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An animal model of trait anxiety: Carioca high freezing rats as a model of generalized anxiety disorder

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Abstract

Despite being one of the main components of anxiety and playing a pivotal role in how an individual perceives and copes with anxiogenic situations or responds to a given treatment, trait anxiety is paradoxically omitted in most animal models of anxiety. This is problematic and particularly more concerning in models that are used to screen drugs and other treatments for specific anxiety disorders and to investigate their neurobiological mechanisms. Our group has been engaged in the search for specific anxiety-related traits in animal models of anxiety. We developed two new lines of rats with strong phenotypic divergence for high (Carioca High-conditioned Freezing [CHF]) and low (Carioca Low-conditioned Freezing [CLF]) trait anxiety as expressed in the contextual fear conditioning paradigm. Here, we summarize key behavioral, pharmacological, physiological, and neurobiological differences in one these lines, the CHF rat line, relative to randomized-cross controls and discuss how far they represent a valid and reliable animal model of generalized anxiety disorder and so high trait anxiety.

Anxiety is characterized by uncomfortable feelings of apprehension, insecurity, and uncertainty, combined with very specific physiological, neural, and behavioral reactions that are typically triggered by the perception of potentially threatening situations in the environment. From an evolutionary perspective, human anxiety likely has its origins in similar defensive reactions that are shared with many other animals, particularly mammals (Blanchard et al., 2001; Graeff, 2010; McNaughton & Corr, 2022), which through evolution have become increasingly complex and sophisticated in their capacity to anticipate and successfully cope with various sources of threat to our physical and emotional well-being.

In some individuals and for many reasons that have been widely investigated, anxiety eventually loses its adaptive function and can become a disorder, although there is no precise cutoff point that delineates adaptive ("normal") and non-adaptive ("pathological") anxiety. In clinical terms, anxiety is considered a disorder when it becomes excessive, persistent, and uncontrollable or in cases where it occurs even when there is little or nothing in the environment to indicate a potential threat. Under these circumstances, anxiety takes on a pathological dimension and often requires specific treatment for its clinical management (Baxter et al., 2013; Öhman & Mineka, 2001).

Pathological anxiety is not limited to a single homogeneous clinical condition; instead, it extends to several qualitatively distinct anxiety disorder categories that depend on their causes and symptoms. According to the *Diagnostic and Statistical Manual of Mental Disorders, fifth edition, Text Revision (DSM-5-TR*; American Psychiatric Association, 2022), the major anxiety disorders include separation anxiety disorder, selective mutism, specific phobia, social anxiety, panic disorder, agoraphobia, and generalized anxiety disorder (GAD), in addition to other anxiety disorders, such as substance/medication-induced anxiety disorder, anxiety disorder due to another medical condition, and other specified and unspecified anxiety disorders.

Among anxiety disorders, GAD is believed to be one of the most prevalent (Ruscio et al., 2017). Such disorder encompasses various signs and symptoms, the most evident of which is a diffuse, persistent, and exacerbated feeling of worry that is not restricted to a particular stimulus but rather involves various circumstances (Crocq, 2017). Worry is impossible to assess in animals, but muscle tension, irritability, fatigue, difficulty concentrating, sleep disturbances, restlessness, and combinations of these symptoms often coexist with this feeling of worry. According to the *DSM*-5, the feeling of worry must be accompanied by at least three of these symptoms on most days for at least 6 months to satisfy the criteria for GAD.

Anxiety has two forms: state anxiety and trait anxiety (Spielberger, 1972). State anxiety is a transient state of anxiety that occurs at a given moment in a specific context. It is an emotional state of anxiety that is directly related to the perception of a potential threat, such that its intensity tends to increase in the presence of the threat and ceases when it is no longer present.

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Trait anxiety refers to an individual's vulnerability to express anxiety over time in different situations, a relatively stable component of personality that involves an intricate interplay between genetic and environmental factors for its expression (Gottschalk & Domschke, 2017). Importantly, individuals vary considerably in their anxious personality traits and coping styles (Knowles & Olatunji, 2020; Myles et al., 2020). Moreover, extreme variations in certain anxiety traits have been linked to specific anxiety disorders (Knowles & Olatunji, 2020), this link has received great clinical and research interest.

1. Animal models of trait anxiety

Anxiety-like behavior is not exclusive to humans. Many nonhuman animals also have biological mechanisms that enable them to anticipate and successfully cope with various threat-related stimuli in the environment. Except for phenomenological and highly subjective aspects that are inherent to reports of human anxiety, almost all other anxiety-related components show great similarity across mammalian species and have long been used to study anxiety in laboratory settings (for review, see Graeff, 2010; Steimer, 2011). Since the seminal work of Hall (1934) that showed that rats with high and low levels of emotionality exhibit different patterns of exploration in an open field (i.e., more emotionality, less exploration), no other emotion has been more studied in animal models than anxiety and its related disorders.

