Pontifícia Universidade Católica
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Vitor de Castro Gomes

TESE DE DOUTORADO

Genetic selection of two new rat lines displaying different levels of conditioned freezing behavior

Thesis presented to the Departmento de Psicologia, PUC-Rio as partial fulfillment of the requirements for the degree of Doutor em Psicologia Clínica in the Departamento de Psicologia do Centro de Teologia e Ciências Humanas da PUC-Rio.

Advisor: Prof. Jesus Landeira Fernandez

Rio de Janeiro
March 2012

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Abstract

Gomes, Vitor de Castro, Fernandez, Landeira-Fernandez, J. (Advisor). **Genetic selection of two new rat lines displaying different levels of conditioned freezing behavior.** Rio de Janeiro, 2012. 160p. PhD Thesis – Departamento de Psicologia, Pontifícia Universidade Católica do Rio de Janeiro.

Bidirectional selective breeding of a defensive response or any other phenotypic characteristic is a technique in which animals are bred to modify the frequency of the genes that underlie 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. In the present work we employed the conditioned freezing in response to contextual cues previously associated with footshock as the phenotype criterion for developing two new rat lines. The basic protocol consisted of mating male and female albino Wistar rats with the highest and lowest conditioned freezing in response to the contextual cues of the experimental chamber where animals were exposed to three unsignaled electric footshocks on the previous day. Study 1 presents the initial results of fourteen generations of selective breeding. We found that after three generations, reliable differences between these two lines were already present, indicating a strong heritable component of this type of learning. The lines were named Carioca High conditioned Freezing (CHF) and Carioca Low conditioned Freezing (CLF). Also, we introduced a third group of randomly selected animals (RND) in our selective breeding program. In Study 2, we investigated the different patterns of fear extinction and reacquisition in these two new lines. Finally, in Study 3, results showed dissociation between contextual and phasic fear between CHF and CLF rats.

Keywords

Animal models of anxiety; Amygdala; Emotionality; Strains; Genetic selection.

Resumo

Gomes, Vitor de Castro, Fernandez, Landeira-Fernandez, J. (Advisor). **Seleção genética de duas novas linhagens de ratos selecionados com diferentes níveis de comportamento de congelamento condicionado.** Rio de Janeiro, 2012. 160p. Tese de Doutorado – Departamento de Psicologia, Pontifícia Universidade Católica do Rio de Janeiro.

Criação seletiva bidirecional de uma resposta defensiva ou qualquer outra característica fenotípica é uma técnica na qual animais são criados com o objetivo de modificar a frequência dos genes que estão subjacentes a um fenótipo em particular. O acasalamento de animais de uma determinada população com base nos extremos opostos de uma característica observável vai propagar, após diversas gerações, este fenótipo particular em direções opostas, levando-se à criação de duas linhagens contrastantes. No presente trabalho empregamos o congelamento condicionado em resposta a estímulos contextuais previamente associados com choques elétricos nas patas como critério de seleção para o desenvolvimento de duas novas linhagens de ratos. O protocolo básico consistiu de acasalamento entre machos e fêmeas Wistar com as maiores e as menores taxas de congelamento condicionado em resposta a sinais contextuais da câmara experimental onde os animais foram expostos a três choques elétricos não sinalizados no dia anterior. O Estudo 1 apresenta os resultados iniciais de quatorze gerações de criação seletiva. Os resultados mostraram que diferenças significativas entre estas duas linhagens foram encontradas após 3 gerações, indicando um forte componente hereditário deste tipo de aprendizagem. As linhagens foram denominadas Cariocas com Alto Congelamento Condicionado (CAC) e Cariocas com Baixo Congelamento condicionado (CBC). Além disso, nós introduzimos um terceiro grupo de animais aleatoriamente selecionados (CTRL) em nosso programa de criação seletiva. No Estudo 2 investigamos os diferentes padrões de extinção e da reaquisição do medo condicionado nestas duas novas linhagens. Por fim, no Estudo 3, nossos resultados sugeriram uma dissociação entre o medo contextual e o medo discreto entre animais CAC e CBC.

Palavras-chave

Modelos animais de ansiedade; Emocionalidade; Linhagens; Seleção genética

SUMMARY

"Spirit" comes from the Latin word "to breathe." Despite usage to the contrary, there is no necessary implication in the word "spiritual" that we are talking of anything other than matter (including the matter of which the brain is made), or anything outside the realm of science. On occasion, I will feel free to use the word. Science is not only compatible with spirituality; it is profound source of spirituality. when we recognize our place in as immensity of light years and in the passage of ages, when we grasp the intricacy, beauty, and subtlety of life, then that soaring feeling, that sense of elation and humility combined, is surely spiritual. So are our emotions in the presence of great art or music or literature, or of acts of exemplary selfless courage. The notion that science and spirituality are somehow mutually exclusive does a disservice to both."

(Carl Sagan, *The demon haunted world*)

1
Introduction

Fear and anxiety are complex concepts. Both terms have been used to describe a set of highly orchestrated neural events that involve sensory processing and motor responses triggered by threatening situations. These events are mediated by central neural circuitries and peripheral neuroendocrine pathways and clearly have adaptive value. Sensory systems function as alarm signals to warn of real or potential danger, producing a shift to a state of high vigilance that prepares the individual to avoid or escape from a wide variety of dangerous situations. Most of these reactions are not exclusive to our species. Because of their importance for survival, fear and anxiety traits are believed to have been selected in animal evolution and shaped by natural selection for their crucial role in protecting individuals who face adverse environments (Coutinho et al., 2010; Gross & Hen, 2004; Marks & Nesse, 1994).

However, these highly adaptive events can be disabling when the individual experiences them excessively or when they occur in the absence of any threatening stimuli. In these cases, they represent a pathological condition termed an anxiety disorder. Often chronic in nature, these disorders are among the most prevalent mental health problems across the individual life span, producing severe impairments in social and occupational functioning.

According to an evolutionary perspective, an anxiety disorder reflects a malfunctioning of the neural circuits responsible for detecting, organizing, or expressing adaptive defense reactions (Jacobson & Cryan, 2010). Humans and nonhuman mammals share approximately the same behavioral defense strategies, reflected by activation of similar underlying neural circuitry. Therefore, animal models of anxiety can be extremely helpful for better understanding the behavioral, neural, and genetic substrates involved in these pathologies. The purpose of this thesis is to present two new rat lines that might be a useful model of generalized anxiety disorder (GAD). Before we discuss this model, defining how anxiety disorders are currently classified is important.

1.1
Clinical Aspects of Anxiety Disorders

The concept of anxiety disorders has changed dramatically over the years as more clinical and experimental evidence has been collected. In the clinical setting, anxiety disorders have departed from a single construct that ranged in intensity from normal to pathological or neurotic levels. A major shift in this view occurred with Klein's pioneering work (Klein, 1964; Klein & Fink, 1962), which showed that imipramine had a selective effect in the treatment of panic disorder. Moreover, certain anxiety disorders have been suggested to differ from each other in the primary object or specificity of threat. Fear of a circumscribed and well-defined object is a characteristic of specific phobias, whereas diffuse and chronic sustained anxiety is the main feature of GAD.

The 3rd edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-III; American Psychiatric Association, 1980) introduced the current descriptive symptom-based approach to mental disorders with well-defined, explicit diagnostic criteria. This new classification incorporated distinct nosological entities, such as panic disorder, specific and social phobias, GAD, posttraumatic stress disorder, and obsessive-compulsive disorder. In the DSM-III, GAD was left as a residual diagnosis of worry, to be made only in the absence of other anxiety and depressive syndromes. Consequently, this residual category carried low diagnostic reliability.

With the publication of the DSM-IV (American Psychiatric Association, 1994) and International Classification of Diseases and Related Health Problems (ICD-10; World Health Organization, 1992), these anxiety disorder categories remained basically the same. However, the diagnosis of GAD shifted from a residual category in the DSM-III to an independent anxiety disorder type in the DSM-IV. Free-floating anxiety was associated with the worry construct, which in turn produced several symptoms, such as muscle tension, fatigue, restlessness, concentration difficulties, and irritability. According to the DSM-IV, excessive and unrelenting worry is generally associated with impairments in academic, social, and personal functioning and related to multiple domains or activities. To

be considered a pathological feature of GAD, worry must occur more days than not for a period of at least 6 months.

1.2
Animal Models of Anxiety

In the experimental setting, most of the studies that investigate the etiological mechanisms that underlie anxiety disorders have been performed using animal models. Defensive reactions of the laboratory rat (*Rattus norvegicus*) have been employed as the main system for modeling human anxiety. In his classic work, Calvin Hall (1934) employed the word "emotionality" to describe a set of defensive reactions that an animal presents in a potentially dangerous situation, such as an open field. Since then, several other animal models of anxiety were developed. An important issue regarding these models is the fact that most of them depend on the animal's locomotor activity. This is why a pure measure of emotionality, devoid of non-emotional confounding factors, such as locomotor activity, might be a difficult task to achieve (Ramos, 2008). Therefore, using different animal models of anxiety might help to exclude non-emotionality factors that might interact with an anxiety-related response. Moreover, examining whether a given result in one model can be generalized to other models, and thus estimating the extent to which the expression of different defensive responses might be mediated by a single emotional trait, is also possible.

As in the clinical setting, the traditional view that highlighted these experimental studies was that animal defensive responses were mediated by a single and general anxiety construct (Broadhurst, 1975; Gray, 1979; Hall, 1934). Nevertheless, as new data were collected, it became clear that animal defensive behavior is mediated by a complex and multidimensional construct (Aguilar et al., 2002; Belzung & Le Pape, 1994; Ramos et al., 1997; Torrejais et al., 2008). These diverse dimensions found in animal models of anxiety may indicate that clinically defined anxiety disorders could be associated with a particular animal model. However, the adoption of descriptive and operational criteria from the modern classification systems imposed a validity problem among the several anxiety disorder categories. The DSM-IV and ICD-10 are not primarily based upon

etiology, neurobiology, epidemiology, genetics, or responses to medications, but rather on phenomenological descriptions of clinical data that have imprecise similarity or correlate with each other within and between individuals (Gould & Gottesman, 2006). Therefore, unsurprising are the several problems that are encountered when attempting to use the current systems of mental disorder classification as a guide for developing viable animal models.

These models basically consist of exposing an animal to an innate or learned aversive environment while assessing one or a set of defensive behaviors. Typically, each animal model has been validated with pharmacological agents with well-know anxiolytic or anxiogenic properties in clinical settings. Below we summarize 11 models classified according to the innate or learned characteristics regarding a threatening situation.

1.3
Innate Aversive Paradigms

1.3.1
Open field test

The open field is one of the most popular animal models of anxiety, probably because of the simplicity of the apparatus and the easily identifiable and well-defined defensive reactions observed in animals in this situation. The apparatus consists of a large square or circular arena surrounded by walls so that the animal cannot escape. The floor of the arena is marked with squares or concentric lines to quantify the animal's locomotion. This test was first employed by Hall (1934), who used defecation in the open field as a measure of timidity or emotionality because of its relationship with the autonomous nervous system. General locomotor activity, especially locomotion in the central illuminated area of the arena (which is aversive to the animal), became another index of emotionality. In addition to ambulation, other behavioral measures include grooming, freezing, and rearing on the walls or in the space. The main definition of emotional reactivity in this model is the association between low ambulation

and a high rate of defecation. The effect of anxiolytic and anxiogenic drugs in the open field has been widely demonstrated (e.g., Prut and Belzung, 2003).

1.3.2
Elevated plus-maze

Inspired by an earlier elevated Y-maze (Montgomery, 1955), the elevated plus maze was first introduced in 1984 (Handley and Mithani, 1984) and subsequently validated for use with rats (Pellow et al., 1985). The elevated plus maze is based on the natural fear of rodents for open and elevated alleys. The apparatus consists of four elevated arms that are arranged in a "cross-like" pattern, with two opposite open arms with a minimal lip and two closed arms with high walls. At their intersection, a central platform allows access to all four arms. This region is also called the "decision making" area. The rat is placed on the central platform, and total exploration or locomotion is measured as the total number of entries in the open and closed arms for 5 min. The percentage of open arm entries and percentage of time spent in the open arms are used as anxiety indices, whereas the number of closed arm entries is used as an index of locomotor activity. In fact, rats confined in the open arms show more physiological and behavioral signs of fear, such as higher defecation rates and decreased locomotion, than when confined in the closed arms. Moreover, factor analysis studies indicated that this paradigm reliably dissociates the anxiety-like (open arm entries) from locomotor (close arm entries) effects of several anxiolytic and anxiogenic agents (Cruz et al., 1994). Because of its ability to dissociate the emotional effects from motor effects of drugs, the elevated plus maze is one of the most widely used animal models for screening anxiolytic drugs (Pellow et al., 1985).

1.3.3
Light-Dark box test

The light-dark box test, also known as the light-dark transition test, was first described by Crawley and Goodwin (1980) to investigate the anxiolytic properties of drugs in mice. The model is based on the innate aversion that rodents have to

brightly illuminated areas. The apparatus consists of equally sized compartments connected by a small door. One compartment is brightly illuminated, and the other is dark. Usually, the animal is placed in the dark compartment, and several parameters, such as the distance traveled in each side of the box, total number of transitions between the light and dark compartment, latency to enter the light compartment, and time spent in each compartment, can be measured. The total number of transitions appears to be an index of locomotor activity, whereas the latency to first enter the light compartment or total time spent in the light compartment are considered emotional measures. The anxiolytic effects of benzodiazepines and 5-hydroxytryptamine-1A ($5\text{-}HT_{1A}$) receptor agonist compounds have been detected in this animal model (Bourin and Hascoët, 2003).

1.3.4
Social interaction test

Initially developed by File and Hyde (1978), the social interaction test is based on the fact that the occurrence of pairs of male rats that perform social behaviors depends on the aversiveness (i.e., high light level or novelty of the situation) of the experimental condition. The frequency of and time spent by pairs of males engaging in social interaction can be divided into two categories: aggressive behaviors (e.g., kicking, jumping on, wrestling, and boxing) and non-aggressive behaviors (e.g., sniffing, following, grooming). Because the behavior of one rat influences the behavior of the other, the pair of rats is treated as a unit, and only one score for the pair is used. In a typical protocol, the pair of animals is placed in an arena with the floor divided into squares so that general activity can be measured. All of the animals are individually acclimated to the arena at least 1 day prior to testing. An increase in social interaction without a concomitant increase in motor activity is indicative of an anxiolytic-like effect, whereas a specific decrease in social interaction indicates an anxiogenic-like effect (File and Seth, 2003). The social interaction test has been widely validated with anxiolytic and anxiogenic drugs and is able to distinguish between anxiolytic and sedative effects (File, 1985).

1.3.5
Ultrasonic vocalization

Zippelius and Schleidt (1956) observed that infant mice produced ultrasonic vocalizations (USVs) when separated from their mothers and littermates. These USVs, which can trigger rodent maternal search and retrieval behaviors (Brunelli, 2005), are whistle-like sounds characterized by frequencies ranging between 30 and 90 kHz, with duration of 10-200 ms and intensity of 60-100 dB. Maternal isolation is a stressful event for rodent pups, producing cardiovascular changes, increased autonomic nervous system activity, and activation of the hypothalamic-pituitary-adrenal axis. Ultrasonic vocalizations may also be emitted during other stressful events, such as frustrated non-reward, opiate withdrawal, and cold ambient temperatures.

In this paradigm, when a rat pup between 4 and 16 days of age is separated from its mother and littermates for a brief period of time, it typically emits a so-called 40-kHz vocalization. The test can be performed under two different stress conditions. Pups are placed in isolation in either a warm (37°C) or cold (18°C) environment for 5 min. The total number and duration of ultrasonic calls emitted by the pups during this period is used as an index of anxiety. Anxiolytic compounds reduced the number and cumulative duration of USV (Portfors, 2007). Pharmacological and behavioral studies have also indicated that USV in isolated rat pups might represent an important model of separation anxiety in early development (Ditcher et al., 1996; Insel and Winslow, 1991).

1.4
Learned aversive paradigms

1.4.1
Habituation and sensitization of the acoustic startle response

The acoustic startle response is a reflex characterized by a short-latency sequence of facial and skeletal muscle contractions following an unexpected and intense acoustic stimulus. This is a defensive response because its behavioral

pattern consists of reactions that are likely to prevent a predator attack or other possible injury from the environment. The acoustic startle response is mediated by neural circuitry located in the lower brainstem. Auditory stimuli are processed by cochlear nuclei, which send ascending projections to the caudal pontine nucleus, with descending projections to motor neurons in the spinal cord (Lee et al., 1996).

The amplitude of the acoustic startle response can be measured automatically with special sensors beneath the rat cage and can be modified by various non-associative and associative learning processes. Habituation and sensitization represent two forms of non-associative learning that can bidirectionally modulate the amplitude of this response. Habituation refers to a decrease of the startle reflex magnitude as a function of repeated presentation of the acoustic stimulus (Prosser and Hunter, 1936). Sensitization refers to an increase of the startle reflex amplitude in response to repeated presentation of the acoustic stimulus caused by presentation of an aversive stimulus, such as a footshock (Davis, 1989). An increase of the startle reflex appears to reflect a state of diffuse fear associated with arousal and vigilance produced by the footshock that is presented without any relationship to the acoustic stimuli (Groves and Thompson, 1970). Several reports indicated that limbic structures, such as the amygdaloid complex, play an important role in the sensitizing effects of electric footshocks (Hitchcock et al., 1989). Moreover, this sensitization effect appears to be mediated by γ-aminobutyric acid (GABA) receptors in the basolateral amygdala (Van Nobelen and Kokkinidis, 2006).

1.4.2
Fear-potentiated startle

In addition to sensitization, the amplitude of the acoustic startle response can be enhanced when it is elicited in the presence, rather than absence, of a fear-eliciting conditioned stimulus (CS), such as a light, that was previously associated with an aversive unconditioned stimulus (US), such as a footshock. According to this associative learning paradigm, developed by Brown et al. (1951), a rat is initially exposed to the CS-US pairing. Rats are later tested for fear responses to the CS by eliciting the startle reflex with a series of brief and intense acoustic

stimuli presented in the presence or absence of the CS. An increase in the amplitude of the startle reflex in the presence of the CS has been termed a fear-potentiated startle response. This effect has been replicated using either an auditory or visual CS, when the startle reflex is elicited by either an acoustic or air puff stimulus (Davis, 1986). Several studies indicate that anxiolytic drugs can block fear-potentiated startle in rats (e.g., Davis, 1986, 1993), suggesting that this is an adequate animal model of anxiety.

1.4.3
Avoidance Responses

Avoidance learning involves the acquisition of a response that prevents the occurrence of a future aversive event. There are two forms of avoidance learning: active and passive. In both situations, the animal is required to perform (active avoidance) or suppress (passive avoidance) a response to prevent an aversive event that was scheduled to occur.

1.4.3.1
Active Avoidance

The active avoidance learning procedure has numerous variations. One of the first studies, Mowrer and Lamoreaux (1942, 1946) employed an apparatus known as a shuttle box (Mowrer, 1940; Mowrer and Miller, 1942), which consists of a box divided into two equal compartments by a hurdle, over which the subject can jump to shuttle from one compartment to the other. Variations of the shuttle box replace the hurdle with a doorway between the compartments so the animal can cross from one side of the box to the other. An electric footshock (US) can be delivered to the animal's paws through the grid floor of the box, and a lamp or speaker can present a warning signal (CS).

In the two-way shuttle box avoidance procedure, the animal is placed in one of the compartments. After a predetermined length of time, a CS is presented, and the animal must go to the other compartment before the occurrence of the US. After a short period of time, the CS is presented again, and the subject must return

to the original compartment to avoid the aversive event. If the shuttle response does not occur in the presence of the CS, then the US remains on until an escape response of going to the other compartment occurs. Therefore, in each trial, the onset of the CS precedes the onset of the US. An avoidance response during the CS terminates the CS and cancels the US, whereas an escape response after the onset of the US terminates both the CS and US.

Subjects first learn to escape from the US. As training continues, the escape response begins to occur in the presence of the CS, which turns off the CS and prevents the delivery of the aversive event. Notice that the subject can avoid the footshock by shuttling either way, from the left to right compartment or vice versa. For this reason, the procedure is called two-way avoidance.

The primary measure of learning in this task is an increase in avoidance responses. The acquisition of high rates of this response might require 100 or more trials because of the complex nature of this type of learning. The two-way avoidance procedure involves a conflict situation, given that both compartments have aversive and safety functions. Typically, animals that are less emotionally reactive to this aversive procedure exhibit better learning than animals that are more "afraid" of this situation. Indeed, higher levels of electrical footshock are associated with lower two-way avoidance performance (Levine, 1966; McAllister et al., 1971). Accordingly, anxiolytic drugs enhance, whereas anxiogenic compounds impair, the acquisition of two-way avoidance (Fernández-Teruel et al., 1991; Savić et al., 2005).

The fact that no one side of the shuttle box is always safe can be overcome by testing the animal in a one-way avoidance task. In this paradigm, the animal is always placed in the same compartment where the CS and US occur. In the other compartment, neither the CS nor US appears. An avoidance response is defined when the animal shifts from the danger compartment to the safe compartment in the presence of the CS, whereas an escape response is defined when the animal shuttles from one side to the other in the presence of the US. In the one-way avoidance apparatus, contextual cues associated with the start or dangerous compartment are clearly different from the goal or safe compartment. Although the acquisition of the one-way avoidance response is rapidly observed, this

paradigm involves confounding variables, such as handling stimuli necessary to move the animal from the safe to dangerous compartment between trials.

In the case of one-way avoidance learning, the anxiety index is exactly the opposite from two-way avoidance. The more "afraid" the animal is when learning the situation, the better the acquisition of the one-way avoidance response. For example, one-way avoidance is generally better with higher footshock intensities (Dieter, 1976), in contrast to the acquisition of two-way avoidance (Levine, 1966; McAllister et al., 1971). The effects of anxiolytic drugs on one-way avoidance performance have been inconsistent. Although classic anxiolytic drugs, such as benzodiazepines, did not cause any effect in one-way active avoidance acquisition (Gray and McNaughton, 2000), Sanger et al. (1989) found that 5-HT_{1A} receptor agonists (but not imipramine) impaired the acquisition of one-way avoidance.

1.4.3.2
Passive Avoidance

The passive avoidance response is a rapid learning process that involves single training and test sessions. This paradigm has two versions: step-through and step-down. In step-through passive avoidance, the animal is placed in a bright compartment, and the latency to enter the dark compartment is recorded. After entering this compartment, the animal receives an electric footshock. During the test session, generally 24 h after the training session, the animal is returned to the bright compartment, and the latency to enter the dark compartment, which at this point is not electrified, is measured. In step-down passive avoidance, the animal is placed on a small platform located approximately 4 to 8 cm above the grid. When the animal steps down from the platform with its four paws on the grid, it receives an electric footshock. In the test session, the animal is placed back on the platform, and the step down latency is measured.

Aversive learning is inferred from the delay of the step-through or step-down responses that were made before training. Because delaying a response is an active process, this paradigm has also been termed inhibitory, rather than passive, avoidance. An animal that is more "afraid" has a longer latency (i.e., better inhibitory avoidance). Pharmacological results corroborate this premise.

Benzodiazepines and various 5-HT_{1A} receptor agonist compounds with anxiolytic properties impair passive avoidance performance (Anglade et al., 1994; Misane et al., 1998).

1.4.4
Conditioned emotional response

Initially developed by Estes and Skinner (1941), the conditioned emotional response was one of the first animal models that measured the learned aspects of fear. In a typical experiment, food-deprived rats are initially trained to lever press for food for intermittent reinforcement. After giving a sufficient number of training sessions to establish stable lever pressing, a CS, such as a light or tone, is paired with a US, such as an electric footshock. After a small number of pairings, the animal returns to the appetitive operant condition, and the CS is presented. A variation of this procedure is to measure the disruptive effects of the CS on some consummatory response, such as a thirsty rat licking for water on a water tube. Aversive learning is measured by suppression according to the ratio $a/(a + b)$, in which "*a*" represents the number of responses made during the CS, and "*b*" represents the number of responses made during a period that immediately preceded the onset of the CS and had the same duration as the CS. If the CS did not acquire any associative learning, then suppression does not occur, and the ratio is 0.5. The more "afraid" the animal is of the CS, the lower the ratio. Maximal conditioning to the CS produces total suppression of the response, and the ratio is 0.0. Several reports indicate that anxiolytic drugs alleviate the suppressive effect of the CS (e.g., Davis, 1990).

1.5
Contextual fear conditioning as a model of generalized anxiety disorder

Regardless of the variability of animal models available for the study of current clinically defined anxiety disorders, fear conditioning has been historically associated with one of the main causes of pathological anxiety (i.e., neurosis;

Pavlov, 1927; Watson & Rayner, 1920). In a typical fear conditioning experiment, a discrete and emotionally neutral stimulus, such as a light or tone, reliably signals the occurrence of an aversive stimulus (Unconditioned Stimulus – US), such as an electric footshock. After a few pairings between these two stimuli, the previously harmless stimulus becomes a potent conditioned stimulus (CS) and acquires the ability to elicit several fear reactions, including defensive behaviors (freezing), autonomic (i.e., increases in blood pressure and heart rate) and endocrine (hormone release) responses, as well as modifications in pain sensitivity (analgesia) and reflex expression (eyeblink response and fear potentiated startle).

Another form of fear conditioning is to make the aversive stimulus unpredictable. According to this alternative procedure, a rat is exposed to a novel chamber and, after a few minutes, a brief and unsignaled footshock is delivered. When returned to the same chamber in the absence of the aversive stimulus, the animal presents a permanent fear reaction to contextual cues previously associated with the footshock. Contextual fear conditioning represents one of the simplest and most rapid forms of producing aversive learning (Landeira-Fernandez, 1996).

Considerable evidence from animal and human experiments indicates that fear conditioning in response to a discrete CS or to contextual cues are mediated by different neural circuitries (Indovina et al., 2011; Ferreira et al., 2003; Kim & Fanselow, 1992; LeDoux, 2000; Pohlack et al., 2011). These results support the hypothesis of at least two dimensions of fear conditioning, and each dimension might be related to clinically distinct anxiety disorders. Specific phobias, characterized by cue-specific or phasic fear reactivity, might be modeled by aversive conditioning in response to a discrete CS (Grillon, 2002; Grillon and Davis, 1997). GAD, in contrast, is characterized by persistent and diffuse or non-cue-specific anxiety and might be modeled by contextual fear conditioning (Brandão et al., 2008; Grillon and Davis, 1997).

When the CS-US pairings occur in a certain context, aversive conditioning is simultaneously acquired for both the CS and contextual cues. Conditioning to contextual cues can impose measurement problems with regard to the amount of aversiveness to the discrete CS. Therefore, conditioning to the CS is assessed by placing the animal in a context different from training. To prevent generalization from the training context to the context were the CS was tested, fear extinction of

the training context or pre-exposure to the test context is important to guarantee a low level of freezing in response to the context were the discrete CS was tested (Jacobs et al., 2010).

1.5.1
The neurocircuitry of contextual fear conditioning

The neural circuitry responsible for fear conditioning for both auditory, visual, olfactory or contextual stimuli are mapped and very well understood (Maren, 2001; Romanski et al., 1993; LeDoux, 2003). Although different pathways may participate in processing dangerous stimuli, they all seem to converge in the amygdalae (LeDoux, 2000). In this regard, the amygdaloid nuclei can be roughly divided into two main subsystems: the basolateral complex (BLA) (which in turn, is formed by the lateral (LA), basolateral (BL) and basomedial (BM) nuclei) and the central nucleus (CE). The BLA receives and integrates sensory information from a wide variety of sources. These include the medial and ventral subdivisions of the thalamic medial geniculate nucleus (MGm and MGV, for an auditory stimulus); the perirhinal cortex (PRh, for a visual stimuli); primary auditory cortex (TE) and the insular cortex (INS, for gustatory and somatosensory information); the thalamic posterior intralaminar nucleus (PIN, somatosensory information), the hippocampal formation (spatial and contextual information), which include area CA1, the ventral subiculum (vSUB), the entorhinal cortex (ENT) and the piriform cortex (PIR, for olfactory stimulus). As a result, the BLA is a place of sensory convergence and a possible site for CS-US association within the amygdala. Intra-amygdaloid circuitry sends the CS-US association to the CE, where different projections to the hypothalamus and brainstem mediate fear responses such as potentiated acoustic startle (nucleus reticularis pontinis caudalis, RPC), increased hear rate and blood pressure (lateral hypothalamus, LH; dorsal motor nucleus of vagus, DMN), increased respiration (parabrachial nucleus, PB), glucocorticoid release (paraventricular nucleus of the hypothalamus, PVN; bed nucleus of the stria terminalis) and freezing response (periaqueductal gray, PAG). Figure 1 illustrates the circuitry.

More specifically, the neural circuitry responsible for contextual fear conditioning involves multimodal sensory information that reaches the basolateral amygdala (BLA) through direct projections from the hippocampus. Indeed, long-term potentiation (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 in the 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 information flow within the amygdaloid region involves projections from the BLA to the central amygdala (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.

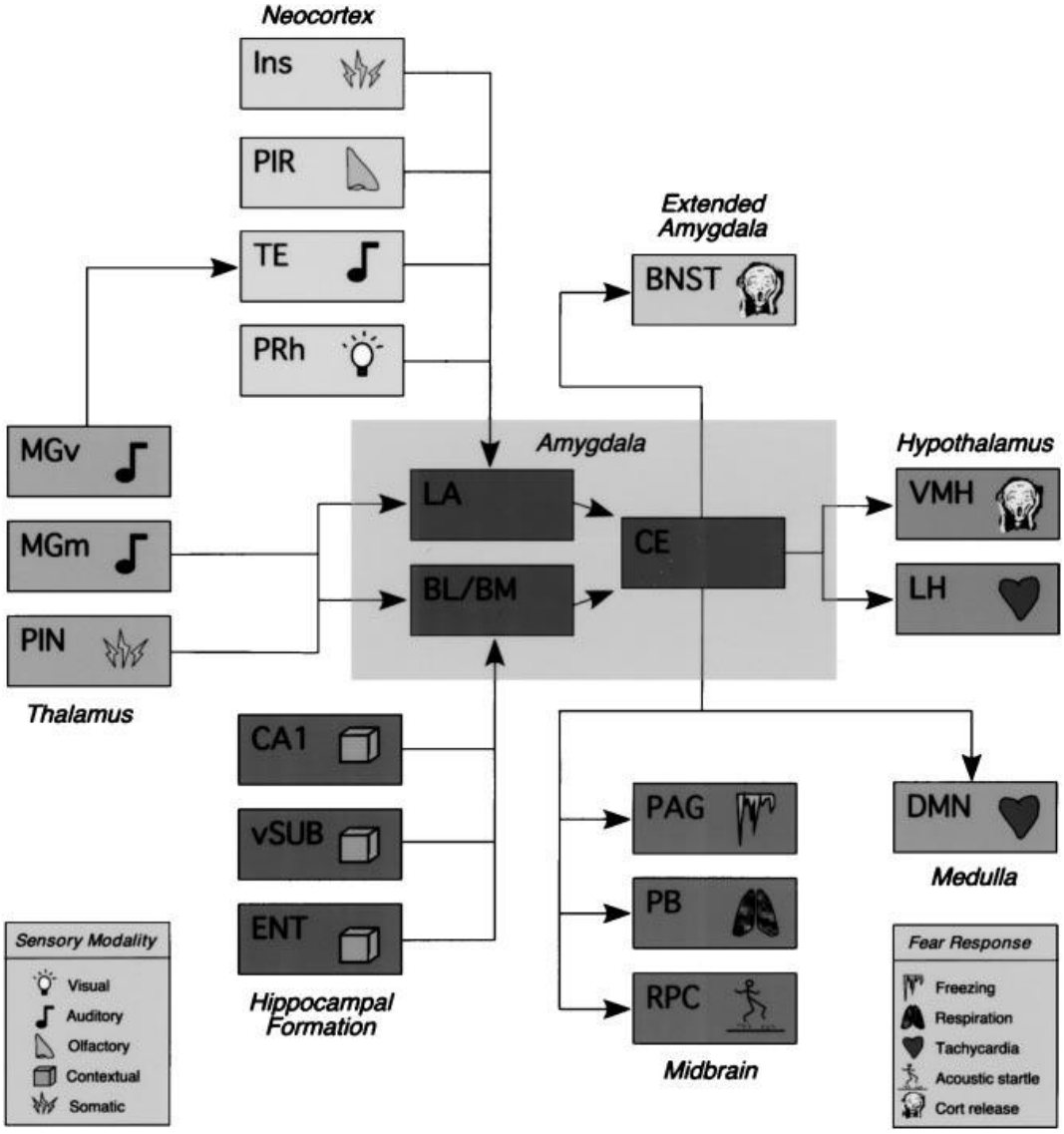

Figure 1: Anatomy of fear conditioning circuits in the brain. To simplify, all projections are drawn as unidirectional connections, although in several cases these connections are reciprocal. LA (lateral amygdala); BL (basolateral amygdala), BM (basomedial amygdala); BLA (basolateral complex); CE (central nucleus); MGm and MGv (thalamic medial and ventral geniculate nucleus); perirhinal cortex (PRh); primary auditory cortex (TE); insular cortex (INS); thalamic posterior intralaminar nucleus (PIN); vSUB (ventral subiculum); ENT (entorhinal cortex); PIR (piriform cortex); PAG (periaqueductal gray); RPC (nucleus reticularis ponits caudalis); LH (lateral hypothalamus); DMN (dorsal motor nucleus of the vagus); PB (parabrachial nucleus); PVN (paraventricular nucleus of the hypothalamus); BNST (bed nucleus of the stria terminalis).(Figure extracted from Maren, 2001) Annu. Rev. Neurosci. 2001.24:897-931. Downloaded from arjournals.annualreviews.org by CAPES

1.5.2
The conditioned freezing response

Freezing behavior is defined as a crouching posture (Blanchard and Blanchard, 1969) and cessation of motor activity, including whisker and nose movements (Bolles and Riley, 1973; Bindra and Anchel, 1963), with the exception of movements necessary for respiration (Bolles and Collier, 1976; Fanselow, 1980). This response is an efficient behavioral defense reaction against predation because predators have difficulty detecting an immobile target, and movement can function as a releasing stimulus that precipitates a predator attack (Fanselow and Lester, 1988).

