4
Study 2

Reacquisition of context fear after extensive extinction training in rats selectively bred for high and low conditioned freezing

4.1
Objectives

This study aimed to investigate the patterns of extinction and reacquisition of contextual aversive conditioning in CHF and CLF rats.

4.2
Subjects

Fifteen CHF (Carioca High-Conditioned Freezing) and 15 CLF (Carioca Low-Conditioned Freezing) male rats from the eighth generation (S_8) of selective breeding were employed, weighing 350-450 g. Room temperature was controlled (24±1 °C) and the light– dark cycle was maintained on a 12-h on–off cycle (07:00– 19:00 h). All experiments took place during the light phase of the cycle. Animals were between 90 and 120 days old at the beginning of the experiment.

4.3
Equipments

Contextual fear conditioning, extinction and fear reacquisition took place in an observation chamber (25×20×20 cm) that was placed inside of a sound-attenuating chest. A red light bulb (25 W) was placed inside the chest and a video camera was mounted in the back of the observation chambers so that the animal's behavior could be observed on a monitor placed outside the experimental chamber. A ventilation fan attached to the chest supplied a background noise of 78 dB (A scale). The floor of the observational chamber was composed of 15 stainless rods with a diameter of 4 mm and spaced 1.5 cm apart (center-to-center),

which were wired to a shock generator and scrambler (AVS, SCR04; São Paulo). An interface with eight channels (Insight; Ribeirão Preto) connected the shock generator to a computer, which allowed the experimenter to apply an electric footshock. An observation program (GeoVision GV800, PCI Systems) was used to record all procedures A digital multimeter (MD-1400 - ICEL, Manaus) was used to calibrate shock intensities before each experiment. An ammonium hydroxide solution (5 %) was used to clean the chamber before and after each test.

4.4
Procedures

This study aimed to investigate the patterns of extinction and reacquisition of contextual aversive conditioning in CHF and CLF rats. The first conditioning session occurred in the phenotyping process of the S_8 generation (see study 1). During this acquisition phase, each animal was placed in the observation chamber for 8 min. At the end of this period, three 0.6 mA unsignaled electrical footshocks were delivered. Each shock lasted 1 s, with an intershock interval of 20 s. The animal was returned to its home cage 3 min after the last shock. A time-sampling procedure was employed to evaluate fear conditioning in response to contextual cues. Every 2 s, the animal was observed, and a well-trained observer recorded episodes of freezing, which were defined as the total absence of movement of the body or vibrissae with the exception of respiration. The 15 CHF rats with top high and 15 CLF rats with bottom low levels of conditioning freezing were then chosen as breeders to create generation S_9, and after mating, they were employed in the present extinction study. 2 months after this initial session of aversive conditioning (phenotyping), the rats received the 1st extinction training, constituted of 12 extinction sessions (1 session/day), in order to investigate the strength of long-term contextual fear memory extinction. Each extinction session consisted of placing the animal for 8 min in the same chamber in which the 3 footshocks had been previously administered. No footshock or other stimulation occurred during this period. In the end of the 12th extinction session, all animals were subjected to a single reacquisition training that was the same as the acquisition procedure previously described. 24 hrs after this reacquisition session, the animals were subjected to a second set of 12 extinction sessions, aiming the

investigation of extinction strength of short-term contextual fear memory in both lines of animals.

