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## Research Report

# Amygdaloid lesions produced similar contextual fear conditioning disruption in the Carioca high- and low-conditioned freezing rats

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## ABSTRACT

Rats selectively bred for high or low levels of emotionality represent an important and powerful tool to investigate the role of genetic variables in the occurrence of different anxiety disorders. In the present study, albino rats were selectively bred for differences in defensive freezing behavior in response to contextual cues previously associated with footshock, an animal model of general anxiety disorder. The results indicate that these two new lines of rats, which we refer to as Carioca High-Freezing (CHF) and Carioca Low-Freezing (CLF), show a reliable difference in conditioned freezing after three generations of selection. CHF and CLF rats did not present any differences during baseline or post-shock periods. Males from both lines consistently exhibit more conditioned freezing to contextual cues than females. A second experiment used male rats from the fourth generation to investigate the participation of the amygdala during contextual fear conditioning in the CHF and CLF lines. The results indicate that post-training amygdaloid electrolytic lesions lead to similar disruptions in conditioned freezing behavior in both animal lines.

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## 1. Introduction

Anxiety disorders represent the most prevalent mental health problem over the course of an individual's life span (The World Health Organization, World Mental Health Survey Consortium, 2004). They constitute a heterogeneous group of interrelated nosological categories associated with excessive and irrational fear in conjunction with intense physiological arousal. Different patterns of animal defensive behavior have been successfully used to investigate the underlying pathophysiological mechanisms involved in anxiety disorders. Understanding these neural mechanisms,

and consequently the etiology of these disorders, will aid the design of new and more effective forms of therapy for anxiety management.

Defensive freezing behavior is an immobile and crouching posture that animals adopt when facing potentially threatening or dangerous situations (Fanselow, 1984a). For example, in a typical contextual fear conditioning experiment, a rat is exposed to a novel chamber and a few minutes later a brief, unsignaled footshock is presented. Shortly after the shock or some time later (hours, days, or months), the animal freezes when returned to the same chamber in the absence of the aversive stimulus (Gale et al., 2004). Several studies indicate

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that this defensive freezing posture is a conditioned response to contextual cues associated with the footshock and represents one of the most useful animal models for generalized anxiety disorder (see Brandão et al., 2008 for a review). For example, conditioned freezing is weakened by anxiety-reducing drugs (Fanselow et al., 1991) and enhanced by anxiety-inducing drugs (Conti et al., 1990; Izumi et al., 1999), indicating its ability to predict the validity of pharmacological substances that modulate general anxiety disorder in humans.

Selective breeding is a laboratory technique in which animals are bred in order to modify the frequency of genes underlying a particular phenotype. Mating animals within a population based on the opposite extremes of an observable characteristic will push, over many generations, this particular phenotype in opposite directions, leading to two separately bred lines. This technique has been widely employed to investigate how genes can influence a broad variety of behavioral traits, including defensive reactions associated with emotionality.

The development of bidirectional lines of animals with high and low levels of emotionality started in the middle of the 20th century, and since then a relatively large number of different lines have been described in the literature (see Ramos and Mormède, 2006 for a review). Unconditioned and conditioned emotional responses have been employed as the criteria for mating selection in rats. Among the unconditioned fear paradigms are ambulation and defecation in the open field, such as in the Maudsley reactive and non-reactive rats (Broadhurst, 1958; Hall, 1938) or open arm entrance in the elevated plus-maze, as in the high- and low-anxiety related behavior rats (Liebsch et al., 1998a; 1998b). Among paradigms of conditioned fear, active avoidance behavior has served as the main selection criteria for selectively breeding animals with high and low levels of emotionality. Examples in the literature are the high- and low-avoidance rats referred to as Roman (Bignami, 1965), Syrakuse (Brush et al., 1979), Koltushi (Ryzhova et al., 1983), and Hatano (Ohta et al., 1995).

A few studies have indicated that conditioned freezing is a highly heritable response that can be rapidly selected. Radcliffe et al. (2000) and, more recently, Ponder et al. (2007, 2008) succeeded in producing two mouse lines exhibiting high and low levels of conditioned freezing after a single generation of selective breeding. Despite this encouraging result, there have been no published attempts to produce rat lines exhibiting high and low levels of conditioned freezing. This is an important issue, especially because rats are the predominant species used in laboratory studies to investigate the neurobiology of conditioned fear. Therefore, one of the purposes of the present study was to start a selective breeding program to develop two lines of rats with extremely high or low levels of defensive freezing response to contextual cues previously associated with footshock.

