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4   # Sex differences in the immediate extinction deficit and renewal of

5   # extinguished fear in rats

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Abstract

Extinction learning is central to exposure-based behavioral therapies for reducing fear and anxiety in humans. However, patients with fear and anxiety disorders are often resistant to extinction. Moreover, trauma and stress-related disorders are highly prone to relapse and are twice as likely to occur in females compared to males, suggesting that females may be more susceptible to extinction deficits and fear relapse phenomena. In this report, we tested this hypothesis by examining sex differences in a stress-induced extinction learning impairment, the immediate extinction deficit (IED), and renewal, a common form of fear relapse. In contrast to our hypothesis, there were no sex differences in the magnitude of the immediate extinction deficit in two different rat strains (Long-Evans and Wistar). However, we did observe a sex difference in the renewal of fear when the extinguished conditioned stimulus was presented outside the extinction context. Male Wistar rats exhibited significantly greater renewal than female rats, a sex difference that has previously been reported after appetitive extinction. Collectively, these data reveal that stress-induced extinction impairments are similar in male and female rats, though the context-dependence of extinction is more pronounced in males.

Introduction

Clinical disorders caused by trauma exposure (e.g., post-traumatic stress disorder, PTSD) afflict millions of men and women in the United States every year [1]. Importantly, women are twice as likely as men to develop PTSD, suggesting that biological sex may influence the neural and behavioral processes underlying the pathophysiology of stress- and trauma-related disorders [2]. Consistent with this, a substantial body of work has revealed sex differences in learning and

45 memory processes that may contribute to the development and maintenance of PTSD. For

46 example, there are robust sex differences in Pavlovian fear conditioning in rats [3–5]. In this form

47 of learning, a neutral conditioned stimulus (CS), such as an acoustic tone, is arranged to precede

48 and predict a noxious unconditioned stimulus (US), such as an electric footshock. After as little as

49 a single conditioning trial, presentation of the CS (or placement in the conditioning context) elicits

50 anticipatory, conditioned fear responses (CRs), including freezing behavior [6–9]. Several studies

51 have revealed that male rats exhibit greater acquisition of conditioned freezing to the

52 environmental stimuli associated with shock (i.e., the conditioning context) relative to females

53 [5,10–15], whereas freezing to discrete auditory CSs is similar in males and females [5,15,16].

54 Interestingly, there are also sex differences in hippocampal and amygdala synaptic plasticity

55 mechanisms thought to underlie these forms of learning [5,17]. These results suggest that sex

56 differences in the neural and behavioral mechanisms of aversive learning and memory may

57 contribute to the pathophysiology of PTSD.

58 Another form of learning implicated in the maintenance of PTSD is extinction. Extinction

59 is a form of learning in which repeated non-reinforced presentations of a conditioned stimulus

60 (CS) reduce the magnitude reduction of the CRs acquired during Pavlovian conditioning [18,19].

61 Importantly, extinction is central to cognitive-behavioral therapies for PTSD, including prolonged

62 exposure therapy [20]. In prolonged exposure therapy, trauma-related stimuli are repeatedly

63 presented within a safe setting until those stimuli no longer elicit fear. Although extinction-based

64 therapy can be highly effective at reducing pathological fear, the durability of extinction memory

65 can be compromised by a variety of factors [21,22]. For instance, suppression of a CR after

66 extinction is typically limited to the setting or context in which extinction learning occurred,

67 resulting in renewal of the CR outside of the extinction context [23]. Furthermore, stress (acute or

68   chronic) can considerably reduce extinction learning and long-term retention, thereby fear relapse

69   [24]. For example, the "immediate extinction deficit" (IED) occurs when extinction training is

70   conducted shortly after fear conditioning (a stressor), rendering impairments in long-term

71   extinction memory and relapse of conditioned fear [25,26]. Interestingly, immediate extinction

72   occurs against the background of high levels of generalized contextual fear, which has been

73   proposed to play an important role in the IED [26] (though see [27]).

74   Although extinction learning plays a central role in behavioral therapies for trauma- and

75   stressor-related disorders, the interaction of sex differences in contextual fear conditioning, on the

76   one hand, with extinction learning and memory, on the other, is unclear. In addition to sex

77   differences in contextual fear conditioning, there is considerable evidence that stress-related neural

78   circuitry in females are more sensitive to those in males [28–31]. These sex differences might

79   make females more susceptible to stress-induced extinction learning impairments, such as the IED.

80   Moreover, sex differences in contextual conditioning and stress responsivity might influence the

81   relapse of extinguished fear, including renewal. Therefore, in this study, we characterized the IED

82   in male and female rats using two common laboratory rat strains (Long Evans and Wistar) and

83   standard Pavlovian fear conditioning and extinction procedures. Surprisingly, we found that there

84   was no sex difference in the IED in either rat strain; both male and female rats exhibited poor

85   extinction retention relative to animals undergoing delayed extinction and exhibited similar levels

86   of conditioned freezing compared with non-extinguished controls. In contrast, male Wistar rats

87   showed greater renewal of fear to an extinguished CS in a novel context compared to females. Sex

88   differences in renewal were not due to greater contextual fear in male rats. These results reveal

89   that male and female rats are similarly susceptible to stress-induced extinction impairments, but

90   that males may be more susceptible to fear renewal.

91

Materials and methods

Ethics statement

94 This study was carried out in strict accordance with the recommendations of the National Institutes

95 of Health and Texas A&M University. These experiments were approved by the Texas A&M

96 University Animal Care and Use Committee (Animal Use Protocol Number: 2020-0305).

Subjects

98 A total of 64 adult female and male Long-Evans and Wistar rats were used in this study. Long-

99 Evans Blue Spruce rats (male, n = 16; female, n = 16) were obtained from Envigo (Indianapolis,

100 IN) and weighed 200-224 g upon arrival. Wistar rats (male, n = 16; female, n = 16) were bred in-

101 house; they were 11-16 weeks of age upon behavioral testing. The Wistar rats used in this study

102 were derived from *Crh*-Cre rats [32] obtained from the laboratory of Robert Messing at the

103 University of Texas at Austin. These rats were bred in our laboratory with commercially

104 supplied wild-type Wistar rats (Envigo, Indianapolis, IN). The Wistar rats were genotyped and

105 randomly assigned to groups (extinction type) within each litter; both Cre$^+$ and Cre$^-$ rats were

106 included in the experiments and their behavior was identical. All rats were individually housed in

107 a temperature- and humidity-controlled vivarium, with a 14:10 hour light/dark cycle and ad

108 libitum access to food and water. Behavioral testing was conducted during the light phase. Rats

109 were handled 1 minute per day for 5 days prior to testing to acclimate them to the experimenter.

Procedure

111 All behavioral procedures took place in a standard rodent conditioning chamber with two

112 aluminum walls, two Plexiglas walls and a Plexiglas ceiling (Med Associates, St. Albans, VT).

113   The chamber was outfitted with a speaker mounted to the upper corner of one wall for delivery of

114   auditory stimuli. The grid floor was composed of stainless-steel rods for delivery of scrambled

115   footshock. Load-cell force transducers located underneath each chamber measured displacement

116   of the chamber in response to motor activity; these voltages (+/-10 V) were acquired at 5 Hz and

117   transformed to absolute values (scale of 0-100). A freezing bout was defined as five consecutive

118   values below 10 (freezing threshold, corresponding to one second of freezing).