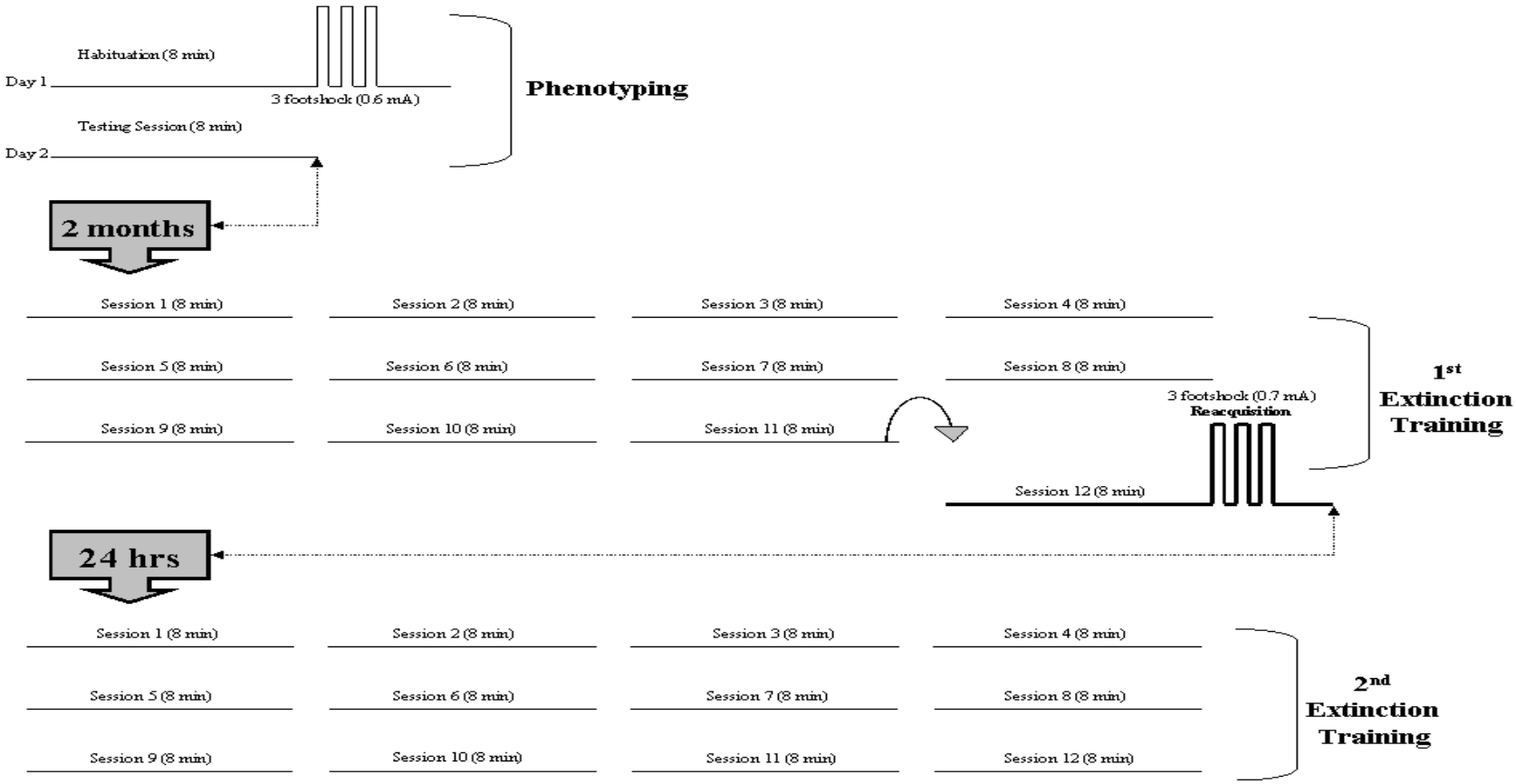

Figure 21: Extinction Procedure. *Note*: 1 session / day.

4.5
Results

Figure 14 show the means (+SEM) percentage of conditioned freezing in the first aversive conditioning training of the 15 CHF and 15 CLF rats previously selected for this experiment. The behavioral comparison of CHF and CLF rats in the testing session was performed through a Student's t-test. The analysis showed significant differences between CHF and CLF rats (T_{28}=4.55; p<0.001).