Several studies showed that freezing is the most reliable measure of aversive contextual conditioning. This defensive response is a direct function of shock intensity (Sigmundi et al., 1980) and depends on the association between the cues of the experimental chamber and the footshock (Landeira-Fernandez et al., 2006). For example, when the footshock is presented simultaneously with the rat's placement in the chamber, no contextual fear conditioning is observed (Landeira-Fernandez et al., 1995). What makes freezing a very attractive index is that fear conditioning can be evaluated directly without any form of food or water deprivation or any form of operant response acquisition. Also, freezing is considered an unconditioned response when triggered by an innate threatening situation. For example, rats freeze when exposed to innately recognized predators, such as a cat (Griffith, 1920).

Conditioned freezing in response to contextual cues previously associated with footshocks has been pharmacologically validated as an adequate model of anxiety disorder. Accordingly, classic anxiolytic benzodiazepines, such as midazolam and diazepam (Fanselow and Helmstetter, 1988), and non-benzodiazepine anxiolytics, such as the serotonin-1A (5-hydroxytryptamine-1A [5-HT_{1A}]) receptor agonist ipsapirone (Inoue, Tsuchiya, Koyama, 1996) and 5-HT reuptake inhibitors citalopram and fluvoxamine (Hashimoto et al., 1996), reduced the amount of conditioned freezing. On the other hand, anxiogenic substances, such the benzodiazepine inverse agonist dimethoxy-β-carboline, produced

freezing behavior similar to that elicited by fear conditioning (Fanselow et al., 1991).

Freezing can also be employed to measure fear conditioning in response to a discrete CS, such as a light or tone (Sigmundi and Bolles, 1983). Freezing in response to contextual cues and a discrete CS previously associated with footshock is mediated by different neural circuitries (Kim and Fanselow, 1992). For that reason, freezing triggered by contextual cues and a discrete CS should be evaluated differently (for a review, see Brandão et al, 2008).

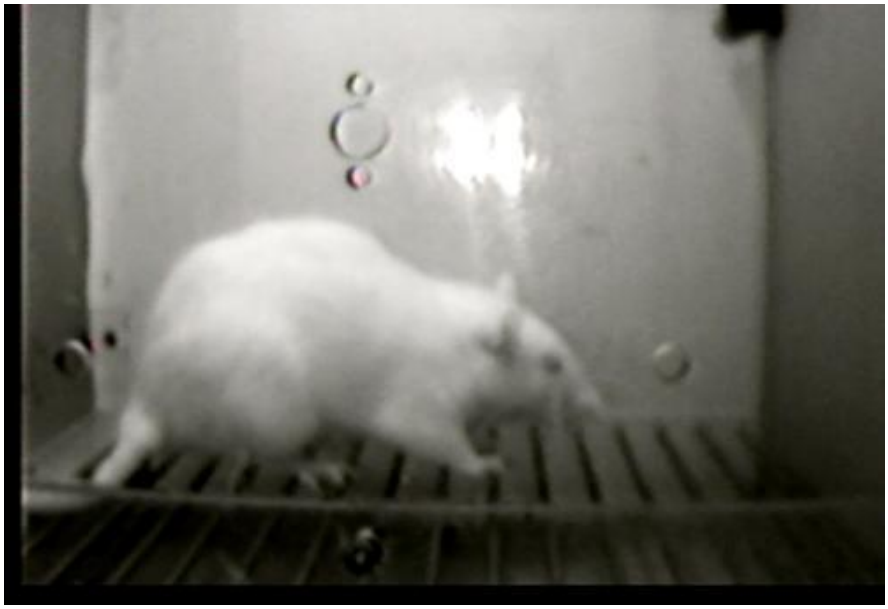

Figure 2: Typical conditioned freezing response in a rat.

1.5.3
Fear Extinction

A behavioral phenomenon that has received considerable interest of late, especially for the use in the treatment of human anxiety disorders, is the fear extinction learning. Extinction can be defined as the decrease of conditional responding (in this case decrease of CRs) following several presentations of a CS in the absence of the US. Extinction was first described by Ivan Pavlov (1927) in

his classic studies employing salivary response in dogs, and since then has received considerable experimental attention. Indeed, there is a large amount of data demonstrating that extinction is itself new learning (in this sense, inhibitory learning) that comes to inhibit or suppress the expression of Pavlovian CRs. Differently from the excitatory memories formed through conditioning, the inhibitory memories established through extinction procedures tend to be relatively volatile, i.e. the extinction weakens over time, promoting the spontaneous recovery of excitatory CRs as time elapses after extinction. Additionally, extinction memories are context-dependent, e.g. CR expression is restricted only in the context in which CS-alone presentations occurred. After extinction, CSs will continue to produce vigorous CRs when they are encountered outside of the extinction context.

Experimental investigation at the behavioral and systems level showed that most forms of extinction learning do not involve the forgetting or reversal of learned fear association (Bouton, 1993 and Rescorla & Heth, 1975). In fact, like other forms of learning, extinction consists of three phases: acquisition, consolidation and retrieval, each of which depends on specific neural structures (amygdala, prefrontal cortex and hippocampus, respectively).

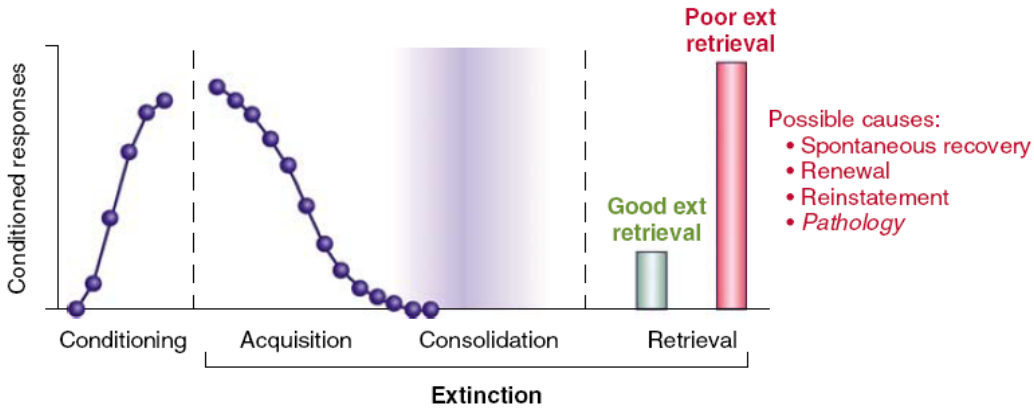

Figure 3: Fear extinction paradigm, with both conditioning, acquisition and consolidation phases (adapted from Quirk, 2008).

1.6
Fear and Anxiety: a genetic approach

Following involvement in a traumatic or stressful incident most people do not develop an anxiety disorder. Individuals differ greatly in their capacity to develop fearful associations, including conditioned fear. In this perspective, it is widely established that, in addition to the neural circuitry responsible for emotional defensive reactions, the environment and genetically determined predisposing factors also play a significant role in the pathogenesis of stress-related disorders (Gordon & Hen, 2004). Notably, the predisposition to develop an anxiety disorder is inherited, meaning that is partially influenced by the genotype of the individual (Stein et al., 2002). A major challenge for the neuroscience of anxiety disorders is to understand why some individuals endure a transition from normal emotional defensive reactions to the pathological and exaggerated patterns characteristics of the generalized anxiety disorders, as well as correlated illness like phobias, obsessive-compulsive disorders, panic disorders, post-traumatic stress disorder (PTSD) and impairments in fear extinction (Gross & Hen, 2004).

Several strategies have been developed in the past 60 years in order to analyze these inter-individual differences and susceptibility to psychiatry disorders, including the GAD. A genetic approach can be employed to investigate and dissect the individual variability between and within populations, searching for the molecular bases (genes and gene products), which underlie such variability. In this sense, these genetic models of anxiety disorders might be a useful tool for understanding why some individuals present adequate emotional reactions and others endure an exaggerated pattern of anxiety responses in the absence of a fear-provoking context (Finn et al, 2003; Grahame, 2000). These strategies include the use of inbred strains, multiple marker strains, animals obtained from gene targeting technologies and quantitative trait locus mapping (QTL), among others (Wood & Toth, 2001; Rudolph & Mohler; 2004; Lesch, 2001; Finn et al, 2003; Clément et al, 2002). Discussing these techniques is beyond the scope of this thesis, but some of those approaches will be considered for future studies. Here, we focused on the so-called Selection Experiments, in particular the “Artificial Selection” procedure.

1.6.1
Selection Experiments

The more newly developed tools of "genetic engineering" (e.g. knockouts, transgenesis) frequently attempt to modify only one or maybe a few gene loci and then analyze the phenotypic outcome. However, in the wild, sexual and natural selection act most directly on complex phenotypes (e.g. life history traits, behavior), which are mostly highly polygenic (affect by a myriad of genes, most of them with relatively small effects). Hence, from an evolutionary perspective, selection experiments may offer a major advantage, being more representative of the kind of genetic changes that occur in nature.

Selection experiments have a long history (Bell, 1997; Falconer, 1992; Falconer and Mackay, 1996; Garland and Carter, 1994; Gibbs, 1999; Hill and Caballero, 1992; Hill and Mackay, 1989; Robertson, 1980; Roff, 1997; Rose et al., 1990; Travisano and Rainey, 2000) and have occurred in non-laboratory contexts since human beings first developed agriculture, including the domestication of several plants and animals (for dog domestication, see Morey, 1994, Vila et al, 1997, Trut, 1999).

Analogously, in a scientific context, evolutionary scientists and behavioral psychologists often use selection experiments. By allowing the alteration of phenotypes at higher and lower levels of certain biological organizations, and then determining quite accurately what other traits change as a result, this is a powerful tool to dissect the basis of such variability.

One interesting example of a selection study is the "Laboratory Natural Selection". In this situation, a freely breeding population is exposed to changed environmental conditions, such as different temperatures or salinities. Assuming that the genetic variance exists for relevant traits, the organisms will then adapt to the new conditions. These kinds of experiments are more common with non-vertebrates, e.g. Drosophila (Gibbs, 1999; Rose, 1984; Rose et al., 1996; Zera and Harshman, 2001), bacteria (Mongold et al., 1996; Travisano and Rainey, 2000; Travisano et al., 1995) and also viruses, but have been employed with vertebrates. A curious variant of laboratory natural selection is termed laboratory culling (Rose et al., 1990). In this type of experiment, a given population is exposed to a

lethal stress until a fraction of the population dies. The remaining is allowed to breed. The majority of experiments employ non-vertebrates, such as Drosophila (Rose et al, 1990), but very rarely with vertebrates, mainly because of ethical considerations.

1.6.2
Artificial Selection

Artificial Selection (or Genetic Selection) is a technique in which every individual of a given population is measured, at each generation, for a particular phenotype trait or a combination of traits (behavior, body size or major component of fitness, e.g. fecundity). The top and bottom fraction of individuals are then chosen as the breeders to produce the next generation. Mating animals within a heterogeneous population, based on the opposite extremes of an observable characteristic, will propagate this particular phenotype in opposite directions over many generations. The selection aggregates increasing and decreasing alleles in the "high" and "low" lines, respectively, leading to two separately and well-contrasted breeding lines. The assumption is that after several generations of selection, the phenotypic contrast between the high and low lines will be maximized based on the effects of the genes that facilitate either the high or the low phenotypes and were polymorphic within the initial founding population. Additionally, genes that do not influence the selected phenotype and are not physically linked to the relevant genes (i.e., do not map in the same chromosome region) will vary randomly within each of the two lines across generations. Due the fact that any finite population will undergo genetic changes caused by random genetic events, an experiment that involves selection in both directions (bidirectional) must involve at least three lines: one selected for high phenotypic values, one randomly bred as a control, and one selected for low phenotypic values (Garland, 2003). Once they have diverged, selected and control lines can be compared with respect to associated traits that are thought to cause differences at the phenotypical level (Rose et al., 1984; Schlager et al., 1983). Differently from laboratory natural selection and laboratory culling studies (see

above), artificial selection allows the researcher to make very detailed choices on what is exactly under selection. In this way, very particular aspects of performance, morphology, physiology and behavior can be targeted (Emlen, 1996, Weber, 1990; Wilkinson, 1993).

Although very useful and relatively simple to perform, some considerations should be made when using the artificial selection technique. Firstly, the consistency of response and replication of experimental lines are crucial to ensuring that differences can be attributed to the effects of selection, instead of founder effects and/or random genetic drift, which can occur in combination with unique mutations. Nevertheless, many early selection studies employed a single line or two, selected according to opposite phenotypes (Falconer, 1992). Still, selection experiments are often performed without replication (Koch and Britton, 2001; Nakamura et al., 1993). Surely, the high costs and intricate logistics involved in a selective breeding program could discourage replication studies, leading to serious implications. Even lines of organisms that are not under divergent selective pressure may be expected to differentiate genetically, and therefore, phenotypically, because of [1] differences in allele frequencies that occurred at the founding lines, [2] random genetic drift and [3] unique mutations (Garland, 2003). The same observations will apply to lines under divergent selective pressure. Thus, if a phenotypic difference between two selected lines is observed (one for “high” values and another “low”) after several generations, perhaps it may not have been caused by the selective procedure that was imposed. Genetic drift and divergence caused by founder effects, as well as limits to selection caused by the exhaustion of additive genetic variance (Weber, 1996), can be diminished by increasing the sample size within each line, which is often difficult to achieve with rodents. Replication of lines under the same selection criterion (e.g. preference for alcohol) can avoid problems of false correlated responses (DeFries et al., 1978; Henderson, 1989, 1997; Rose et al., 1996). In fact, the limitations of selection studies without replication lines are very similar to those of two species comparisons (Garland and Adolph, 1994).

Finally, it is important to emphasize that some behaviors may perhaps not react to artificial selection, and this negative outcome is often difficult to analyze. Besides, the selective breeding procedure might work for only one of the breeding

lines and a loosening of the selective pressure could lead to a regression to the initial behavioral levels of the parental population (Papini, 2002).

1.7
Behavioral Profile of eight rat genetic models

The development of bidirectional lines or strains of rodents with high and low levels of emotional reactions associated with a threatening situation began in the middle of the 20th century and, since then, a relatively large number of different genetic models based on this strategy has been developed (Ramos and Mormède, 2006). These models might represent powerful tools to study the behavioral, neural, and genetic mechanisms that underlie the different types of anxiety disorders. To that end, evaluating whether the phenotypic differences in a bidirectional selected line or strain indeed reflects differential animal emotionality is important. Moreover, still unclear is whether a genetic model of anxiety shares the same emotional system within a unitary construct or reflects a set of qualitatively different emotional dimensions that in turn might recruit distinct neurobiological and genetic mechanisms.

An important issue in validating a genetic model of anxiety is to analyze whether the behavioral differences between two contrasting lines/strains are also present in other animal models of anxiety. Table 1 shows the results of eight bidirectionally selected lines with distinct selection criteria with regard to innate or learned aversive situations across the 11 animals models of anxiety described in the previous section of this study. To facilitate table interpretation, results from rats selectively bred for high anxiety-related responses are always presented first in relation to the counterpart animals. A white cell designates a congruent result (i.e., animals selectively bred for high anxiety-related responses are indeed more emotionally reactive that their counterparts in the particular animal model of anxiety in which they were tested). A cell filled with a dotted pattern indicates a mixed result. Finally, a black cell represents a contradictory result that challenges some aspect of the genetic model, such as the presence of a motor effect, no differences, or differences in opposite directions between the two groups of animals in a particular animal model of anxiety. The behavioral profile of each of

these eight pairs of lines/strains across the 11 models of anxiety is described below:

1.7.1
Maudsley Reactive and Non-Reactive rats

Broadhurst, at the Maudsley Hospital, University of London, London, United Kingdom, began in 1954 the development of two lines of rats based on the procedure by Hall (1934), who used the number of fecal boli excreted in the open field as a measure of emotionality in rats. The lines were named Maudsley Reactive (MR: high-defecating; i.e., high anxiety-related response) and Maudsley Non-Reactive (MNR: low-defecating; i.e., low anxiety-related response).

After only four generations of mating male and female rats with the highest and lowest rates of defecation in the open field, differences between MR and MNR rats were found to be consistent (Broadhurst, 1957, 1958). Defecation scores among MR rats were close to three for both males and females rats, whereas MNR animals displayed scores close to zero. Selection was discontinued in the 15th generation, but the differences regarding defecation were still present when animals were tested in the 20th generation.

In the early 1960s, Broadhurst distributed these lines to investigators in North America, such as Sudak and Maas (1964) at the National Institute of Health (NIH; sublines designated MR/N and MNR/N), who received animals from the 20th generation. Harrington (1972; 1979; 1981), at the University of Northern Iowa, also received animals from the 25th generation. Harrington actually received one reactive (designed MR/Har) and two separate non-reactive (designed MNR/Har and MNRA/Har) sublines from Broadhurst. The MNRA/Har line, originally named MNR-a by Broadhurst, was initiated when an allelic difference was discovered at the agouti locus in the MNR line in the 8th generation. The Harrington colony was later relocated to Lafayette Clinic, Detroit. From this stock, the Maudsley sublines were sent to Blizard (1981) at Wake Forest University and Satinder (1981) at Lakehead University in Canada (sublines designated MR/Har/Lu and MNR/Har/Lu). Notably, Satinder's Non-reactive subline was derived from MNRA/Har and not MNR/Har, as the designation might

suggest. In 1987, the original MR and MNR lines developed in London were terminated but were reimported later with the MR/Har and MRNA/Har lines and have been employed in numerous studies (Blizard and Adams, 2002).

Several experiments have been conducted with MR and MNR rats, the results of which have been analyzed in important papers (e.g., Blizard, 1981; Broadhurst, 1975). Initial behavioral results suggested that these lines could represent an animal model of a general emotional trait. As shown in Table 1, MR rats were less active in the open field than MNR rats (Imada, 1970). Both MR and MR/Har animals presented a greater suppression ratio in the conditioned emotional response paradigm compared with their respective MNR and MNRA/Har counterparts (Commissaris et al., 1986; Singh, 1959). Interestingly, MR/N animals in the first postnatal week presented a higher USV frequency than MNR/N pups when isolated from their mothers and littermates for a brief period of time (Insel and Hill, 1987). These findings are consistent with the adult characteristics of the Maudsley lines because they were selectively bred for adult expression of high and low levels of emotionality.

However, conflicting or even opposite results in other animal models of anxiety imposed a serious threat to the possibility that the Maudsley lines might indeed represent a genetic model of a general emotional trait. For example, Overstreet et al. (1992) reported a congruent result in which the MR rats spent very little time in the open arms of the elevated plus maze compared with MRNA rats. However, Paterson et al. (2001) did not observe any difference between MR/Har and MRNA/Har rats in the open arms. Instead, a motor effect was found, in which MR/Har animals spent more time in the closed arms compared with MNRA/Har animals. Inconsistent results were also found in the two-avoidance learning paradigm;- While Broadhurst and Levine (1963) found that MR animals perform worse in the two-way avoidance procedure compared with MRN rats, Paterson et al. (2001) did not observe any differences between MR/Har and MNR/Har rats.

Results from the acoustic startle response are in the opposite direction; Commissaris et al. (1988) found that MR/Har rats presented within-session habituation to repeated presentation of a brief acoustic stimulus, whereas MNRA/Har rats exhibited virtually no habituation. In another study, Paterson et

al. (2001) found that MR/Har rats exhibited less sensitization of the acoustic startle response compared with MRNA/Har rats. Finally, Paterson et al. (2001) reported the absence of differences between MR/Har and MRNA/Har animals in the fear-potentiated startle paradigm.

1.7.2
Floripa High and Low rats

In 2003, Ramos, at the Federal University of Santa Catarina, Florianópolis, Brazil, reported the development of two new rat lines selectively bred for high and low locomotion in the central aversive area of the open field (Ramos et al., 2003). Initially, they produced a highly heterogeneous population through an intercross of three rat strains (i.e., Wistar, Hooded, and Lewis) and then initiated selective breeding of male and female rats for the lowest and highest scores of central open field ambulation. These lines were named Floripa[1] Low (L: low locomotion in the central area; i.e., high anxiety-related response) and High (H: high locomotion in the central area; i.e., low anxiety-related response) rat lines. After four generations of selection, a difference between the Floripa L and H rat lines in locomotor activity within the center of an open field was observed. As expected, the L line consistently displayed lower locomotion in the central area of the open field than rats of the H line. Floripa L lines also exhibited lower locomotion in the periphery of the open field (i.e., where the animal concentrates most of its activity) compared with the H line.

Several behavioral studies have investigated the Floripa H and L lines. In the black and white box test, Floripa L rats spent less time in the white compartment than Floripa H rats after four generations (Ramos et al., 2003). Although this result is consistent with the view that Floripa L rats are more emotionally reactive that Floripa H rats, other results contest this possibility. For example, although Hinojosa et al. (2006) found that Floripa L animals presented a higher defecation rate in the open field compared with Floripa H animals, this difference was not detected in an early study (Ramos et al., 2003). Anxiety-related

[1] Floripa is short for the city of Florianópolis

responses observed in these two lines of animals in the elevated plus maze have also been confusing. Ramos et al. (2003) found that, after four generations, Floripa L rats spent less time in the open arms than Floripa H rats. However, in a subsequent study, this difference between the Floripa lines in the open arms was found only in females but not in males (Hinojosa et al., 2006). Finally a motor effect has also been detected in these two lines of animals, both in the elevated plus maze and black and white box paradigm (Ramos et al., 2003).

1.7.3
Tsukuba High and Low Emotional rats

In 1975, Fujita reported the development of two new lines of animals with high and low emotional reactivity at the University of Tsukuba, Ibaraki, Japan (Fujita, 1975, 1984). Similar to the Floripa H and L animals, locomotion was also employed as the selection criterion. However, a different perspective was adopted. In its natural habitat, the rat that easily emerges from its burrow and explores its surroundings might be less anxious or emotionally reactive than another animal that prefers its burrow. An apparatus that simulates this situation in a laboratory setting and used for the bidirectional selection of these two lines consists of a dark starting box (7 cm × 7 cm) with a small exit to a bright straight runway (120 cm long × 20 cm wide × 45 cm high). According to this procedure, each animal is placed in the dark starting box, and 30 s later the door is opened so that the animal has access to the runway. Each test lasts for 5 min, and animals are tested for 3 consecutive days. Male and female rats with the lowest and highest ambulatory activity scores in the runway are then mated.

After 34 generations of inbreeding (brother and sister mating), two strains with significant differences in activity in the runway test were defined as Tsukuba High Emotional (THE: low ambulatory activity in the runway; i.e., high anxiety-related response) and Tsukuba Low Emotional (TLE: high ambulatory activity in the runway; i.e., low anxiety-related response). As expected, THE rats showed higher latencies in leaving the start box, taking more time to arrive at the end of the runway. Most initial research with these strains has been performed in the

strain's country of origin, Japan, and a review with the large amount of physiological and behavioral data obtained was published by Fujita et al. (1994).

Several results suggest that THE animals are more emotionally reactive than TLE animals. Accordingly, Kitaoka and Fujita (1991) reported that THE rats presented lower activity and higher defection compared with TLE animals in the open field paradigm. Moreover, Naito et al. (2000) also found that THE pups emitted higher USV rates in response to isolation distress from day 3 to day 18 compared with TLE pups, indicating a consistent defensive response pattern from early development to adulthood. Finally, THE animals showed lower shuttle avoidance acquisition compared with their counterpart strain (Fujita and Katayama, 1981).

Results from the passive avoidance paradigm are confusing. Miyamoto and Fujita (1997) showed that THE animals had better step-down passive avoidance performance compared with TLE rats. However, no differences between these two strains were found in step-through passive avoidance performance (Wada and Makino, 1997). Finally, divergent results argued against the possibility that THE animals represent an animal model of a general anxiety trait. Employing the conditioned suppression paradigm, Fujii et al. (1989) did not find any differences between the Tsukuba strains in the suppression ratios of licking and an operant response.

1.7.4
High and Low Anxiety-related Behavior rats

In 1998, Landgraff and colleagues (Liebsch et al., 1998a, b), at the Max Planck Institute of Psychiatry, Munich, Germany, reported the creation of two lines of Wistar rat based on open arm entries in the elevated plus maze. The percentage of time spent in the open arms was employed as the main criterion for bidirectional selection. Other open-arm parameters were also employed in the following rank order: percentage of entries into the open arms > number of full open arm entries > latency to first open arm entry. Only animals with average activity scores (distance traveled) were selected (Liebsch et al., 1998a). Beginning in 1993, male and female rats with the lowest and highest proportion of open arm

scores were mated to establish the two lines now termed High Anxiety-related Behavior (HAB: low proportion of open arm scores; i.e., high anxiety-related response) and Low Anxiety-related Behavior (LAB: high proportion of open arm scores; i.e., low anxiety-related response).

Henniger et al. (2000) reported a study with female HAB and LAB rats at the 7th generation in the elevated plus maze. The results indicated that HAB animals displayed a lower percentage of entries into and time spent on the open arms as compared with LAB rats. Importantly, HAB and LAB rats did not differ in the number of closed arm entries. Yilmazer-Hanke et al. (2004) also observed the same pattern of results with male HAB and LAB rats at the 9th generation.

The HAB and LAB lines have been evaluated in various behavioral paradigms of anxiety, and some of these results corroborated the hypothesis that HAB animals are more emotionally reactive than LAB animals. Liebsch et al. (1998b) reported that the HAB line showed a decrease in open field ambulation compared with LAB animals. Wigger et al. (2001) also reported that HAB rat pups exhibited an enhanced frequency of USVs in response to isolation distress on postnatal day 11 and lower open arm exploration in the elevated plus maze throughout adulthood compared with LAB animals, suggesting that the differences in emotionality between these two lines are already present in the early phase of development and remain present during adulthood. Finally, Henniger et al. (2000) investigated whether the anxiety-related response differences in HAB and LAB rats were also present in the light-dark box and social interaction tests. The results indicated that HAB animals spent less time in the light compartment and engaged in less active social interaction than LAB rats. Importantly, this study also found a locomotor activity effect in both the light-dark box and social interaction test, with HAB rats being less active than their LAB counterparts.

Results from learned aversive paradigms, however, did not support the possibility that HAB and LAB animals represent a model of general emotionality. For example, Muigg et al. (2008) reported that HAB and LAB animals presented the same freezing response during the acquisition of an aversive conditioning task in response to a tone paired with an electrical footshock, although HAB rats showed a considerable deficit in the ability to extinguish the conditioned freezing

response to the acoustic stimulus. Yilmazer-Hanke et al. (2004) also reported that HAB and LAB animals did not differ in the freezing response to contextual cues previously associated with footshocks. In this study, Yilmazer-Hanke et al. (2004) also reported divergent results when HAB and LAB animals were tested in the acoustic startle paradigm. As expected, HAB animals presented lower scores on the open arms in the elevated plus maze compared with LAB animals. However, an opposite response pattern was observed in the acoustic startle response paradigm, in which HAB rats also displayed lower fear sensitization than their HAB counterparts.

1.7.5
High and Low Ultrasonic Vocalization rats

To investigate generational and developmental variables associated with anxiety, Brunelli et al. (1996), at Columbia University, New York, USA, reported the creation of two lines of rats selected for different rates of USVs in response to isolation. Rat pups were screened at 10 ±1 days of age in a 2 min isolation test. Male and female pups with the highest and lowest rates of USV were selected for later breeding. After only one generation, the High line presented more USVs than the Low line. After three generations, the Low and High lines diverged significantly from each other in their USV responses rates and from control animals that were mated randomly. This selection program was the first successful study that attempted to selectively breed a neonatal phenotype among rats and has been termed USV High (high neonatal isolation-induced USV; i.e., high anxiety-related response) and USV Low (low neonatal isolation-induced USV; i.e., low anxiety-related response).

Few behavioral studies have investigated USV animals. Results from a modified version of the open field suggested that USV High and Low animals might represent an adult genetic model of anxiety (Zimmerberg et al., 2005). In this study, adult USV rats were placed inside a closed and opaque cylinder that was in turn placed in an open field. The results indicated that USV High animals emerged into the open field later and crossed fewer squares in the central area of the open field than USV Low rats.

However, confusing results with male and female (in either proestrus or diestrus) USV rats were also reported in the social interaction test (Zimmerberg et al., 2005). The results indicated that only proestrus female USV High rats engaged in less social interaction compared with proestrus female USV Low rats. No differences were found between male or diestrus female USV High and Low animals. Confusing results were also reported by Ditcher et al. (1996) in the elevated plus maze. This study indicated that although USV High animals presented a lower percentage of time in the open arms than USV Low animals, no difference was observed between USV High and control unselected animals.

Table 1. Behavioral profile of eight rat genetic models (columns) across 11 animal models of anxiety (lines). The results from rats selectively bred for high anxiety-related responses are always presented first in relation to the counterpart animals. White cells indicate that differences between the two groups are in the expected direction. Cells filled with a dotted pattern indicate mixed results. Black cells indicate that the result challenged some aspect of the genetic model (i.e., motor effect, no differences between the two groups, or differences in the opposite direction). Superscript numbers indicate the bibliographic references of the behavioral result. M, male; F, female; FP, female proestrus; FD, female diestrus.