A large and highly consistent body of literature indicates that the amygdala is critically involved in the regulation of contextual fear conditioning (Fanselow and LeDoux, 1999; Kim and Jung, 2006; Maren, 2005). For example, electrolytic or neurotoxic lesions of the amygdala made before or after training disrupts conditioned freezing (Blanchard and Blanchard, 1972; Cousens and Otto, 1998; Helmstetter, 1992; Kim et al., 1993; Maren et al., 1996; Oliveira et al., 2004). In fact, reversible

inactivation of the amygdala prevents the acquisition of contextual fear conditioning (Helmstetter and Bellgowan, 1994). Moreover, electrical or chemical stimulation of the amygdala can induce defensive freezing behavior (al Maskati and Zbrozyna, 1989; Da Costa Gómez et al., 1996; Kapp et al., 1982; Sajdyk and Shekhar, 1997). Finally, amygdaloid neurons show plasticity during fear conditioning (Ono et al., 1995; Parré and Collins, 2000; Rogan et al., 1997), probably mediated by long-term potentiation (LTP).

Recent results indicate that several genes in the amygdala are differentially expressed when mice are bidirectionally selected for conditioned freezing (Ponder et al., 2007). Therefore, this brain structure may be associated with the bidirectional selection of rats exhibiting high and low levels of conditioned freezing. The present work also investigated this issue as follows. After high- and low-conditioned freezing differences were established through the selective breeding procedure, a second experiment investigated the effect of bilateral lesions of the amygdala on contextual fear conditioning in these two new lines of animals. Electrolytic lesions were performed in both sides of the amygdala. Although this procedure destroy both neuronal cells and fibers of passage, evidence suggests that either electrolytic or neurotoxic lesions, that preserve the fibers of passage, produce similar effects on conditioned freezing in response to contextual cues associated with footshocks (Koo et al., 2004).

## 2. Results

Table 1 shows the distribution of the 448 animals of the S1, S2, and S3 generations. Animal distribution remained relatively constant across both lines. An analysis using the chi-square test showed no significant differences between the 12 groups ( $\chi^2=0.58$ ,  $p>0.7$ ).

Fig. 1 presents the mean ( $\pm$ SEM) percentage of time spent freezing during the baseline (top) and post-shock (bottom) acquisition session periods of the original population (S0) as well as of the three generations (S1, S2 and S3) selected for high (CHF) and low (CLF) rates of conditioned freezing. As can be observed from the upper portion of Fig. 1, freezing during the baseline period was minimal with no significant differences among the groups. This impression was confirmed by a three-way analysis of variance (ANOVA). The first factor, with three levels, was related to the number of generations (S1, S2, and S3). The second factor, with two levels, was related to the breeding line (CHF and CLF). Finally, the third factor, also with two levels, was related to the animal's sex (male and female). This analysis revealed an absence of a three-way interaction [ $F(2,448)=1.41$ ;  $p>0.2$ ]. No two-way interactions between sex and the selected generation [ $F(2,448)=1.34$ ;  $p>0.2$ ], between sex and breeding line [ $F(1,448)=0.15$ ;  $p>0.9$ ] or between selected generation and breeding line [ $F(2,448)=0.84$ ;  $p>0.4$ ] were found. No main effect of sex [ $F(1,448)=1.06$ ;  $p>0.3$ ], breeding line [ $F(1,448)=0.21$ ;  $p>0.6$ ] or selected generation [ $F(2,448)=0.67$ ;  $p>0.5$ ] was also detected.

Freezing behavior during the post-shock period of CHF and CLF lines remained relatively constant across the different generations. The three-way ANOVA revealed an absence of a three-way interaction [ $F(2,448)=1.06$ ;  $p>0.1$ ].

**Table 1 – Distribution of the number of male and female rats of selected lines exhibiting high- and low-conditioned freezing responses along the three selected generations (S1, S2, and S3)**

Selected generation	High Freezing		Low Freezing		Total
	Male	Female	Male	Female	
S1	37	39	34	37	147
S2	37	35	37	34	143
S3	34	45	42	37	158
Total	108	119	113	108	448

No two-way interactions between sex and the selected generation [ $F(2,448)=0.08$ ;  $p>0.9$ ], between sex and breeding line [ $F(1,448)=0.13$ ;  $p>0.9$ ] or between selected generation and breeding line [ $F(2,448)=0.15$ ;  $p>0.8$ ] were detected. No main effect of sex [ $F(1,448)=1.77$ ;  $p>0.1$ ], breeding line [ $F(1,448)=1.84$ ;  $p>0.1$ ] or selected generation [ $F(2,448)=1.56$ ;  $p>0.2$ ] was also found.