119   To assess the IED, we conducted separate experiments in Long-Evans and Wistar rats. In

120   the first experiment, male and female Long-Evans rats underwent either immediate or delayed

121   extinction after auditory fear conditioning. Extinction was followed by a retention test conducted

122   24 hours later. All the behavioral procedures were conducted in the same context (Context A).

123   Animals were transported from the vivarium to the laboratory in black plastic boxes. A metal pan

124   beneath the grid floor of each conditioning chamber was cleaned with a 1% ammonium solution.

125   The room housing the conditioning chambers was illuminated with red light and fans affixed to

126   each chamber were turned on. Doors of the outer sound attenuating cabinets were closed.

127   Conditioning consisted of a 3-min baseline period followed by 5 CS-US pairings. The CS was a

128   10-s white noise (80 dB) paired with a 2-s, 1-mA footshock unconditioned stimulus (US). The

129   intertrial interval (ITI) was 70 seconds. After conditioning, animals were placed in the transport

130   boxes and returned to the vivarium unless undergoing immediate extinction, in which case they

131   remained in the transport boxes until the immediate extinction session began. Either 15-min or 24-

132   hr later, the animals were returned to the conditioning chambers for immediate or delayed

133   extinction, respectively. Extinction consisted of a 3-min baseline period followed by 45 CS-alone

134   presentations (40-s ITI). All animals were returned to the chamber again 48-hr after conditioning

135   for a retrieval test consisting of a second extinction session.

136   In the second experiment, we examined the IED in Wistar rats. Because the IED has not

137   previously been studied in Wistar rats, we used a no-extinction control similar to our previously

138   published work in Long-Evans rats [25]. The conditioning procedures were identical to those used

139   for the Long-Evans rats, except for the use of a stronger footshock US (2 mA). After conditioning,

140   animals received either an immediate or delayed extinction procedure or were simply placed in the

141   conditioning chambers (no-extinction control); extinction retrieval testing was conducted 48 hours

142   after. Conditioning and extinction procedures were conducted in Context A. After the retrieval

143   test, all animals underwent a second extinction session (re-extinction) then were randomly

144   assigned to a retrieval test (5 CS-only presentations) in either Context A (SAME, the extinction

145   context) or Context B (DIFF, a novel context). Animals tested in Context B were transported from

146   the vivarium to the laboratory in white transport boxes. For Context B, the conditioning chambers

147   had a metal pan beneath the grid floor that was cleaned with a 3% acetic acid solution, and the

148   room was illuminated with standard white light. Fans affixed to the sound attenuating cabinets

149   were turned off and doors to the cabinets were left open.

Statistics

151   Data were analyzed with StatView software (SAS Institute). Results are displayed as mean ±

152   standard error the mean (SEM). Analysis of variance (ANOVA) was used to assess percentage of

153   time freezing with repeated measures of trial ($\alpha = 0.05$).

154

Results

Both male and female rats display the immediate extinction deficit

157   We first sought to determine if both male and female rats are similarly susceptible to the IED, a

158   stress-induced extinction impairment (Fig 1A). To this end, Long-Evans rats underwent standard

159   auditory fear conditioning which consisted of 5 CS-footshock (US) pairings. All rats acquired fear

160   to the CS [main effect of Trials: $F_{5,\,135} = 99.8$, $p < .0001$] and there were no differences between

161   males and females [no main effect of Sex: $F_{1,\,27} = 0.60$, $p = 0.45$; Trials x Sex interaction: $F_{5,\,135} =$

162   0.46, $p = .81$] (Fig 1B; Conditioning). Rats next underwent fear extinction either 15 minutes

163   (Immediate) or 24 hours (Delayed) later in the same context, which consisted of 45 CS-alone trials.

164   We found that rats extinguished immediately following fear conditioning showed impaired within-

165   session extinction compared to animals extinguished 24 hours later [main effect of Ext Type: $F_{1,}$

166    $_{27} = 6.21$, $p = 0.02$; Trials x Ext Type interaction: $F_{9,\,243} = 2.87$, $p = 0.003$] (Fig 1B; Extinction).

167   Forty-eight hours after conditioning, all animals were brought back to the extinction context and

168   tested for extinction memory with another 45 CS-alone trials. During retrieval, both groups froze

169   at similar levels to the first five trials, however, following this the Delayed animals reduced their

170   freezing, demonstrating good extinction memory, while high freezing in the Immediate group

171   persisted [Trials x Ext Type interaction: $F_{8,\,216} = 2.90$, $p = .004$] (Fig 1B; Retrieval). Importantly,

172   although females showed a slightly faster reduction in freezing compared to males [$F_{8,\,216} = 1.72$,

173    $p = 0.09$], the immediate extinction procedure produced a similar deficit in extinction retention in

174   both male and female rats [Trials x Ext Type x Sex interaction: $F_{8,\,216} = 0.274$, $p = 0.97$]. These

175   data suggest that male and female Long-Evans rats are similarly susceptible to the immediate

176   extinction deficit.

177

178   **Fig 1. Male (♂□) and female (♀□) Long-Evans exhibit the IED. (A)** Behavioral timeline for

179   the different experimental groups. **(B)** Freezing data show that Long-Evans rats acquired

180 equivalent levels of conditional fear to the auditory CS (Conditioning). Both Immediate and

181 Delayed groups showed a marked reduction in fear to the CS throughout fear extinction, with

182 Delayed animals showing lower levels of fear at the end of extinction training (Extinction).

183 Although all groups of animals showed equivalent levels of fear early in the retrieval test (Block

184 1), immediately extinguished animals demonstrated a reduced rate of re-extinction compared to

185 Delayed animals, indicative of impaired extinction memory (Retrieval). This IED was similar

186 between male and females, though, females showed faster rate of re-extinction compared to males

187 in both groups. All error bars represent ± SEM.

188

189   There are some inconsistencies in the reported findings of sex differences in learning and

190 memory paradigms and it has been suggested that some of these findings may be strain specific

191 [33]. We thus sought to replicate the above findings using a different strain of rats, while adding

192 control groups that do not undergo fear extinction (Fig 2A). Equal numbers of Cre$^+$ and Cre$^-$ male

193 and female Wistar rats (see the *Materials and Methods* section for more detail) were used for the

194 following experiments. Throughout all statistical comparisons, we observed no significant effects

195 of genotype and thus chose to collapse data across genotype to improve statistical power. All rats

196 were first conditioned as previously described and all rats developed conditional responding to the

197 CS [main effect of Trials: $F_{5, 140} = 50.24, p < 0.0001$] (Fig 2B). Female rats showed slightly higher

198 freezing compared to males [main effect of Sex: $F_{1, 28} = 8.65, p < 0.0065$] and, although Immediate

199 and Delayed groups displayed slightly different learning curves [Trial x Ext Type interaction: $F_{5, 140} = 5.01, p = 0.0003$], they displayed equivalent total amounts of freezing [no main effect of Ext

200

201 Type: $F_{1, 28} = 0.02, p = 0.90$] (Fig 2B). Following conditioning, rats either underwent extinction

202 15 minutes (Immediate-Ext) or 24 hours (Delayed-Ext) after conditioning, or they were merely re-

203   exposed to the context 15 minutes (Immediate-NoExt) or 24 later hours (Delayed-NoExt) (Fig