Animal model of anxiety-like behavior	Maudsley Reactive and Non-Reactive rats	Floripa High and Low rats	Tsukuba High and Low Emotional rats	High and Low Anxiety-related Behavior rats	High and Low Ultrasonic Vocalizations rats	Roman High and Low Avoidance rats	Syracuse High and Low Avoidance rats	Carioca High and Low Conditioned Freezing rats
Open field								
Defecation	Parameter employed to create the line	Floripa L = Floripa H[9] Floripa L > Floripa H[10]	THE > TLE[11]	--------	--------	RLA = RHA[31] RLA/Verh > RHA/Verh[32]	SLA/Bru > SLA/Bru[40]	--------
Ambulation	MR < MNR[1]	Parameter employed to create the line	THE < TLE[11]	HAB < LAB[19]	USV High < USV Low[22]	RLA < RHA[31] RLA/Verh < RHA/Verh[32]	SLA/Bru = SHA/Bru[40]	--------
Elevated plus maze								
Open arm	MR < MNRA[5] MR/Har = MNRA/Har[6]	Floripa L < Floripa[9] F: Floripa L < Floripa H[10] M: Floripa L = Floripa H[10]	--------	Parameter employed to create the line	USV High < USV Low[23] USV High = Random[23]	RLA/Verh < RHA/Verh[33] RLA/I < RHA/I[34, 35] RLA/Verh > RHA/Verh[36]	--------	CHF < Control[41]
Closed arm	MR/Har < MNRA/Har[6]	Floripa L < Floripa H[9, 10]	--------	HAB = LAB[17, 18]	--------	RLA/Verh < RHA/Verh[33]	--------	CHF = Control[41]
Light-dark box								
Time in the light compartment	--------	Floripa L < Floripa H[9]	--------	HAB < LAB[17]	--------	RLA/Verh < RHA/Verh[37] RLA/Verh > RHA/Verh[36]	--------	--------
Locomotor activity	--------	Floripa L < Floripa H[9]	--------	HAB < LAB[17]	--------	--------	--------	--------
Social interaction								
Social activity	--------	--------	--------	HAB < LAB[17]	FP: USV High > USV Low[22] DF: USV High = USV Low[22] M: USV High = USV Low[22]	RLA/Verh = RHA/Verh[36, 37]		CHF < Control[41]
Locomotor activity	--------	--------	--------	HAB < LAB[17]		RLA/Verh = RHA/Verh[36, 37] RLA/Verh < RHA/Verh[37]		CHF = Control[41]

Table 1 (*continued*):

Animal model of anxiety-like behavior	Maudsley Reactive and Non-Reactive rats	The Floripa High and Low rats	The Tsukuba High and Low Emotional rats	The High and Low Anxiety-related Behavior rats	The High and Low Ultrasonic Vocalizations rats	The Roman High and Low Avoidance rats	The Syracuse High and Low Avoidance rats	Carioca High and Low Conditioned Freezing rats
Ultrasonic vocalization								
Frequency	MR/N > MNRA/N[4]	--------	THE > TLE[12]	HAB > LAB[20]	Parameter employed to create the line	--------	--------	--------
Acoustic startle response								
Habituation	MR/Har > MNRA/Har[8]	--------	--------	--------	--------	--------	--------	--------
Sensitization	MR/Har < MNRA/Har[6]	--------	--------	HAB < LAB[18]	--------	RLA/Verh > RHA/Verh[24]	--------	--------
Fear-potentiated startle								
Startle amplitude	MR/Har = MNRA/Har[6]	--------	--------	--------	--------	RLA/Verh > RHA/Verh[25]	--------	--------
Active avoidance								
Two-way	MR < MNRA[7] MR/Har = MNRA/Har[6]	--------	THE < TLE[13]	--------	--------	Parameter employed to create the line	Parameter employed to create the line	--------
One-way	--------	--------	--------	--------	--------	Only *1 sec in the safe compartment:* RLA/I < RHA/I[27, 28]	--------	--------
Passive avoidance								
Step-down	--------	--------	THE > TLE[14]	--------	--------	--------	--------	--------
Step-through	--------	--------	THE = TLE[15]	--------	--------	--------	--------	--------

Table 1 (*continued*):

Animal model of anxiety-like behavior	Maudsley Reactive and Non-Reactive rats	The Floripa High and Low rats	The Tsukuba High and Low Emotional rats	The High and Low Anxiety-related Behavior rats	The High and Low Ultrasonic Vocalizations rats	The Roman High and Low Avoidance rats	The Syracuse High and Low Avoidance rats	Carioca High and Low Conditioned Freezing rats
Conditioned emotional response								
Supression ration	MR > MNR[2] MR/Har > MNRA/Har[3]	--------	THE = TLE[16]	--------	--------	RLA/Verh > RHA/Verh[26]	SLA/Bru > SHA/Bru[38, 39]	--------
Conditioned freezing								
Context	--------	--------	--------	HAB = LAB[18]	--------	RLA/Verh > RHA/Verh[25, 29] RLA/I > RHA/I[30]	--------	Parameter employed to create the line
Discrete CS	--------	--------	--------	HAB = LAB[21]	--------	RLA/Verh > RHA/Verh[27, 29]	--------	--------

Note: 1, Imada (1979); 2, Singh (1959); 3, Commissaris et al. (1986); 4, Insel and Hill (1987); 5, Overstreet et al. (1992); 6, Paterson et al. (2001); 7, Broadhurst and Levine (1963); 8, Commissaris et al. (1988); 9, Ramos et al. (2003); 10, Hinojosa et al. (2006); 11, Kitaoka and Fujita (1991); 12, Naito et a(2000); 13, Fujita and Katayama (1981); 14, Miyamoto and Fujita (1997); 15, Wada and Makino (1997); 16, Fujii et al. (1989); 17, Henniger et al. (2000); 18, Yilmazer-Hanke et al. (2004); 19, Liebsch et al. (1998b); 20, Wigger et al. (2001); 21, Muigg et al. (2008); 22, Zimmerberg et al. (2005); 23, Ditcher et al. (1996); 24, Schwegler et al. (1997); 25, López-Aumatell et al. (2009); 26, Ferré et al. (1995); 27, Morón et al. (2010); 28, Torres et al. (2007); 29, Aguilar et al. (2002); 30, Escorihuela et al. (1997); 31, Broadhurst and Bignami (1965); 32, Gentsch et al. (1982); 33, Meyza et al. (2009); 34, Driscoll et al. (1998); 35, Escorihuela et al. (1999); 36, Chaouloff et al. (1994); 37, Steimer and Driscoll (2003); 38, Brush et al. (1988); 39, Gupta and Brush (1998); 40, Brush et al. (1985); 41, Dias et al. (2009).

1.7.6
Roman High and Low Avoidance rats

In 1961, Bignami, at the Istituto Superiore di Sanità, Rome, Italy, started a selective breeding program with Wistar rats for low and high rates of two-way avoidance. The animals were subjected to five daily sessions of 50 trials, with 30 s between trials. Each trial consisted of a visual CS (light) that preceded the onset of a footshock US. The occurrence of a crossing response from one side to the other side of a shuttle box during the CS terminated the CS and avoided the US. If the response occurred after the onset of the US, then both the CS and US were terminated. Male and female rats with the lowest and highest rates of avoidance were selected and mated together while avoiding inbreeding. After five generations, the two selected lines differed markedly (at least threefold differences) in the number of avoidance responses, with no sex differences (Bignami, 1965). The lines were named Roman Low Avoidance (RLA: low rates of two-way avoidance; i.e., high anxiety-related response) and Roman High Avoidance (RHA: high rates of two-way avoidance; i.e., low anxiety-related response).

In 1964, Bignami took a sabbatical to work with Broadhurst and transferred the two lines to England, where they were distributed to various laboratories (Broadhurst and Bignami, 1965). One of the most well-know colonies was established in 1972 at the Institut für Verhaltenswissenschaft, Zürich, Switzerland. The two sublines were named RLA/Verh and RHA/Verh and have been continuously bred since then, initially by Bättig, and later by Driscoll (Driscoll and Bättig, 1982). In parallel with the RLA/Verh and RHA/Verg sublines, an inbreeding program was initiated in 1993, derived through brother and sister matings from the outbred sublines. Since 1997, the inbred RLA/Verh (RLA/I) and RHA/Verh (RLA/I) rats have been maintained at the Universidad Autónoma de Barcelona, Spain, under the direction of Fernández-Teruel (Escorihuela et al., 1999).

Several results from learned aversive paradigms support the hypothesis that RLA rats have a stronger emotional reaction than RHA animals. For example, in the acoustic startle response test, RLA/Verh displayed higher sensitization (Schwegler et al., 1997) and greater fear-potentiated startle (López-Aumatell et al., 2009) compared with RHA/Verh rats. Results from the conditioned emotional response test also indicated that

RLA/Verh rats presented more shock-induced suppression of drinking behavior compared with RHA/Verh rats (Ferré et al., 1995).

Evidence also showed that Roman inbred animals behaved differently during the acquisition of a one-way avoidance response (Morón et al., 2010; Torres et al., 2007). In these experiments, the rats learned to run from a danger compartment, where they received a warning signal followed by an electric footshock, to a safe compartment, where these stimuli were not presented. The results indicated that RLA/I rats exposed for 1 s to the safe compartment showed poorer performance than RHA/I rats. These differences were not observed when the animals were exposed for 30 s to the safe compartment. Because the reinforcing value of the running response among animals that remained in the safe compartment for only 1 s would be very low (fear relief), the one-way avoidance response would be expected to mainly result from the aversive conditioning that occurred in the danger compartment. Accordingly, RLA animals would tend to freeze, whereas RHA animals would tend to flee.

Indeed, conditioned freezing appears to be one of the main differences between the Roman animals. For example, López-Aumatell et al. (2009) found that RLA/Verh animals presented more conditioned freezing in response to contextual cues and to a discrete CS previously associated with electrical footshocks. The same results were also found by Aguilar et al. (2002). Finally, Escorihuela et al. (1997), working with inbred Roman rats, also found that RLA/I rats presented more conditioned freezing in response to contextual cues than RHA/I rats. Therefore, the low two-way avoidance performance of the RLA rats might be attributable to the fact that these animals are more "afraid" of the contextual cues previously associated with footshock. The interaction between conditioned freezing in response to contextual cues and the acquisition of a two-way avoidance response has been recently demonstrated by Vicens-Costa et al. (2011). They found that rats that presented relatively higher levels of context-conditioned freezing during the initial trials of two-way avoidance learning were less likely to acquire this response. Therefore, contextual fear conditioning negatively predicted two-way avoidance acquisition.

The behavioral results from the Roman animals in innate paradigms of anxiety are puzzling. Initial studies in the open field indicated that RLA rats were less active, without any differences in defection, compared with RHA rats (Broadhurst and Bignami, 1965). However, other studies with the Swiss subline indicated that

RLA/Verh rats were less active and defecated more than RHA/Verh rats (Gentsch et al., 1982). Confusing results were also reported in the elevated plus maze. For example, Meyza et al. (2009) reported that RLA/Verh rats entered the open arms less compared with RLA/Verh rats. RLA/I animals also entered the open arms of the elevated plus maze less compared with RHA/I animals (Driscoll et al., 1998; Escorihuela et al., 1999). Surprisingly, opposite results were found by Chaouloff et al. (1994), in which RLA/Verh rats spent more time on the open arms compared with RHA/Verh rats.

Results from the light-dark box test are also confusing. Steimer and Driscoll (2003) reported that RLA/Verh rats were more emotionally reactive, reflected by an increased latency to first enter the light compartment, than their RHA/Verh counterparts. However, Chaouloff et al. (1994) found that RLA/Verh rats were less emotionally reactive, reflected by the time spent in the light compartment, than RHA/Verh rats. Finally, results from the social interaction test are not consistent with the view that Roman animals represent a model of a general anxiety trait since two studies failed to detect differences in anxiety-related response parameters in this paradigm between the two Verh sublines (Chaouloff et al., 1994; Steimer and Driscoll, 2003).

Locomotor activity results from social interaction paradigm are unclear. Chaouloff et al. (1994) did not find any differences between RHA/Verh and RLA/Verh animals. However, Steimer and Driscoll (2003) showed that RLA/Verh rats were less active than RHA/Verh rats. Results from the elevated plus maze indicated that RHA/Verh animals were less active than RLA/Verh rats (Meyza et al., 2009).

1.7.7
Syracuse High and Low Avoidance rats

In 1965, Brush, at the Syracuse University, Syracuse, New York, USA, started a selective breeding program with Long-Evans hooded rats, also based on low and high rates of two-way avoidance (Brush, 1966). Similar to Bignami's Roman lines, Brush's animals were required to cross from one side to the other side of a shuttle box to avoid an electrical footshock. However, Brush's procedure was slightly different from Bignami's and had only a single test session composed of 10 pretest trials, in which the CS was presented alone with an intertrial interval of 120 s. Immediately after the 10

pretest trials were 60 training trails, in which the CS was followed by the US. The warning CS was a compound auditory and visual stimulus that lasted for 5 s, whereas the US was a low-intensity footshock (0.25 mA). Male and female rats with the lowest and the highest avoidance responses during the 60 trials and that met the pretest criteria (response latencies less than 5 s on fewer than five of the 10 pretest trials and on fewer than three of the last five pretest trials) were selected and mated.

In 1979, Brush and colleagues reported the results of 25 consecutive generations (Brush et al., 1979). Similar to the study by Bignami (1965), the two selected strains differed markedly in the number of two-way avoidance responses after five generations, with no sex differences (Bignami, 1965). These strains were named Syracuse Low Avoidance (SLA/Bru: low rates of two-way avoidance; i.e., high anxiety-related response) and Syracuse High Avoidance (SHA/Bru: high rates of two-way avoidance; i.e., low anxiety-related response).

The hypothesis that differences in emotionality might covariate with this selected phenotype received support when the Syracuse animals were tested in the conditioned emotional response paradigm. Brush et al. (1988) and Gupta and Brush (1998), ten years later, found that SLA/Bru rats had greater suppression ratios than SHA/Bru rats. However, results from the open field were incoherent (Brush et al., 1985). SLA/Bru rats showed greater defecation than SHA/Bru animals, but these two lines did not differ in ambulation.

1.7.8
Carioca High and Low Conditioned Freezing rats

Landeira-Fernandez, at the Pontifícia Universidade Católica do Rio de Janeiro, Rio de Janeiro, Brazil, was also interested in developing a rat genetic model of extreme phenotypes for learned fear. Instead of the two-way avoidance paradigm, conditioned freezing in response to contextual cues previously associated with footshock was used as the phenotype criterion for developing the two lines.

The breeding program began in 2006. The contextual fear conditioning protocol involved acquisition and test sessions. During acquisition, Albino Wistar rats were placed in the observation chamber. After 8 min, three unsignaled electrical footshocks were delivered. Twenty-four hours later, each animal was placed again in the original

context, but no footshock or other stimulation occurred. Freezing was recorded for 8 min. The total amount of freezing behavior observed during the test session was used as the criterion for animal mating. Male and female rats with the highest and lowest conditioned freezing scores were selected and mated. Gomes and Landeira-Fernandez (2008) found that after three generations, reliable differences between these two lines were already present, indicating a strong heritable component of this type of learning. Males consistently exhibit more conditioned freezing in response to contextual cues than females. The lines were named Carioca[2] High Conditioned Freezing (CHF: high amount of freezing in response to contextual cues previously associated with footshock; i.e., high anxiety-related response) and Carioca Low Conditioned Freezing (CLF: low amount of freezing in response to contextual cues previously associated with footshock; i.e., low anxiety-related response). This model will be further detailed in Study 1 of this thesis.

The first behavioral results from this ongoing selective breeding program were reported by Dias et al. (2009). They performed a battery of behavioral tests with the fourth generation of CHF lines and control unselected rats using the elevated plus maze and social interaction test, among others. In the elevated plus maze, the results indicated that CHF animals were significantly more emotionally reactive than control rats in terms of both the number of entries into the open arms and percentage of time spent on the open arms. Their time spent engaging in social interaction was also significantly decreased. Importantly, no differences were found in locomotor activity, reflected by the number of entries into the closed arms of the elevated plus maze and number of crossings into the social interaction test arena.

1.8
Problem of locomotor activity

One of the main problems of using animal models of anxiety is the possible interaction between the behavioral measurements of emotionality and the animal's locomotor activity. Indeed, motor effects have been found in Maudsley (measured in the elevated plus maze), Floripa (measured in the elevated plus maze and light-dark box),

[2] Carioca is the name given to those born in Rio de Janeiro.

HAB/LAB (measured in the light-dark box and social interaction test), and Roman (measured in the elevated plus maze) animals. In all of these cases, rats with high anxiety-related responses also exhibited a reduction in locomotor activity. These motor effects might represent an important confounding variable because the differences in emotionality among these animals might be at least partially explained by differences in locomotor activity.

Anxiety and locomotor activity are intimately associated. Most defensive reactions involve a decrease in exploratory ambulation and an increase in freezing behavior. Therefore, discarding any reasonable influence of locomotor activity on the occurrence of anxiety-like responses is almost impossible. Even in paradigms in which anxiety and motor indices are relatively well dissociated, such as in the elevated plus maze, it is unclear how these performance variables may in fact interact in this animal model of anxiety. For example, hypoactivity in the elevated plus maze can overcome the detection of anxiogenic-like effects in some experimental manipulations (e.g., Padovan and Guimarães, 2000) but not in others (e.g., Maisonnette et al., 1993). Moreover, a motor effect in the elevated plus maze can be part of the defense response to an anxiogenic compound (e.g. Cruz et al., 2005).

However, a few procedural manipulations can be conducted to estimate the possible modulatory effect of locomotor activity on defensive reactions. For example, Tsukuba (Fujita et al., 1994), HAB/LAB (Liebsch et al., 1998a) and Roman (Meerlo et al., 1997) animals did not exhibit any motor differences when measured under basal conditions in their home cage using a radiotelemetric system. Therefore, the motor effect observed in these genetic models of anxiety is not associated with general spontaneous locomotor activity but is a reaction to a possible threatening situation.

When locomotor activity plays a fundamental role in the expression of defensive behavior and is part of the natural coping reaction that the animal adopts in response to a threatening situation, employing statistical procedures to test whether the difference between the high and low anxiety groups in the emotional index can be explained by differences in locomotor activity is still possible. Accordingly, when the two groups exhibit a significant difference in both emotional and locomotor activity indices, examining these data with an analysis of covariance (ANCOVA) is important. This analysis is performed on the emotional index, including locomotor activity as a covariate variable. This analysis can lead to two possible conclusions. If the difference

previously detected between the two groups is still present after the ANCOVA, then the conclusion can be made that the anxiety-like behavior was not biased by locomotor activity. However, if the significant difference disappears with the ANCOVA, then locomotor activity is an important confounding variable, and the anxiety-like response difference between the two groups might be attributable to differences in motor activity.

None of the studies reviewed in the present work employed an ANCOVA as a possible statistical procedure to assess the extent to which individual variability in locomotor activity contributed to the anxiety-like effect. This is surprising because this analysis can be easily performed in several innate aversive paradigms that derive an emotional and motor variable for the same animal. For example, in the open field, comparing the number of squares visited in the central area of the open field is possible using the number of squares visited on the periphery of the open field as a covariate variable. In the social interaction test, differences in the total amount of social interaction can be controlled by the locomotor activity measures, reflected by the number of squares visited by the animal. Differences in emotional parameters in the light-dark box, such as the latency to first enter the light compartment or total time spent in the light compartment, can be controlled by a locomotor activity parameter such as the total number of transitions. Finally, the ANCOVA can be used in the elevated plus maze to determine whether the differences in the percentage of time spent on or percentage of entries into the open arms can be explained (covariate) by the number of entries into the closed arms.

1.8.1
Anxiety as a multidimensional construct

One of the main goals of the present short review was to investigate whether a genetic model of rats selectively bred for high and low levels of a particular anxiety-like response would display similar results in other experimental paradigms that also require the expression of a different defensive response. A positive answer to this question would support the traditional view that differences in emotionality reflect a continuum of variation within a single general trait that ranges in intensity from normal to pathological levels (Broadhurst, 1975; Gray, 1979; Hall, 1934).

However, the results found in the present work do not support this view. As shown in Table 1, the Maudsley animals presented inconsistent results in the open arm parameter of the elevated plus maze and acquisition of two-way avoidance learning. Divergent results were also detected in the habituation, sensitization, and fear-potentiation of the acoustic startle response. The Floripa lines also had inconsistent results with regard to open field defecation and the open arm parameters of the elevated plus maze. Tsukuba animals also presented opposite results in the acquisition of passive step-through avoidance and the suppression ratio of the conditioned emotional response.

Results that were opposite to the general trait hypothesis were also found in HAB and LAB lines when tested for fear sensitization of the acoustic startle response and conditioned freezing in response to contextual cues and a CS previously associated with footshock. The USV strains presented inconsistent results in the open arm parameter of the elevated plus maze and social interaction test. The Roman strains also presented opposite results in the social interaction test and inconsistent results in several innate aversive models of anxiety, such as defecation in the open field, the open arm parameters in the elevated plus maze, and time spent in the light compartment of the light-dark box. Finally, Syracuse rats also presented opposite results with regard to ambulation in the open field.

These results clearly argue against the early conceptualization of emotional reactivity as a unitary construct and reinforce a more recent approach that proposed that anxiety is a complex, multidimensional, and dynamic phenomenon (Aguilar et al., 2002; Belzung and Le Pape, 1994; Ramos et al., 1997; Torrejais et al., 2008). In these studies, statistical techniques, such as the factor analysis, have been employed to investigate whether different animal models of anxiety measure the same underlying latent factor. These factor analyses studies indicate that different aversive paradigms may assess different forms of anxiety. For example, File (1992) showed that indices of anxiety derived from the elevated plus maze (i.e., number of entries into and time spent on the open arms), Vogel test (i.e., frequency of punished drinking), and social interaction test (i.e., time spent engaged in social interaction), loaded on three independent factors, suggested the existence of different forms of anxiety generated by each of these paradigms. Similarly, Belzung and Le Pape (1994) found a weak correlation between the measures of anxiety in the elevated plus maze and in light-dark box. For a review of

the similarities and differences between the elevated plus maze, light-dark box, and open field, see Ramos (2008).

These diverse dimensions found in animal models of anxiety might reflect the clinical diversity generally found among human patients, in whom pathological anxiety is classified into several categories (American Psychiatric Association, 1994; World Health Organization, 1993). In fact, the treatment of different anxiety disorders might involve a wide range of pharmacological compounds, with distinct mechanisms of action, such as increasing the effects of GABAergic neurotransmission or modulating serotonergic activity (Outhoff, 2011). Pharmacological studies that have used diverse anxiety tests have also detected the multidimensional aspect of anxiety. For example, experimental paradigms that generate behavioral inhibition caused by conflicts between approach and avoidance tendencies are sensitive to benzodiazepine compounds. These animal models also indicated that substances that decrease serotonergic activity increased anxiety, whereas those that increase serotonergic neurotransmission produced an anxiogenic effect. In contrast, other animal models that require vigorous escape responses to proximal aversive stimuli appeared to be resistant to benzodiazepine drugs, whereas substances that increased serotonergic activity produced an anxiolytic effect (Graeff and Zangrossi, 2010).

Different neural circuitries also appear to be involved in distinct dimensions of anxiety. Gray and McNaughton (2000) argued that the septo-hippocampal system contributes to the cognitive component of anxiety (worry), whereas the amygdaloid complex and its projections to the ventral portion of the periaqueductal gray are critically involved in the regulation of defensive freezing behavior in response to innate or conditioned aversive stimuli (Fanselow, 1994). Active defensive responses to proximal stimuli, generally associated with nociception, appear to involve the dorsal portion of the periaqueductal gray and its ascending projections to forebrain structures related to the sensorial processing of aversive stimuli (Vianna et al., 2001a).

The present review also found a remarkable relationship between anxiety-like responses during early development and adulthood. The USV lines were created to produce a developmental-genetic model system. The hypothesis is that autonomic and behavioral temperamental differences in infancy might cause behavioral or autonomic nervous system dysfunction in adulthood (Brunelli, 2005). The results appear to be encouraging because the USV High and Low lines selected for different rates of USV in

response to isolation during infancy and tested during adulthood presented reliable differences in several animal models, such as the open field, social interaction test, and elevated plus maze. Moreover, MR, THE, and HAB pups consistently presented more USV isolation calls than their respective counterpart lines/strains.

The fact that differences in emotionality in adulthood might be already present early in development converges with results from clinical studies, which have indicated that there is an influence of temperamental factors present in childhood on the development of anxious symptoms during adult life (Kagan and Snidman, 1999). These results are also in agreement with the conceptual distinction between trait and state anxiety. State anxiety refers to a transient condition that is only observable at particular moments and varies in intensity over time. Trait anxiety refers to a relatively permanent and stable characteristic that is less susceptible to influences by a particular state or situation (Cattell and Scheier, 1961).

1.8.2
Phenotype comparisons and possible methodological limitations

Importantly, one needs to be extremely careful when interpreting either the presence or absence of correlations/associations between two phenotypic traits (e.g., behavioral, anatomical, biochemical, etc.) in one or several pairs of selected lines. Therefore, a few genetic considerations about the selection method should be clarified. Firstly, two pairs of rat lines that are selected in different laboratories will differ, not only with regard to the behavioral method used to select them, but also in the genetic characteristics of their initial populations. Therefore, even if the foundation rat lines have the same name (e.g., Wistar), which is obviously often not the case, because they are outbred, each sample of animals screened in the first generation (S0) of each study has different polymorphisms for different genes. Selection can only act upon the genes that vary (i.e., are polymorphic) in that specific population. Behaviors are almost always polygenic (i.e., they are influenced by a myriad of genes). Thus, if two genes, A and B, are equally relevant to a trait, but each of them is polymorphic only in one of the two starting populations, future differences between the lines will be related to gene A only in one line pair and related to gene B only in the other line pair. Therefore, if genes A and B act through different neurobiological mechanisms, then the two analogous

genetic models (e.g., Maudsley and Roman) may display emotional similarities that are attributable to different underlying mechanisms. In conclusion, two traits that are correlated in one model and uncorrelated in another model, although they effectively share biological pathways, should not be surprising. Thus, two final lines could be equally fearful, for example, through different biological mechanisms.

Secondly, because of practical reasons, selection experiments in rodents can only be performed in relatively small samples of larger foundation populations. In such small samples, totally avoiding two genetic phenomena, namely genetic drift and inbreeding (Falconer and MacKay, 1996), is virtually impossible. Both these factors can produce significant increases or decreases in allele frequency, possibly leading to fixation, differentially in either the high or low selected line (e.g., 100% of allele "A" in the high line and 100% of allele "a" in the low line), and this may occur in any gene that has absolutely no effect on the selected trait. Consequently, these lines may differ in innumerable behavioral, anatomical, and biochemical traits that have nothing to do with the desired phenotype (e.g., emotionality), similar to any random pair of unselected inbred strains. Thus, significant correlated traits may be spurious unless they are proven to appear in different independent selected studies, which was the case for several behaviors discussed above, or in different replicate lines of the same study (Crabbe, 1999).

Finally, the importance of linked genes should not be overlooked. Because genes lie in chromosomes and because the starting rat populations may not be highly outbred, two neighboring polymorphic genes, if in linkage disequilibrium, tend to pass their alleles on to the following generations as a "package" (i.e., allele "A" together with allele "B" and allele "a" together with allele "b"). If only the A/a variation is relevant to the selected phenotype, then the final high/low lines will differ also for the B/b polymorphism and all of the cascading phenotypes influenced by B/b, thus creating an additional false positive result and possibly leading the neuroscientist to believe that fearfulness somehow relates to all of these accidental phenotypic differences.

2
Objectives

The main objective of the present work was to develop a bidirectional selective breeding program employing Wistar rats, using as selection criterion the conditioned freezing behavior in response to contextual cues previously associated with footshocks. In addition, we propose the introduction of a third group of randomly mated rats (RND) in the selective breeding program, which could serve as a control for the selected lines. In order to contextualize this newly developed model, we decided to revise, based on the literature, the behavioral results of 8 selected lines with contrasting levels of anxiety-related responses in 11 animal tests of anxiety. Once a confident behavioral divergence between CHF (Carioca High Freezing) and CLF (Carioca Low Freezing) selected lines was established, we also aimed to verify several aspects of the fear conditioning paradigm, such as extinction and reacquisition of conditioned freezing behavior, as well as the dissociation between contextual X phasic fear, in these two new animal lines. It is our hypothesis that these selected lines could be a suitable model in the understanding the pathophysiology of fear learning, hence expanding our knowledge of the genetic basis of conditioned fear.

3
Study 1

Genetic selection of two new rat lines displaying different levels of conditioned freezing behavior

3.1
Objectives

The main objective of the present work was to develop a bidirectional selective breeding program using Wistar rats, employing the conditioned freezing behavior in response to contextual cues previously associated with footshocks as selection criterion. Also, we aimed to introduce a third group of randomly mated rats (RND) in the selective breeding program, which could serve as a control for the selected lines.

3.2
Subjects

Albino Wistar rats were employed as subjects. The initial stock of these animals was obtained in 1995 from a local producer (Oswaldo Cruz Foundation), and since then they have been maintained in the colony room of the PUC-Rio Psychology Department with controlled room temperature (24 ± 1°C) and in a 12 h/12 h light/dark cycle (07:00-19:00 h). The selective breeding procedure described in this work began in March of 2006. Experiments occurred always during the light phase of the cycle. 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*. The animals were between 75 and 85 days of age at the beginning of the experiment. For five days before 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 the PUC-Rio Psychology Department ethics committee and conformed to 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).

3.3
Equipments

Contextual fear conditioning occurred in four observation chambers of Plexiglas (25 × 20 × 20 cm), each one placed inside a sound-attenuating box. A red light bulb (25 W) was placed inside the box, and a digital video camera was mounted in the back of the observation chamber so the animal's behavior could be observed on a monitor outside the experimental chamber. An observation program (GeoVision GV800, PCI Systems) was used to record all procedures (Figure 4). A ventilation fan attached to the box supplied background noise of 78 dB (A scale). The floor of the observation chamber consisted of 15 stainless steel rods (4 mm diameter) spaced 1.5 cm apart (center-to-center), which were wired to a shock generator and scrambler (Insight, São Paulo, Brazil). An interface with eight channels (Insight, São Paulo, Brazil) connected the shock generator to a computer, which allowed the analyst to apply an electric footshock. 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 subject.

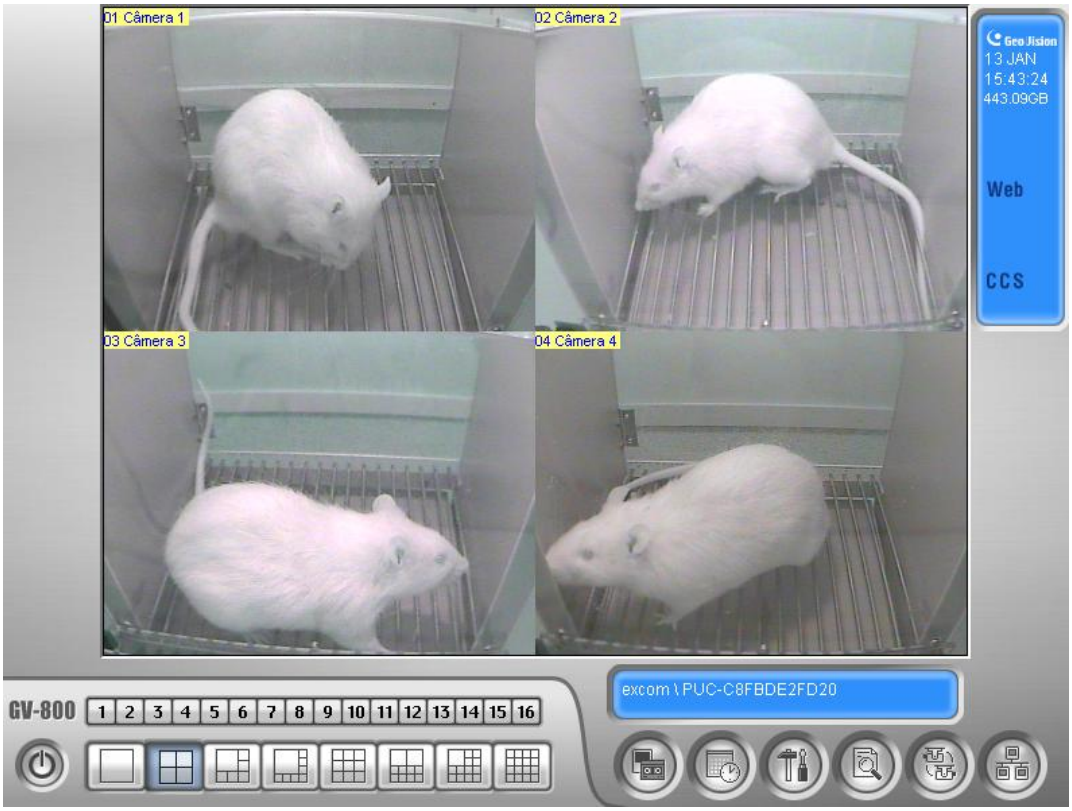

Figure 4 - Video recording program used for behavioral register.