Conditioned freezing scored during the test session was also analyzed by the three-way ANOVA. This analysis indicated an absence of a three-way interaction [ $F(2,448)=0.17$ ;  $p>0.8$ ]. No two-way interactions were found, either between sex and the selected generation [ $F(2,448)=0.28$ ;  $p>0.7$ ], or between sex and breeding line [ $F(1,448)=0.22$ ;  $p>0.6$ ]. However, ANOVA did reveal a reliable two-way interaction between breeding lines along the three different generations [ $F(2,448)=7.55$ ;  $p<0.001$ ]. The analysis also revealed a significant main effect of sex [ $F(1,448)=13.10$ ;  $p<0.001$ ] and breeding line [ $F(1,448)=14.77$ ;  $p<0.001$ ] but no main effect of selected generation [ $F(2,448)=0.68$ ;  $p>0.5$ ].

The presence of a main effect of sex indicated that importance of this variable in this study. Therefore, male and female results were analyzed separately. Fig. 2 presents the mean ( $\pm$ SEM) percentage of time spent freezing in male (top) and female (bottom) rats during the test session of the original population (S0), as well as of the three generations (S1, S2 and S3) selected for high (CHF) and low (CLF) rates of conditioned freezing. Pairwise Student's t-test comparisons between CHF and CLF were performed for each selected generation among male and female rats. These analyses allowed us to identify in which generation the two lines of male and female rats presented a reliable difference in conditioned freezing. The results indicate that CHF and CLF males did not show any significant differences in the first (S1) or second (S2) generations [S1:  $t(69)=0.37$ ,  $p>0.7$ –S2:  $t(72)=10.92$ ;  $p>0.3$ ]. However, a reliable difference between the two male lines was detected in the third (S3) generation [ $t(74)=4.47$ ;  $p<0.001$ ]. The same pattern was observed among females. A reliable difference between CHF and CLF lines within females was detected in S3 [ $t(80)=5.31$ ,  $p<0.001$ ], but not in S1 [ $t(74)=0.55$ ;  $p>0.5$ ] or S2 [ $t(67)=0.92$ ;  $p>0.3$ ]. Therefore, selective breeding led to divergence of conditioned freezing in both male and female rats by the third selected generation.

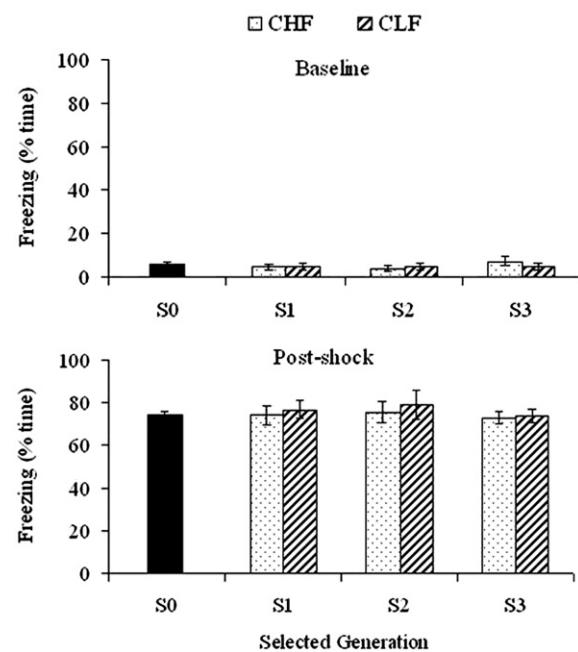
### 3. Results

Of the 48 male rats subjected to surgery, nine animals were excluded due to death (1) or misplaced lesions (8). The final

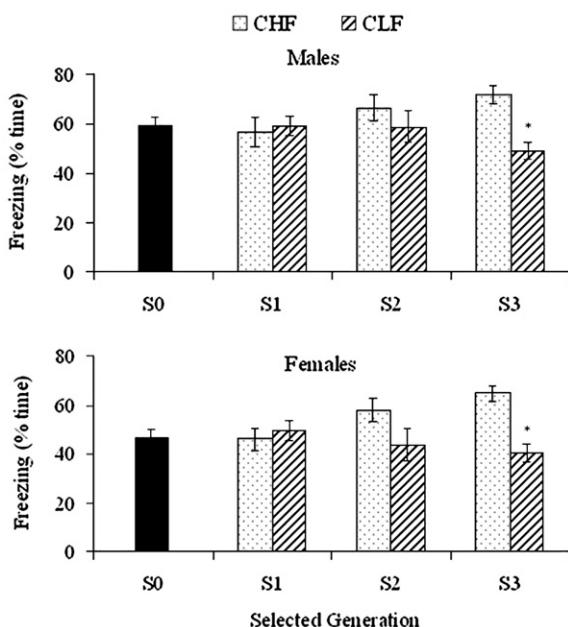
sizes of each of the six groups were as follows: CHF-amygdala lesion group,  $n=9$ ; CHF-sham lesion group,  $n=11$ ; CLF-amygdala lesion group,  $n=7$ ; and CLF-sham lesion group,  $n=12$ .