204   2A). Although female rats showed more freezing during conditioning, we did not observe any sex

205   differences during extinction [no main effect of Sex: $F_{1, 24} = 0.03$, $p = 0.86$; Block x Sex interaction:

206    $F_{9, 216} = 0.61$, $p = 0.79$; Ext Type x Sex interaction: $F_{1, 24} = 0.61$, $p = 0.44$; or Block x Ext Type x

207   Sex interaction: $F_{9, 216} = 0.76$, $p = 0.66$]. Animals that underwent extinction showed very high

208   levels of CS-evoked freezing compared to control animals that were exposed to the context [main

209   effect of Ext: $F_{1, 24} = 68.38$, $p < 0.0001$; Block x Ext interaction: $F_{9, 216} = 17.21$, $p < 0.0001$] (Fig

210   2C-D). Like the previous experiment, all groups showed equally high freezing during the first 5

211   trials of retrieval testing; however, delayed extinction animals exhibited a clear decrease in

212   freezing in the second block of 5 trials whereas high freezing persisted in all other groups [Block

213   x Ext x Ext Type interaction: $F_{8,192} = 3.50$, $p = 0.0009$] (Fig 2C-D). This effectively demonstrates

214   that undergoing extinction training immediately following conditioning impairs extinction

215   retention to that of animals that never underwent extinction at all. Importantly, we once again show

216   no sex differences in the IED when comparing across extinction groups [no Block x Ext x Ext

217   Type x Sex interaction: $F_{8,192} = 1.88$, $p = 0.065$] or in any other comparison during retrieval testing

218   (all p-values > .09). Collectively, we show that male and female rats, across strains, are equally

219   susceptible to stress-induced extinction impairments.

220

221   **Fig 2. Male (♂□) and female (♀□) Wistar rats exhibit an IED. (A)** Behavioral timeline for the

222   different experimental groups. **(B)** All groups acquired similar levels of conditioned freezing prior

223   to extinction. **(C)** Wistar rats that underwent extinction training showed a reduction in fear across

224   extinction trials with immediately extinguished rats reaching lower levels of freezing than delayed

225   animals. Despite this, immediate extinction animals still showed impaired extinction during

226   retrieval testing as indicated by reduced rate of re-extinction. This effect was similar in both males

227   and females. **(D)** Comparatively, No-Extinction animals exhibited high levels of conditioned

228   freezing during retrieval testing that was similar among all groups. All error bars represent ± SEM.

229

230   Male, but not female, rats exhibit renewal of extinguished fear

231   Similar to stress-induced impairments in extinction learning, contextual processing is thought to

232   be central to trauma-related disorders such as PTSD [34,35]. Previous work has demonstrated sex

233   differences in the renewal of extinguished CRs after appetitive conditioning [36], however, it is

234   currently unknown if this is true for aversively conditioned CSs. To test this, Wistar rats from the

235   previous experiment underwent an additional extinction session to completely extinguish any

236   remaining fear (Fig 3). The animals were then reassigned to new groups for renewal testing and

237   were counterbalanced for previous group assignments. After the second extinction session, male

238   and female rats were either placed back into the extinction context (A; retrieval context) or they

239   were placed into a novel context (B; renewal context) where they were presented 5 CS-alone trials.

240   During the second extinction session all rats showed moderate levels of fear during the first five-

241   trial block but quickly reduced freezing to baseline levels [main effect of trials: $F_{9,252} = 34.09$, $p <$

242   0.0001], and there were no sex or group differences during extinction [Trials x Sex interaction:

243    $F_{9,252} = 1.579$, $p = 0.12$; Trials x Context interaction: $F_{9,252} = 1.169$, $p = 0.32$; Trials x Sex x Context

244   interaction: $F_{9,252} = 0.654$, $p = 0.75$] (Fig 3; Extinction II). During testing, both male and female

245   rats placed back into the extinction context showed very low levels of freezing. However, males,

246   but not females, placed into the novel context showed a renewal of conditioned freezing [Trials x

247   Sex x Context interaction: $F_{5,140} = 2.60$, $p = 0.028$], particularly during the first two trials (Fig 3;

248   Renewal Test). These data demonstrate that female rats do not display renewal of freezing to an

249 extinguished aversive CS. We suggest that differences in the contextual regulation of aversive

250 memory such as these may underlie susceptibilities to anxiety- and trauma-based disorders.

251

252 **Fig 3. Male (♂□) but not female (♀□) Wistar rats exhibit renewal of extinguished fear.**

253 Freezing data showing that male and female Wistar rats displayed similar levels of fear during a

254 second extinction session after the prior extinction retrieval test (left). However, only male rats

255 displayed fear renewal the following day when presented the extinguished CS in a novel context

256 (DIFF, right) relative to animals tested in the extinction context (SAME). This was particularly

257 evident during the first two trials of testing. All error bars represent ± SEM.

258

Discussion

259

260 In this study we investigated sex differences in failures of extinction learning and retrieval. We

261 found that male and female rats are equally susceptible to the IED—a stress-induced extinction

262 impairment— in two different strains of animals. Interestingly, we also show that females, but not

263 males, exhibit impairments in the renewal of extinguished fear, suggestive of a sex difference in

264 contextual processing. This finding is in line with previous work showing sex differences in the

265 renewal of appetitive conditioning [36]. Our work extends previous literature and suggests that

266 differences in contextual processing may be a critical factor accounting for the disparity in

267 susceptibility for stress and trauma-related disorders across sexes.

268   Although both male and female rats show comparable deficits in immediate extinction,

269 female rats showed a more rapid (but non-significant) reduction in conditioned freezing during

270 retrieval testing, regardless of the extinction procedure (immediate or delayed). It is unlikely that

271 this rate difference is a consequence of sex differences in conditioning insofar as Long-Evans rats

272 showed no sex differences in conditioning, whereas Wistar females showed greater levels of

273 freezing during conditioning. A potential explanation for sex differences in the (re)extinction of

274 fear is estrous cycle phase. For example, extinction is impaired in females that undergo extinction

275 training during metestrus but enhanced in females trained in estrus or proestrus [37]. Ovarian

276 steroids underlie the more rapid reduction in freezing during extinction retrieval in females [38,39],

277 which was observed under both low- and high-stress conditions. This suggests that stress does not

278 block the adaptive effects of high estrogen and progesterone on retrieval facilitation. From another

279 perspective, however, changes in hormonal state across extinction training and retrieval testing in

280 females might be expected to impair extinction retrieval and facilitate renewal because extinction

281 retrieval is highly context-dependent [23]. Yet the opposite was true: female rats showed superior

282 extinction retrieval and did not exhibit renewal (at least in Wistar rats). It would be important in

283 future work to examine the role that estrous cycle and gonadal steroids may play in these processes.