3.4
Procedures

In order to develop a line of rats with a high rate of conditioned freezing, termed Carioca[3] 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 (S_0). The contextual fear conditioning protocol involved an acquisition session 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, with each shock lasting 1 s and with an intershock interval of 20 s. The initial shock strength employed in the first five generations of selective breeding was 1.0 mA. In order to avoid ceiling effects, this footshock intensity was reduced in our breeding program in the 6th generation to 0.7 mA and in the 8th generation on forward to 0.6 mA. The animal was then returned to its home cage 3 min

[3] Carioca is the name given to those born in Rio de Janeiro.

after the last shock. The testing session occurred approximately 24 h after training. This test consisted of placing the animal for 8 min in the same chamber in which the three footshocks had been administered in 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 2 s the animal was observed and a well-trained observer (VCG), blind to the experimental conditions, recorded episodes of freezing, defined as the total absence of movement of the body or vibrissae except for movements required for respiration. In the first four generations (S_1-S_4), each rat was observed one at a time. In the S_5-S_8 generations, rats were observed in pairs; finally, in the subsequent generations (S_9-S_{14}), rats were observed in groups of four. Moreover, in S_{14} the freezing was manually scored by a different observer (CEB), in order to investigate the impact of an independent observation. Figure 5 shows the contextual fear conditioning paradigm.

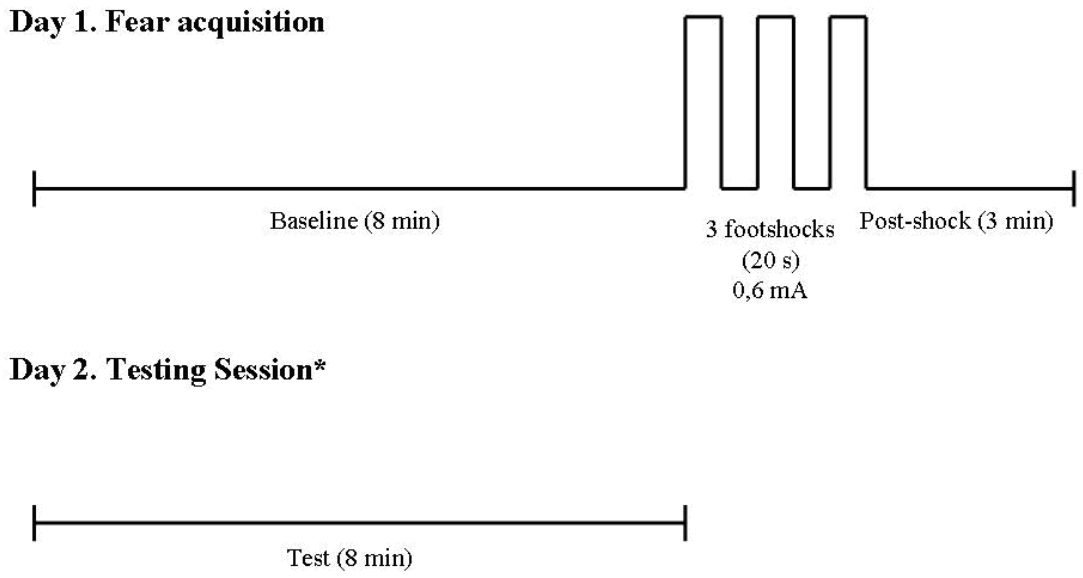

Figure 5 - Contextual fear conditioning procedure used for phenotyping. *Freezing registered in the testing session was employed as mating criteria.

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 3 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 scores, as well as the 10 male and 10 female rats with the lowest conditioned freezing rates, 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 (S_1). The same procedure was used for the production of new generations of selected animals (S_2 to S_{14}). Mating always occurred within each line. One exception occurred in S_2, when one female from the CLF line with the highest score of conditioned freezing was bred with a male from the CHF line that also had the highest score of conditioned freezing. The high- and low-family breeders were chosen after all animals from a given generation had been phenotyped. However, the number of breeding pairs varied through generations. Also, due to fertility problems, an additional crossing in the fifth generation, and two additional crossings in the seventh generation were made, and the resulting offspring was then incorporated to its respective generation. Also, an additional cross was made in the generation S_{12}, but the offspring was only employed in the third study of the present thesis.

From the fourth generation onwards, a third group of males and females were introduced in our breeding program. These animals underwent the same procedures employed to produce the high and low freezing lines. The only difference is that this third group, termed “Randomly Mated” (RND), did not receive any selection pressure after phenotyping, i.e. the mating was randomized among animals. The objective was to create a parallel control group for the selected lines, with an intermediate conditioned freezing response. The initial population of RND rats was produced from 16 breeding pairs of Wistar rats randomly bred in our colony. This stock had the same origin of the High and Low selected rats of our laboratory. Brother–sister breeding pairs were always avoided in all groups to reduce inbreeding, thus reducing genetic variability, 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). Table 2 shows the number of breeding pairs in each generation of selective breeding, as well the rate of females who gave birth.

Generation	High Freezing		Randomly Selected		Low Freezing	
	Breeding pairs	% Fertilized females	Breeding pairs	% Fertilized females	Breeding pairs	% Fertilized females
S_0	10	100%	-	-	10	100%
S_1	11	100%	-	-	11	91%
S_2	10	80%	-	-	10	80%
S_3	14	86%	-	-	14	100%
S_4	16	63%	16	69%	16	63%
S_5	15	60%	10	90%	15	47%
S_5/F_1	10*	80%	-	-	10*	50%
S_6	15	100%	9	100%	15	87%
S_7	15	40%	15	87%	15	46%
S_7/F_1	9*	33%	-	-	6*	22%
S_7/F_2	4*	80%	-	-	5*	50%
S_8	15	86%	10	80%	15	80%
S_9	15	66%	14	64%	15	73%
S_{10}	15	86%	14	71%	15	93%
S_{11}	15	100%	15	80%	15	73%
S_{12}	15	93%	15	93%	15	93%
S_{13}	15	93%	15	93%	15	93%
S_{14}	15	93%	15	93%	15	93%

Table 2 - Fertility rate throughout generations. *Same Breeders.

Despite the limited space of our facilities, the number of individuals of each population of the CHF and CLF selected lines was kept unaltered in all generations, except in cases of natural deaths. For the RND rats, however, no more than 70 rats were maintained in the first five generations (S_5-S_{10}), and after that the population was kept unchanged.

Generation	High Freezing		Randomly Selected		Low Freezing	
	Males	Females	Males	Females	Males	Females
S_1 (n=147)	37	39	-	-	34	37
S_2 (n=143)	37	35	-	-	37	34
S_3 (n=158)	34	45	-	-	42	37
S_4 (n=151)	46	31	-	-	33	41
S_5 (n=217)	29	37	40	16	49	46
S_6 (n=175)	29	23	26	24	39	34
S_7 (n=308)	64	61	17	25	73	68
S_8 (n=184)	28	35	30	34	32	25
S_9 (n=279)	70	56	22	38	51	42
S_{10} (n=250)	44	37	42	40	37	50
S_{11} (n=353)	60	56	60	46	62	69
S_{12} (n=345)	67	55	54	70	49	50
S_{13} (n=338)	59	42	57	51	67	62
S_{14} (n=425)	56	69	86	73	69	72
Total (n=3473)	**660**	**621**	**434**	**417**	**674**	**667**

Table 3 - Distribution of the number of male and female rats behaviorally characterized among high conditioned freezing, randomly selected and low conditioned freezing animals along fourteen selected generations. *Note:* For S_0, N=120 (60 males and 60 females).

Body weight. To assess possible lines differences in growth rates, litters were weighed from S_9 to S_{13}, at 7 days of age, and individual animals were weighed on postnatal days (PND) 21 and 42, and also on the training day. Due to practical reasons, animals did not go to the conditioning training in the same day. In this case, weight on the conditioning day was considered in the range of 75-85 day old animals.

3.5
Statistics

Selective Breeding

Behavioral data from the S_0 population were compared by a Student's t-test only regarding sex effects, because the High and Low conditioned freezing lines did not exist yet. Since the number of selected groups varied during the selective breeding process, as well the intensity of footshocks, starting in S_1 behavioral data were analyzed separately for each generation. For the first four generations of selective breeding, a two-way ANOVA was used, either for baseline, post-shock or conditioned freezing: the first factor, with 2 levels, was breeding line (CHF and CLF), and the second factor, with 2 levels, was the animal's sex (male and female). For the other selected generations (S_5-S_{14}), a two-way ANOVA for each dependent variable was also conducted. But, in this case, the first factor, breeding line, had 3 levels (CHF, CLF and RND), and the second factor, with 2 levels, was related to the animal's sex (male and female). Additionally, an ANCOVA was also conducted, with the post-shock freezing as a covariant factor, to evaluate whether the breeding line effect on conditioned freezing during the test session was attributable to possible post-shock differences that these animals presented during the training session. The level of significance employed was of 0.05. Fisher's Least Significant Differences (LSD) test was used for post-hoc comparisons.

3.6
Results

Selective Breeding

The S_0 Population

Data of baseline and post-shock freezing were not registered in the S_0 generation. The results of the Student's t test regarding conditioned freezing in the testing session showed no significant differences among males and females (t_{118}= 0.018; p>0.05).

S_1 generation

In the first-generation offspring of selective breeding, the results of baseline freezing showed the absence of two-way interaction ($F_{1,142}$=0.077; p=0.781), and no main effects for line ($F_{1,142}$=0.043; p=0.835) and sex ($F_{1,142}$=0.114; p=0.735). For post-shock freezing, the analysis showed no two-way interaction ($F_{1,142}$=0.107; p=0.743), and no main effects for line ($F_{1,142}$=0.588; p=0.444) or sex ($F_{1,142}$=0.218; p=0.641). For conditioned freezing, the ANOVA showed a lack of two-way interaction ($F_{1,142}$=0.032; p=0.857), as well as a lack of main effects for line ($F_{1,142}$=0.283; p=0.595); however main effects for sex were observed ($F_{1,142}$=5.197; p<0.05).

The ANCOVA results showed no significant two-way interaction ($F_{1,141}$=0.3; p=0.864), and no main effects for line ($F_{1,141}$=0.263; p=0.609), and main effects for sex ($F_{1,141}$=5.2; p<0.0.5)

S_2 generation

Results of the baseline freezing in the second generation of selective breeding showed the absence of two-way interaction ($F_{1,139}$=0.9; p=0.344), and no main effects for line ($F_{1,139}$=0.109; p=0.741) or sex ($F_{1,139}$=0.043; p=0.835). For post-shock freezing, the analysis showed the absence of two-way interaction ($F_{1,139}$=1.66; p=0.199), and no main effects for line ($F_{1,139}$=1.418; p=0.235) or sex ($F_{1,139}$=0.602; p=0.439).

For conditioned freezing, results showed the absence of two-way interaction ($F_{1,139}$=0.274; p=0.6), and no main effects for line ($F_{1,139}$=3.298; p=0.071); however, results showed main effects for sex ($F_{1,139}$=4.044; p=0.046).

For the ANCOVA: absence of two-way interaction ($F_{1,138}$=0.273; p=0.602), and no main effects for line ($F_{1,138}$=3.232; p=0.74), but main effects for sex ($F_{1,138}$=3.991; p<0.05).

S_3 generation

In the third generation, results from baseline freezing showed a lack of two-way interaction ($F_{1,155}$=1.613; p=0.205), and the absence of main effects for line ($F_{1,155}$=1.463; p=0.228) and sex ($F_{1,155}$=3.104; p=0.08). The analysis of post-shock freezing showed the absence of two-way interaction ($F_{1,155}$=0.52; p=0.471), and no main effects for line ($F_{1,155}$=0.157; p=0.692) or sex ($F_{1,155}$=1.2; p=0.275).

The analysis of conditioned freezing found the absence of two-way interaction ($F_{1,155}$=0.207; p=0.65). However, main effects for line ($F_{1,155}$=43.758; p<0.001) and sex ($F_{1,155}$=4.072; p<0.05) were observed. Post-hoc comparisons found significant differences between CHF and CFL animals, for both males and females (all p <0.001).

For the ANCOVA, an absence of two-way interaction ($F_{1,154}$=0.217; p=0.642), and main effects for line ($F_{1,154}$=43.541; p<0.001) and sex ($F_{1,154}$=4.09; p<0.05) were found.

S_4 generation

Results of the baseline freezing in the fourth generation of selective breeding showed the absence of two-way interaction ($F_{1,147}$=0.762; p=0.383), and no main effects for line ($F_{1,147}$=0.179; p=0.672) or sex ($F_{1,147}$=0.388; p=0.534). For post-shock freezing, the absence of two-way interaction ($F_{1,147}$=0.066; p=0.797), and main effects for line ($F_{1,147}$=6.2; p<0.05), but not for sex ($F_{1,147}$=3.516; p=0.063) were observed.

For conditioned freezing, the analysis showed the absence of two-way interaction ($F_{1,147}$=0.093; p=0.76); however, main effects for line ($F_{1,147}$=60.160; p<0.001) and sex ($F_{1,147}$=10.393; p<0.001) were observed. Pairwise post-hoc comparisons showed that CHF differed from CLF animals for both males and females (all p <0.001).

For the ANCOVA, the absence of two-way interaction ($F_{1,146}$=0.55; p=0.815) and main effects for line ($F_{1,146}$=51.426; p<0.001) and sex ($F_{1,146}$=15.913; p<0.001) were observed.

S_5 generation

The analysis of baseline freezing in the fifth generation showed the absence of two-way interaction ($F_{2,211}$=0.38249; p=0.682) and no main effects for line ($F_{2,211}$=0.48182; p=0.618) or sex (F1,211=0.505; p=0.477). For post-shock freezing, the absence of two-way interaction ($F_{2,11}$=1.824; p=0.163) was observed; and results also showed a main effect for line ($F_{2,211}$=13.101; p<0.001), but not for sex ($F_{2,211}$=1.24; p=0.266). Fisher LSD post-hoc comparisons showed that male CHF animals presented significantly more post-shock freezing responses than males CLF and RND animals, and that female CHF rats presented more post-shock freezing responses than female RND animals (all p <0.001).

For conditioned freezing, ANOVA showed the absence of two-way interaction ($F_{2,11}$= 0.821; p=0.441), and main effects for line ($F_{2,211}$=26.400; p<0.001) or sex ($F_{1,211}$=28.259; p<0.001). Fisher LSD post-hoc comparisons showed that male CHF animals presented significantly more conditioned freezing than male CLF and RND animals (all p <0.001).

For the ANCOVA, the absence of two-way interaction ($F_{2,210}$=1.252; p=0.288), and main effects for line ($F_{1,210}$=17.771; p<0.001) and sex ($F_{1,210}$=32.418; p<0.001) were observed.

S_6 generation

In the sixth generation, ANOVA results for baseline freezing showed the absence of two-way interaction ($F_{2,169}$= 0.898; p=0.409), and a lack of main effects for line ($F_{2,169}$=1.042; p=0.354) and sex ($F_{1,169}$=0.033; p=0.854). The same patterns of results was found for post-shock freezing, with results showing no two-way interaction ($F_{2,169}$=0.898; p=0.409), and no main effects for line ($F_{2,169}$=1.042; p=0.354) or sex ($F_{1,169}$=0.033; p=0.854).

The analysis of conditioned freezing behavior showed no two-way interaction ($F_{2,169}$=0.153; p=0.858), but results showed main effects for line ($F_{2,169}$=7.422; p<0.001) and sex ($F_{1,169}$=14,321; p<0.001). Fisher LSD post-hoc comparisons showed that male CHF animals only differed from male RND animals, but not from male CLF animals. The same pattern of results was found for females (all p <0.001).

The ANCOVA showed a non-significant two-way interaction ($F_{2,168}$=173.703; p=0.71). Main effects for line ($F_{2,168}$=5.882; p<0.001) and sex ($F_{1,168}$=13,165; p<0.001) were also observed.

S_7 generation

In the seventh generation of selective breeding, the results for baseline freezing demonstrated the absence of two-way interaction ($F_{2,302}$=1.025; p=0.359), and the absence of main effects for line ($F_{2,302}$=1.307; p=0.272) and sex ($F_{1,302}$=0.048; p=0,826). The analysis of post-shock freezing also showed the absence of two-way interaction ($F_{2,302}$=0.522; p=0.593) and main effects for line ($F_{2,302}$=12.123; p<0.001) and sex ($F_{1,302}$=4.508; p<0.05). Post-hoc comparisons showed that, for males, CLF differed from CHF and RND animals. The same results were found for females (all p <0.001).

The results for conditioned freezing showed no two-way interaction ($F_{2,302}$=2.850; p=0.594); however main effects were observed for line ($F_{2,302}$=14.152; p<0.001) and sex ($F_{1,302}$=14.233; p<0.001). Fisher LSD post-hoc comparisons showed that CHF animals significantly differ from CLF and RND animals for males. For females, CHF differed only from CLF animals (all p <0.001).

The ANCOVA performed with the post-shock freezing as a covariant factor showed the absence of a two-way interaction ($F_{2,301}$=2.409; p=0.92). Main effects were also found for line ($F_{2,301}$=10.632; p<0.001) and sex ($F_{1,301}$=10.526; p<0.001).

S_8 generation

For baseline freezing, the results showed neither a two-way interaction ($F_{2,178}$=2.007; p=0.127) nor main effects for line ($F_{2,178}$=0.613; p=0.542) and sex ($F_{1,178}$=1.159; p=0.283). For post-shock freezing, the absence of two-way interaction was verified ($F_{2,178}$=0.997; p=0.371); also, main effects for line ($F_{2,178}$=4.581; p=0.011),

but not for sex ($F_{1,178}$=0.416; p=0.519) were found. Post-hoc pairwise comparisons showed differences for females, with CLF animals being significantly different from CHF and RND animals (all p <0.05).

For context freezing, ANOVA showed no two-way interaction ($F_{2,178}$=0.620; p=0.538), but main effects for line ($F_{2,178}$=3.371; p<0.05) and sex ($F_{1,178}$=8.003; p<0.05) were found. Post-hoc pairwise comparisons showed differences only between male CLF and male RND animals (p<0.05).

The ANCOVA showed a non-significant two-way interaction ($F_{2,177}$=0.617; p=0.541), and main effects for sex ($F_{1,177}$=7.643; p<0.05), but not for line ($F_{2,177}$=1.362,; p=0.259).

S_9 generation

The analysis for baseline freezing showed neither a two-way interaction ($F_{2,273}$=0.265; p=0.766) nor main effects for line ($F_{2,273}$=0.243; p=0.784) or sex ($F_{1,273}$=1.316; p=0.252). For post-shock freezing, the absence of a two-way interaction ($F_{2,273}$=1.956; p=0.143) and main effects for line (F2,273=5.550; p<0.05) but not for sex ($F_{1,273}$=0.920; p=0.338) were noted. Pairwise post-hoc comparisons showed that CHF animals differed from CLF animals, which differed from RND animals. For females, CHF differed from CLF animals (all p <0.05).

For conditioned freezing, the analysis showed the absence of a two-way interaction ($F_{2,273}$=0.597; p=0.551), and main effects for line ($F_{2,273}$=28.206; p<0.001) and sex ($F_{1,273}$=6.463; p<0.05). Post-hoc pairwise comparisons showed that CHF animals differed from CLF and RND animals, for both males and females (all p <0.001).

The ANCOVA analysis showed the absence of a two-way interaction ($F_{2,272}$=0.437; p=0.646) and main effects for line ($F_{2,272}$=26.042; p<0.001) and sex ($F_{1,272}$=9.152; p<0.001).

S_{10} generation

For baseline freezing, results showed the absence of a two-way interaction ($F_{2,244}$=1.796; p=0.168), and no main effects for line ($F_{2,244}$=0.542; p=0.582) or sex

($F_{1,244}$=0.000; p=1.000). For post-shock freezing, the analysis showed the absence of a two-way interaction ($F_{2,244}$=0.391; p=0.676), and no main effects for sex ($F_{1,244}$=3.197; p=0.075); however, main effects for line ($F_{2,244}$=13.927; p<0.001) were found. Post-hoc comparisons showed that CHF animals differed from CLF and RND animals, for both males and females.

For conditioned freezing, the results showed a two-way interaction ($F_{2,244}$=5.552; p<0.05), and main effects for line ($F_{2,244}$=18.902; p<0.001) and sex ($F_{1,244}$=13.516; p<0.001). Post-hoc comparisons found differences between CHF and CLF, RND animals, for both males e females (all p <0.001).

The ANCOVA performed with post-shock freezing as a covariant factor showed the presence of a significant two-way interaction ($F_{2,243}$=6.301; p<0.05), and also main effects for line ($F_{2,243}$=10.88; p<0.001) and sex ($F_{1,243}$=10.856; p<0.001).

S_{11} generation

For baseline freezing, ANOVA showed an absence of two-way interaction ($F_{2,347}$=0.462; p=0.629), and no main effects for line ($F_{2,347}$=2.727; p=0.066) or sex ($F_{1,347}$=2.498; p=0.114). For post-shock freezing, results showed the presence of a two-way interaction ($F_{2,347}$=6.763; p<0.001), and main effects for line ($F_{2,347}$=10.399; p<0.001) and sex ($F_{1,347}$=5.383; p<0.05). Post-hoc comparisons showed significant differences only for females, with RND animals differing from CHF and CLF animals (all p <0.001).

Results for conditioned freezing showed the absence of a two-way interaction ($F_{2,347}$=0.474; p=0.622) and main effects for line ($F_{2,347}$=23.121; p<0.001) and sex ($F_{1,347}$=33.379; p<0.001) were noted. Post-hoc comparisons showed that CHF animals differed from RND and CLF animals, and that RND differed from CLF animals, for males; for females, CHF animals significantly differed from RND and CLF animals (all p <0.05).

The ANCOVA performed with post-shock freezing as a covariant factor showed the absence of a two-way interaction ($F_{2,346}$=1.724; p=0.18), and main effects for line ($F_{2,346}$=15.761; p<0.001) and sex ($F_{1,346}$=27.801; p<0.001).

S_{12} generation

The results for baseline freezing showed the absence of a two-way interaction ($F_{2,339}$=2.875; p=0.057), and no main effects for line ($F_{2,339}$=2621; p=0.074) or sex ($F_{1,339}$=0.075; p=0.783). For post-shock freezing, the results showed the absence of a two-way interaction ($F_{2,339}$=0.43; p=0.65), but main effects for line ($F_{2,339}$=20.269; p<0.001) and sex ($F_{1,339}$=14.023; p<0.001) were observed. For males, post-hoc comparisons showed that CHF animals differed from CLF, but not from RND animals. Moreover, CLF differed from RND animals. The same pattern of results was found for females (all p <0.001).

For conditioned freezing, the results showed the absence of a two-way interaction ($F_{2,339}$=0.071; p=0.931), but main effects for line ($F_{2,339}$=18.128; p<0.001) and sex ($F_{1,339}$=41.856; p<0.001) were observed. For males, post-hoc comparisons showed that CHF animals differed from CLF, but not from RND animals. Furthermore CLF differed from RND animals. For females, CHF differed from CLF and RND animals. Finally, CLF differed from RND animals (all p <0.001).

The ANCOVA performed with post-shock freezing as a covariant factor showed a non-significant two-way interaction ($F_{2,338}$=0.195; p=0.823) and main effects for line ($F_{2,338}$=10.297; p<0.001) and sex ($F_{1,338}$=31.055; p<0.001) were also verified.

S_{13} generation

For baseline freezing, the results showed the absence of a two-way interaction ($F_{2,332}$=0.279; p=0.756), and no main effects for line ($F_{2,332}$=0.393; p=0.531) or sex ($F_{2,332}$=1.309; p=0.271) were found. For post-shock freezing, the absence of a two way interaction ($F_{2,332}$=0.322; p=0.724), and main effects for line ($F_{2,332}$=5.257; p<0.05), but not for sex ($F_{2,332}$=0.525; p=0.469) were noted. Post-hoc comparisons showed significant differences only for males, with CHF animals differing from CLF animals (p<0.05).

For conditioned freezing, a two-way interaction ($F_{2,332}$=3.207; p<0.05), and main effects for line ($F_{2,332}$=36.372; p<0.001) and sex ($F_{2,332}$=20.8; p=0.001) were observed. Pairwise post-hoc comparisons showed that, for males, CHF differed from CLF and

RND animals; for females, CHF differed from CLF and RND animals. Finally CLF differed from RND animals (all p <0.001).

The ANCOVA performed with post-shock freezing as a covariant factor showed a non-significant two-way interaction ($F_{2,331}$=0.324; p<0.723), and main effects for line ($F_{2,331}$=5.154; p<0.05), but not for sex ($F_{1,331}$=0.514; p=474).

S_{14} generation

Baseline results from S_{14} showed the absence of a two-way interaction ($F_{2,419}$=1.99; p=0.14), and main effects for line ($F_{2,419}$=17.76; p<0.001), but not for sex ($F_{1,419}$=1.53; p=0.21). Post-hoc comparisons showed that CLF differed from CHF and RND rats, for males (all p <0.001). For females, CHF differed from RND and CLF rats (all p <0.001). The analysis of post-shock freezing showed a non-significant two-way interaction ($F_{2,419}$=2.84; p=0.06), and main effects for line ($F_{2,419}$=17.12; p<0.001), but not for sex ($F_{1,419}$=0.28; p<0.6). Post-hoc comparisons indicate that, for males, CLF differed from CHF and RND animals (all p <0.05). The same pattern of results was found for females.

The analysis of conditioned freezing showed the presence of a significant two-way interaction ($F_{2,419}$=3.994; p<0.05), and main effects for line ($F_{1,419}$=67.23; p<0.001) and sex ($F_{1,419}$=25,65; p<0.001). Post-hoc comparisons showed that CHF, CLF and RND rats differed significantly among themselves (all p <0.05). The same pattern of results was found for females.

The ANCOVA results showed the absence of a significant two-way interaction ($F_{2,418}$=2.577; p=0.77), and main effects for line ($F_{2,418}$=54.065; p<0.001) and sex ($F_{1,418}$=30.06; p<0.001).

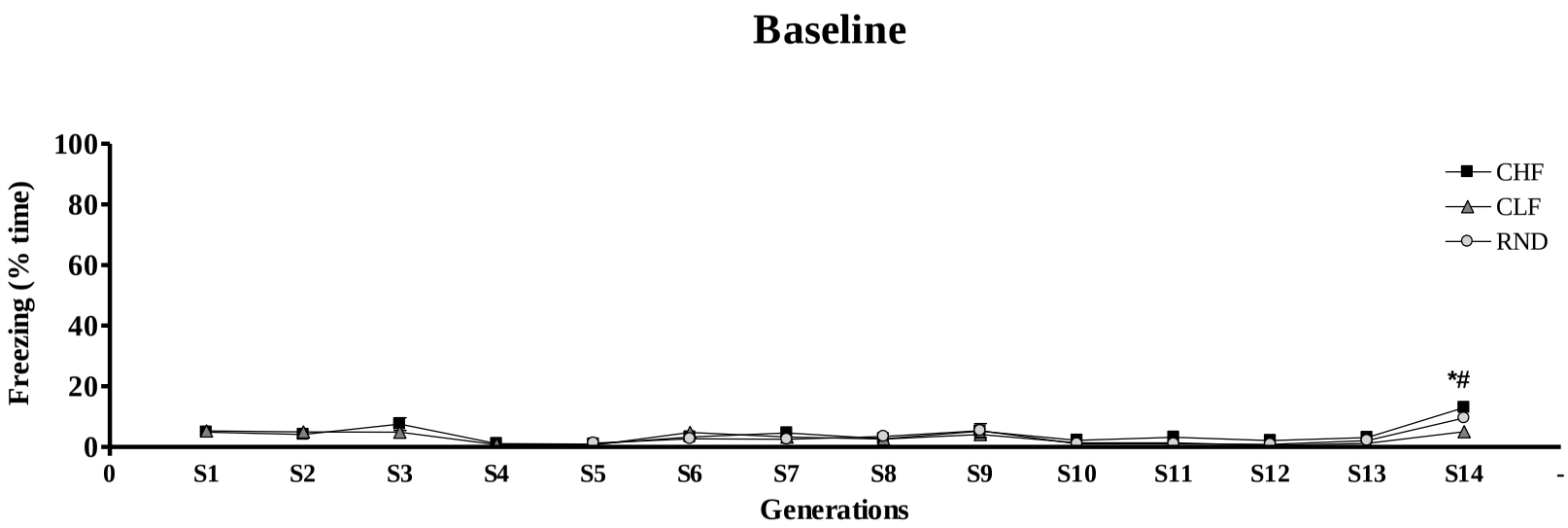

Figure 6 - Mean (± SEM) percentage of time spent freezing during the baseline acquisition session period of fourteen generations (S_1-S_{14}) of male and female rats selected for high (CHF) and low (CLF) levels of conditioned freezing, as well for randomly selected (RND) rats; * indicates significant differences between CHF and CLF rats; # indicates significant differences between CHF and RND rats (all $p < 0.05$).

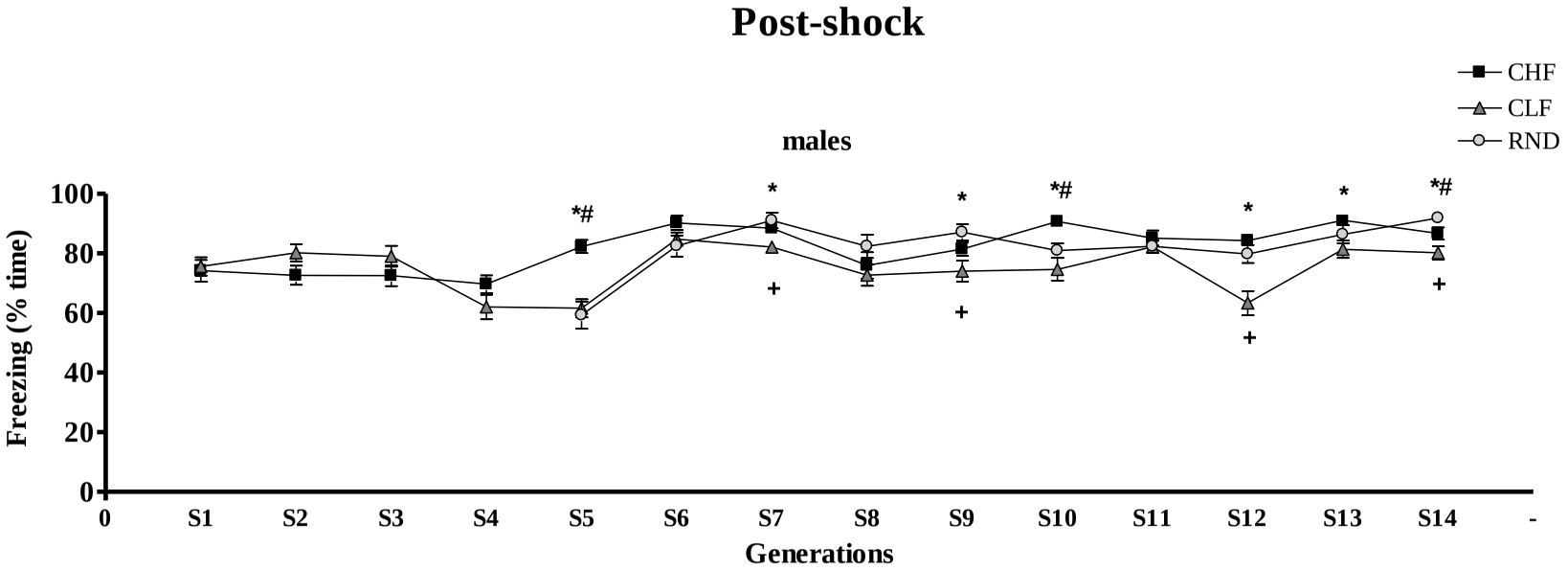

Figure 7 - Mean (± SEM) percentage of time spent freezing during the post shock acquisition session period for males of fourteen generations (S_1-S_{14}) of rats selected for high (CHF) and low (CLF) levels of conditioned freezing, as well for randomly selected (RND) rats; * indicates significant differences between CHF and CLF rats; # indicates significant differences between CHF and RND rats; + indicates significant differences between CLF and RND rats (all $p < 0.05$).