Dozens of animal models of elicited anxiety have been validated, and many have been recognized as valuable or indispensable tools for studying defensive behaviors and searching for more effective and targeted treatments for specific anxiety disorders (Steimer, 2011). However, despite its obvious clinical important, trait anxiety is paradoxically omitted or rarely addressed in most of these studies. This is particularly concerning when attempting to model or simulate anxiety in anxiolytic screening studies and when investigating neurobiological mechanisms that underlie defensive behaviors and their possible associations with anxiety *disorders*. One reason omission of a trait perspective is problematic is that behavioral tests of anxiety in humans and animals always involve an interplay between a trait anxiety component (which reflects an individual's vulnerability or susceptibility to anxiety) and the situation that elicits state anxiety at the time of testing. Thus, when extreme forms of these traits are ignored, it is difficult, if not impossible, to dissociate adaptive defensive reactions from eventual maladaptive defensive reactions that are supposedly associated with specific anxiety disorders. Moreover, trait anxiety is not directly observable. Instead, it is inferred as a tendency to anxiety that can only be phenotypically observed and assessed through a standardized anxiety-related measure (e.g., behavioral, physiological, and neural correlates) at the time of testing. Finally, interactions between trait and state anxiety have been found to influence both the direction and magnitude of a given treatment (Griebel et al., 2000; Rao & Sadananda, 2016).

One strategy to manipulate trait anxiety in animal models is bidirectional selective breeding for extremes in anxiety-related parameters (for review, see Steimer & Driscoll, 2003), such as high anxiety-related behavior (HAB) and low anxiety-related behavior (LAB) rats and mice (Carboni et al., 2022; Landgraf & Wigger, 2003; Liebsch et al., 1998), Roman high- and low-avoidance rats (Bignami, 1965; Giorgi et al., 2019), Naples high- and low-excitability rats (Sadile et al., 1984; Pellicano & Sadile, 2006), the Syracuse (high- and low-avoidance) rat strains (Brush, 2003; Brush et al., 1999), the Maudsley reactive and non-reactive strains (selected for emotional defecation; Broadhurst, 1960, 1975), the Tsukuba (high and low runway activity) rat strains (Blizard et al., 2005; Fujii et al., 1989; who also differ on defecation), Floripa H and L rat lines (Izídio, & Ramos, 2007; Ramos et al., 2003), aggressive and non-aggressive mice (Benus et al., 1991; Miczek et al., 2015), and Carioca High-conditioned Freezing (CHF) and Carioca Low-conditioned Freezing (CLF) rats. The latter were developed by our group and associated laboratories, whose data and foundations as an animal model of GAD are discussed below.

2. CHF rat line as a model of GAD

Starting with a highly heterogeneous population of Wistar rats, our groups used a selective breeding protocol that has been in progress for the last two decades, to develop two new lines of rats. The lines differ in strong phenotypic divergence for high (CHF) and low (CLF) trait anxiety, respectively, with selection based on conditioned freezing scores in the well-known contextual fear conditioning paradigm (Bolles & Fanselow, 1982). The basic contextual fear conditioning protocol for our breeding separation involves two phases, trial and test sessions, that occur on two consecutive days. On the first day (conditioning trial), the rats are placed in a conditioning chamber. After 8 min (pre-shock period/baseline), they are exposed to three unavoidable mild electric footshocks. Twenty-four hours later, the rats are placed in the same conditioning chamber (context), but no shock is delivered. In this second exposure (test session), a trained observer records the occurrence of freezing behavior for 8 min according to a time-sampling schedule. Rats were scored every 2 s as either freezing or not freezing. Freezing was defined as a crouching, immobile posture with no movement other than that required for breathing. Contextual freezing behavior is then converted to a percentage as an anxiety-like measure. The breeding protocol, which we have uninterruptedly conducted in 42 successive generations since 2006, consists of the selective mating of male and female rats with their respective highest and lowest percentages of contextual freezing behavior. To better interpret differences between these two rat lines, a group of Wistar rats (CTL), composed of the offspring of randomized cross-breeding populations, is used as an additional control group in most of our studies. All animals were phenotyped at 2–3 months of age.

Over the past 16 years, more than 13 000 animals have already been phenotypically selected based on this protocol. Figure 1 presents the conditioned freezing behavior of our breeding lines across the 42 generations. As we reported in our first study (Castro-Gomes & Landeira-Fernandez, 2008), CHF and CLF rats exhibited reliable differences in conditioned freezing after the first three generations of selection. Males from both lines consistently exhibit more conditioned freezing in response to contextual cues than females. We also noted that the shock parameters that we employed (i.e., three 1 mA, 1 s unsignaled electrical footshocks with an inter-shock interval of 20 s) were very high. After the fourth generation, the shock intensity was reduced until it reached 0·4 mA in the 12th generation. In the 13th and 14th generations, we increased the shock intensity to 0·5 mA and 0·6 mA, respectively. This shock intensity has remained until the present generation. Male and female CHF, CLF, and CTL animals systematically exhibit clear differences across the remained generations.