284 　　　　Given that the IED is a stress-induced deficit driven by the locus coeruleus (LC) [26,40,41],

285 a sexually-dimorphic structure [28], it is perhaps surprising that we did not observe sex differences

286 in the IED. Recent work has revealed that the IED is driven by LC-derived norepinephrine (LC-

287 NE) which excites neurons in the basolateral nucleus of the amygdala (BLA) [40,42,43] and, in

288 turn, impairs the infralimbic (IL) division of the medial prefrontal cortex [44–46]. The IL is critical

289 to the formation of long-term extinction memories [47–49]. Although LC-NE release within the

290 prefrontal cortex enhances arousal and is necessary for successful extinction learning [50], high

291 levels of LC-NE may act to impair prefrontal function [40,51]. Substantial work has demonstrated

292 that LC neurons in females compared to males are genetically distinct [52], have greater dendritic

293 morphology [53], and are 10-30x more sensitive to activation by corticotropin-releasing factor

294 (CRF), irrespective of cycling hormones [54]. Moreover, the central nucleus of the amygdala

295   (CeA) is a large source of CRF input that is directly excitatory to LC-NE neurons [55–59], and

296   CeA CRF+ cells have recently been shown to be necessary and sufficient to drive the IED [60].

297   All of this would suggest that females should be highly susceptible to stress-induced extinction

298   impairments. Nonetheless, it has also been found that prior stress sensitizes LC neurons in male

299   rats [61,62], but not females, which abolishes sex differences in LC sensitivity [54]. Thus, we

300   suggest that high levels of physiological stress (such as that from fear conditioning) may result in

301   a ceiling effect such that male and female rats are equally susceptible to stress-induced

302   impairments in extinction learning. Given this, it would be worthwhile to know if female rats are

303   susceptible to the IED under conditions that are typically not stressful enough to cause extinction

304   impairments in male rats, such as weaker shocks or fewer conditioning trials [40,63]. Although

305   acute stress from fear conditioning in rodents does not appear to result in obvious sex differences

306   in the IED procedure, it is still possible that the wide-range of sex differences in the LC-NE system

307   plays a central role in the development of chronic stress and/or psychiatric disorder, such as PTSD,

308   in humans.

309   Our finding that male, but not female, rats exhibit renewal of extinguished fear mirrors

310   work in both appetitive and aversive conditioning [36,64]. Anderson and Petrovich (2015)

311   investigated the renewal of food seeking using an ABA design in Long-Evans rats and found that

312   male, but not female, renewed previously extinguished conditional responding to the food cup

313   when tested outside of the extinction context [36]. They additionally found that renewal returned

314   in ovariectomized female rats that received estradiol replacement throughout behavioral training

315   and testing, but not in ovariectomized females without estradiol replacement. This suggests that

316   levels of estradiol may play a critical role in renewal in female rats [36]. Contrary to this, Hilz and

317   colleagues have shown that renewal in females only occurs when they are extinguished in the

318   proestrous phase (high levels of estrogen) and tested in metestrous/diestrous phase (low levels of

319   estrogen), suggesting that renewal in intact females relies on a shift in interoceptive hormonal state

320   [65,66]. Further adding to this theory, Park and colleagues show that female rats display renewal

321   at a juvenile age (P18) before hormone cycling begins [67,68]. In aversive conditioning, estrous

322   cycle phase has been shown to modulate fear renewal in female rats. Female rats that undergo

323   auditory fear extinction during metestrus and diestrus, but not in proestrus and estrus, show fear

324   renewal to the CS in a novel context [64]. Taken together, we suggest that renewal in female rats

325   depends heavily on both interoceptive and exteroceptive context.

326   Interestingly, a previous report found that adult female rats did exhibit fear renewal when

327   freezing in an alternate context is compared to the last five trials of extinction from the previous

328   day [69]. This is more akin to a different from of fear relapse called spontaneous recovery [70].

329   Moreover, the within-subjects comparison for extinction retrieval was conducted one hour after

330   renewal testing in all animals allowing for the possibility that renewal testing further extinguished

331   animals prior to retrieval testing [69]. Collectively, there is still much work to be done to determine

332   the factors that regulate sex differences in fear relapse after extinction.

333   Indeed, sex differences in brain areas critical for the context-dependence of extinction may

334   underlie differences in renewal. The hippocampal formation encodes and transmits spatial

335   information to various limbic regions, including the mPFC and amygdala, primarily through its

336   ventral subregion (vHPC) that is critical for guiding contextually appropriate behaviors. The vHPC

337   sends monosynaptic projections to the lateral and central nuclei of the amygdala [71,72], and a

338   dense feedforward inhibitory circuit to the mPFC [73,74], each of which have been implicated in

339   context-dependent fear renewal. Appetitive renewal similarly involves the activation of mPFC-

340   projecting vHPC neurons [75], modulation of the mPFC [76,77], and amygdala activation

341   [65,66,77]. Interestingly, sex differences in the recruitment of these neural circuits mirrored the

342   behavioral sex differences seen in appetitive renewal [65,66,75–77]. It seems likely that the failure

343   to recruit these circuits when confronted with an extinguished CS outside of the extinction context

344   underlies impaired renewal often observed in female rats.

345   In summary, we first show that both male and female rats are equally susceptible to the

346   IED despite well-known sex differences in the neural circuits underlying the IED. We speculate

347   that enhanced basal excitability of the LC-NE system in females may result in increased

348   susceptibility to mild stressors compared to male rats, whereas strong acute stressors result in the

349   sensitization of the male LC-NE system, abolishing potential sex differences in extinction learning.

350   We next show that female rats do not display context-mediated fear renewal, similar to reports in

351   appetitive literature. We argue that renewal of conditional responding in female rats may depend

352   heavily on interoceptive, in addition to exteroceptive, contexts. Changes in interoceptive context

353   are monitored by the insular cortex [78], and it's possible that insular projections to the amygdala

354   and para-hippocampal regions may gate the renewal of extinguished fear. Alternatively, cycling

355   hormones such as estradiol may modulate the excitability of limbic circuits underlying renewal.

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References

1. Kessler RC, Petukhova M, Sampson NA, Zaslavsky AM, Wittchen H-U. Twelve-month and lifetime prevalence and lifetime morbid risk of anxiety and mood disorders in the United States. Int J Methods Psychiatr Res. 2012;21: 169–184. doi:10.1002/mpr.1359

2. Steel Z, Marnane C, Iranpour C, Chey T, Jackson JW, Patel V, et al. The global prevalence of common mental disorders: a systematic review and meta-analysis 1980-2013. Int J Epidemiol. 2014;43: 476–493. doi:10.1093/ije/dyu038

3. Dalla C, Shors TJ. Sex differences in learning processes of classical and operant conditioning. Physiol Behav. 2009;97: 229–238. doi:10.1016/j.physbeh.2009.02.035

4. Day HLL, Stevenson CW. The neurobiological basis of sex differences in learned fear and its inhibition. Eur J Neurosci. 2020;52: 2466–2486. doi:10.1111/ejn.14602

5. Maren S, De Oca B, Fanselow MS. Sex differences in hippocampal long-term potentiation (LTP) and Pavlovian fear conditioning in rats: positive correlation between LTP and contextual learning. Brain Res. 1994;661: 25–34. doi:10.1016/0006-8993(94)91176-2

6. Pavlov PI. Conditioned reflexes: An investigation of the physiological activity of the cerebral cortex. Ann Neurosci. 1927;17: 136–141. doi:10.5214/ans.0972-7531.1017309

7. Maren S. Neurobiology of Pavlovian fear conditioning. Annu Rev Neurosci. 2001;24: 897–931. doi:10.1146/annurev.neuro.24.1.897

8. Fendt M, Fanselow MS. The neuroanatomical and neurochemical basis of conditioned fear. Neurosci Biobehav Rev. 1999;23: 743–760. doi:10.1016/S0149-7634(99)00016-0