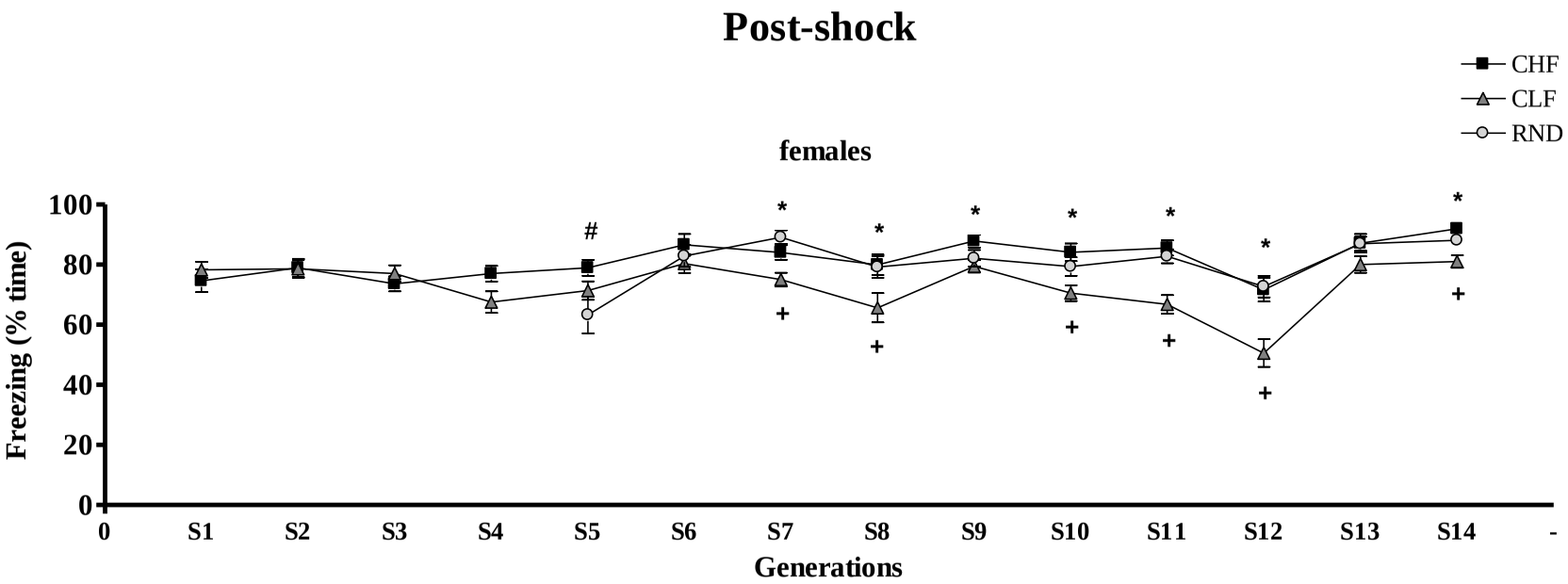

Figure 8 - Mean (± SEM) percentage of time spent freezing during the post shock acquisition session period for females of fourteen generations (S_1-S_{14}) of rats selected for high (CHF) and low (CLF) levels of conditioned freezing, as well for randomly selected (RND) rats; * indicates significant differences between CHF and CLF rats; # indicates significant differences between CHF and RND rats; + indicates significant differences between CLF and RND rats (all $p < 0.05$).

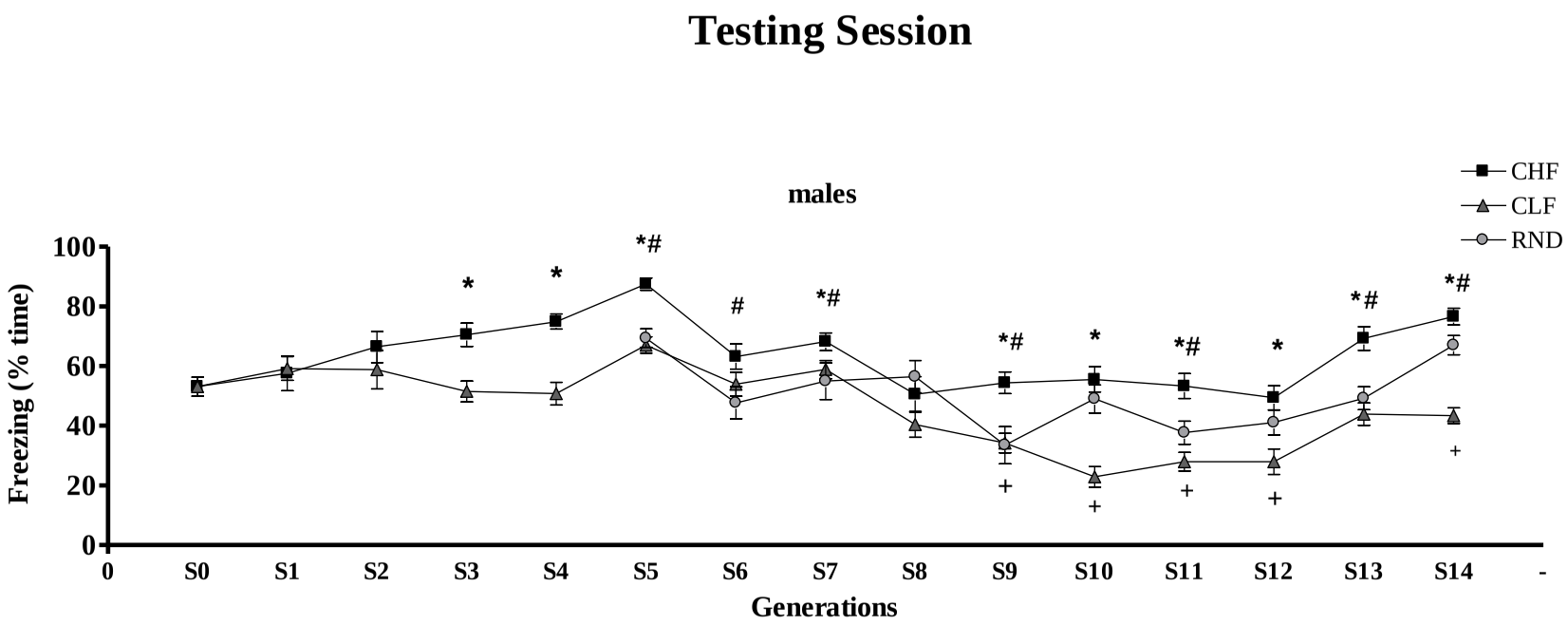

Figure 9 - Mean (± SEM) percentage of conditioned freezing during the testing session period for males rats selected for high (CHF) and low (CLF) levels of conditioned freezing, as well for randomly selected (RND) rats, in relation to the S_0 generation and the next fourteen generations (S_1-S_{14}); * indicates significant differences between CHF and CLF rats; # indicates significant differences between CHF and RND rats; + indicates significant differences between CLF and RND rats (all $p < 0.05$).

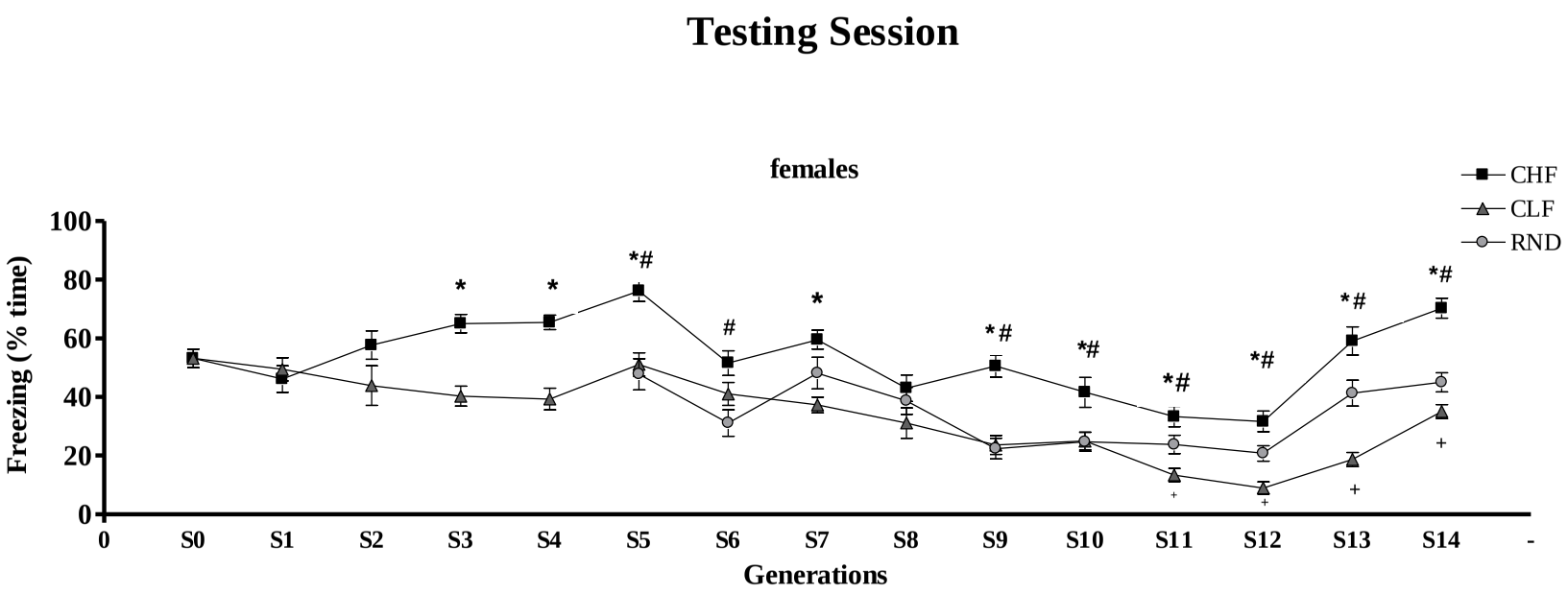

Figure 10- Mean (± SEM) percentage of conditioned freezing during the testing session period for males rats selected for high (CHF) and low (CLF) levels of conditioned freezing, as well for randomly selected (RND) rats, in relation to the S_0 generation and the next fourteen generations (S_1-S_{14}); * indicates significant differences between CHF and CLF rats; # indicates significant differences between CHF and RND rats; + indicates significant differences between CLF and RND rats; (all $p < 0.05$).

Different footshock intensities

The impact of different footshock intensities in CHF and CLF animals was also evaluated. The lines of each generation were clustered according to their respective footshock intensity (S_1-S_5: 1.0 mA; S_6-S_7: 0.7 mA; S_8-S_{14}: 0.6 mA). An initial three-way ANOVA for shock intensity (0.6; 0.7 and 1.0 mA), selected line (CHF and CLF) and for sex (male and female) was performed. It was found a significant two-way interaction for shock X line ($F_{2,2628}$=10.7; $p<0.001$), a significant two-way interaction for line X sex ($F_{1,2628}$=4.9; $p<0.05$), and main effects for line ($F_{1,2628}$=218.8; $p<0.001$) and sex ($F_{1,2628}$=101.6; $p<0.001$). However, it was found an absence of a significant three-way interaction regarding shock X line X sex ($F_{2,2628}$=0.53; $p>0.05$).

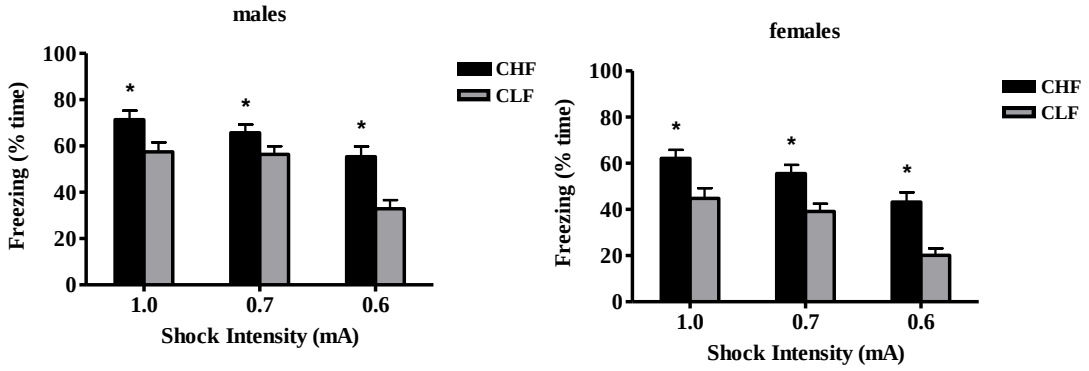

Figure 11: Mean (±SEM) percentage of conditioned freezing at different footshock intensities in CHF and CLF rats, among males and females; * denotes significant differences ($p<0.05$)

A subsequent two-way ANOVA only for shock intensity (0.6; 0.7 and 1.0 mA) and selected line (CHF and CLF) was then performed. Results showed a significant two-way interaction ($F_{2,2634}$=10.69; $p<0.001$), and main effects for shock intensity ($F_{2,2634}$=115.82; $p<0.001$) and line ($F_{1,2634}$=207.67; $p<0.001$). Significant differences between CHF and CLF were found in all shock levels (all $p<0.05$). Moreover, it was observed higher differences between CHF and CLF rats at the shock intensity of 0.6 mA (Figure 11). Indeed, this impression was

confirmed by a Delta comparison between CHF and CLF rats, among males and females, at every shock level (Figure 12).

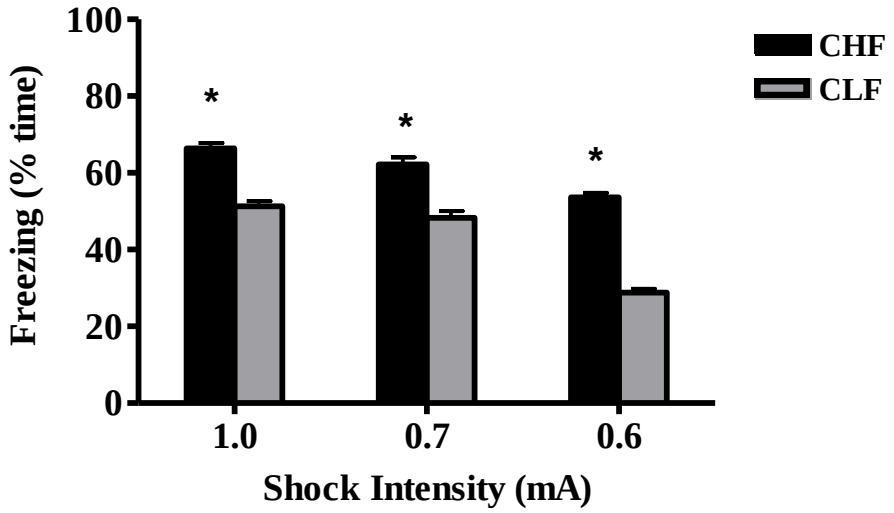

Figure 12: Mean (±SEM) percentage of conditioned freezing at different footshock intensities in CHF and CLF rats; * denotes significant differences ($p<0.05$)

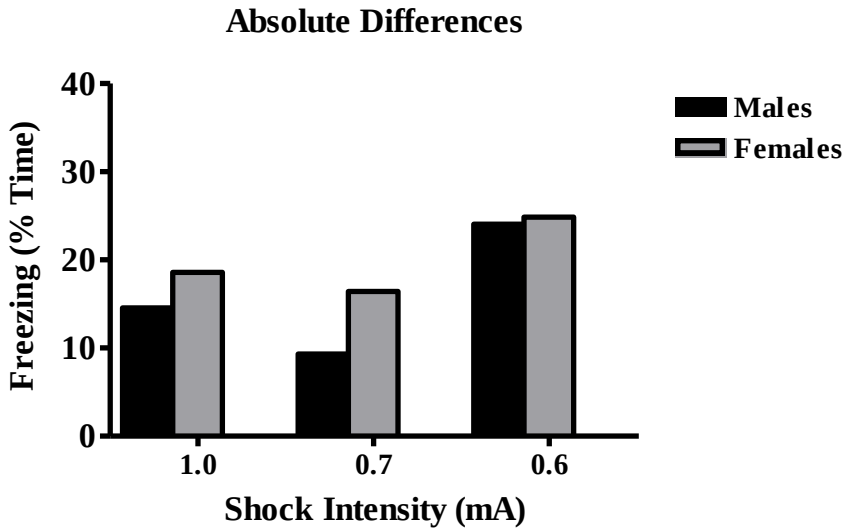

Figure 13: Absolute differences of conditioned freezing between CHF and CLF rats, among males and females, at different shock intensities.

Impact of a randomly selected (RND) control group

To asses the influence of RND animals in the selective breeding program since their introduction, conditioned freezing was analyzed through a two-way ANOVA, including only generations S_5 to S_{14}, but pooling together data from all these generations. The first factor, with 3 levels, was breeding line (CHF, CLF and RND); the second factor, with two levels, was related to the animal's sex (male and female). A significant two-way interaction ($F_{2,2868}$=4.87; $p<0.05$), and main effects for line ($F_{2,2868}$=152.173; $p<0.001$) and sex ($F_{1,2868}$=182.85; $p<0.001$) were observed. Post-hoc comparisons showed that CHF and CLF differed from each other and from RND animals, for both males and females (all $p <0.001$). Figure 13 shows the mean (+SEM) of conditioned freezing of CHF, CLF and RND rats, for males and females.

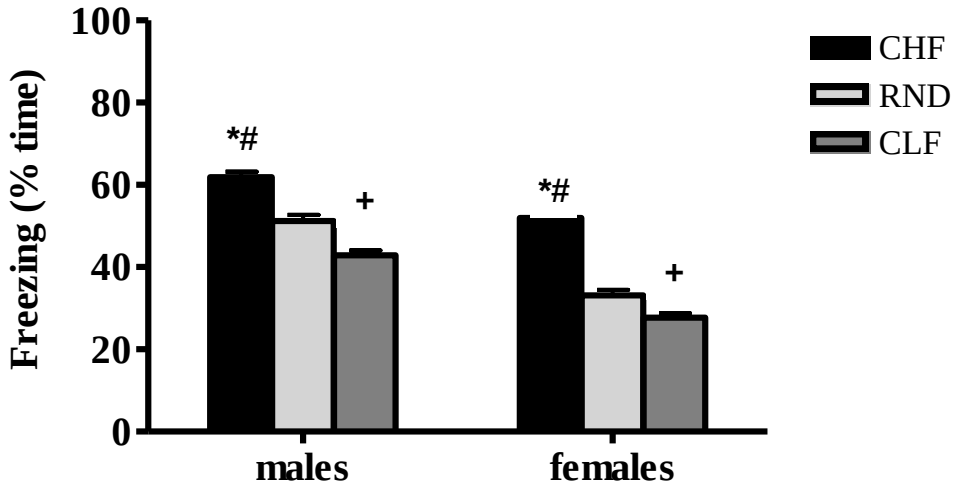

Figure 14: Mean (+SEM) percentage of conditioned freezing in generations S_5-S_{14}, all pooled together, of CHF, CLF and RND rats, for males and females. * indicates significant differences between CHF and CLF rats; # indicates significant differences between CHF and RND rats; + indicates significant differences between CLF and RND rats (all $p < 0.05$).

Sex Differences

Sex differences were evaluated for post-shock freezing and conditioned freezing. For post-shock freezing, males rats froze more in the post shock acquisition period (79.1% ± 0.49) than females (78.4% ± 0.52). A Student's t test analysis showed significant differences between the groups (t_{3471}=2.064; $p<0.05$). The same pattern of results was found for conditioned freezing registered in the training session. In general, male rats froze 53.38 (± 0.72), whereas females froze 39.78% (± 0.71) in testing sessions. Student's t test indicates a significant difference between the two groups (t_{3471}=13.0425; $p<0.001$).

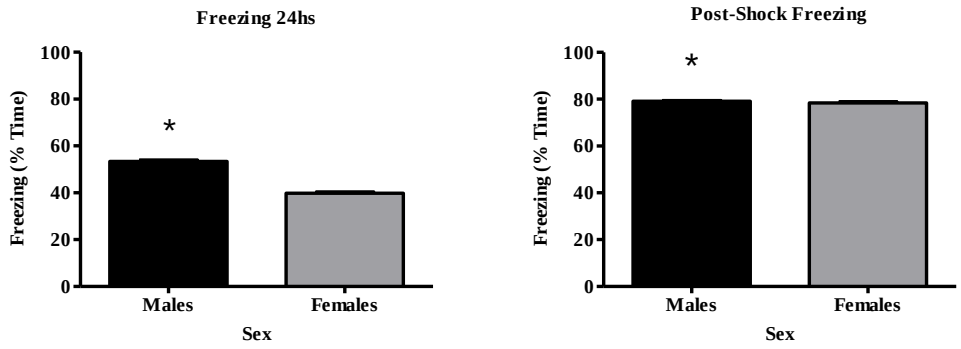

Figure 15: Mean (± SEM) of conditioned freezing registered 24hs after the training session (left) and of freezing registered during the post-shock acquisition period (right) among males (n=1768) and females (n=1705) rats; * indicates significant differences ($p<0.05$).

Absolute differences between CHF, CLF and RND animals through generations

In order to evaluate the strength of the selection procedure, absolute differences in conditioned freezing between CHF, CLF and RND animals, among males and females, was assessed through a Delta comparison of each generation of selective breeding (CHF x CLF; CHF x RND; CLF x RND).

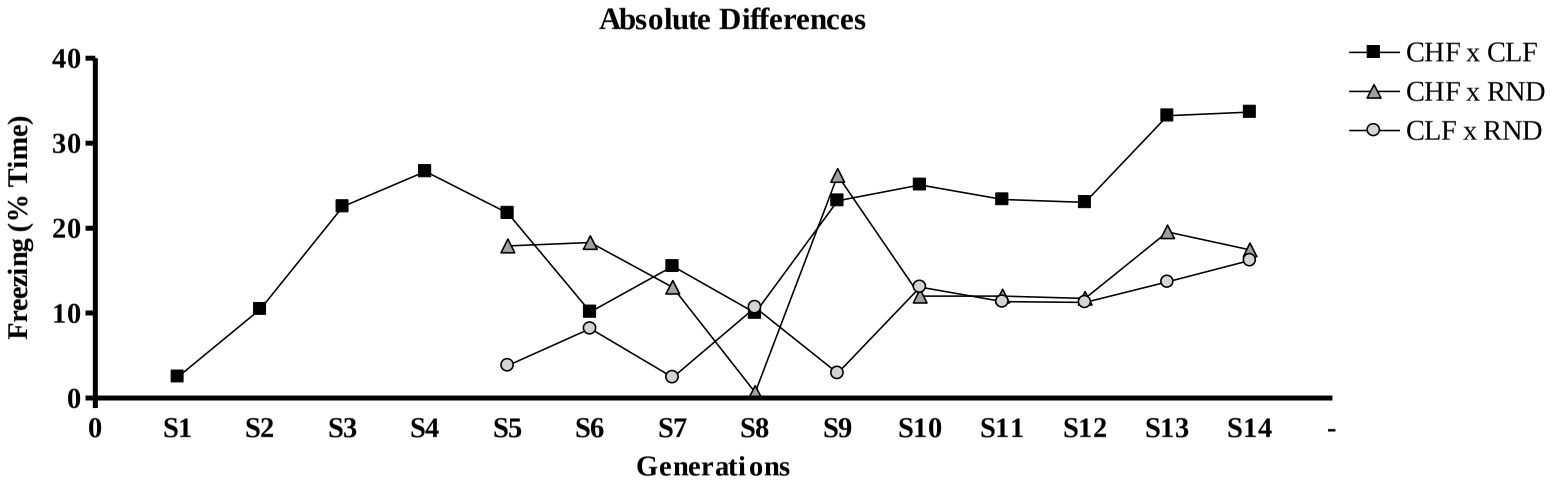

Figure 16: Absolute differences in conditioned freezing behavior between selected lines along thirteen generations of selective breeding.

To evaluate the absolute differences accumulated between the comparison groups during thirteen generations of selective breeding, we performed a One-Way ANOVA, with the Delta means for each comparison group as a factor. It was found a significant interaction ($F_{2,31}$=6.12; $p<0.05$), and post-hoc analysis showed that the comparison between CHF x CLF and between CHF x RND rats differed significantly from the CLF x RND comparison ($p<0.05$).

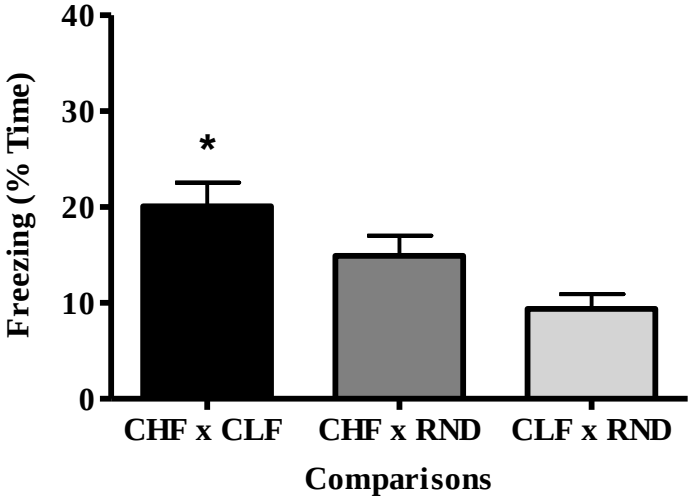

Figure 17: Mean (± SEM) of absolute differences of conditioned freezing between selected lines; * denotes significant difference between CHF x CLF and CLF x RND comparisons ($P<0.05$).

Heritability

The heritability ratio (h^2b), also called broad heritability, measures the degree of phenotypic variation (V_P) due to genetic factors for a single population under the limits of environmental variability during the study. In this sense, h^2b measures the variation observed in the phenotype, i.e., expresses the proportion of variance due to the genetic component. However, in selection experiments, researchers are more interested in the improvement of one specific trait (e.g. Freezing Behavior), which is regulated by the effects of additive genes. This more limited estimate has been called narrow heritability (h^2n). The basic formula to

calculate the narrow heritability is: $h^2n=R/S$, where "R" is the "Genetic Gain", obtained by subtracting the mean of one generation from the mean of the previous generation; and "S" is the selection differential, obtained by subtracting the mean of the selected individuals from the mean of its respective generation. Thus, to obtain the effects of additive genes, we use the formula $R=h^2n \times S$ (Klug & Cummings, 1991).

By applying this formula we evaluate the degree of heritability for freezing behavior, as well as the estimates of Genetic Gain in CHF and CLF rats, among males and females, during the selective breeding procedure. Due to variations in the shock intensity during the phenotyping process, only generations under the same protocol regarding shock intensity were compared. Table… shows the generations of CHF and CLF rats evaluated and their respective previous generation. Figure… shows the h^2n ratio of CHF and CLF during the selective breeding process and figure… shows the Genetic Gain observed in CHF and CLF lines across the selective breeding procedure.

Comparisons

Generation	Previous Generation	Shock Intensity
S_1	S_0	1.0mA
S_2	S_1	1.0mA
S_3	S_2	1.0mA
S_4	S_3	1.0mA
S_5	S_4	1.0mA
S_7	S_6	0.7mA
S_9	S_8	0.6mA
S_{10}	S_9	0.6mA
S_{11}	S_{10}	0.6mA
S_{12}	S_{11}	0.6mA
S_{13}	S_{12}	0.6mA
S_{14}	S_{13}	0.6mA

Table 4: Generations of CHF and CLF rats employed in the estimation of heritability and genetic gain.

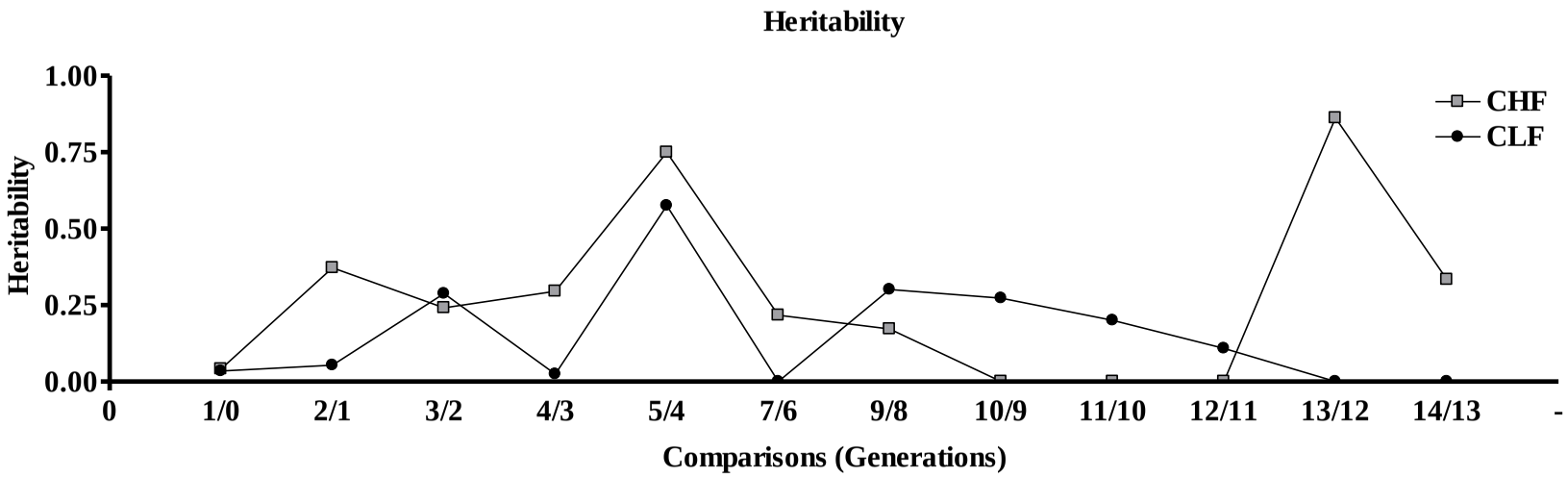

Figure 18: Estimates of narrow heritability (h^2) of freezing behavior for CHF and CLF rats along 14 generations of selective breeding.