Fig. 3 shows a representative histological section of a bilateral electrolytic lesion of the amygdala. Fig. 4 presents a composite of the representative areas of the smallest and largest lesions in the amygdala. Histological examination of the bilateral electrolytic lesions in the amygdala showed that the induced damage was usually symmetrical and affected most of the basolateral (BLA) and central nucleus (CEA) of the amygdala, as well as lateral portions of the amygdala and small portions of the ventral striatum.

Fig. 5 depicts the mean ( $\pm$ SEM) percentage of time that sham- and amygdaloid-lesioned animals exhibited freezing during the test session. These results were analyzed by a  $2 \times 2$  ANOVA. The first factor, with two levels, was related to the type of lesion (amygdala or sham). The second factor, with also two levels, was related to the two breeding lines (CHF or CLF). The ANOVA revealed no significant interaction between these two factors [ $F(1,39)=3.1$ ;  $p>0.08$ ] and a significant effect of lesion [ $F(1,39)=18.0$ ;  $p<0.001$ ] and breeding line [ $F(1,39)=17.9$ ;  $p<0.001$ ]. Pairwise comparison indicated that CHF animals froze more than CLF animals, and amygdala lesion caused a reduction in their conditioned freezing ( $p<0.01$  in all cases). The mean percentage difference between sham and lesioned animals within each line of animals was calculated in order to estimate the effect of amygdaloid lesions on conditioned freezing in each of the lines. These analyses revealed that



**Fig. 1 – Mean ( $\pm$ SEM) percentage of time spent freezing during the baseline (top) and post-shock (bottom) acquisition session periods of the original population (S0) as well as of the three generations (S1, S2 and S3) selected for high (CHF) and low (CLF) levels of conditioned freezing. Original population was composed by 120 animals (60 males and 60 females). Number of animals in each group across the three generations is presented in Table 1.**



**Fig. 2 – Mean (±SEM) percent of conditioned freezing during the testing session among male (top) and female (bottom) rats selected for high- or low-conditioned freezing in relation to the original population (S0) and the next three generations (S1, S2, and S3). Original population was composed by 120 animals (60 males and 60 females). Number of male and females rats in each group across the three generations is presented in Table 1. Asterisk indicated  $p < 0.001$ .**

amygdaloid lesions reduced the percentage of conditioned freezing by 60.6% in the CHF line and by 63.0% in the CLF line.

#### 4. Discussion

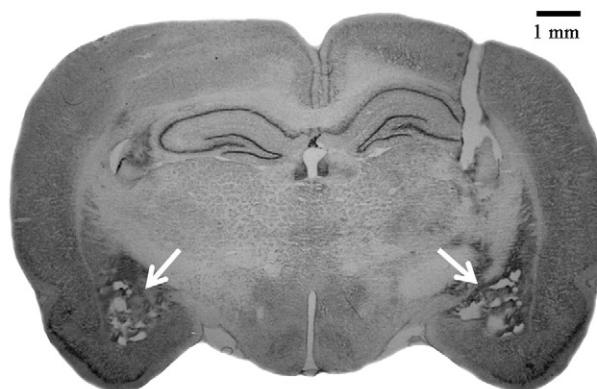
This work presents initial results from two new lines of animals that were selectively bred for high or low levels of freezing in response to contextual cues previously associated with footshock. Results from this ongoing selective breeding program in our laboratory indicated a progressive divergence of the conditioned freezing phenotype in both male and female rats. Differences between CHF and CLF lines became clear after three breeding generations. No differences in freezing behavior were observed during baseline or post-shock periods of the acquisition sessions. These results represent the first successful attempt to select rats with reliable and selective differences in conditioned freezing. It extends the results of previous report, which also achieved a bidirectional short-term selection of this conditioned response among mice (Ponder et al., 2007; 2008; Radcliffe et al., 2000).