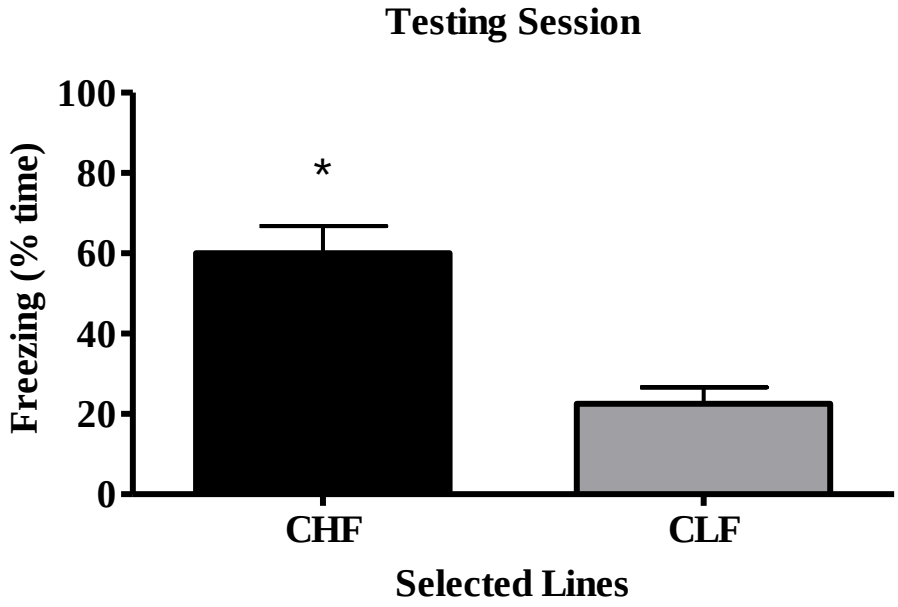

Figure 22: Mean (+SEM) percentage of conditioned freezing in the first aversive conditioning training of the 15 CHF and 15 CLF rats previously selected for this experiment; * denotes significant difference (p<0.05).

In order to evaluate the impact of the fear extinction procedures, a 2X12 ANOVA with repeated measures (line X sessions) was performed, for both 1st and 2nd extinction training. The within factor was testing session (12 sessions) and the between factor was line (CHF and CLF). Results of the 1st extinction training indicate the presence of a significant two-way interaction ($F_{11,308}$=2.704; p<0.05),

and main effects for session ($F_{11,308}$=8.01; p<0.001), but not for line ($F_{1,28}$=0.77; p=0.388). Fisher LSD's post-hoc comparisons showed that CHF and CLF rats differed significantly in the first two sessions of extinction training (all p <0.001). Both rat lines reached the same asymptotic level of contextual fear extinction in the twelfth session (Figure 15)

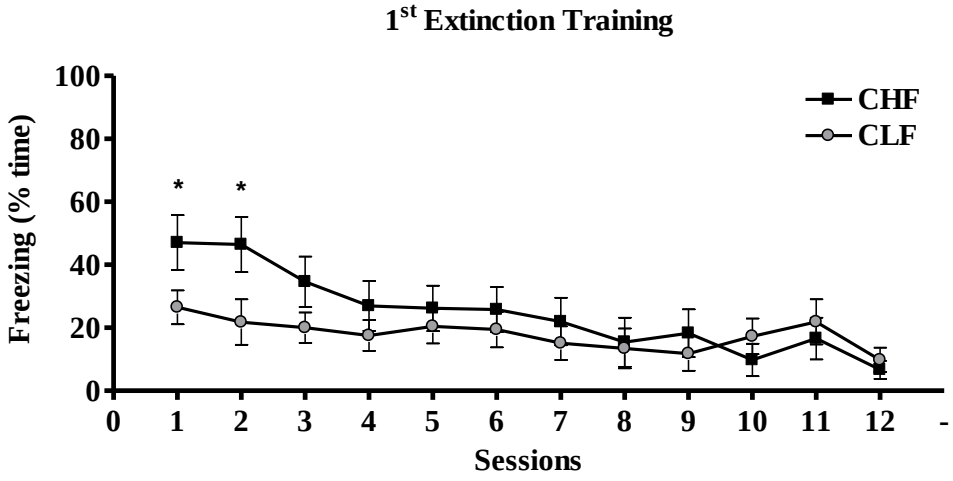

Figure 23: Mean (+ SEM) percentage of conditioned freezing of CHF and CLF rats along 12 extinction sessions in the 1st extinction training two months after initial fear conditioning training; * denotes significant differences between CHF and CLF (p<0.05).

Repeated measurements of the ANOVA results for the second extinction training showed an absence of a two-way interaction ($F_{11,308}$=1.09; p=0.365), and main effects for session ($F_{11,308}$=6.98; p<0.001), but not for line ($F_{1,28}$=1.23; p=0.275). However, it was observed a non-significant trend, with CHF rats showing more conditioned freezing along sessions than CLF rats in the 2nd extinction training (Figure16).