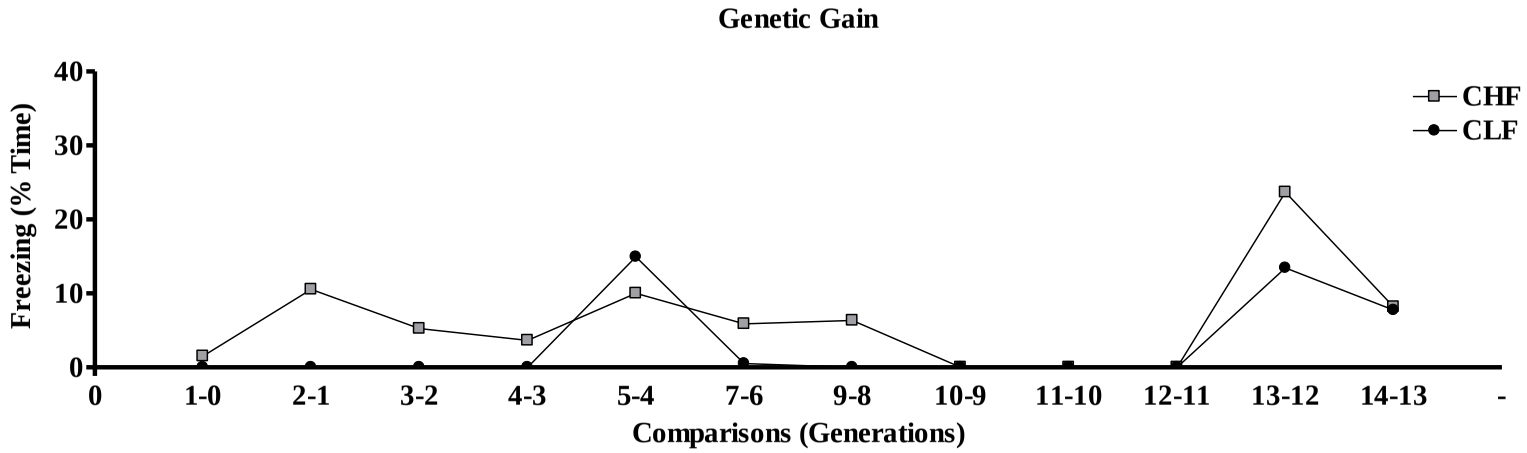

Figure 19: Estimates of Genetic Gain for CHF and CLF rats along 14 generations of selective breeding.

Body Weight

Figure 12 shows the mean (± SEM) of litter weight through 5 generations of selective breeding (S_9-S_{13}). Weight at PND 7 was analyzed using a one-way ANOVA for each generation. Non-significant differences between CHF, CLF and RND in all generations were observed (all $p < 0.05$).

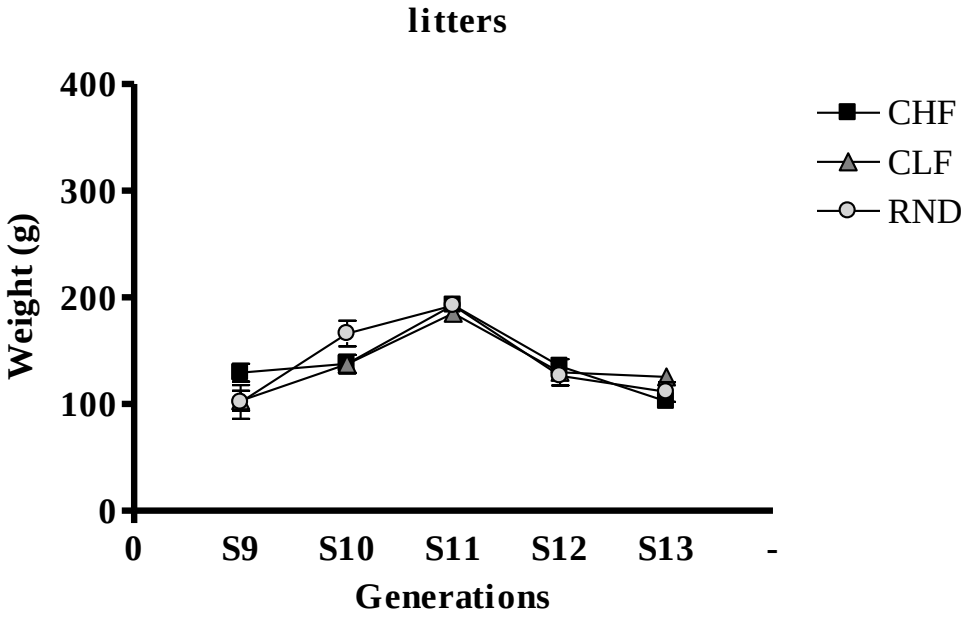

Figure 20 - Mean (± SEM) of litter weight from generation S9-S13 (all $p < 0.05$).

Rats are sexually dimorphic and body differences are expected. However, the pattern of change in body weight for males and females in the present study was similar. So, data for males and females was presented together.

Generation	Age (Days)	High Freezing		Randomly Selected		Low Freezing	
		Mean Weight (g)	(±) SEM	Mean Weight (g)	(±) SEM	Mean Weight (g)	(±) SEM
S_9	21	40,24	0,72	43,93	0,84	43,44	1,04
	42	134,06	1,57	133,09	1,82	129,59	2,27
	75-85	289,59	6,35	278,01	7,39	271,7	9,21
S_{10}	21	39,76	0,64	38,93	0,62	40,48	0,63
	42	128,37	9,2	139,64	8,88	125,07	9,15
	75-85	249,21	5,86	234,56	5,65	253	5,82
S_{11}	21	48,62	0,69	45,16	0,64	54,14	0,72
	42	135,35	2,04	132,06	1,91	149,54	2,13
	75-85	228,04	4,53	222,14	4,25	243,74	4,72
S_{12}	21	33,3	0,47	32,56	0,52	34,58	0,46
	42	117,59	1,43	118,95	1,58	111,9	1,42
	75-85	229,43	4,61	220	5,12	222,59	4,57
S_{13}	21	38,81	0,56	37,88	0,49	44,49	0,54
	42	176,8	7,09	161,25	6,27	166,53	6,86
	75-85	258,74	5,43	250,91	4,8	257,06	5,24

Table 5: Mean (± SEM) of body weight in S_9-S_{13} generations among CHF, RND and CLF rats.

To assess changes in body development among the lines, data from PND 21 and PND 42, as well as from the training day, were analyzed using a linear regression model correlating weight X age for each generation. Results showed a steady increase of body weight during development in all groups. Table 5 shows the linear model for each generation and line.

Generation	High Freezing		Randomly Selected		Low Freezing	
	Linear Regression Model	R^2	Linear Regression Model	R^2	Linear Regression Model	R^2
S_9	y = 124.68x - 94.72	0.98	y = 117.04x - 82.403	0.9814	y = 114.13x - 80.017	0.9804
S_{10}	y = 104.73x - 70.337	0.9922	y = 106.26x - 73.003	0.9863	y = 97.815x - 57.92	0.9997
S_{11}	y = 89.71x - 42.083	0.9996	y = 94.8x - 40.46	0.9999	y = 88.49x - 43.86	0.9999
S_{12}	y = 98.065x - 69.357	0.9935	y = 94.005x - 64.987	0.9896	y = 93.72x - 63.603	0.998
S_{13}	y = 109.97x - 61.813	0.9788	y = 106.29x - 56.543	0.9927	y = 106.52x - 63.017	0.9917

Table 6: Linear trend model for generation S_9-S_{13} in CHF, RND and CLF rats.

3.8
Discussion

In any selection experiment aiming at the study of emotionality, the main objective is to produce groups of animals with opposite behavioral responses to the same threatening environmental stimuli, and then investigate their genetic underpinnings. Emotionality is a construct that mirrors the emotional reactivity of an animal to its environment. Therefore, the use of multiple measures of emotionally is crucial to dissect the appropriate overlay between the different dimensions of this construct. This was the case of several studies described in the previous section of this work, which employed innate (Broadhurst, 1957; Ramos et al., 2003; Fujita, 1975; Liebsch et al., 1998a; Brunelli et al., 1996) or learned fear responses (Bignami, 1965; Brush, 1966; Gomes & Landeira-Fernandez, 2008).

In this sense, the main purpose of the present study was to develop a bidirectional selective breeding program employing Wistar rats, using the conditioned freezing in response to contextual cues previously associated with footshocks as selection criterion. The hypothesis is that the phenotypical differences in learned fear may be associated with functionally different conditioned fear circuits. The preliminary results of this ongoing procedure in our laboratory represent the first successful attempt to select rats with reliable and selective differences in conditioning freezing, and extend the findings of our previous report (Gomes & Landeira-Fernandez, 2008). Also, we introduced a third group of randomly mated rats (RND) in the selective breeding program, which may serve as a control group for the selected lines. Results from this continuous selective breeding program in our laboratory indicate 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. Reports from mouse studies have indicated that only one generation was sufficient to differentiate high- and low- conditioned freezing lines (Ponder et al., 2007; 2008; Radcliffe et al., 2000), whereas the present results detected a reliable difference after three generations. This result may suggest subtle differences between the two species. The present CHF and CLF lines are

particularly meaningful since most behavioral, pharmacological, and neuroanatomical experiments studying conditioned fear have been conducted using rats.

Very low levels of freezing behavior were observed among all groups during the baseline periods of the acquisition sessions (Figure 6). ANOVA results did not show significant differences between all groups. The only exception was in S_{14}, with CHF animals demonstrating more unconditioned freezing than the other groups. This analysis clearly demonstrates that differences in conditioning freezing observed between CHF and CLF animals in the testing session were not related with the initial levels of animal activity during the baseline period. It also reveals that handling for 5 days results in very low levels of unconditioned freezing responses prior to the occurrence of footshocks.

However, results of post-shock freezing registered in the acquisition sessions are still unclear. Although the ANOVA analysis showed sex effects only in the S_5, S_7, S_{11} and S_{12} generations, in general males rats froze more in the post shock acquisition period (79.1% $\pm$ 0.49) than females (78.4% $\pm$ 0.52). A Student's t test analysis showed significant differences between the groups (t_{3471}=2.064; $p<0.05$). Also, the ANOVA results showed differences in the amount of post-shock freezing between CHF and CLF animals. These differences were not observed in our original report that employed the first three generations of these two lines (Gomes & Landeira-Fernandez, 2008), but it was detected in a recent work (Gomes et al, 2011a). Furthermore, differences between these two lines and the RND group were also observed, for both males and females (see Figure 9 and 10).

Three possibilities may explain these discrepant results. One is that the footshock intensity used to phenotype animals until the present generation (0.6 mA) was much lower than the intensity used during the first five generations (1.0 mA). Therefore, the higher footshock intensity could lead to a ceiling effect so that differences in post-shock freezing behavior might not be observed. Indeed, the footshock intensity was reduced in our breeding program in the 6th generation to 0.7 mA and in the 8th generation to the present intensity (0.6 mA) to prevent possible ceiling effects produced by this relatively strong (1.0 mA) footshock intensity. A second possibility could be related to the fact that freezing observed

immediately after footshock reflects associative learning between contextual cues and the aversive footshock (Fanselow, 1980, 1990; Vianna et al., 2001b). For example, when the footshock is presented simultaneously with the rat's placement in the chamber, no contextual fear conditioning is observed (Landeira-Fernandez et al., 1995). Moreover, placing the animal in a different context from the one in which the footshock was delivered did not produce any freezing behavior (Fanselow, 1980). Therefore, differences in the post-shock freezing between CHF and CLF animals could be a consequence of the fact that CHF rats have a greater propensity for exhibiting higher conditioned freezing responses compared with CLF animals because of the continuous bidirectional selection over different generations. A third possible explanation for these incongruent results might be related to differences in pain sensitivity between these two lines. This is an important issue because freezing observed immediately after footshock is closely related to pain sensitivity and shock intensity (Cordero et al., 1998; Fanselow, 1984b). According to this possibility, selection for high and low conditioned freezing might independently lead to co-selection of other contributing factors that are not genetically linked but contribute to the phenotype that is being selected, such as differences in pain sensitivity to footshock. However, further studies are necessary to thoroughly test this possibility.

The major finding of the present study was the divergence between CHF and CLF rats in the conditioned freezing behavior in response to contextual cues previously associated with footshocks, along fourteen generations of selective breeding. Differences became significant after three generations and have stabilized (at least onefold) since then. These data reveal that conditioned fear is a highly heritable trait and can be rapidly, bidirectionally selected after a few generations.

We evaluate the degree of narrow heritability (h^2n) of freezing behavior in CHF and CLF rats, among males and females, during the selective breeding procedure (see Results). Figure 18 shows the h^2n ratio of CHF and CLF during the selective breeding process. In the first five generations of selective breeding, we observed a high degree of heritability of freezing behavior for CHF and CLF rats, but this rate decreased in the subsequent generations. However, for CHF rats, the degree of heritability increased in the S_{13} and S_{14} generations again, suggesting

that the genetic gain observed in these two generations (Figure 19) could be explained by the effects of the continuous selective breeding process, whereas this explanation maybe not suitable for CLF rats, since they showed low levels of heritability in generations S_{13} and S_{14}. In this sense, we believe that we need more generations of selective breeding in order to make the characteristics of this recently developed model more clearer and consistent.

Even with stable differences between CHF and CLF rats observed in the majority of generations, two exceptions occurred during the phenotyping process. Although an observed trend of divergent levels of freezing between CHF and CLF rats was noted, in S_6 and S_8, no statistically significant difference was observed between CHF and CLF, for both males and females. A possible explanation for this inconsistency might be related with the fertility problems found (most probably to inbreeding depression effects) in generations S_5 and S_7, which gave rise to S_6 and S_8, respectively. To circumvent this problem, in S_5 an additional cross was made, whereas in S_7 two additional crosses were made. The inclusion of offspring with different days of birth may be affected by subtle and involuntary alterations in the experimental context. Another, more plausible, hypothesis is related to the decreases occurred in the footshock intensities in our program, specifically in S_6 and S_8. As discussed above, the conditioned freezing response is particularly sensitive to shock strength (Fanselow and Bolles, 1979).

Indeed, a crucial variable of the present study is footshock intensity. As discussed above, the footshock intensity was reduced in our breeding program in the 6th generation to 0.7 mA and in the 8th generation to 0.6 mA, in order to prevent possible ceiling effects produced by the relatively strong intensity of 1 mA. Most importantly, strong footshock magnitudes could affect the selective breeding process as well, recruiting alternate pathways capable of mediating fear-related responses that are not directly associated with the conditioned freezing response (Ponnusamy et al, 2007). We evaluated the impact of different footshock intensities in CHF and CLF animals (see Results). The lines of each generation were clustered according to their respective footshock intensity (S_1-S_5: 1.0 mA; S_6-S_7: 0.7 mA; S_8-S_{14}: 0.6 mA). An initial three-way ANOVA for shock intensity (0.6; 0.7 and 1.0 mA), selected line (CHF and CLF) and for sex (male and female) was performed, but it was found an absence of a significant three-way interaction

regarding shock X line X sex. A subsequent two-way ANOVA only for shock intensity (0.6; 0.7 and 1.0 mA) and selected line (CHF and CLF) showed a significant two-way interaction, and main effects for shock intensity and line. Importantly, significant differences between CHF and CLF rats were found in all shock levels (all $p<0.05$). Moreover, it was observed higher differences between CHF and CLF rats at the shock intensity of 0.6 mA (Figure 13). A Delta comparison between CHF and CLF rats at every shock level confirmed this impression (Figure…). In this regard, results suggest that the footshock intensity of 0.6 mA could be the ideal for our phenotyping process.

Inter-chamber footshock reliability is essential for minimizing errors within experimental groups. Variations in footshock intensity (US) between chambers add additional variables that make interpreting the data difficult (Chang et al, 2009). For this reason, all conditioning chambers were calibrated with a multimeter before each experiment. However, a recurrent problem employing rodents in fear conditioned experiments is the accumulation of animal waste (i.e. urine, feces) in the stainless steel rods of conditioned chambers, as well in the scrambler circuit. This could affect the experimental procedure itself, reducing footshock intensities and leading to a reduction in freezing levels in all experimental groups. In this context, we employed new conditioned boxes in S_{13} and S_{14}, which probably led to an increase in the freezing levels observed in all groups. However, the behavioral divergence already observed between the selected lines in previous generations was maintained.

Another important feature of the present selective breeding program is the development of a randomly selected group of animals (RND), with intermediate levels of freezing behavior. This group of animals might serve as a control for the high- and low- conditioned freezing lines. The RND group was introduced when CHF and CLF rats were in the fifth generation of selective breeding (S_5). Based on data collected so far, RND animals in our selective breeding program showed promising results. A significant interaction was observed, with RND animals presenting intermediate freezing levels, in S_{10} and S_{14} for males and S_{12}, S_{13} and S_{14} for females.

We assessed the influence of RND animals in the selective breeding program since their introduction through a two-way ANOVA, including only

generations S_5 to S_{14}, but pooling together data from all these generations. It was found a significant two-way interaction, and main effects for line and sex. Importantly, post-hoc comparisons showed that CHF and CLF differed from each other and from RND animals, for both males and females (all $p < 0.001$). We evaluate absolute differences in conditioned freezing between CHF, CLF and RND animals, among males and females through a Delta comparison of each generation of selective breeding (Figure 16) and also absolute differences accumulated during thirteen generations of selective breeding. ANOVA results showed that the comparison between CHF x CLF and between CHF x RND rats differed significantly from the CLF x RND comparison ($p<0.05$).

These findings suggest that RND animals are presenting intermediate levels of freezing compared with the high and low lines. This is an important issue since, despite the importance of a randomly selected control group most of the selective programs reviewed in this work did not employ a group of random controls (Broadhurst, 1957, 1958; Ramos et al., 2003; Fujita, 1975, 1984; Liebsch et al., 1998a, b; Bignami, 1965). The only exception was the High and Low USV lines, widely divergent from each other and from the respective Random line, which has maintained N:NIH strain USV rates overall from generation to generation. However, in general, it was observed that High USV lines demonstrate more behavioral measures consistent with an "anxious" phenotype, whereas the Low line usually shows few behavioral differences from random control animals (Brunelli, 2005).

Due the fact that conditioned freezing response is a function of shock intensity, dependent on the association between conditioned and unconditioned stimuli, and is sensitive to a series of manipulations that interfere with its associative strength (Fanselow and Bolles, 1979; Landeira-Fernandez, 1996; Landeira-Fernandez et al., 1995), it is possible that differences in contextual fear conditioning between CHF and CLF animals might reflect differences in the pain sensitivity of these two groups. Previous studies indicate 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). In this regard, an ANCOVA was performed, with post-shock freezing as a covariant factor, for each generation of selective breeding, in order to evaluate whether the breeding

line effect on conditioned freezing during the test session was attributable to post-shock differences that these animals presented during the training session. The ANCOVA results showed the absence of significant results in all generations (see Results). The only exception occurred in S_{10}, where a significant two-way interaction was observed ($F_{2,243}$=6.301; $p<0.05$). In this regard, an additional ANCOVA was performed, with post-shock freezing response as a covariant factor. But, in this case, the RND line was excluded from the analysis. The first factor, with 2 levels, was breeding line (CHF and CLF), and the second factor, with 2 levels, was the animal's sex (male and female). In this situation, results showed a non-significant two-way interaction ($F_{1,163}$=3.837; $p>0.05$), and main effects for line ($F_{1,163}$=21.042; $p<0.001$) but not for sex ($F_{1,163}$=1.023; $p=0.313$). Together, these findings weaken the possibility of differences in pain sensitivity being responsible for differences in conditioned fear, suggesting dissociation between the post-shock freezing response and the conditioned freezing response in the present study. Also, we propose that these two forms of freezing behavior might be mediated by a distinct set of genes that in turn regulates different neural mechanisms associated with each form of learning. In accordance to 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). However, future studies are important to further evaluate whether CHF and CLF rats might present differences in pain sensitivity.

A possible criticism regarding the present selective breeding procedure is related to the accurate measurement of the conditioned freezing response itself. In the present study, freezing behavior was scored manually by an experienced observer (VCG), blind to the experimental conditions. However, freezing manual score is a long and tedious method that often requires multiple independent observations, being seriously susceptible to potential bias. Indeed, in order to ensure robust measures of freezing behavior, several studies have attempted to develop automated methods for the analysis of rodent motion during fear conditioning procedures (Contarino et al., 2002; Fitch et al., 2002; Marchand et al., 2003; Takahashi, 2004; Kopec et al, 2007; Anagnostaras et al, 2000), including our own (Gomes et al, 2009). Although some advantages were observed, these algorithms have some drawbacks. For example, several require

very sophisticated hardware that measure animal activity indirectly (e.g. photo-beam interruption of force-transduction). Others show poor time resolution, produce non-linear results, or only score motion and not freezing. Unfortunately, neither of these systems was suitable for our current experimental conditions. However, if more robust methods of automated freezing register were to be developed in the future, they would be incorporated in our selective breeding program in order to strengthen the phenotyping process.

Finally, another important aspect regarding the accurate measurement of conditioned freezing is the use of independent observations. Indeed, the data collected in S_{14} came from a different observer (CEB) from the other first thirteen generations. Results were very consistent, with the same behavioral pattern observed in the majority of generations.

Body Weight

Another interesting finding of the present study is related to body development among all rat groups. Body weight began to be measured in S_9. Litters were weighed at PND 7, and individual animals were weighed in PND 21, PND 42 and on the training day. Rats were sexually dimorphic and differences in body weight were as expected. Results showed a steady increase of body weight during development in all groups. This suggests that the continuous selective breeding process has not affected, so far, growth rates and body development measured as body weight. However, it is not unusual to observe differences in body weight in artificial selection experiments. For example, it was observed that Low line USV birth weights became significantly lower than both High and Random line weights since the 14^{th} generation of selective breeding. However, by the time of weaning Low line weights were not different from the High and Random lines for either sex, up to adulthood. The prenatal and/or genetic mechanisms underlying this long-term reduction in Low USV line birth weight are not yet known. The hypothesis is that the Low line fetuses may be genetically programmed for smaller sizes, or that the Low Line maternal uterine environment might be in some way unfavorable for fetus growth and development (Brunelli, 2005).

Sex Differences

Results from Study 1 also indicate that male rats consistently exhibit more conditioned freezing in the testing session than females during the development of the CHF and CLF lines, with the same being true for the RND control group. As a whole, male rats froze 53.38 (± 0.72), whereas females froze 39.78% (± 0.71) in testing sessions. Student's t test indicates a significant difference between the two groups (t_{3471}=13.0425; $p<0.001$). Significant differences between males and females were also found in post-shock freezing (see above). Sex 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). According to Steimer & Driscoll (2005), most of experimental studies involving anxiety and stress in rodents employed male animals. Basically, this preference is associated to the negative effects caused by physiological and behavioral variations that females present due the estrous cycle. Such variations are related to fluctuations of the hormones estrogen and progesterone. For example, it was observed that females from "Roman High Avoidance" rats are more active and less anxious in the proestrus in comparison to females in the diestrus. Besides, sex hormones might influence other tasks associated with learning and cognitive performance.

Sexual differences in anxiety tests employing rodents were firstly observed in the open field. In this model, males usually show less locomotor activity and higher defecation levels than females. Such results are traditionally interpreted as an indicative that males are more "fearful" or "anxious" than females. However, these differences could arise from other reasons, such as differences in metabolism levels, for example. Tests carried in other three anxiety models (Social Interaction, Elevated Plus Maze and Vogel Conflict Test) also indicate sexual differences. Nevertheless, the differences varied throughout the tests, with females demonstrating less anxiety in the Elevated Plus Maze, and being more anxious in the Vogel Conflict Test. Blanchard and colleagues (1991) showed that

females are more anxious than males in situations of potential danger, such as in the presence of a cat (for a review, see Palanza, 2001).

It has been suggested that these differences may be related to sexual dimorphisms 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 the marked sex differences observed in the present study are associated with a higher magnitude of male hippocampus LTP compared to female rats.

Conditioned fear and the interaction between two-way avoidance and freezing responses

Historically, the pavlovian fear conditioning was closely associated with one of the main causes of pathological anxiety (i.e. neurosis; Pavlov, 1927; Watson & Rayner, 1920). Surprisingly, the two-way avoidance response has been the main conditioned phenotype criterion employed for developing bidirectionally selected lines or strains based on learned aversive paradigms. That is the case for the Roman and Syracuse animals (discussed in the introduction section of this work) and other lines, such as Australian (Bammer, 1983), Koltushi (Ryzhova et al., 1983), and Hatano (Ohta et al., 1995) animals.

The use of the two-way avoidance response as the phenotype criterion for the development of so many genetic models of fear conditioning is curious because the learning mechanisms involved in the acquisition of this response are still unclear. In fact, two-way avoidance learning represents one of the oldest theoretical debates in behavioral sciences (for an elegant review of this debate, see Bolles, 1972). The two-factor theory (Mowrer and Lamoreaux, 1946) was one of the first attempts to address this issue. This theory posits that two different forms of learning are responsible for the acquisition of two-way avoidance. Initially, an animal undergoes classic aversive conditioning between the CS and US. Subsequently, the animal learns the instrumental response of crossing from one compartment to the other to terminate the CS and thus negatively reinforces the

response through a fear reduction process. According to this theory, the more "afraid" the animal is of the CS (respondent learning), the better the acquisition of the two-way avoidance response (instrumental learning). Therefore, an instrumental response is employed to measure the amount of fear triggered by the CS.

Although the two-factor theory has the appealing feature of integrating respondent and operant learning processes, several results have raised serious criticisms regarding this theoretical framework. As previously discussed, higher electrical footshocks are associated with lower two-way avoidance performance (Levine, 1966; McAllister et al., 1971). Moreover, manipulations that decrease contextual fear conditioning enhance the acquisition of the two-way avoidance response (Dieter, 1977). Finally, two-way avoidance can be bidirectionally modulated pharmacologically. Anxiolytic drugs enhance, whereas anxiogenic compounds impair, the acquisition of this response (Fernández-Teruel et al., 1991; Savić et al., 2005). However, these results contrast with the two-factor theory and indicate that the less emotionally reactive the animal is to the aversive situation, the better the animal will learn the shuttle box response. This fact imposes a certain problem with this animal model of learned fear, since the inability to learn the two-way avoidance response (i.e., a negative result) is employed as an index of the presence of a conditioned fear reaction. An alternative view of the two-way avoidance learning process is based on the fact that fear becomes classically conditioned, not only in response to a discrete CS that signals the occurrence of the footshock but also in response to contextual cues of each compartment where the US is presented (Landeira-Fernandez, 1996). Freezing in response to contextual cues and the CS might interfere with two-way avoidance acquisition. In fact, recent results indicate that animals tend to freeze when required to go to the compartment where they were previously shocked (Vicens-Costa et al., 2011).

On the other hand, the Carioca lines employed a much simpler procedure that involved contextual aversive conditioning, with freezing used as a direct and prominent measure of conditioned fear. Contextual fear conditioning is a useful paradigm for studying long-term memory in animals and has been widely shown to be a reliable behavioral index of associative fear (Fanselow, 1984). Moreover, contextual fear conditioning in rats is a highly heritable trait that can be rapidly

and bidirectionally selected (Gomes & Landeira-Fernandez, 2008). Anatomical and electrophysiological studies have described the neural circuitry involved in both CS and contextual fear conditioning, including the entire extent of sensory inputs to endocrine, autonomic, and behavioral outputs (Delgado et al., 2008; Fanselow, 1994; LeDoux, 2000). Long-term potentiation in the amygdala has also been shown to mediate the formation of fear conditioning (Goosens and Maren, 2004; Sigurdsson et al., 2007). Finally, isomorphism appears to exist between the freezing response to contextual stimuli paired with electrical shocks and generalized anxiety disorder (for a review, see Brandão et al., 2008). Therefore, the Carioca lines might represent an ideal system for studying the molecular and cellular bases of conditioned fear.

Genetics of Pavlovian fear phenotypes

Several studies employing phenotypic differences in conditioned fear are just beginning to identify how divergent conditioned fear memories responses may be related with different sets of genes. Clinical studies with human PTSD patients, for example, consistently indicate that they are more “conditionable”, have greater emotional valence for conditioned stimuli and take longer to extinguish fear once established than non-PTSD subjects (Peri et al., 2000; Orr et al., 2007; Blechert et al., 2005; Norrholm et al., 2011). Corroborating genetic studies of fear learning recurrently mention the elucidation of genes involved in human anxiety disorders as a goal (Moldin, 2000)

The individual variability in traits related with associative fear has been determined in rats and mice through the use of selected lines and inbred strains. The major advantage of employing inbred strains is that the differences in fear related behavior between strains provide direct evidence of variability. This was confirmed by several studies demonstrating that diverse inbred strains of mice differ in their performance in cued and contextual fear conditioning (Gerlai, 1998; Nguyen et al, 2000; Owen et al, 1997b; Paylor et al, 1994; Stiedl et al, 1999; Bolivar et al, 2001). Furthermore, a number of quantitative trait loci (QTL) have been identified as influencing fear conditioning in mice. For example, QTLs were located on chromosomes 1, 10 and 16 for freezing in response to cued stimulus

(Caldarone et al, 1997; Owen et al, 1997; Valentinuzzi et al, 1998; Wehner et al, 1997). Besides, selection studies with mice showed that significant differences in conditioned fear can be achieved using selective breeding, which also establishes their genetic underpinnings (Radcliffe et al, 2000; Ponder et al, 2007a; 2008).

Phenotypical differences in neural networks underlying fear responses are likely to contribute to phenotypical differences in conditioned fear behavior. Evidence indicates that differences in neuronal structure present in the fear circuitry are associated with differences in fear behaviors. An important study reported by Wellman et al (2007) used a 5-HTT transporter (5-HTT) knockout mouse as a model, and demonstrated a phenotype deficit in fear extinction recall. Moreover, associated structural changes in the medial prefrontal cortex (MPFC) and amygdala were also found, when neurons in these pathways showed increased dendrite length and increased spine density, respectively. The same authors (Izquierdo et al, 2006) also produced a reduced fear extinction phenotype using behavioral stress. In this case, a brief and uncontrollable stress led to dendrite retraction in neurons located in the mPFC, with an associated reduction in fear extinction.

However, most of the studies related to genetics of conditioned fear employed mice as subjects. It is our hypothesis that the recently developed CHF and CLF animals could be a suitable model in the search for the underlying genes related with conditioned fear response in rats. The combination of a variety of genetic approaches, such as selective breeding, with a large body of data on the cellular plasticity mechanisms and neural networks of conditioned fear shows great potential in attempting to explain why some individuals are capable to form longer lasting and stronger conditioned fear memories and to what extent this defensive outcome is related to other anxiety-related responses. The last 20 years have seen the precise identification of the input and output circuits of the amygdala, as well as emerging data regarding synaptic plasticity which encodes fear memories. Undoubtedly, the combination of the data on neural and cellular mechanism of conditioned fear and genetics of fear phenotypes is fundamental to our understanding of fear pathologies, in particular the Human Anxiety Disorder (Johnson et al, 2012).

In sum, the present study introduces two new lines of rats bidirectionally selected for their enhanced (CHF) or reduced (CLF) contextual fear conditioning, as measured by freezing behavior. A divergence between these two lines was observed after three generations, indicating a strong heritable component of this trait. A random (RND) line of randomly selected rats was also used as a control group for the CHF and CLF lines.

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.