The CHF rat line has been identified as a valid and reliable animal model of GAD. We present a brief summary of how the

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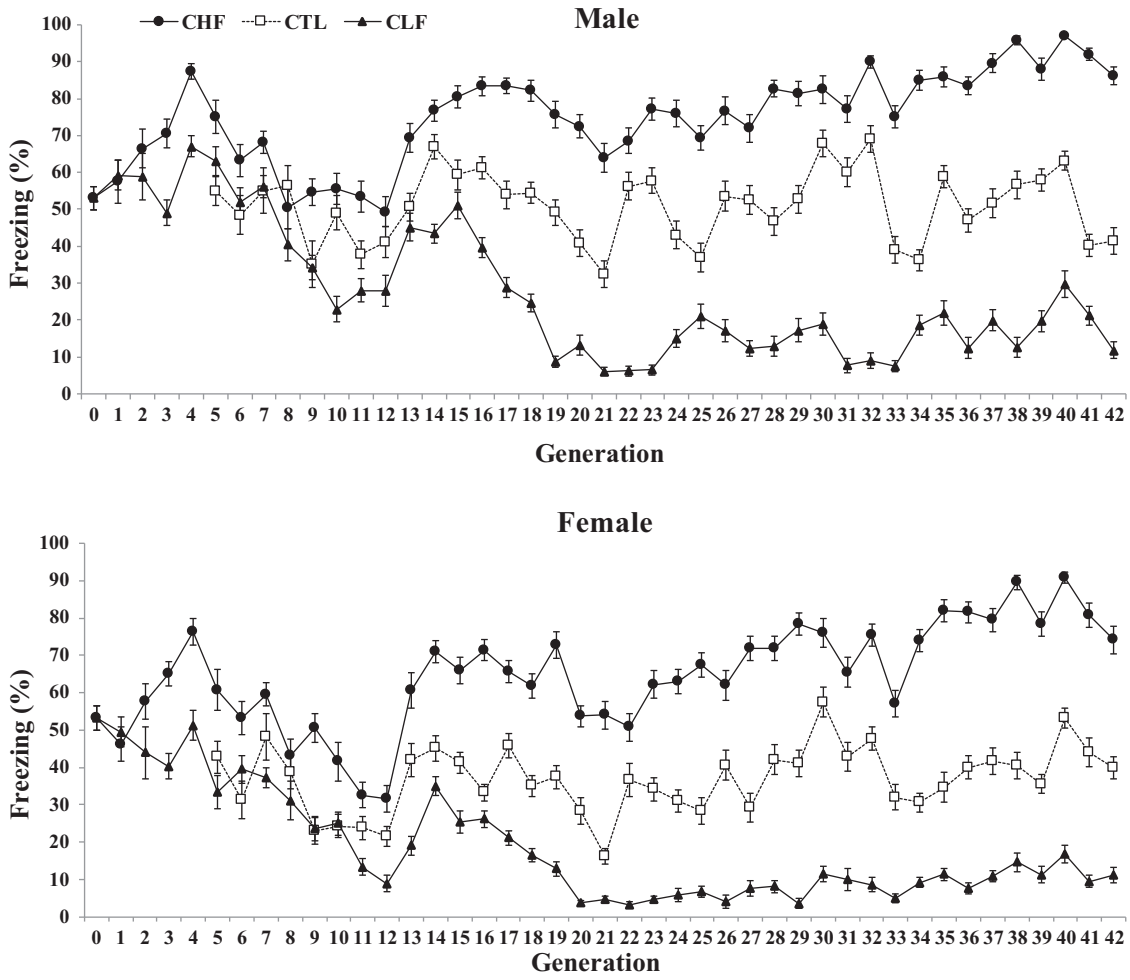

Figure 1. Mean ± SEM percentage of the time spent freezing in male (top) and female (bottom) Carioca High Freezing (CHF) and Carioca Low Freezing (CLF) rats across 42 generations. Control animals (CTL) started in the fifth generation of the two breeding lines.

main behavioral, pharmacological, physiological, and neurobiological findings from these animals constitute an animal model of GAD. Notably, no animal model of anxiety fully recapitulates all aspects of clinical anxiety in humans. Importantly, however, this is not the intention of such models. Typically, the validity of an animal model of anxiety or some other psychiatric condition is estimated by considering its degree of face validity, predictive validity, and construct validity (Treit, 1985; Willner, 1984), although there is often certain parsimony with regard to the need to fully meet these three criteria, depending on the purpose of the study.