9. LeDoux JE. Emotion circuits in the brain. Annu Rev Neurosci. 2000;23: 155–184. doi:10.1146/annurev.neuro.23.1.155

10. Wiltgen BJ, Sanders MJ, Behne NS, Fanselow MS. Sex differences, context preexposure, and the immediate shock deficit in Pavlovian context conditioning with mice. Behav Neurosci. 2001;115: 26–32. doi:10.1037/0735-7044.115.1.26

11. Gresack JE, Schafe GE, Orr PT, Frick KM. Sex differences in contextual fear conditioning are associated with differential ventral hippocampal extracellular signal-regulated kinase activation. Neuroscience. 2009;159: 451–467. doi:10.1016/j.neuroscience.2009.01.009

12. Ribeiro AM, Barbosa FF, Godinho MR, Fernandes VS, Munguba H, Melo TG, et al. Sex differences in aversive memory in rats: possible role of extinction and reactive emotional factors. Brain Cogn. 2010;74: 145–151. doi:10.1016/j.bandc.2010.07.012

396 13. Daviu N, Andero R, Armario A, Nadal R. Sex differences in the behavioural and
397   hypothalamic-pituitary-adrenal response to contextual fear conditioning in rats. Horm
398   Behav. 2014;66: 713–723. doi:10.1016/j.yhbeh.2014.09.015

399 14. Colon L, Odynocki N, Santarelli A, Poulos AM. Sexual differentiation of contextual fear
400   responses. Learn Mem. 2018;25: 230–240. doi:10.1101/lm.047159.117

401 15. Cossio R, Carreira MB, Vásquez CE, Britton GB. Sex differences and estrous cycle
402   effects on foreground contextual fear conditioning. Physiol Behav. 2016;163: 305–311.
403   doi:10.1016/j.physbeh.2016.05.026

404 16. Totty MS, Warren N, Huddleston I, Ramanathan KR, Ressler RL, Oleksiak CR, et al.
405   Behavioral and brain mechanisms mediating conditioned flight behavior in rats. Sci Rep.
406   2021;11: 8215. doi:10.1038/s41598-021-87559-3

407 17. Blume SR, Freedberg M, Vantrease JE, Chan R, Padival M, Record MJ, et al. Sex- and
408   Estrus-Dependent Differences in Rat Basolateral Amygdala. J Neurosci. 2017;37: 10567–
409   10586. doi:10.1523/JNEUROSCI.0758-17.2017

410 18. Bouton ME. Context and behavioral processes in extinction. Learn Mem. 2004;11: 485–
411   494. doi:10.1101/lm.78804

412 19. Bouton ME, Maren S, McNally GP. Behavioral and neurobiological mechanisms of
413   pavlovian and instrumental extinction learning. Physiol Rev. 2021;101: 611–681.
414   doi:10.1152/physrev.00016.2020

415 20. VanElzakker MB, Dahlgren MK, Davis FC, Dubois S, Shin LM. From Pavlov to PTSD:
416   the extinction of conditioned fear in rodents, humans, and anxiety disorders. Neurobiol
417   Learn Mem. 2014;113: 3–18. doi:10.1016/j.nlm.2013.11.014

418 21. Goode TD, Maren S. Animal models of fear relapse. ILAR J. 2014;55: 246–258.
419   doi:10.1093/ilar/ilu008

420 22. Bouton ME. Context, ambiguity, and unlearning: sources of relapse after behavioral
421   extinction. Biol Psychiatry. 2002;52: 976–986. doi:10.1016/s0006-3223(02)01546-9

422 23. Bouton ME, Bolles RC. Contextual control of the extinction of conditioned fear. Learn
423   Motiv. 1979;10: 445–466. doi:10.1016/0023-9690(79)90057-2

424 24. Maren S, Holmes A. Stress and fear extinction. Neuropsychopharmacology. 2016;41: 58–
425   79. doi:10.1038/npp.2015.180

426 25. Maren S, Chang C. Recent fear is resistant to extinction. Proc Natl Acad Sci USA.
427   2006;103: 18020–18025. doi:10.1073/pnas.0608398103

428   26.   Maren S. Nature and causes of the immediate extinction deficit: a brief review. Neurobiol
429   Learn Mem. 2014;113: 19–24. doi:10.1016/j.nlm.2013.10.012

430   27.   Merz CJ, Wolf OT. The immediate extinction deficit occurs in a nonemotional learning
431   paradigm. Learn Mem. 2019;26: 39–45. doi:10.1101/lm.048223.118

432   28.   Bangasser DA, Cuarenta A. Sex differences in anxiety and depression: circuits and
433   mechanisms. Nat Rev Neurosci. 2021;22: 674–684. doi:10.1038/s41583-021-00513-0

434   29.   Bangasser DA, Wiersielis KR, Khantsis S. Sex differences in the locus coeruleus-
435   norepinephrine system and its regulation by stress. Brain Res. 2016;1641: 177–188.
436   doi:10.1016/j.brainres.2015.11.021

437   30.   Bangasser DA, Wiersielis KR. Sex differences in stress responses: a critical role for
438   corticotropin-releasing factor. Hormones (Athens). 2018;17: 5–13. doi:10.1007/s42000-
439   018-0002-z

440   31.   Bale TL, Epperson CN. Sex differences and stress across the lifespan. Nat Neurosci.
441   2015;18: 1413–1420. doi:10.1038/nn.4112

442   32.   Pomrenze MB, Millan EZ, Hopf FW, Keiflin R, Maiya R, Blasio A, et al. A transgenic
443   rat for investigating the anatomy and function of corticotrophin releasing factor circuits.
444   Front Neurosci. 2015;9: 487. doi:10.3389/fnins.2015.00487

445   33.   Colom-Lapetina J, Begley SL, Johnson ME, Bean KJ, Kuwamoto WN, Shansky RM.
446   Strain-dependent sex differences in a long-term forced swim paradigm. Behav Neurosci.
447   2017;131: 428–436. doi:10.1037/bne0000215

448   34.   Liberzon I, Abelson JL. Context Processing and the Neurobiology of Post-Traumatic
449   Stress Disorder. Neuron. 2016;92: 14–30. doi:10.1016/j.neuron.2016.09.039

450   35.   Maren S, Phan KL, Liberzon I. The contextual brain: implications for fear conditioning,
451   extinction and psychopathology. Nat Rev Neurosci. 2013;14: 417–428.
452   doi:10.1038/nrn3492

453   36.   Anderson LC, Petrovich GD. Renewal of conditioned responding to food cues in rats:
454   Sex differences and relevance of estradiol. Physiol Behav. 2015;151: 338–344.
455   doi:10.1016/j.physbeh.2015.07.035

456   37.   Milad MR, Igoe SA, Lebron-Milad K, Novales JE. Estrous cycle phase and gonadal
457   hormones influence conditioned fear extinction. Neuroscience. 2009;164: 887–895.
458   doi:10.1016/j.neuroscience.2009.09.011

459   38.   Gupta RR, Sen S, Diepenhorst LL, Rudick CN, Maren S. Estrogen modulates sexually
460   dimorphic contextual fear conditioning and hippocampal long-term potentiation (LTP) in
461   rats. Brain Res. 2001;888: 356–365.