Reports from mouse studies have indicated that only one generation was sufficient to differentiate high- and low-conditioned freezing lines, whereas the present results detected a reliable difference after three generations. This result may suggest subtle differences between the two species. As a whole, these data reveal that conditioned fear is a highly

heritable trait and can be rapidly, bidirectionally selected after a few generations. The present CHF and CLF lines are particularly important since most behavioral, pharmacological, and neuroanatomical experiments studying conditioned fear have been conducted using rats.

The active avoidance paradigm has been widely used in genetic research as the main rat model of conditioned fear. Avoidance is a complex form of learning that involves the acquisition of both associative fear and an operant response (Gray, 1975; Mowrer, 1947; 1960). The interaction between these two learning processes may interfere with the measurement of emotional processes mediated by associative learning. For example, manipulations that decrease conditioned fear – such as a reduction in shock intensity (McAllister et al., 1971), anxiolytic drugs (Fernandez-Teruel et al., 1991), and a decrease in contextual fear conditioning (Dieter, 1977) – enhance the acquisition of an active avoidance response. On the other hand, freezing is a more direct and prominent measure of conditioned fear since it does not involve the acquisition of an operant response. This defensive response is a function of shock intensity, depends on the association between conditioned and unconditioned stimuli, and is sensitive to a series of manipulations that interferes with its associative strength (Fanselow and Bolles, 1979; Landeira-Fernandez, 1996; Landeira-Fernandez et al., 1995). Therefore, in studies investigating the genetic mechanisms of conditioned fear, lines of animals selectively bred for high and low levels of conditioned freezing may represent a better model than the bidirectional selection of the active avoidance response.

It is possible that differences in contextual fear conditioning between CHF and CLF animals might reflect differences in pain sensitivity of these two new lines of animals. This is an important issue since freezing observed immediately after footshock as well as 24 h after conditioning are closely related to pain sensitivity and shock intensity (Cordero et al., 1998; Fanselow, 1984b). The fact that CHF and CLF rats did not present any differences in post-shock freezing during the acquisition sessions of the contextual fear conditioning weakens this possibility. However, future studies are important to further evaluate whether CHF and CLF rats might present differences in pain sensitivity.