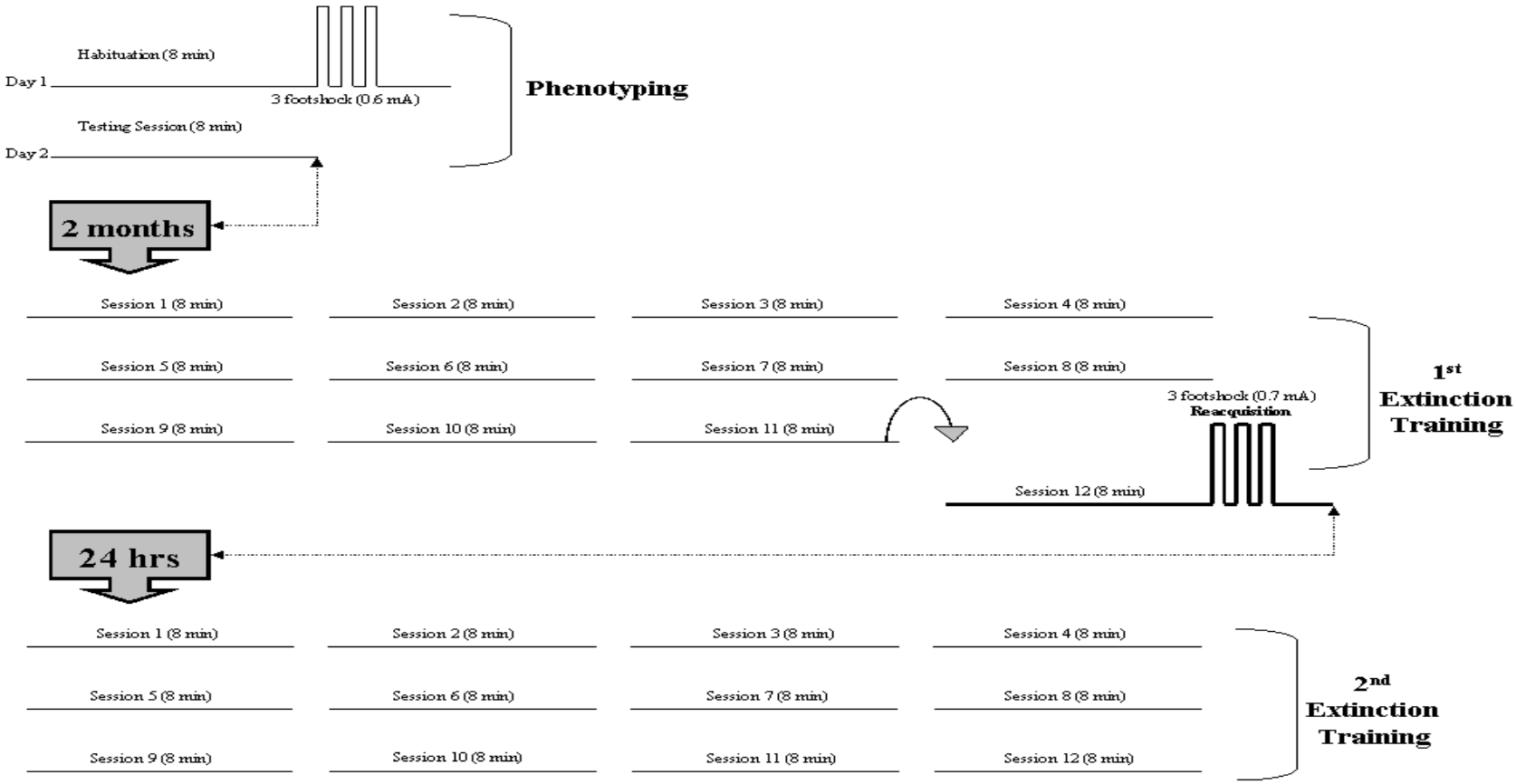

Figure 21: Extinction Procedure. *Note*: 1 session / day.

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).

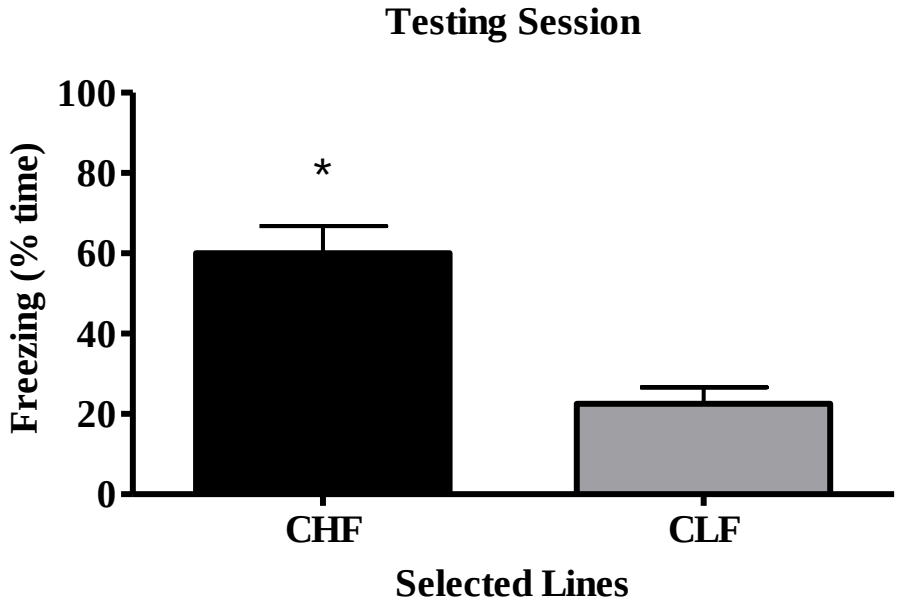

Figure 22: Mean (+SEM) percentage of conditioned freezing in the first aversive conditioning training of the 15 CHF and 15 CLF rats previously selected for this experiment; * denotes significant difference (p<0.05).

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)

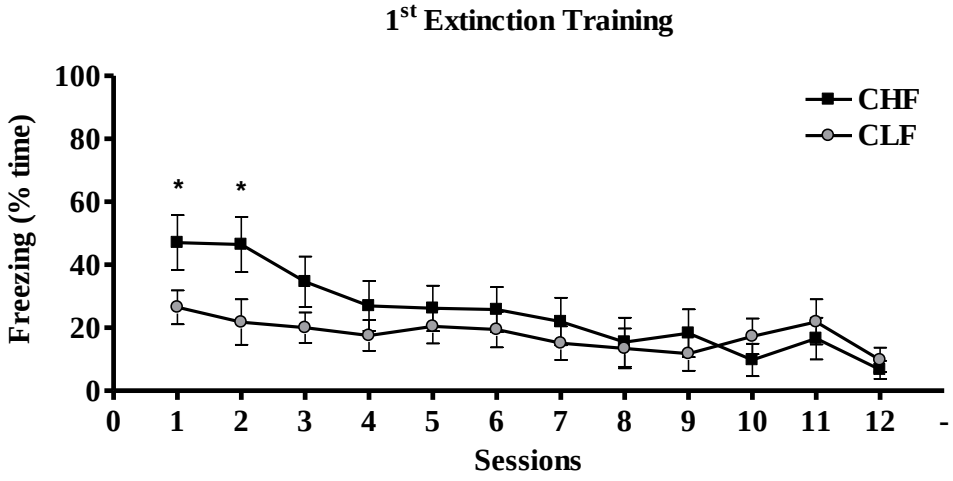

Figure 23: Mean (+ SEM) percentage of conditioned freezing of CHF and CLF rats along 12 extinction sessions in the 1st extinction training two months after initial fear conditioning training; * denotes significant differences between CHF and CLF (p<0.05).

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).

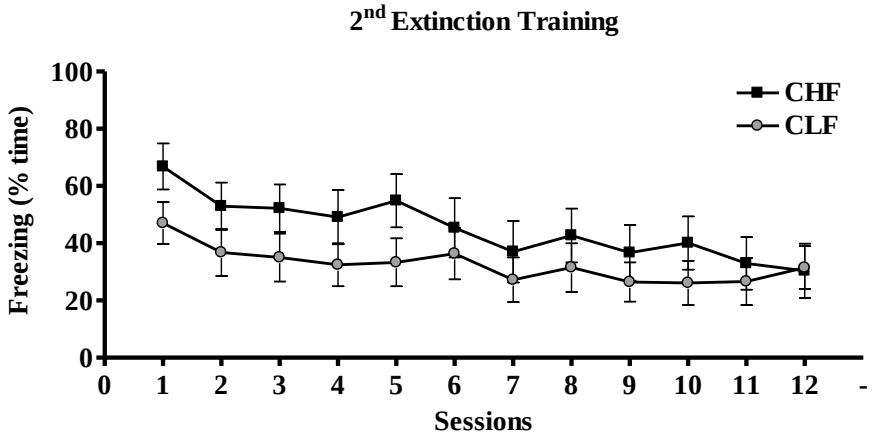

Figure 24: Mean (+ SEM) percentage of conditioned freezing of CHF and CLF rats along 12 extinction sessions in the 2nd extinction training 24hs after reacquisition training ($p<0.05$).

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 $p<0.05$).

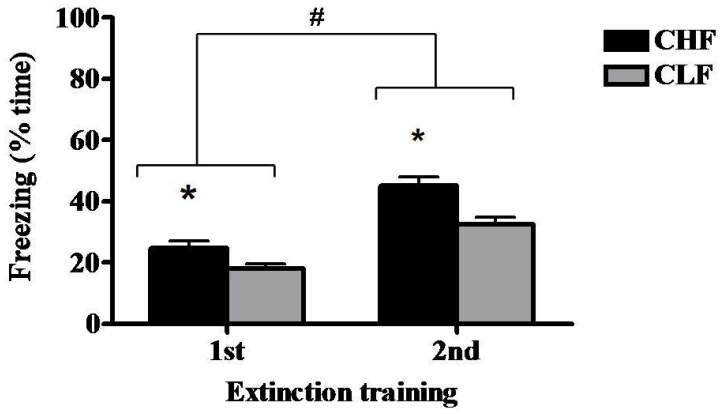

Figure 25: Mean (± SEM) percentage of conditioned freezing in 1st and 2nd extinction training among CHF and CLF rats ($p<0.05$).

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).

5
Study 3

Dissociation between contextual X cued auditory fear conditioning in rats selective bred for high and low conditioned freezing

5.1
Objectives

The objective of the present study was to evaluate the CHF and CLF lines in both contextual and discrete fear-conditioned paradigms.

5.2
Subjects

The experiment was performed using adult female CHF (n=23) and CLF (n=26) rats, from S_{12}/F_2 generation, aged 15-19 weeks and weighing 350-450 g at the time of experiment. Animals were born and maintained in the colony room of the Psychology Department at PUC-Rio with controlled room temperature (24 ± 1°C) and a 12 h/12 h light/dark cycle (07:00-19:00 h). Animals were housed in groups of three to five, according to their respective lines, in polycarbonate cages (18 × 31 × 38 cm) with food and water available *ad libitum*. Fear conditioning occurred during the light phase of the cycle. For 5 days before the fear conditioning experiment, the animals were handled once daily for a period of 2 min.

5.3
Equipments

Both contextual and auditory cued fear conditioning procedures occurred in four observation chambers (25 × 20 × 20 cm), each placed inside a sound-attenuating box. A video camera was mounted in the back of the observation chamber so the animal's behavior could be observed on a monitor outside the

experimental chamber. Freezing behavior, defined as the absence of all non-respiratory movements, was used as a measure of fear. The floor of each chamber consisted of 15 stainless steel rods (4 mm diameter) spaced 1.5 cm apart (center-to-center), which were wired to a shock generator and scrambler (Insight, São Paulo, Brazil). An interface with eight channels (Insight) connected the shock generator to a computer allowed the analyst to apply an electric footshock. A speaker mounted outside a grating in one wall of the chamber was used for the delivery of acoustic CS (conditioned stimulus; pure tone, 90 dB, 2 mHz, 30 s). An ammonium hydroxide solution (5%) and a peppermint scent solution (10%) were used to clean the chamber before and after each subject (odors from these solutions were also used to establish unique olfactory contexts).

Although the same equipments described above were used for all procedures, we create two distinct contexts (A and B), in order to avoid generalizations among experimental days. Table 7 shows the specific adjustments for each experimental set. For the first context (Context A), a 15 w red house-light mounted above the conditioning chamber was turned on. The chambers were cleaned with a 5 % ammonium solution. To provide a distinct odor, stainless steel pans containing a thin layer of this solution were placed underneath the grid floors before the rats were placed inside. Rats were transported from their home cages to this context in white plastic boxes.

For the second context (Context B), 15 w red fluorescent lights were turned on providing illumination, ventilation fans were kept off and the chambers were cleaned with a 10% peppermint scent solution. Also, stainless steel pans containing a thin layer of this same solution were placed underneath the grid floors before the rats were placed inside to provide a distinct odor. In order to create a distinct context configuration, two opposite wooden plaques, in a 65^{o} angle were placed in the chamber. Rats were transported from their home cages to this context in black plastic boxes.

	Context A	Context B
15 w red house-light	On	On
Ventilation Fans	On	Off
Cleaning Solution	5% ammonium	10% peppermint scent
Rats Transportation	White plastic box	Black plastic box
Extra Walls	----------	Two opposite wooden plaques

Table 7: Context configuration adjustment for Contexts A and B.

5.4
Procedures

Day 1

For the acquisition of cued fear conditioning, rats were transferred from the animal facilities in white plastic boxes, placed in Context A and habituated in the observation chamber for 4 min. After this period, fear acquisition was elicited by presenting audible cues (CS) that terminated with electric footshocks (US, 0.6 mA, 1 s, four times). A random stimulus-free period (2-5 min) separated and followed the shocks. After the electric shocks, rats were left in the acquisition chamber for a period of 2 min.

Day 2

For testing of the contextual fear conditioning, 24 h later rats were transferred from the animal facilities in white plastic boxes and placed in Context A for a period of 12 minutes. No footshock or other stimulation occurred during this period.

Day 3

For testing of the cued auditory fear conditioning, animals were transferred from the animal facilities in black plastic boxes and placed in Context B. After a period of 4 minutes of habituation, a CS audible cue (pure tone, 90 dB, 2 kHZ) was presented for 8 minutes. Rats were then returned to their home cages. Figure 18 shows the behavioral paradigm of the experiment.

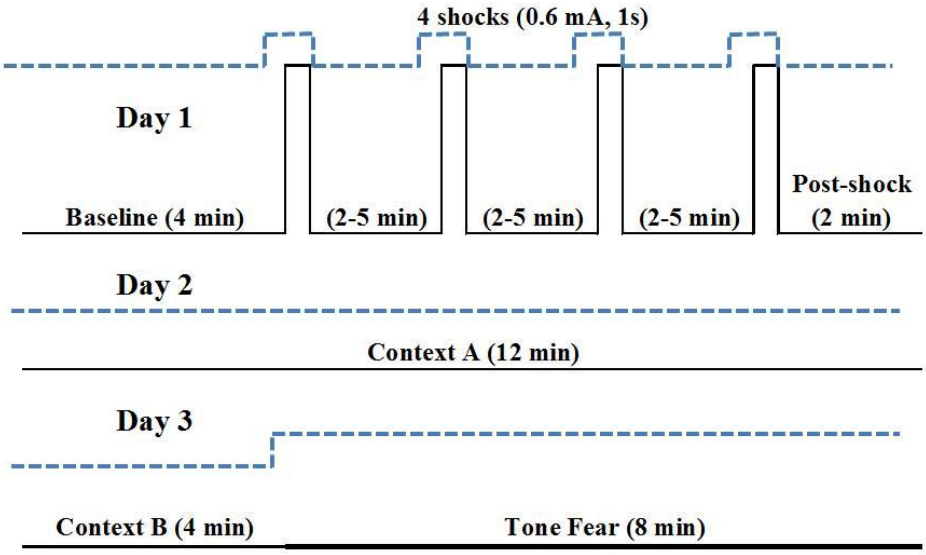

Figure 26: Contextual fear conditioning procedure used for cued auditory fear conditioning; ----- represents auditory conditioned stimulus (CS); —— represents the footshock unconditioned stimulus (US).

5.5
Results and discussion

Figure 27 shows the means (± SEM) percentage of conditioned freezing observed in the acquisition session (Day 1). The Student's t-test showed no significant differences between CHF and CLF animals in the baseline acquisition period (t_{47}=0.89; p=0.38), but analyses indicate significant differences in post-shock freezing (t_{47}=3.63; p<0.001).

Figure 28 shows the means (± SEM) percentage of conditioned freezing registered in Context A on the second day of experiments. Significant differences between CHF and CLF animals were observed in the levels of conditioned freezing behavior (t_{47}=2.57; p<0.05). These results replicate the behavioral pattern observed in CHF and CLF rats in Studies 1 and 2 of the present thesis, and also of previous report for females (Gomes & Landeira-Fernandez, 2008).

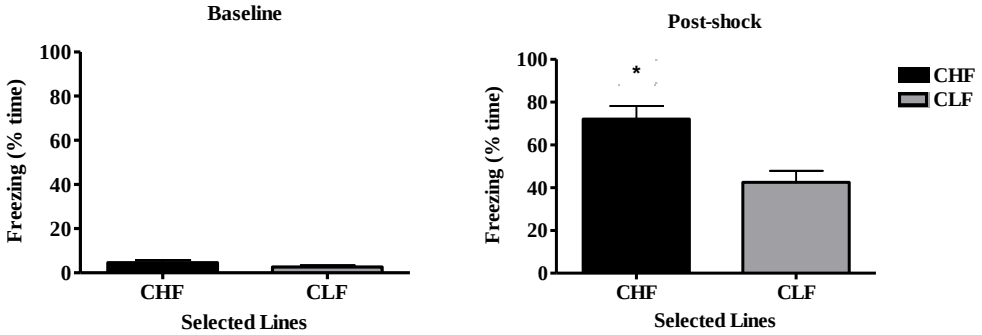

Figure 27: Means ± SEM of conditioned freezing observed on Day 1 in baseline and post-shock acquisition periods of CHF and CLF female rats; * denotes significant differences between CHF and CLF rats (P<0.001).

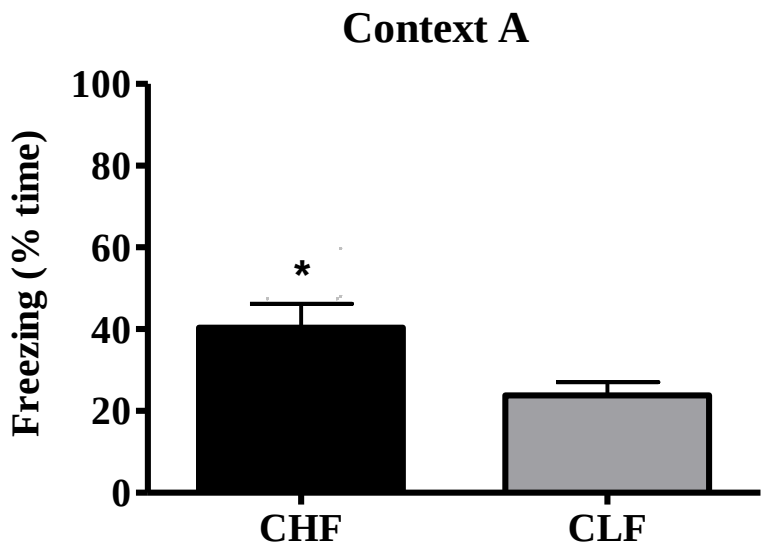

Figure 28: Means ± SEM of conditioned freezing along the 12 minutes of Context A retrieval on Day 2 for CHF and CLF female rats; * denotes significant difference (p<0.001).

Figure 29 indicates the means (± SEM) percentage of conditioned freezing in the altered context as well as freezing associated to auditory CS registered on the third day of experiments. Very low levels of freezing in Context B on the third day of experiments were observed. The Student's t-test showed no significant differences between CHF and CLF animals (t_{47}=1.68; p>0.05). Importantly, these results indicate the absence of generalization to the changed environment of Context B.

The purpose of this study was to evaluate the dissociation between contextual and cued auditory fear conditioning. In this sense, results showed non-significant differences between CHF and CLF rats (t_{47}=0.33; p=0.74) in tone fear.

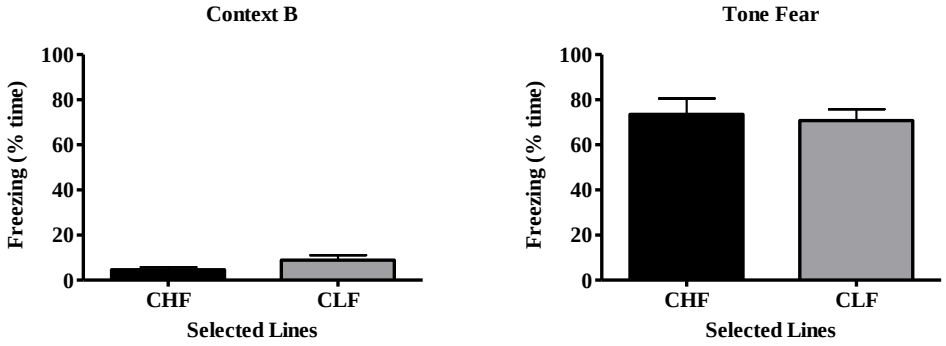

Figure 29: Means ± SEM of conditioned freezing observed on Day 3 in Context B and Tone fear periods of CHF and CLF female rats (P>0.001).

By using a classical cued fear conditioned paradigm, we found that CHF rats, with an innate predisposition to present higher levels of contextual fear conditioning measured as freezing behavior, demonstrated the same levels of freezing in response to a cued auditory stimulus in comparison to CLF rats, for females. Low levels of freezing observed in both lines in the altered context (Context B) prior the occurrence of the tone indicate no generalization. These results suggest that the continuous selective breeding for freezing in response to contextual cues in CHF and CLF rats may not be influencing the neural circuitry underlying freezing behavior in response to discrete/phasic stimuli in these lines of animals. This finding is in agreement with several reports which indicate that fear conditioning in response to a discrete CS and contextual cues is mediated by different neural circuitries (Indovina et al., 2011; Ferreira et al., 2003; Kim &

Fanselow, 1992; LeDoux, 2000; Pohlack et al., 2011), and supports the hypothesis of at least two dimensions of fear conditioning, each related to clinically distinct anxiety disorders. Specific phobias, characterized by cue-specific or phasic fear reactivity, might be modeled by aversive conditioning in response to a discrete CS (Grillon, 2002; Grillon and Davis, 1997). GAD, on the other hand, is characterized by persistent and diffuse or non-cue-specific anxiety and can be modeled by contextual fear conditioning (Brandão et al., 2008; Grillon and Davis, 1997)

Nonetheless, this lack of significant differences in the freezing behavior in response to a discrete CS may be related to strength of the fear conditioning procedure itself. For example, Muigg et al (2008) tested rat lines selectively bred for high (HAB) and low (LAB) anxiety-related behavior in a classical cued fear conditioning task utilizing freezing responses as a measure of fear. In the same manner as our results, they found that cued fear acquisition was similar in both lines. However, they intended to produce similar levels of freezing in both lines in order to study the fear extinction. In this sense, they employed a relatively strong conditioning paradigm, including five CS/US pairings, and a stronger shock intensity (0.7 mA) than we used in the present experiment. Thus, it is possible, through a massive conditioning procedure, to produce similar levels of freezing in two lines selectively bred for high an low anxiety related responses. Moreover, LAB rats show an enhanced (baseline and fear-potentiated) startle response, as compared to HAB rats (Yilmazer-Hanke, Wigger, Faber-Zuschratter, Linke, & Schwegler, 2004).

A divergent result was reported by López-Aumatell et al (2009). In this study, they employed inbred strains (RLA-I and RHA-I) derived from the swiss sublines of the Roman High- (RHA-Verh) and Roman Low- (RLA-Verh) Avoidance, in a fear conditioning procedure. The results indicate that, compared to RHA-I rats, RLA-I animals display higher levels of conditioned fear to contextual and to a visual CS. However, they did not employ an altered context to present the cued visual CS. The fear of a visual CS was measured in the same context where the rats were trained in the previous day. In this sense, it is possible that the behavioral divergence in response to a visual CS may lead to the rise of generalization, and not particularly to the visual CS.

In addition, a possible criticism of these results is related to the intensity of the tone used in the present experiment. Although we used tone intensity (90 dB) in the range of most reported studies, it is not clear whether this intensity is influencing the conditioned freezing response. Indeed, the amount of tone fear observed is much higher than the contextual fear, for both lines. In this sense, further studies employing different tone intensities are necessary to investigate the auditory fear conditioned among CHF and CLF rat lines. Moreover, it is important to employ different CS (visual, olfactory) to dissect possible differences between contextual and discrete fear conditioning in the Carioca lines.

6
Conclusions and prospects for the future

Anxiety disorders are among the most prevalent mental health problems across the individual life span. Early clinical and experimental conceptualizations of anxiety departed from a single or unitary general trait model. More recent theories have favored the view that anxiety is a complex, multidimensional, and dynamic phenomenon. Animal modeling has been crucial in dissecting the pathophysiological mechanisms and designing more effective therapies. Contextual fear conditioning shows a clear isomorphism with GAD, whereas discrete fear conditioning appears to be a valid animal model for specific phobias (Davis et al, 2010).

Bidirectional selection for high and low anxiety-like behavior is a valuable tool for understanding the neural substrates of anxiety disorders. The development of bidirectional lines or strains of animals with high and low levels of emotional reactions associated with a threatening situation began in the middle of the 20th century. Since then, a relatively large number of different genetic models based on this strategy have been developed. These models might represent powerful tools to study the behavioral, neural, and genetic mechanisms that underlie the different types of anxiety disorders. The present work shows the initial results of two new lines of Wistar rats, named CHF and CLF, which were selectively bred for high and low levels of freezing in response to contextual cues previously associated with footshocks. After three generations of breeding, CHF rats were considered to have a greater propensity for exhibiting higher contextual conditioned freezing responses compared with CLF animals. The present phenotype results of the first fourteen generations indicated that CHF and CLF lines differed from each other and from a RND control line. CHF and CLF animals also presented differences in freezing triggered immediately after the occurrence of footshocks. Studies conducted in the present thesis also revealed different patterns of fear extinction and reacquisition, as well as differences in phasic fear between these two groups of animals. However, it has not been established yet that such differences in fear-related responses are due to differences in the formation of learned fearful

associations vs. trivial differences related to other aspects of the paradigm (e.g. shock sensitivity)

An important issue in the process of developing a new genetic animal model of anxiety is to evaluate whether the pair of contrasting rat lines selectively bred for high and low anxiety-related responses also display convergent results in other threatening situations that also require the activation of defensive responses. In the present work we present a behavioral characterization of 8 selected rat lines in 11 animal tests of anxiety. As shown in Table 1, none of the eight models, including the most traditional ones, such as the Maudsley and Roman animals, were evaluated in all 11 paradigms. Therefore, additional experiments are necessary to further evaluate the behavioral profile of each of these pairs of contrasting lines/strains selectively bred for high and low anxiety-related behavior

The first behavioral results from our ongoing selective breeding program were reported by Dias et al. (2009). They performed a battery of behavioral tests that evaluated the emotional and cognitive aspects of the 4th generation of the CHF and RND lines. To evaluate anxiety-related behaviors, the CHF and RND lines were tested in the elevated plus maze and social interaction test. CHF animals were significantly more emotionally reactive than RDN rats in terms of both the number of entries into and time spent in the open arms of the elevated plus maze. The time spent engaged in social interaction behavior was also significantly decreased. Importantly, no differences were found in locomotor activity, measured by the number of entries into the closed arms of the elevated plus maze and number of crossings in the social interaction test arena. Therefore, motor activity did not account for the differences between CHF and RDN animals. Dias et al. (2009) also found an absence of differences between the CHF and RND lines in the forced swim test, suggesting that the anxiety trait selected in the CHF line did not interact with affective disorder traits, such as those for depression. The cognitive aspects of CHF rats were evaluated in the object recognition task. The results from this test indicated no difference between the two groups. These negative results indicate that our breeding procedure, which increased the occurrence of conditioned freezing in response to contextual cues previously associated with footshocks, may be not interfering with other emotional or memory systems. Although these results are extremely encouraging,

additional experiments are necessary to further evaluate the behavioral profile of each of these lines.

In this sense, we emphasize the importance of performing exhaustive behavioral exploratory studies with the recently developed CHF and CLF lines. Various aspects such as locomotion and defecation in the open field test, USV's frequency in pups and adult individuals, time in the light compartment of the ligh-dark box as well locomotor activity in this test, habituation and sensitization of the acoustic startle response, startle amplitude in the fear potentiated startle test, acquisition of two-way and one-way active and passive avoidance responses and suppression ratio of conditioned emotional response, among several other tests, need to be performed in order to delineate a complete behavioral profile of CHF and CLF rats. Moreover, it is also important to evaluate these lines in other animal tests that are not directly related to emotional responses, like spatial memory, and other cognitive functions like attentional shifting (Hatcher et al, 2005), for instance.

Recently, Galvão et al. (2011) exposed CHF and CLF animals from the 9th generation to the dorsal periaqueductal gray matter (dPAG) electrical stimulation paradigm. Empirical research has successfully employed electrical stimulation of the dPAG as a useful animal model of both panic attack (i.e., the acute reaction that might trigger the panic disorder condition) and panic disorder (i.e., the chronic or continuous condition that characterizes the full expression of this anxiety disorder). A stepwise increase in the electrical current intensity used to stimulate the dPAG in rats produces a suppression of spontaneous locomotor activity (i.e., freezing) accompanied by piloerection and exophthalmus at lower intensities. As stimulation continues, active escape behaviors, such as running and jumping, appear at higher intensities (Brandão et at., 1982). After the termination of the dPAG electrical stimulation at the escape threshold, the animal engages in a long-lasting freezing response (Vianna et al., 2001a). Freezing and escape responses triggered by electrical stimulation of the dPAG represent a model of panic attack, whereas dPAG post-stimulation freezing at the aversive escape threshold appears to be a model of panic disorder (for review, see Brandão et al., 2008).

Results indicated that CHF animals had a higher dPAG electrical stimulation aversive threshold for producing freezing and escape reactions than

CLF animals. However, CHF animals displayed more freezing behavior immediately after dPAG electrical stimulation at the escape threshold compared with CLF animals. Thus, although CHF animals were more resistant to the expression of freezing and escape behavior in response to dPAG stimulation, they were more prone to freezing after the occurrence of the dPAG aversive stimulation compared with CLF animals. These results are consistent with the interpretation that, although anticipatory anxiety might exert an inhibitory effect on the expression of panic attack, it might also facilitate the pathogenesis of panic disorder.

Also, differential behavior such as high and low conditioned freezing may be expected to lead to differential protein expression, which can be analyzed by 2D electrophoresis (Ditzen et al., 2006; Kwon et al., 2011a). This procedure provides valuable information to compare the variations occurring within the proteome of organisms, which may, for example, reflect a response to biological perturbations or external stimuli resulting in different expression of proteins or redistribution of specific proteins within cells. A preliminary study employing a simple sample clean-up protocol of whole rat brain among CHF and CLF female rats showed satisfactory 2D gel electrophoresis results, with very well-resolved protein spots. These initial results from our lab (Gomes et al, 2011b) showed that at least 7 protein spots were differentially expressed ($p < 0.05$) between both rat groups, using tools such as 3D spot analysis on the imaging software. The differentially expressed protein spots suggest that behavioral differences might be reflected in the brain protein expression of CHF and CLF lines. However, further studies are of interest in order to identify these proteins and verify if any further differences in these genetically selected rats exist.

Moreover, oxidative stress has been linked with pathological manifestations of many neurological disorders and there is strong evidence in literature that social phobia, depression, and other anxiety-related phenotypes are in part related to oxidative stress such as increased reactive species production (Bouayed et al, 2009; Hovatta et al, 2010). The hippocampus and amygdala seem to be strongly affected by the deleterious effects of oxidative insult. We conducted a preliminary evaluation of the oxidative stress status of various brain structures like cortex, hippocampus and cerebellum, using 2,7- dichlorofluorescin diacetate (DCFH-DA), a sensor of reactive oxygen species (ROS) in males from the S_{12}/F_1

generation of CHF and CLF rats. Results revealed that the free radical concentration was significantly ($p<0.05$) higher in all brain structures from CHF as compared with CLF. This initial finding further showed that hippocampus has the highest ($p<0.05$) free radical concentration as compared with cortex and cerebellum in CHF, indicating that hippocampus may be the prime target of the deleterious effects of ROS. The levels of malondialdehyde (MDA), an earlier marker of lipid peroxidation, were also measured in all three structures and it was found that CHF has significantly ($p<0.05$) higher rate of lipid peroxidation that CLF. Consistent with the DCF assay, the hippocampus showed higher level of MDA as compared with other brain structures. These findings suggest the prominent presence and involvement of redox system which may play a significant role in initiating or exacerbating anxiety and related disorders (Gomes et al, 2011c).

Another way to approach the behavioral divergence between CHF and CLF rats is through the detailed study of neurotransmitter systems related to defensive responses in these animals. For example, the known dual role of the neurotransmitter serotonin (5-HT) in anxiety disorders was tested in CHF and CLF rats. A preliminary study (León et al, 2011) investigated the effect of Ketanserina - a 5-HT_{2A} antagonist - in animals of both lines tested in the EPM. It was found an anxiogenic effect in CHF rats, while an ansiolytic effect in CLF rats, suggesting differential serotonergic expression between selected lines. However, the pattern of expression of other classes of neurotransmitters, like GABA, NMDA, glutamate or dopamine, for example, still needs to be further investigated in these groups of animals.