39. Nagaya N, Acca GM, Maren S. Allopregnanolone in the bed nucleus of the stria terminalis modulates contextual fear in rats. Front Behav Neurosci. 2015;9: 205. doi:10.3389/fnbeh.2015.00205

40. Giustino TF, Ramanathan KR, Totty MS, Miles OW, Maren S. Locus Coeruleus Norepinephrine Drives Stress-Induced Increases in Basolateral Amygdala Firing and Impairs Extinction Learning. J Neurosci. 2020;40: 907–916. doi:10.1523/JNEUROSCI.1092-19.2019

41. Totty MS, Payne MR, Maren S. Event boundaries do not cause the immediate extinction deficit after Pavlovian fear conditioning in rats. Sci Rep. 2019;9: 9459. doi:10.1038/s41598-019-46010-4

42. Giustino TF, Seemann JR, Acca GM, Goode TD, Fitzgerald PJ, Maren S. β-Adrenoceptor Blockade in the Basolateral Amygdala, But Not the Medial Prefrontal Cortex, Rescues the Immediate Extinction Deficit. Neuropsychopharmacology. 2017;42: 2537–2544. doi:10.1038/npp.2017.89

43. McCall JG, Siuda ER, Bhatti DL, Lawson LA, McElligott ZA, Stuber GD, et al. Locus coeruleus to basolateral amygdala noradrenergic projections promote anxiety-like behavior. eLife. 2017;6. doi:10.7554/eLife.18247

44. Fitzgerald PJ, Giustino TF, Seemann JR, Maren S. Noradrenergic blockade stabilizes prefrontal activity and enables fear extinction under stress. Proc Natl Acad Sci USA. 2015;112: E3729-37. doi:10.1073/pnas.1500682112

45. Kim SC, Jo YS, Kim IH, Kim H, Choi J-S. Lack of medial prefrontal cortex activation underlies the immediate extinction deficit. J Neurosci. 2010;30: 832–837. doi:10.1523/JNEUROSCI.4145-09.2010

46. Hong J, Kim D. Freezing response-independent facilitation of fear extinction memory in the prefrontal cortex. Sci Rep. 2017;7: 5363. doi:10.1038/s41598-017-04335-y

47. Do-Monte FH, Manzano-Nieves G, Quiñones-Laracuente K, Ramos-Medina L, Quirk GJ. Revisiting the role of infralimbic cortex in fear extinction with optogenetics. J Neurosci. 2015;35: 3607–3615. doi:10.1523/JNEUROSCI.3137-14.2015

48. Quirk GJ, Mueller D. Neural mechanisms of extinction learning and retrieval. Neuropsychopharmacology. 2008;33: 56–72. doi:10.1038/sj.npp.1301555

49. Maren S, Quirk GJ. Neuronal signalling of fear memory. Nat Rev Neurosci. 2004;5: 844–852. doi:10.1038/nrn1535

50. Uematsu A, Tan BZ, Ycu EA, Cuevas JS, Koivumaa J, Junyent F, et al. Modular organization of the brainstem noradrenaline system coordinates opposing learning states. Nat Neurosci. 2017;20: 1602–1611. doi:10.1038/nn.4642

497 51. Snyder K, Wang W-W, Han R, McFadden K, Valentino RJ. Corticotropin-releasing
498   factor in the norepinephrine nucleus, locus coeruleus, facilitates behavioral flexibility.
499   Neuropsychopharmacology. 2012;37: 520–530. doi:10.1038/npp.2011.218

500 52. Mulvey B, Bhatti DL, Gyawali S, Lake AM, Kriaucionis S, Ford CP, et al. Molecular and
501   functional sex differences of noradrenergic neurons in the mouse locus coeruleus. Cell
502   Rep. 2018;23: 2225–2235. doi:10.1016/j.celrep.2018.04.054

503 53. Bangasser DA, Zhang X, Garachh V, Hanhauser E, Valentino RJ. Sexual dimorphism in
504   locus coeruleus dendritic morphology: a structural basis for sex differences in emotional
505   arousal. Physiol Behav. 2011;103: 342–351. doi:10.1016/j.physbeh.2011.02.037

506 54. Curtis AL, Bethea T, Valentino RJ. Sexually dimorphic responses of the brain
507   norepinephrine system to stress and corticotropin-releasing factor.
508   Neuropsychopharmacology. 2006;31: 544–554. doi:10.1038/sj.npp.1300875

509 55. McCall JG, Al-Hasani R, Siuda ER, Hong DY, Norris AJ, Ford CP, et al. CRH
510   Engagement of the Locus Coeruleus Noradrenergic System Mediates Stress-Induced
511   Anxiety. Neuron. 2015;87: 605–620. doi:10.1016/j.neuron.2015.07.002

512 56. Van Bockstaele EJ, Colago EE, Valentino RJ. Amygdaloid corticotropin-releasing factor
513   targets locus coeruleus dendrites: substrate for the co-ordination of emotional and
514   cognitive limbs of the stress response. J Neuroendocrinol. 1998;10: 743–757.
515   doi:10.1046/j.1365-2826.1998.00254.x

516 57. Valentino RJ, Rudoy C, Saunders A, Liu XB, Van Bockstaele EJ. Corticotropin-releasing
517   factor is preferentially colocalized with excitatory rather than inhibitory amino acids in
518   axon terminals in the peri-locus coeruleus region. Neuroscience. 2001;106: 375–384.
519   doi:10.1016/s0306-4522(01)00279-2

520 58. Reyes BAS, Drolet G, Van Bockstaele EJ. Dynorphin and stress-related peptides in rat
521   locus coeruleus: contribution of amygdalar efferents. J Comp Neurol. 2008;508: 663–675.
522   doi:10.1002/cne.21683

523 59. Bouret S, Duvel A, Onat S, Sara SJ. Phasic activation of locus ceruleus neurons by the
524   central nucleus of the amygdala. J Neurosci. 2003;23: 3491–3497.

525 60. Jo YS, Namboodiri VMK, Stuber GD, Zweifel LS. Persistent activation of central
526   amygdala CRF neurons helps drive the immediate fear extinction deficit. Nat Commun.
527   2020;11: 422. doi:10.1038/s41467-020-14393-y

528 61. Curtis AL, Pavcovich LA, Grigoriadis DE, Valentino RJ. Previous stress alters
529   corticotropin-releasing factor neurotransmission in the locus coeruleus. Neuroscience.
530   1995;65: 541–550. doi:10.1016/0306-4522(94)00496-r

531 62. Curtis AL, Pavcovich LA, Valentino RJ. Long-term regulation of locus ceruleus
532   sensitivity to corticotropin-releasing factor by swim stress. J Pharmacol Exp Ther.
533   1999;289: 1211–1219.