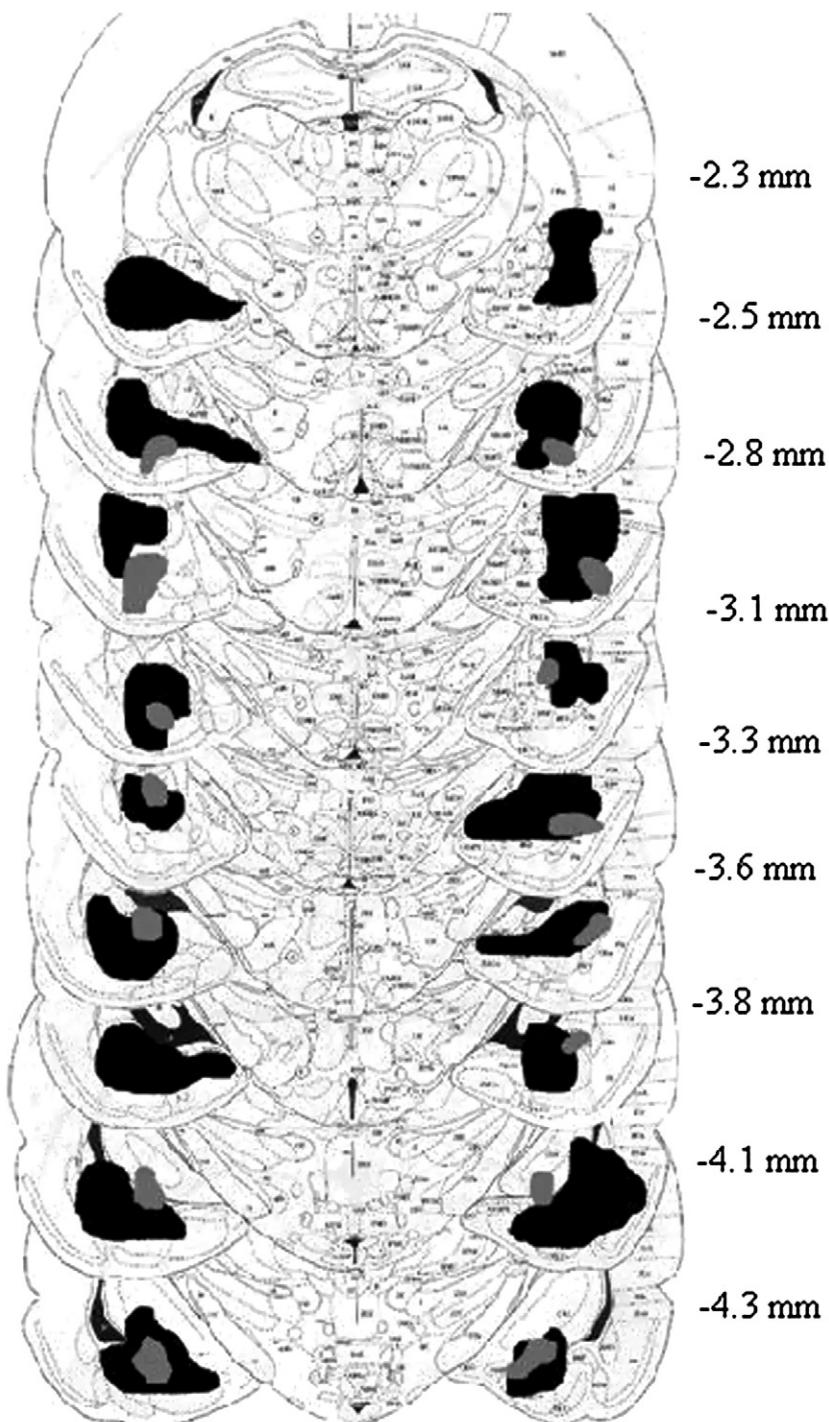


**Fig. 3 – Representative photomicrographs with arrows showing a typical example of a bilateral electrolytic lesion in the amygdala.**

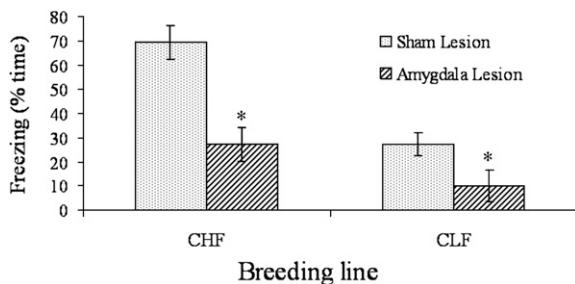
Previous studies indicated that post-shock freezing and freezing observed 24 h after contextual fear conditioning are mediated by associative learning (see Landeira-Fernandez, 1996 for a review). The fact that CHF and CLF animals presented differences in conditioned freezing observed 24 h conditioning but not immediately after footshocks suggests that these two forms of freezing behavior might be mediated by distinct set of genes which in turn regulates different

neural mechanism associated with each form of learning. In accordance with this view, it has been shown that freezing 24 h after conditioning but not post-shock freezing, is mediated by N-methyl D-aspartate receptors (Kim et al., 1991; Kim et al., 1992).

Results from Experiment 1 also indicate that male rats consistently exhibit more conditioned freezing than females during the development of the CHF and CLF lines. Sex



**Fig. 4 – Composite of coronal sections adapted from the Paxinos and Watson (1986) rat brain atlas. Numbers indicate the distance in millimeters from bregma. The figure shows the smallest (black) and largest (gray) damaged areas in the amygdala-lesioned animals.**



**Fig. 5 – Mean percent (±SEM) of conditioned freezing during the testing session among Carioca High-Freezing (CHF) and Carioca Low-Freezing (CLF) rats. Asterisk indicated  $p < 0.01$ .**

differences favoring males have been observed in contextual fear conditioning (Maren et al., 1994; Markus & Zecevic, 1997) as well as in other spatial learning such as in the 12-arm radial maze (Williams et al., 1990) and the Morris water maze (Roof, 1993). It has been suggested that these differences may be related to sexual dimorphism observed in hippocampal anatomy and physiology. Indeed, electrophysiological studies have found that male rats that acquired contextual fear more rapidly than female rats also showed a higher magnitude of LTP induced at perforant path synapses in the dentate gyrus of the hippocampal formation (Maren et al., 1994; Maren, 1995). Therefore, it is possible that marked sex differences observed in the present study are associated with greater magnitude in male hippocampus LTP compared to female rats.

The second experiment used fourth-generation male rats from our selective breeding procedure to investigate the effect of bilateral lesions of the amygdala on contextual fear conditioning in the CHF and CLF lines. In agreement with previous reports (Blanchard and Blanchard, 1972; Cousins and Otto, 1998; Kim et al., 1993; Maren et al., 1996; Oliveira et al., 2004), electrolytic lesions of the amygdala caused a substantial reduction in the amount of conditioned freezing. Interestingly, this deleterious effect was similar in both lines of animals (~60%), indicating that the high and low rates of conditioned freezing induced by our selective breeding procedure are regulated by an amygdala-dependent neural pathway. These results are in agreement with other reports (Maren, 1998; 2001; Zimmerman et al., 2007), which also found that post-training lesions of BLA or CEA caused similar disruption of conditioned freezing in rats with different levels of training.