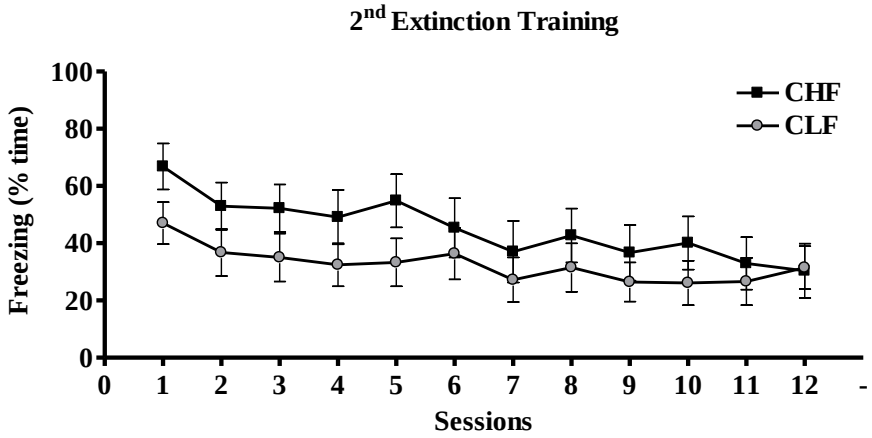

Figure 24: Mean (+ SEM) percentage of conditioned freezing of CHF and CLF rats along 12 extinction sessions in the 2nd extinction training 24hs after reacquisition training ($p<0.05$).

To evaluate the influence of each extinction training set among the rat lines during 24 extinction sessions, a two-way ANOVA was performed. The first factor was breeding line (CHF and CLF) and the second factor was related to extinction training (1st and 2nd). Results showed an absence of a two-way interaction ($F_{1,716}=1.59$; $p=0.206$), and main effects for line ($F_{1,716}=17.356$; $p<0.001$) and extinction training ($F_{1,716}=57.25$; $p<0.001$). Pairwise post-hoc comparisons showed that CHF and CLF rats differed significantly in both 1st and 2nd extinction trainings. Moreover, comparisons within lines indicated that both CHF and CLF rats froze more in the 2nd extinction training (all $p<0.05$).

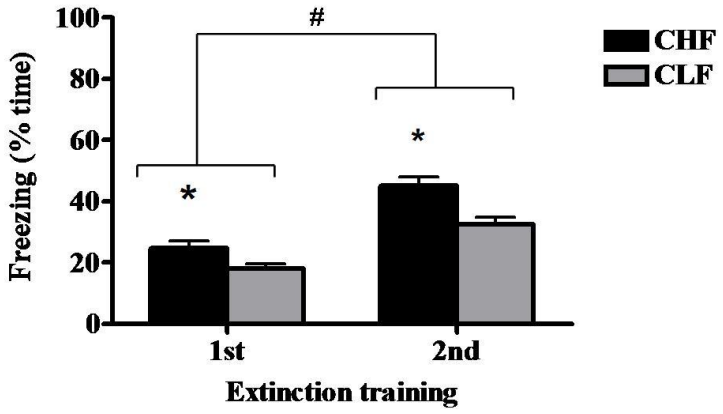

Figure 25: Mean (± SEM) percentage of conditioned freezing in 1st and 2nd extinction training among CHF and CLF rats ($p<0.05$).

4.6
Discussion

Impaired extinction of conditioned fear memories is a main feature of many anxiety disorders, including PTSD and specific phobias. Like other types of learning, extinction learning occurs in three phases: acquisition, consolidation, and retrieval. The proper regulation of emotional expression under changeable environmental conditions is essential for mental health (Quirk, 2008). Indeed, a substantial proportion of anxiety patients do not react effectively to standard behavioral treatments and/or pharmacological (Pull, 2007).

In this sense, the main objective of the present study was to evaluate the patterns of extinction and reacquisition of contextual fear in rats selectively bred for high (CHF) and low (CLF) conditioned freezing responses. We observed robust differences between groups of rats in conditioned freezing during the first aversive training (testing session), confirming effects already observed in Study 1 of this thesis. The analysis of the freezing behavior registered in the 1st extinction training set showed that initial conditioned freezing differences between CHF and CLF rats was maintained 2 months after the initial aversive training. Most importantly, results showed a robust long-term memory formation particularly for CHF rats. Although this was an observed tendency verified on most days, the differences between lines disappeared after two extinction sessions. Both lines reached the same asymptote of freezing behavior in the last (12th) extinction session. Interestingly, although differences in freezing behavior disappeared after 12 extinction sessions, the divergences between CHF and CLF rats reappeared after a single reacquisition training session.

