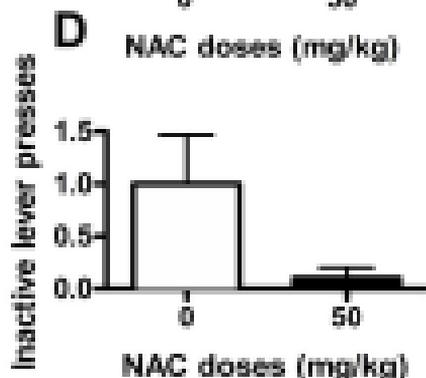
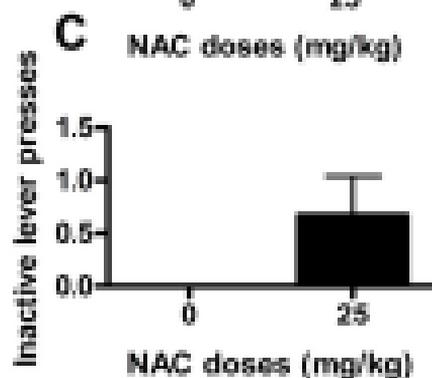
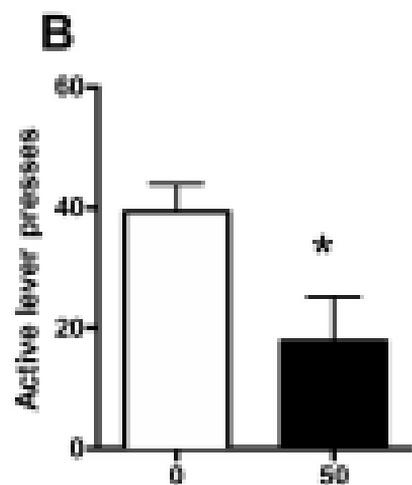
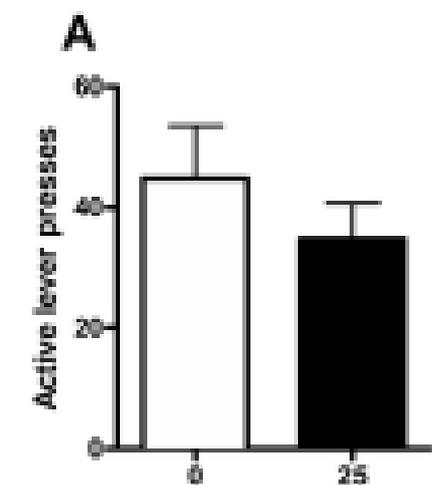
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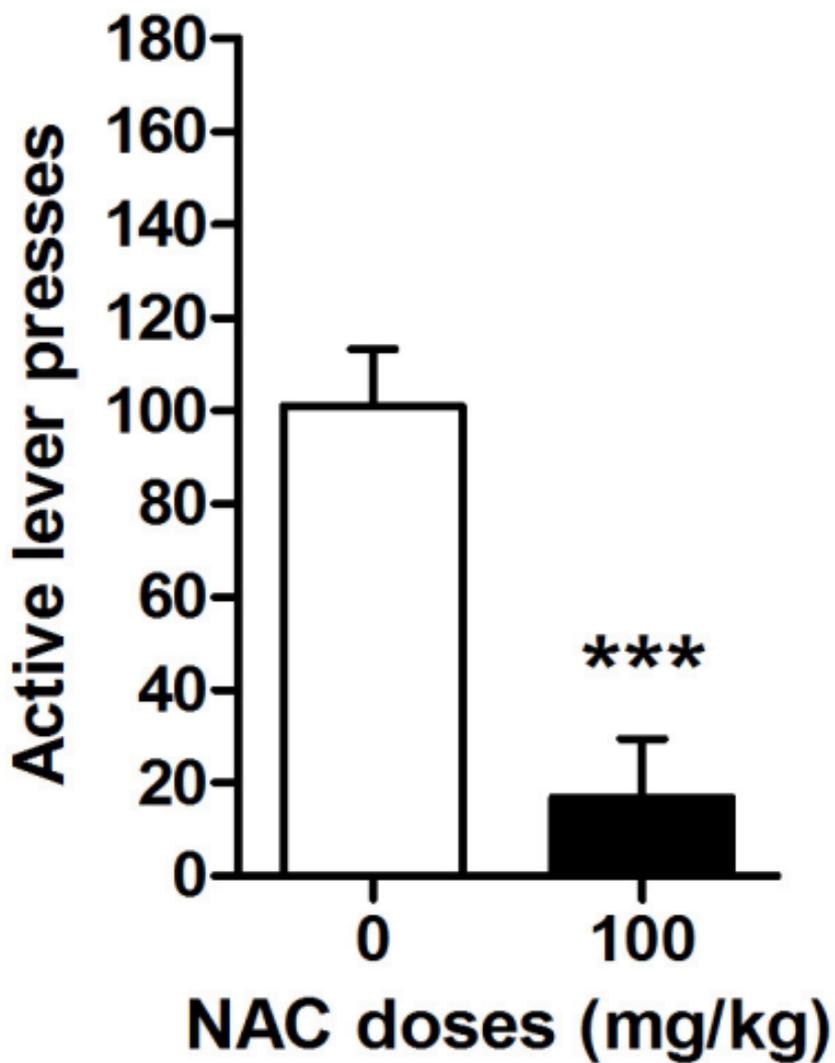