The neural circuitry responsible for contextual fear conditioning involves multimodal sensory information that reaches the BLA through direct projections from the hippocampus. Indeed, LTP has been observed along this hippocampal–amygdaloid pathway (Maren and Fanselow, 1995). Moreover, ascending serotonergic projections from the median raphe nucleus to the hippocampus seem to be part of the pathway that regulates contextual fear conditioning (Silva et al., 2002). The ventral portion of the medial prefrontal cortex (Resstel et al., 2006) and the perirhinal and postrhinal cortices (Bucci et al., 2000; Corodimas and LeDoux, 1995; Sacchetti et al., 1999) are also thought to be involved contextual fear conditioning. Direct projections from these cortical areas to the hippocampus and to the BLA may provide higher-order processing of polymodal sensory information. The informa-

tion flow within the amygdaloid region involves projections from the BLA to the CEA, which constitutes the main output region of the amygdala. Efferent projections from the CEA to the brain stem and hypothalamic areas give rise to distinct behavioral and autonomic reactions involved in this type of conditioning. The motor output of the conditioned freezing response is related to efferents from the CEA to the ventral portion of the periaqueductal gray, which in turn sends projections to motoneuron cell groups in the spinal cord.

The results presented here confirm that the amygdaloid region plays a pivotal role in contextual fear conditioning. Although different pathways may participate in processing dangerous stimuli, they all seem to converge in the amygdala. In this way, CHF and CLF animals bearing lesions within this area show a similar disruption in conditioned freezing. Unfortunately, this study cannot clarify whether other brain structures along these neural pathways might play a differential role in acquisition and expression of the different levels of conditioned freezing induced by our selective breeding procedure. Therefore, further studies are necessary to investigate more completely the contribution of each of these neural structures underlying contextual fear conditioning in these two new lines of animals.

In sum, the present report introduces two new lines of rats bidirectionally selected for their enhanced (CHF) or reduced (CLF) contextual fear conditioning, as measured by freezing behavior. Divergence between these two lines was observed after three generations, indicating a strong heritable component of this trait. The amygdala seems to be crucial for the expression of contextual fear conditioning presented by CHF and CLF lines regardless of their high or low levels of conditioned response since post-training electrolytic lesion within this area produced a similar disruption in conditioned freezing in both lines of animals.

## 5. Experimental Procedures 1

### 5.1. Methods

#### 5.1.1. Subjects

Albino Wistar rats were employed as subjects. The initial matrix of these animals was obtained in 1995 from a local farmer (Oswaldo Cruz Foundation), and since then they have been maintained in the colony room of the PUC-Rio Psychology Department. The selective breeding described in this work began in March of 2006.

Six to eight days after birth, animals were marked by amputation of one toe from each foot and a small cut in one of the ears. Upon weaning at 21 days of age, animals were separated by sex and housed in groups of five to seven, according to their respective lines, in polycarbonate cages measuring 18×31×38 cm, with food and water always provided *ad libitum*.

Room temperature was controlled ( $24 \pm 1^\circ\text{C}$ ) 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 to 120 days of age at the beginning of the experiment. For five days leading up to the experiment, the animals were handled once daily for a period

of 2 min. All experimental protocols employed in this work were approved by a local ethic committee and were conformed with the Brazilian Society of Neuroscience and Behavior Guidelines for Care and Use of Laboratory Animals (SBNeC), which are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals (revised in 1996).

#### 5.1.2. Apparatus

Contextual fear conditioning took place in an observation chamber ( $25 \times 20 \times 20$  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. Ammonium hydroxide solution (5%) was used to clean the chamber before and after each subject.

#### 5.1.3. General procedure

In order to develop a line of rats with a high rate of conditioned freezing, termed Carioca<sup>1</sup> High-Freezing (CHF), and another line of rats with a low rate of conditioned freezing, named Carioca Low-Freezing (CLF), 120 animals (60 males and 60 females) randomly bred in our colony room were used. These animals constituted the initial generation (S0).

The contextual fear conditioning protocol involved an acquisition and a testing session. During acquisition, each animal was placed in the observation chamber for 8 min. At the end of this period, three unsignaled electrical footshocks were delivered at a strength of 1 mA, with each shock lasting 1 s and with an intershock interval of 20 s. The animal was returned to its home cage 2 min after the last shock.