As previously described, extinction is a process of inhibitory learning; which, in the present context, would be the inhibition of fear memories. The first phase of the extinction process, namely acquisition, is characterized by a decrease in conditioned responses (CRs) to the continuous presentation of a conditioned stimulus (CS) without the presence of the unconditioned stimulus (US). However, when the rat goes back to the original context where the US was delivered, the retrieval of the CR initiates a process of reconsolidation, which in turn is necessary for maintenance of the conditioning memory (Nader et al., 2000; Tronson and Taylor, 2007; Dudai, 2002). Interestingly, extinction learning

demands many of the same cellular processes as reconsolidation, such as protein synthesis, NMDArs, β-adrenergic receptors, PKA and MAPk. However, an extinction session may initially trigger CR reconsolidation, but as the session progresses, extinction itself is gradually acquired and consolidated.

ANOVA results from the 1st extinction training set showed a significant two-way interaction ($F_{11,308}$=2.704; $p<0.05$), indicating that acquisition, consolidation and retrieval of inhibitory memories were different for CHF and CLF rats. However, it should be noted that both lines started the 1st extinction training set with different levels of freezing, which could lead to a floor effect in CLF rats during the extinction sessions. The same interaction effect was not observed in the 2nd extinction training ($F_{11,308}$=1.09; $p=0.365$).

In fact, although the reacquisition training has recovered the initial divergence between CHF and CLF rats, freezing levels of both lines were higher than the 1st extinction training (Figure 17). Moreover, the freezing observed in CHF rats in the 2nd extinction was higher than all the others groups. This is an important finding, and is in accordance with human data reported in the meta-analysis of Lissek et al (2005), in which patients with anxiety disorders showed persistently elevated levels of conditioned fear responses during extinction training when compared with normal controls. Moreover, these results suggest that CHF rats present impaired fear extinction, given that after 12 extinction sessions they reached the same asymptote than CLF rats, but only one reacquisition training was sufficient to reestablishe the initial behavioral divergence of both lines.

One possible explanation of this impaired extinction presented by CHF rats may be related with functional differences in the neural circuitry underlying fear memories. Indeed, in the molecular level, systemic drug studies of the acquisition phase have focused in the N-methyl-D-aspartate receptor (NMDAr) molecule. For example, the systemic administration of the NMDAr antagonist MK801 prevented extinction (Baker and Azorlosa, 1996; Cox and Westbrook, 1994) and, more recently, it was shown that a selective antagonist of the Nr2B subunit of the NMDAr (ifenprodil) blocked acquisition of extinction within a session (Sotres-Bayon et al, 2007). In the systemic level, it is becoming clear that acquisition of extinction is controlled by calcium-triggered cascades in the basolateral complex of amygdala (BLA). (Azad et al, 2003; Cannich et al, 2004),

as well by opioid receptors located in the ventrolateral periaqueductal gray matter (vlPAG), since the blocking of *u*-opioid receptors with naloxone in this region prevented acquisition of extinction (McNally et al, 2004b, 2005). Moreover, the BLA is an important site for extinction consolidation. For example, Berlau and McGaugh (2006) showed that, after the increase of the BLA activity with the $GABA_A$ antagonist bicuculine, the extinction was facilitated in a norepinephrine – dependent manner. Importantly, one of the initial observations regarding the mechanisms of extinction was that selective lesions on the medial prefrontal cortex (vmPFC) mitigate extinction of conditioned fear (Morgan et al, 1993). Studies employing lesions in the infralimbic region (IL), an important site of connection between the vmPFC and the BLA, showed that rats could acquire extinction within a session, but had difficult recovering extinction the following day (Quirk et al, 2000). Similar results were observed in several studies employing lesions in the vmPFC (Lebron et al, 2004; Morgan et al, 2003; Weible et al, 2000; Fernandez 2003).

Another hypothesis is directly related to chronic stress. Obviously, many mental disorders are compounded by high levels of chronic stress, which in turn may impair extinction. For example, it was found that chronic stress (daily restraint over a period of 7−20 days) decreases dendritic branching and spine count in the hippocampus (McEwen, 2001) and mPFC (Radley et al., 2004; Cook and Wellman, 2004; Brown et al., 2005; Radley et al., 2006), and this pattern of effects would be expected to increase conditioning and impair extinction. Indeed, a recent extinction study (Muigg et al, 2008) showed that rats selectively bred for high anxiety-related behavior (HAB) demonstrated impaired extinction of conditioned fear memories in comparison to their non-anxious counterparts (LAB).