534 63. Myers KM, Ressler KJ, Davis M. Different mechanisms of fear extinction dependent on
535   length of time since fear acquisition. Learn Mem. 2006;13: 216–223.
536   doi:10.1101/lm.119806

537 64. Bouchet CA, Lloyd BA, Loetz EC, Farmer CE, Ostrovskyy M, Haddad N, et al. Acute
538   exercise enhances the consolidation of fear extinction memory and reduces conditioned
539   fear relapse in a sex-dependent manner. Learn Mem. 2017;24: 358–368.
540   doi:10.1101/lm.045195.117

541 65. Hilz EN, Smith RW, Hong YJ, Monfils MH, Lee HJ. Mapping the estrous cycle to
542   context-specific extinction memory. Behav Neurosci. 2019;133: 614–623.
543   doi:10.1037/bne0000343

544 66. Hilz EN, Agee LA, Jun D, Monfils MH, Lee HJ. Expression patterns of Arc mRNA after
545   renewal of appetitive behavior in female rats. BioRxiv. 2021.
546   doi:10.1101/2021.07.20.453088

547 67. Park CHJ, Ganella DE, Kim JH. Context fear learning and renewal of extinguished fear
548   are dissociated in juvenile female rats. Dev Psychobiol. 2020;62: 123–129.
549   doi:10.1002/dev.21888

550 68. Park CHJ, Ganella DE, Kim JH. Juvenile female rats, but not male rats, show renewal,
551   reinstatement, and spontaneous recovery following extinction of conditioned fear. Learn
552   Mem. 2017;24: 630–636. doi:10.1101/lm.045831.117

553 69. Adkins JM, Lynch J, Gray M, Jasnow AM. Presynaptic GABAB receptor inhibition sex
554   dependently enhances fear extinction and attenuates fear renewal. Psychopharmacology
555   (Berl). 2021. doi:10.1007/s00213-021-05831-w

556 70. Rescorla RA. Spontaneous recovery. Learn Mem. 2004;11: 501–509.
557   doi:10.1101/lm.77504

558 71. Li W-G, Wu Y-J, Gu X, Fan H-R, Wang Q, Zhu J-J, et al. Input associativity underlies
559   fear memory renewal. Natl Sci Rev. 2021;8: nwab004. doi:10.1093/nsr/nwab004

560 72. Xu C, Krabbe S, Gründemann J, Botta P, Fadok JP, Osakada F, et al. Distinct
561   hippocampal pathways mediate dissociable roles of context in memory retrieval. Cell.
562   2016;167: 961-972.e16. doi:10.1016/j.cell.2016.09.051

563 73. Marek R, Jin J, Goode TD, Giustino TF, Wang Q, Acca GM, et al. Hippocampus-driven
564   feed-forward inhibition of the prefrontal cortex mediates relapse of extinguished fear. Nat
565   Neurosci. 2018;21: 384–392. doi:10.1038/s41593-018-0073-9

566   74.   Jin J, Maren S. Fear renewal preferentially activates ventral hippocampal neurons
567   projecting to both amygdala and prefrontal cortex in rats. Sci Rep. 2015;5: 8388.
568   doi:10.1038/srep08388

569   75.   Anderson LC, Petrovich GD. Distinct recruitment of the hippocampal, thalamic, and
570   amygdalar neurons projecting to the prelimbic cortex in male and female rats during
571   context-mediated renewal of responding to food cues. Neurobiol Learn Mem. 2018;150:
572   25–35. doi:10.1016/j.nlm.2018.02.013

573   76.   Anderson LC, Petrovich GD. Ventromedial prefrontal cortex mediates sex differences in
574   persistent cognitive drive for food. Sci Rep. 2018;8: 2230. doi:10.1038/s41598-018-
575   20553-4

576   77.   Anderson LC, Petrovich GD. Sex specific recruitment of a medial prefrontal cortex-
577   hippocampal-thalamic system during context-dependent renewal of responding to food
578   cues in rats. Neurobiol Learn Mem. 2017;139: 11–21. doi:10.1016/j.nlm.2016.12.004