Also, it is also important to investigate if higher levels of anxiety-related behavior in CHF rats are leading to metabolic changes, or vice-versa. Preliminary evaluation of total cholesterol, triglycerides, fasting glucose, oxygen consumption as well as body composition in CHF rats showed interesting results. In comparison to RND animals, male CHF rats from S_9 generation demonstrated increased serum concentration of corticoesterone, cholesterol and triglycerides, while the serum concentration of testosterone was decreased. Moreover, a significant increase in fat compartments, both epididymal and retroperitoneal in CHF rats was also observed (Mousovich-Neto et al, 2011). However, this is just

the beginning of metabolic studies, which also include the investigation of differential responses of the HPA axis, employing the Carioca lines.

The investigation at the neural systemic level is also of major importance to evaluate possible differences in the neural circuitry underlying conditioned fear responses in both CHF and CLF rats. In fact, the first study employing this recently developed model investigated the effect of bilateral lesions of the amygdaloid complex on contextual fear conditioning in CHF and CLF rats from S_4 generation. In agreement with previous reports (Blanchard and Blanchard, 1972; Cousens and Otto, 1998; Kim et al., 1993; Maren et al., 1996; Oliveira et al., 2004), we found that electrolytic lesions of the amygdala caused a substantial reduction in the amount of conditioned freezing. Interestingly, this deleterious effect was similar in both animal lines ($\pm$ 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. Unfortunately, our first 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 (Gomes & Landeira-Fernandez, 2008). Therefore, further studies are necessary to investigate more completely the contribution of each of these neural structures underlying contextual fear conditioning (including the subjacent micro-circuitry) in these two lines of animals.

Most importantly, before we denote the CHF and CLF rats as a genuine genetic model, there is a strong need to investigate if divergences observed in the conditioned freezing behavior and in other above cited aspects are, indeed, related to selected genetic differences in these groups of animals. Such differences may arise from other effects common to any other group of unselected animals, like random genetic drift for example. As previously discussed in this work, a secure step to avoid false correlations and misinterpretation in experiments employing selected lines is the development of replicate lines under the same selection criterion. Although this is not in our near horizon, this type of study should not be discarded. Another well recognized and simple method to investigate innate

differences in anxiety-related behaviors is to evaluate if maternal effects and early-life experiences are influencing the behavioral divergence observed in adult individuals. Indeed, anxious mothers are supposed to raise anxious offspring (Weaver et al, 2006). In this sense, we are beginning to employ cross fostering procedures aiming at the investigation of maternal effects in the divergent anxious behavior patterns between CHF, CLF and RND rats. Also, an initial inter-groups (CHF X CLF) cross-breeding study performed by Meirelles et al (2011) showed promising results. They found that the resulting F_1 offspring of CHF X CLF crossing demonstrated intermediate levels of freezing responses in a contextual fear paradigm, independently of neonatal influences. A subsequent F_2 inter-cross generation may allow us to study Mendelian aspects of heritability.

Finally, we need to clarify genetics. Identifying genes, through gene expression analysis, that are differentially expressed in neural structures subjacent to fear conditioning like amygdala and hippocampus, as well as the proper identification of the chromosome regions that may underlie the behavioral differences in response to selection using quantitative trait locus (QTL) analysis must be employed to genetically characterize this newly developed model.

Undoubtedly, much work remains. However, it is our hypothesis that, after a set of proper studies covering several aspects related to its biological organization, the CHF and CLF lines may be considered a suitable model in the understanding of the pathophysiology of fear learning, hence expanding our knowledge of the human generalized anxiety disorder.

7

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Annex

GOMES, Vitor de Castro, SILVA, Carlos Eduardo Barroso, LANDEIRA-FERNANDEZ, J. (2011). The Carioca High and Low Conditioned Freezing Lines: A New Animal Model of Generalized Anxiety Disorder, Anxiety Disorders. In: Vladimir Kalinin (Ed.). **Anxiety Disorders.** InTech.

The Carioca High and Low Conditioned Freezing Lines: A New Animal Model of Generalized Anxiety Disorder

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1. Introduction

Fear and anxiety are complex concepts. Both terms have been used to describe a set of highly orchestrated neural events that involve sensory processing and motor responses triggered by threatening situations. These events are mediated by central neural circuitries and peripheral neuroendocrine pathways and clearly have adaptive value. Sensory systems function as alerting signals to warn of real or potential danger, producing a shift to a state of high vigilance that prepares the individual to avoid or escape from a wide variety of dangerous situations. Most of these reactions are not exclusive to our species. Because of their importance for survival, fear and anxiety traits are believed to have been selected in human evolution and shaped by natural selection for their crucial role in protecting individuals who face adverse environments (Coutinho et al., 2010; Gross & Hen, 2004; Marks & Nesse, 1994).

However, these highly adaptive events can be disabling when the individual experiences them excessively or when they occur in the absence of threatening stimuli. In these cases, they represent a pathological condition termed an anxiety disorder. Often chronic in nature, these disorders are among the most prevalent mental health problems across the individual life span, producing severe impairments in social and occupational functioning.

According to an evolutionary perspective, an anxiety disorder reflects a malfunctioning of the neural circuits responsible for detecting, organizing, or expressing adaptive defense reactions (Jacobson & Cryan, 2010). Humans and nonhuman mammals share approximately the same behavioral defense strategies, reflected by activation of similar underlying neural circuitry. Therefore, animal models of anxiety can be extremely helpful for better understanding the behavioral, neural, and genetic substrates involved in these pathologies. The purpose of the present chapter is to present two new lines of rats that might be a

useful model of generalized anxiety disorder (GAD). Before we discuss this model, defining how anxiety disorders are currently classified is important.

2. Clinical aspects of anxiety

The concept of anxiety disorders has changed dramatically over the years as more clinical and experimental evidence has been collected. In the clinical setting, anxiety disorders departed from a single construct that ranged in intensity from normal to pathological or neurotic levels. A major shift in this view occurred with Klein's pioneering work (Klein, 1964; Klein & Fink, 1962), which showed that imipramine had a selective effect in the treatment of panic disorder. Moreover, certain anxiety disorders have been suggested to differ from each other in the primary object or specificity of threat. Fear of a circumscribed and well-defined object is a characteristic of specific phobias, whereas diffuse and chronic sustained anxiety is the main feature of GAD.

The 3rd edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-III; American Psychiatric Association, 1980) introduced the current descriptive symptom-based approach to mental disorders with well-defined, explicit diagnostic criteria. This new classification incorporated distinct nosological entities, such as panic disorder, specific and social phobias, GAD, posttraumatic stress disorder, and obsessive-compulsive disorder. In the DSM-III, GAD was left as a residual diagnosis of worry, to be made only in the absence of other anxiety and depressive syndromes. Consequently, this residual category carried low diagnostic reliability. With the publication of the DSM-IV (American Psychiatric Association, 1994) and International Classification of Diseases and Related Health Problems (ICD-10; World Health Organization, 1992), these anxiety disorder categories remained basically the same. However, the diagnosis of GAD shifted from a residual category in the DSM-III to an independent anxiety disorder type in the DSM-IV. Free-floating anxiety was associated with the worry construct, which in turn produced several symptoms, such as muscle tension, fatigue, restlessness, concentration difficulties, and irritability. According to the DSM-IV, excessive and unrelenting worry is generally associated with impairments in academic, social, and personal functioning and related to multiple domains or activities. To be considered a pathological feature of GAD, worry must occur more days than not for a period of at least 6 months.

3. Animal models of anxiety

In the experimental setting, most of the studies that investigate the etiological mechanisms that underlie anxiety disorders have been performed using animal models. Defensive reactions of the laboratory rat (Rattus norvegicus) have been employed as the main system for modeling human anxiety. Defecation in the open field was probably one of the first animal models of anxiety (Hall, 1934). Since then, several other animal models of anxiety have been developed. As in the clinical setting, the traditional view that highlighted these experimental studies was that animal defensive responses were mediated by a single and general anxiety construct (Broadhurst, 1975; Gray, 1979; Hall, 1934). Nevertheless, as new data were collected, it became clear that animal defensive behavior is

mediated by a complex and multidimensional construct (Aguilar et al., 2002; Belzung & Le Pape, 1994; Ramos et al., 1997; Torrejais et al., 2008). In these studies, statistical techniques, such as factor analysis, were employed to investigate whether different animal models of anxiety measure the same underlying latent factor. The results clearly indicated that different animal models assessed distinct forms of anxiety. For example, File (1992) showed that indices of anxiety derived from the elevated plus maze (i.e., the number of entries into and time spent on the open arms of the maze), Vogel conflict test (i.e., frequency of punished drinking), and social interaction test (i.e., time spent engaged in social interaction), loaded on three independent factors, suggesting the existence of different forms of anxiety generated by each of these paradigms.

Pharmacological studies that employed diverse animal models also confirmed the multidimensional aspect of anxiety. For example, benzodiazepine compounds produced an anxiolytic effect in animal models that generate behavioral inhibition caused by the conflict between approach and avoidance tendencies (Maki et al., 2000). These animal models also indicated that substances that decrease serotonergic neurotransmission increase anxiety, whereas compounds that increase serotonergic neurotransmission decrease anxiety (Graeff, 1997). In contrast, other animal models that require vigorous escape responses to proximal aversive stimuli appear to be resistant to benzodiazepine drugs, whereas substances that increase serotonergic activity produce an anxiolytic effect (Graeff and Zangrossi, 2010). Different neural circuitries appear to be involved in distinct dimensions of anxiety. Gray and McNaughton (2000) argued that the septo-hippocampal system contributes to the cognitive component (worry), and the amygdaloid complex and its projections to the ventral portion of the periaqueductal gray (PAG) are critically involved in the regulation of inhibitory behavior in response to innate or conditioned aversive stimuli (Fanselow, 1994). Active defensive behaviors in response to proximal stimuli, generally associated with nociception, appear to involve the dorsal portion of the PAG (dPAG) and its ascending projections to forebrain structures related to the sensorial processing of aversive stimuli (Oliveira et al., 2004).

These diverse dimensions found in animal models of anxiety may indicate that clinically defined anxiety disorders could be associated with a particular animal model. However, the adoption of descriptive and operational criteria from the modern classification systems imposed a validity problem among the several anxiety disorder categories. The DSM-IV and ICD-10 are not primarily based upon etiology, neurobiology, epidemiology, genetics, or responses to medications, but rather on phenomenological descriptions of clinical data that have imprecise similarity or correlate with each other within and between individuals (Gould & Gottesman, 2006). Therefore, unsurprising are the several problems that are encountered when attempting to use the current systems of mental disorder classification as a guide for developing viable animal models.

4. Contextual fear conditioning as a model of generalized anxiety disorder

Regardless of the difficulty developing animal models for current clinically defined anxiety disorders, fear conditioning has been historically associated with one of the main causes of pathological anxiety (i.e., neurosis; Pavlov, 1927;

Watson & Rayner, 1920). In a typical fear conditioning experiment, a discrete and emotionally neutral stimulus, such as a light or tone, reliably signals the occurrence of an aversive stimulus, such as an electric footshock. After a few pairings between these two stimuli, the previously harmless stimulus becomes a potent conditioned stimulus (CS) and acquires the ability to elicit several fear reactions. Another form of fear conditioning is to make the aversive stimulus unpredictable. According to this alternative procedure, a rat is exposed to a novel chamber and, after a few minutes, a brief and unsignaled footshock is delivered. When returned to the same chamber in the absence of the aversive stimulus, the animal presents a permanent fear reaction to contextual cues previously associated with the footshock. Considerable evidence from animal and human experiments indicate that fear conditioning in response to a discrete CS and contextual cues is mediated by different neural circuitries (Indovina et al., 2011; Ferreira et al., 2003; Kim & Fanselow, 1992; LeDoux, 2000; Pohlack et al., 2011). These results support the hypothesis of at least two dimensions of fear conditioning, and each dimension might be related to clinically distinct anxiety disorders. Specific phobias, characterized by cue-specific or phasic fear reactivity, might be modeled by aversive conditioning in response to a discrete CS (Grillon, 2002; Grillon and Davis, 1997). GAD, in contrast, is characterized by persistent and diffuse or non-cue-specific anxiety and might be modeled by contextual fear conditioning (Brandão et al., 2008; Grillon and Davis, 1997). Contextual fear conditioning represents one of the simplest and most rapid forms of producing aversive learning (Landeira-Fernandez, 1996). Defensive freezing behavior has been argued to be the most reliable measure of contextual fear conditioning (Fanselow, 1984a). This defensive response is a direct function of shock intensity (Sigmundi et al., 1980) and depends on the association between the cues of the experimental chamber and footshock (Landeira-Fernandez et al., 2006).

Conditioned freezing in response to contextual cues previously associated with footshock has been pharmacologically validated as an adequate model of anxiety disorder. Accordingly, classic anxiolytic benzodiazepines, such as midazolam and diazepam (Fanselow and Helmstetter, 1988), and non-benzodiazepine anxiolytics, such as the serotonin-1A (5-hydroxytryptamine-1A [5-HT1A]) receptor agonist ipsapirone (Inoue, Tsuchiya, Koyama, 1996) and 5-HT reuptake inhibitors citalopram and fluvoxamine (Hashimoto et al., 1996), reduced the amount of conditioned freezing. Furthermore, anxiogenic substances, such the benzodiazepine inverse agonist dimethoxy-β-carboline, produced freezing behavior similar to that elicited by fear conditioning (Fanselow et al., 1991).

5. The Carioca High and Low conditioned Freezing rats

Bidirectional selective breeding of a defensive response or any other phenotypic characteristic is a technique in which animals are bred to modify the frequency of the genes that underlie 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 various behavioral traits, including defensive

reactions associated with emotionality. In particular, genetic animal models of anxiety disorders might be a useful tool for understanding why some individuals present adequate emotional reactions and others endure an exaggerated pattern of anxiety responses in the absence of a fear-provoking context.

The view that anxiety does not reflect a single or unitary process emphasizes the importance of developing different genetic models with distinct phenotype criteria. In fact, the development of bidirectional lines of animals with high and low levels of emotionality began in the middle of the 20th century. Since then, a relatively large number of different lines have been described in the literature (for review, see Ramos and Mormède, 2006). Innate and learned animal models have been employed for mating selection in rats. Among the innate models are defecation (Maudsley animals; Broadhurst, 1957, 1958) and ambulation in the center of an open field apparatus (Floripa animals; Ramos et al., 2003), ambulation on a runway (Tsukuba animals; Fujita, 1984), open arm parameters in the elevated plus maze (HAB and LAB animals; Liebsch et al., 1998a, b), and infant isolation- induced ultrasonic vocalizations (USV animals; Brunelli & Hofer, 1996). Surprisingly, the two-way-avoidance response has been the main conditioned phenotype criterion used for developing bidirectionally selected rat lines based on learned aversive paradigms. That is the case for Roman (Bignami, 1965), Syracuse (Brush et al., 1979), Australian (Bammer, 1983), Koltushi (Ryzhova et al., 1983), and Hatano (Ohta et al., 1995) animals. Our group in the Psychology Department at Pontifícia Universidade Católica do Rio de Janeiro (PUC-Rio) was also interested in developing a rat genetic model of extreme phenotypes of learned fear. Instead of the two-way avoidance paradigm, conditioned freezing in response to contextual cues previously associated with footshock was employed as the phenotype criterion for developing the two lines. The breeding program began in 2006. The basic protocol consisted of mating male and female albino Wistar rats with the highest and lowest conditioned freezing in response to the contextual cues of the experimental chamber where animals were exposed to three unsignaled electric footshocks on the previous day. Gomes and Landeira-Fernandez (2008) found that after three generations, reliable differences between these two lines were already present, indicating a strong heritable component of this type of learning. The lines were named Carioca1[1] High conditioned Freezing (CHF) and Carioca Low conditioned Freezing (CLF). These two lines represent the most recent rat genetic model in the field of anxiety.

6. Phenotype results of the 12th generation

To illustrate the development of our breeding lines, we present the phenotype results of the 12th generation of the CHF and CLF lines recently collected in our laboratory. A random (RND) line of randomly selected rats was also used as a control group for the CHF and CLF lines. Phenotyping was performed on a total of 122 animals from the CHF line (67 males and 55 females), 124 animals from the RND line (54 males and 70 females), and 99 animals from the CLF line (49 males and 50 females).

[1] Carioca is the name given to those born in Rio de Janeiro.

Animals were born and maintained in the colony room of the PUC-Rio Psychology Department with controlled room temperature (24 ± 1°C) and a 12 h/12 h light/dark cycle (07:00-19:00 h). To assign a control number for each animal, amputation of one toe from each foot and a small incision in one of the ears was performed 6 to 8 days after birth. Upon weaning at 21 days of age, each animal was separated by sex and housed in groups of five to seven, according to their respective lines, in polycarbonate cages (18 × 31 × 38 cm) with food and water available ad libitum. Phenotyping occurred during the light phase of the cycle. The animals were between 75 and 80 days of age at the beginning of the experiment. For 5 days before the contextual fear conditioning experiment, the animals were handled once daily for a period of 2 min.

Contextual fear conditioning occurred in four observation chambers (25 × 20 × 20 cm), each placed inside a sound-attenuating box. A red light bulb (25 W) was placed inside the box, and a video camera was mounted in the back of the observation chamber so the animal's behavior could be observed on a monitor outside the experimental chamber. A ventilation fan attached to the box supplied background noise of 78 dB (A scale). The floor of the observation chamber consisted of 15 stainless steel rods (4 mm diameter) spaced 1.5 cm apart (center-to-center), which were wired to a shock generator and scrambler (Insight, São Paulo, Brazil). An interface with eight channels (Insight) 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. The contextual fear conditioning protocol involved one acquisition session and one test session. During acquisition, each animal was placed in the observation chamber for 8 min. At the end of this period, three unsignaled 0.6 mA, 1 s electric footshocks were delivered with an intershock interval of 20 s. Three minutes after the last footshock (post-shock interval), the animal was returned to its home cage. The test session occurred approximately 24 h after training. This test consisted of placing the animal for 8 min in the same chamber in which the three footshocks were delivered on the previous day. No footshock or other stimulation occurred during this period. A time-sampling procedure was used 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, defined as the total absence of movement of the body or vibrissa, with the exception of movements required for respiration. Previous results from our laboratory indicated that male rats consistently exhibited more conditioned freezing in response to contextual cues than female animals (Gomes and Landeira-Fernandez, 2008). Therefore, male and female results are presented separately. Fig. 1 presents the mean ± standard error of the mean (SEM) percentage of time spent freezing among male and female rats of the CHF, RND, and CLF lines during the post-shock period. The results were analyzed using a two-way analysis of variance (ANOVA). The first factor, with two levels, was related to the animal's sex (male and female). The second factor, with three levels, was related to the breeding line (CHF, RDN, and CLF).

This analysis revealed an absence of a two-way interaction ($F_{2,339} = 0.44$, $p > 0.6$). A main effect of sex was found ($F_{1,339} = 14.02$, $p < 0.001$). As shown in Fig. 1, male rats expressed more freezing behavior than female rats across all three levels of the breeding line factors. A main effect of breeding line was also detected ($F_{2,339} = 20.27$, $p < 0.001$). Pairwise post hoc comparisons performed

with Fisher's Least Significant Difference test indicated that CLF animals expressed lower freezing behavior compared with CHF and RND animals (all $p < 0.001$). Finally, CHF and RND animals did not differ significantly from each other ($p > 0.4$).

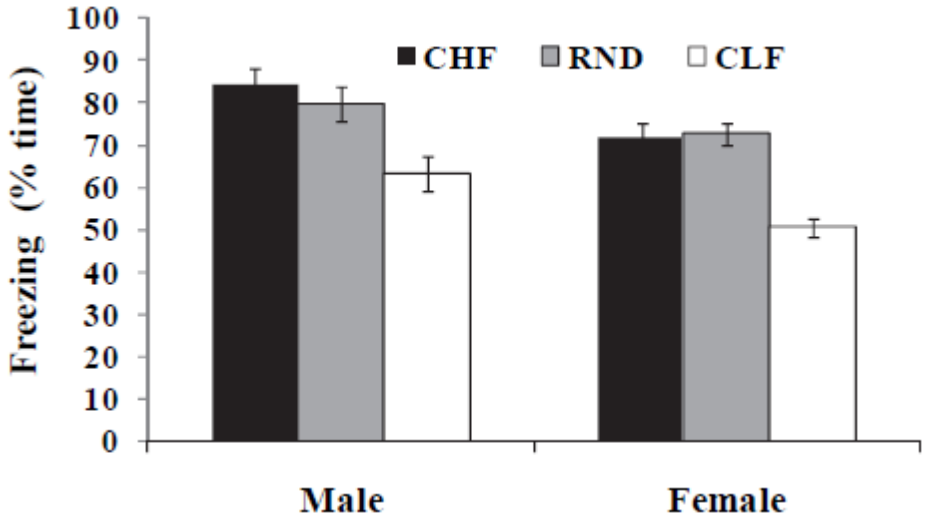

Fig. 1. Mean ± SEM percentage of time spent freezing among male and female rats of the high (CHF), random (RND), and low (CLF) lines during the post-shock period of the training session.

The differences observed in the amount of post-shock freezing behavior between CLF and CHL animals were not observed in our original report that employed the 3rd generation of these two lines (Gomes and Landeira-Fernandez, 2008). Three possibilities may explain these discrepant results. One is that the footshock intensity used to phenotype animals of the present generation (0.6 mA) was much lower than the intensity used during the first three generations (1.0 mA) reported by Gomes and Landeira-Fenandez (2008). Therefore, the higher footshock intensity could lead to a ceiling effect so that differences in post-shock freezing behavior may not be observed. Indeed, the footshock intensity was reduced in our breeding program in the 7th generation to 0.7 mA and in the 8th generation to the present intensity to prevent possible ceiling effects produced by this relatively strong (1.0 mA) footshock intensity.

A second possibility could be related to the fact that freezing observed immediately after footshock reflects associative learning between contextual cues and the aversive footshock (Fanselow, 1980, 1990; Vianna et al., 2001b). For example, when the footshock is presented simultaneously with the rat's placement in the chamber, no contextual fear conditioning is observed (Landeira-Fernandez et al., 1995). Moreover, placing the animal in a different context from the one in which the footshock was delivered did not produce any freezing behavior (Fanselow, 1980). Therefore, differences between CHF and CLF animals in post-shock freezing could be a consequence of the fact that CHF rats have a greater propensity for exhibiting higher conditioned freezing responses compared with CLF animals because of the continuous bidirectional selection over different generations.

A third possible explanation for these incongruent results might be related to differences in pain sensitivity between these two lines. This is an important issue because freezing observed immediately after footshock is closely related to pain sensitivity and shock intensity (Fanselow, 1984b). According to this possibility, selection for high and low conditioned freezing might independently lead to co-selection of other contributing factors that are not genetically linked but contribute to the phenotype that is being selected, such as differences in pain sensitivity to footshock. Further studies are necessary to test this possibility.

Fig. 2 presents the mean and SEM percentage of time spent freezing among male and female rats of the high (CHF), random (RND), and low (CLF) lines during the 8 min test session. Conditioned freezing in response to contextual cues previously associated with footshock was also analyzed using a two-way ANOVA. This analysis indicated an absence of a two- way interaction ($F_{2,339}$ = 0.07, $p > 0.9$). A main effect of sex was found ($F_{1,339}$ = 41.85, $p < 0.001$). As shown in Fig. 2, male rats froze more than female rats across all three levels of the breeding line factors. A main effect of breeding line was also detected ($F_{2,339}$ = 18.13, $p < 0.001$). Fig. 2 also shows that the CHF line expressed the highest amount of conditioned freezing, and the CLF line expressed the lowest amount of freezing. The RND line presented intermediate levels of freezing. These results were confirmed by pairwise post hoc comparisons. CHL animals froze more than RND and CLF animals, and CLF rats froze less than CHF and RDN animals (all $p < 0.01$).

Electric footshock induced a reliable difference between CHF and CLF animals, and we evaluated whether the breeding line effect on conditioned freezing during the test session was attributable to post-shock differences that these animals presented during the training session. An analysis of covariance, with post-shock as a covariant factor, was performed. The results from this analysis confirmed an absence of an interaction ($F_{2,338}$ = 0.19, $p > 0.8$) and main effects of sex ($F_{1,338}$ = 31.14, $p < 0.001$) and breeding line ($F_{2,338}$ = 10.23, $p < 0.001$).

These results confirmed previous findings from our original report (Gomes and Landeira- Fernandez, 2008) and extend these results to a control group of animals that were randomly mated.

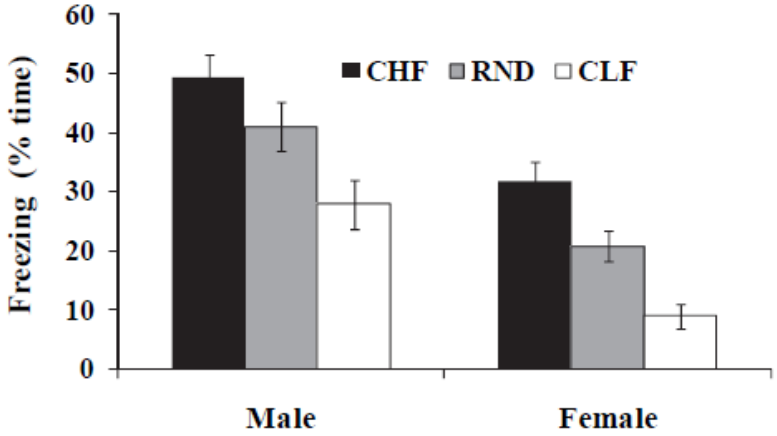

Fig. 2. Mean ± SEM percentage of time spent freezing among male and female rats of the high (CHF), random (RND), and low (CLF) lines during the test session 24 h after training.

7. Behavioral validation of the Carioca lines

An important issue in the processes of developing a new genetic animal model of anxiety is to evaluate whether the pair of contrasting lines of rats selectively bred for high and low anxiety-related responses also display convergent results in other threatening situations that also require the activation of defensive responses. The first behavioral results from this ongoing selective breeding program were reported by Dias et al. (2009). They performed a battery of behavioral tests that evaluated the emotional and cognitive aspects of the 4th generation of the CHF and RND lines. To evaluate anxiety-related behaviors, the CHF and RND lines were tested in the elevated plus maze and social interaction test. CHF animals were significantly more emotionally reactive than RDN rats in terms of both the number of entries into and time spent on the open arms of the elevated plus maze. The time spent engaged in social interaction behavior was also significantly decreased. Importantly, no differences were found in locomotor activity, measured by the number of entries into the closed arms of the elevated plus maze and number of crossings in the social interaction test arena. Therefore, motor activity did not account for the differences between CHF and RDN animals.

Dias et al. (2009) also found an absence of differences between the CHF and RND lines in the forced swim test, suggesting that the anxiety trait selected in the CHF line did not interact with affective disorder traits, such as those for depression. The cognitive aspects of CHF rats were evaluated in the object recognition task. The results from this test indicated no difference between the two groups. These negative results indicated that our breeding procedure, which increased the occurrence of conditioned freezing in response to contextual cues previously associated with footshock, did not interfere with other emotional or memory systems. Although these results are extremely encouraging, additional experiments are necessary to further evaluate the behavioral profile of each of these lines.

8. Panic-related behaviours in the Carioca lines

Panic disorder is a complex anxiety disorder that involves both recurrent, unexpected panic attacks and persistent concern about having additional attacks (American Psychiatric Association, 1994). Although the occurrence of a panic attack is a hallmark of panic disorder, the chronic conditioning of this anxiety disorder is defined by the constant and persistent fear of experiencing further attacks or worry about the possible consequences of a panic attack.

The clinical concept of panic attack and panic disorder is well described in the literature (Freire et al., 2010). However, the relationship between an anticipatory anxiety trait present in GAD with panic attack and the development of panic disorder remains a subject of intense debate (Battaglia and Ogliari, 2005; Bouton et al., 2001; Stein et al., 2010). The distinction between panic disorder and GAD stemmed from Klein´s original observations (Klein, 1964; Klein and Fink, 1962), in which chronic administration of the antidepressant imipramine improved panic disorder, which was resistant to benzodiazepine anxiolytics at doses that improved GAD. This pharmacological distinction between these two anxiety disorder categories has been further qualified. Chronic imipramine also improves

GAD (Kahn et al. 1986), and high-potency benzodiazepines, such as alprazolam, are effective in panic disorder when chronically administered (Schweizer et al. 1993).

Empirical research has successfully employed electrical stimulation of the dPAG as a useful animal model of both panic attack (i.e., the acute reaction that might trigger the panic disorder condition) and panic disorder (i.e., the chronic or continuous condition that characterizes the full expression of this anxiety disorder). A stepwise increase in the electrical current intensity used to stimulate the dPAG in rats produces a suppression of spontaneous locomotor activity (i.e., freezing) accompanied by piloerection and exophthalmus at lower intensities. As stimulation continues, active escape behaviors, such as running and jumping, appear at higher intensities (Brandão et at., 1982). After the termination of the dPAG electrical stimulation at the escape threshold, the animal engages in a long-lasting freezing response (Vianna et al., 2001a). Freezing and escape responses triggered by electrical stimulation of the dPAG represent a model of panic attack, whereas dPAG post-stimulation freezing at the aversive escape threshold appears to be a model of panic disorder (for review, see Brandão et al., 2008).

Recently, Galvão et al. (*in press*) exposed CHF and CLF animals from the 9th generation to the dPAG electrical stimulation paradigm. The results indicated that CHF animals had a higher dPAG electrical stimulation aversive threshold for producing freezing and escape reactions than CLF animals. However, CHF animals displayed more freezing behavior immediately after dPAG electrical stimulation at the escape threshold compared with CLF animals. Thus, although CHF animals were more resistant to the expression of freezing and escape behavior in response to dPAG stimulation, they were more prone to freezing after the occurrence of the dPAG aversive stimulation compared with CLF animals. These results are consistent with the interpretation that although anticipatory anxiety might exert an inhibitory effect on the expression of panic attack, it might also facilitate the pathogenesis of panic disorder.

9. Conclusions

Anxiety disorders are among the most prevalent mental health problems across the individual life span. Early clinical and experimental conceptualizations of anxiety departed from a single or unitary general trait model. More recent theories have favored the view that anxiety is a complex, multidimensional, and dynamic phenomenon. Animal modeling has been crucial in dissecting the pathophysiological mechanisms and designing more effective therapies. Contextual fear conditioning has clear isomorphism with GAD, whereas electrical stimulation of the dPAG appears to be a valid animal model of panic attack and panic disorders.

Bidirectional selection for high and low anxiety-like behavior is a valuable tool for understanding the neural substrates of anxiety disorders. Our laboratory recently developed two new lines of Wistar rats, CHF and CLF, that were selectively bred for high and low levels of freezing in response to contextual cues previously associated with footshock. After three generations of breeding, CHF rats were considered to have a greater propensity for exhibiting higher conditioned freezing responses compared with CLF animals. The present

phenotype results of our 12th generation indicated that CHF and CLF lines differed from each other and from a RND control line. CHF and CLF animals also presented a difference in freezing triggered immediately after the occurrence of footshock.

The results from the 4th generation also indicated that CHF animals were more "anxious" than RND rats in the elevated plus maze and social interaction test. Motor activity did not account for the differences between the CHF and RND lines. The absence of reliable differences between CHF and RND animals in the forced swim test and object recognition task indicated that the breeding procedure, which increased the occurrence of conditioned freezing in response to contextual cues, did not interfere with other emotional or memory systems. Finally, exposure of CHL and CLF animals to electrical stimulation of the dPAG suggested that the component of anticipatory anxiety present in GAD might exert an inhibitory effect on the expression of panic attack, whereas it might also facilitate the pathogenesis of panic disorder.

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