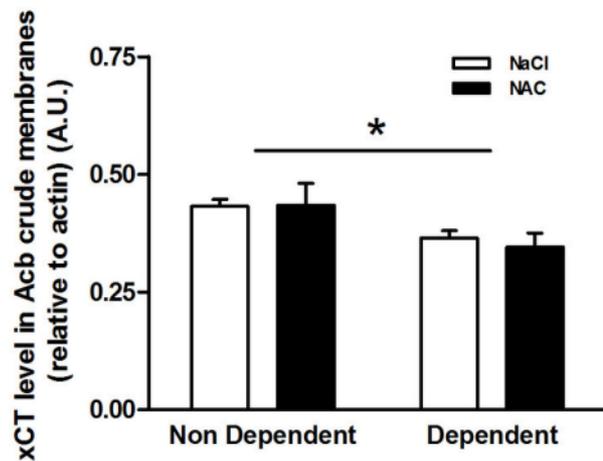
A**B****C****D**

A**B****C**

A**B****C****D**





ANon DependentDependent

NaCl

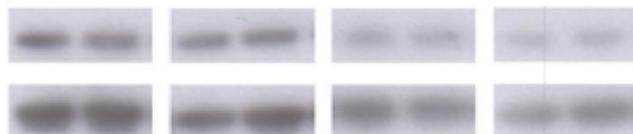
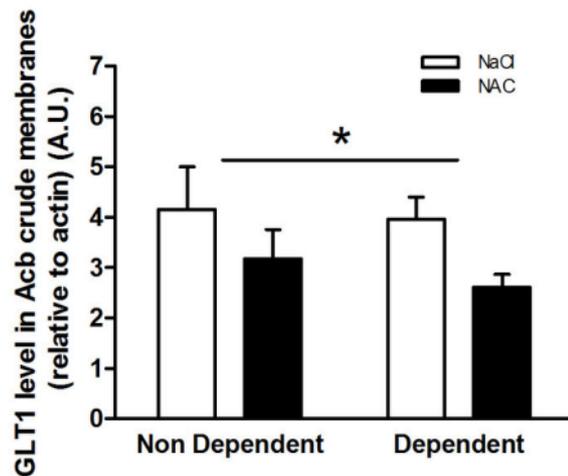
NAC

NaCl

NAC

xCT

Actin

**B**Non DependentDependent

NaCl

NAC

NaCl

NAC

GLUT1

Actin