The testing session occurred approximately 24 h after training. This test consisted of placing the animal for eight min in the same chamber in which the three footshocks had been administered on the previous day. No footshock or other stimulation occurred during this period. A time-sampling procedure was employed to evaluate fear conditioning to contextual cues. Every two seconds, 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 vibrissa except for movement required for respiration. The agreement between observers with respect to the scoring of freezing episodes in our laboratory is higher than 0.95.

At the acquisition session, freezing was scored during the 8-min baseline period prior to the occurrence of the first footshock as well as during the 2-min post-shock period immediately after the occurrence of the third footshock. Freezing was also scored during the 8-min test session. The total amount of freezing behavior observed during the test session was used as the criterion for animal mating. The 10

male and 10 female rats with the highest conditioned freezing score, as well as the 10 male and 10 female rats with the lowest conditioned freezing rate were selected to breed the CHF and CLF lines, respectively. From the 10 CHF families, 76 animals were born, while the 10 CLF families gave rise to 71 animals. These animals were the first-generation offspring of our breeding procedure (S1). The same procedure was used for the production of two new generations of selected animals (S2 and S3). The 10 high- and low-family breeders were chosen after all animals from a given generation had been phenotyped. Mating always occurred within each line. One exception occurred in S2, when one female from the CLF line with the highest rate of conditioned freezing was bred with a male from the CHF line that also had the highest rate of conditioned freezing. Brother-sister breeding pairs were avoided in order to reduce inbreeding, which could lead a reduction in the animal's fertility and random changes in the development of the selected lines due to genetic drift (Falconer and MacKay, 1996).

## 6. Experimental Procedures 2

### 6.1. Methods

#### 6.1.1. Subjects

CHF and CLF animals from the fourth generation (S4) were used as subjects. This new generation of animals was created from S3 following the same procedure described in Experiment 1. The S4 population consisted of 77 animals from the CHF line (46 males and 31 females), and 74 animals from the CLF line (33 males and 41 females).

#### 6.1.2. Equipment and procedure

All animals were phenotyped for contextual fear conditioning using the equipment previously described. The conditioning protocol was slightly different from Experiment 1. Each animal was placed in an observation chamber and three minutes later, three unsignaled electrical footshocks with strength of 1 mA and a duration of 1 s were delivered 20 s apart. One minute after the last shock, the animal was returned to its home cage. Approximately 24 h after the training session, the animal was placed back in the same observation chamber for a 4-min test session in the absence of any stimulation.

The 24 male and 24 female rats from the CHF with the highest conditioned freezing scores as well as the 24 male and 24 female rats from the CLF with the lowest amount of conditioned freezing were mated for one week. After this period, all 48 male rats (24 animals from each line) were housed in groups of six, according to their respective lines. Approximately three weeks later, half of the 24 CHF and CLF animals received bilateral electrolytic lesions in the amygdala whereas the other half of the animals received sham lesions. One week after surgery, each animal was returned to the conditioning chamber for a 3-min test session in the absence of any stimulation.

#### 6.1.3. Surgery

Under aseptic conditions, animals were anaesthetized with tribromoethanol (250 mg/kg, i.p.) and fixed in a stereotaxic

<sup>1</sup> Carioca is the name given to those born in Rio de Janeiro.

frame (David Kopf, Tujunga, CA). The upper incisor bar was set at 3.3 mm below the interaural line such that the skull was horizontal between bregma and lambda. Bilateral electrolytic lesions of the amygdala were made by passing a 5 mA anodal current for 20 s through a stainless steel insect pin (size 00) insulated with baked epoxylite except for the cut tip. A cathode clamped to the tail completed the circuit. The current was delivered by a lesion-generating device (DelVechio, Ribeirão Preto, Brazil). Based on the rat brain atlas of Paxinos and Watson (1986), the stereotaxic coordinates were 3.2 mm posterior to bregma, 4.2 mm lateral to each side of the midline, and 5.8 mm ventral to the dura of the brain. Sham-lesion animals were submitted to the same surgical procedure except that no current was delivered.

#### 6.1.4. Histology

At the end of the experiment, animals were overdosed with cloral hydrate (1 ml/100 g, i.p.) and perfused through the left ventricle of the heart with 0.9% saline followed by a formalin (4%) solution containing potassium ferrocyanide (1%). After transcardiac perfusion, the brain was removed and placed in a 10% solution of phosphate-buffered saline containing 30% sucrose for at least one week. Serial 60 µm brain sections were cut using a cryostat microtome, thaw-mounted on gelatinized slides, and stained with cresyl violet in order to localize the electrolytic lesions according to the rat brain atlas of Paxinos and Watson (1986).

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