579   78.   Namkung H, Kim S-H, Sawa A. The insula: an underestimated brain area in clinical
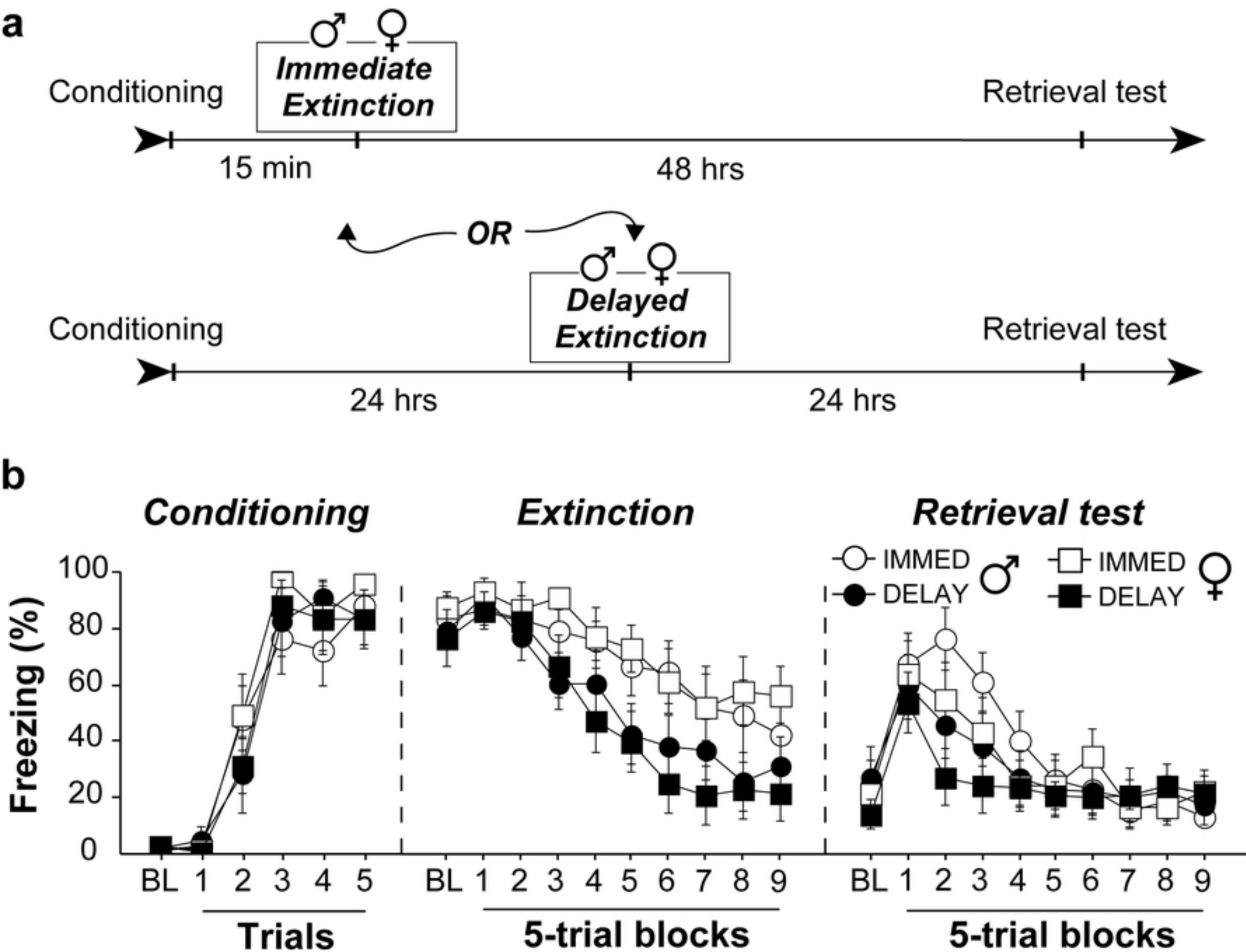580   neuroscience, psychiatry, and neurology. Trends Neurosci. 2017;40: 200–207.
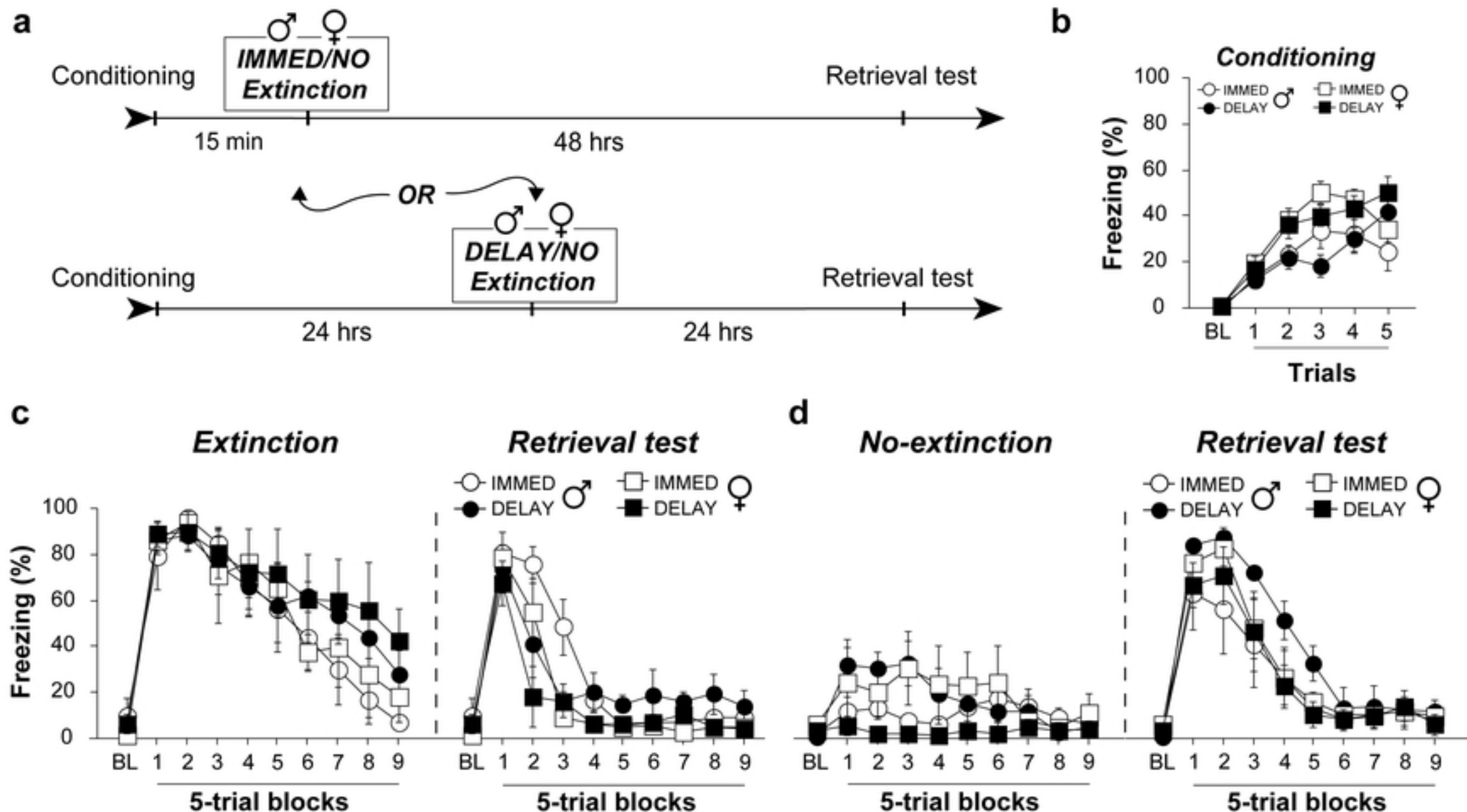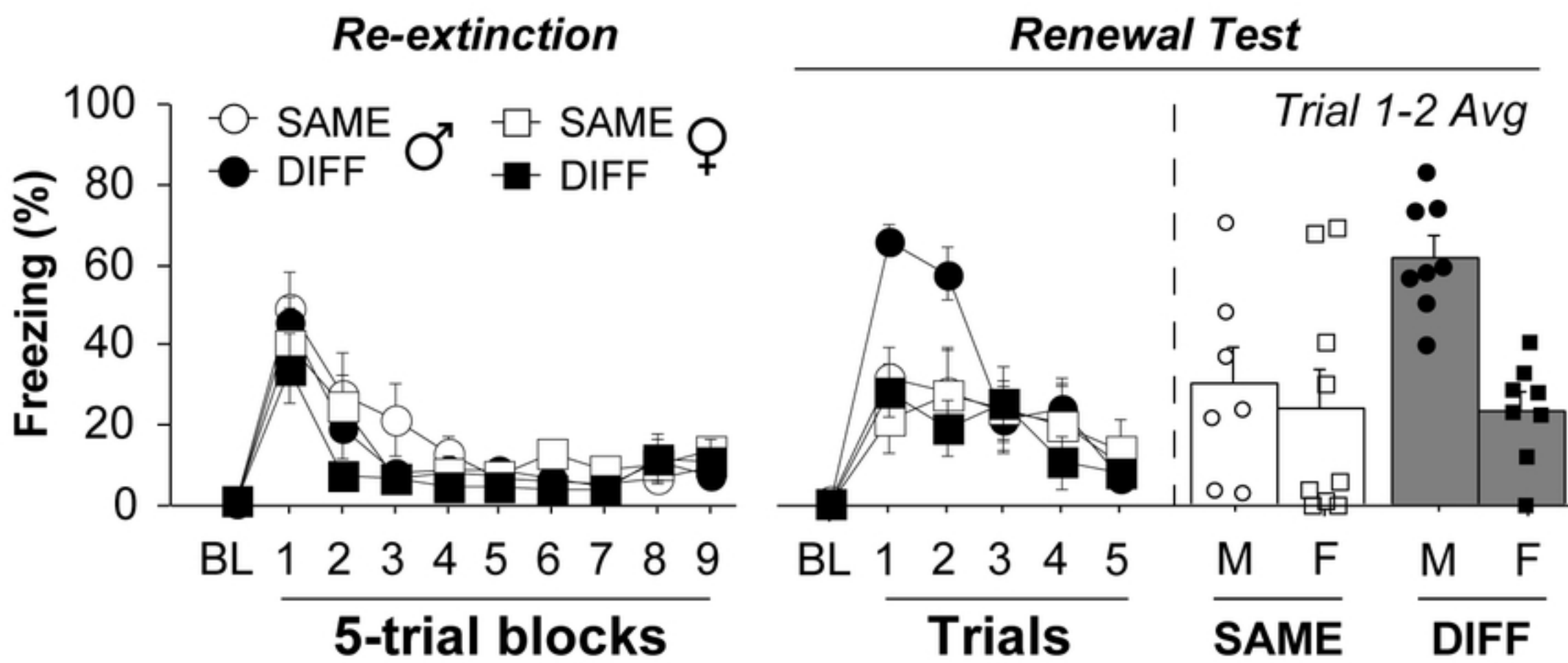581   doi:10.1016/j.tins.2017.02.002

Figure 1

Figure 2

Figure 3