Table 1 summarizes the major correspondence between findings from the CHF rat line and some of the main features of GAD in humans. One of the first behavioral findings from the CHF line was that these animals expressed their corresponding "anxious" characteristics not only in the contextual fear conditioning paradigm but also in the elevated plus maze (Cavaliere et al., 2020; Dias et al., 2009; Léon et al., 2017; Salviano et al., 2014), one of the best-known animal models of anxiety (Cruz et al., 1994; Handley & Mithani, 1984; Pellow et al., 1985). The elevated plus maze is based on the naturally occurring approach-avoidance conflict in rodents that is related to their motivation to explore new environments and innate fear of heights and open spaces (Treit et al., 1993). Importantly, this anxious behavioral profile that is observed in CHF rats in the elevated plus maze (i.e., decrease in open-arm exploration) was detected without significant changes in the total number of arm entries (open + closed arm entries) or absolute number of closed arm entries, thus indicating that the phenotyping protocol that is used for the selection of successive generations of these animals based on contextual fear conditioning does not produce significant general locomotor impairments, a key point in animal models of anxiety that require locomotor activity.

A substantial body of evidence (Brandão et al., 2008; Fanselow, 2000; Luyten et al., 2011; Phillips & LeDoux, 1992) indicates that conditioned freezing behavior in response to a context but not to an explicit cue (e.g., a tone that is previously associated with an aversive stimulus) share several behavioral, physiological, and neurobiological characteristics with GAD. Likewise, the elevated plus maze, at least in its usual form of a single 5-min session, also exhibits features of an animal model of GAD. An intricate relationship has been suggested between a type of behavior that is supposedly related to generalized anxiety, which would occur during the first 5 min of a single exposure to the test, with another type of anxiety (specific phobia) that results from a longer exposure (10 min) or second 5-min exposure to the test (File & Zangrossi, 1993). This view is consistent with findings that anxiolytics increase open-arm exploration during a single 5-min session in the

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Table 1. Correspondence between CHF rat line findings and some main features of generalized anxiety disorder in humans

CHF rat line	Reference	GAD features	Reference
High and diffuse anxiety in contextual fear conditioning, elevated plus maze, and avoidance behavior in the elevated T-maze	Dias et al. (2009); Cavaliere et al. (2020); Léon et al. (2017); Salviano et al. (2014)	Excessive anxiety and worry	American Psychiatric Association (2013); Crocq (2017)
Immobility/freezing behavior	Castro-Gomes and Landeira-Fernandez (2008)	Muscle tension	American Psychiatric Association (2013); Crocq (2017)
Different pattern of acquisition/extinction in response to context and cue	Macedo-Souza et al. (2020); Lages et al. (2021a)	The focus of anxiety is not confined to a specific situation or features of other anxiety disorders (e.g., panic, specific phobia, etc.)	American Psychiatric Association (2013); Crocq (2017)
Increase corticosterone serum levels	Mousovich-Neto et al. (2015)	Elevated cortisol	Lenze et al. (2011)
Benzodiazepine and serotonergic anxiolytics attenuate freezing behavior in CHF but not CLF rats	Cavaliere et al. (2020); León et al. (2017)	Benzodiazepine and serotonergic anxiolytics attenuate GAD symptoms	Gomez et al. (2018); Reinhold et al. (2011)
Higher alcohol intake	Bezerra-Karounis et al. (2020)	Comorbidities or associations with alcohol abuse	Kushner et al. (1990); Smith and Randall (2012)

elevated plus maze but lose this anxiolytic-like effect (i.e., "one trial tolerance") when given in a single 10-min session or second 5-min session (File & Zangrossi, 1993). According to these authors, this mimics a condition that is supposedly related to specific phobia, for which anxiolytics are known to be ineffective (Bandelow, Michaelis, & Wedekind, 2017). Therefore, high trait anxiety in CHF rats exposed for 5 min to the elevated plus maze further corroborates the proposition that these animals are a model of GAD.

Other more recent behavioral findings also corroborate this view. For example, one of the main characteristics of GAD is its chronic course. Accordingly, Lages et al. (2021a) showed that CLF rats were unable to consolidate aversive memories, whereas CHF rats exhibited considerable percentages of freezing behavior even after multiple exposures to the context that was previously associated with the aversive stimulus, with the additional interesting feature of being susceptible to extinction. In another study that compared patterns of freezing behavior in CHF and CLF rats in response to the context or cue (a tone that was previously associated with an electric footshock), Macêdo-Souza et al. (2020) showed that CHF rats froze more than CTL rats and these more than CLF when exposed to the context, that is associated to generalized anxiety disorder (Luyten et al., 2011) but not to the cue that is supposed linked to specific phobia (Garcia, 2017; Grillon et al., 2006).

Potentially threatening situations are also known to activate the hypothalamic–pituitary–adrenal (HPA) axis (Hinds & Sanchez, 2022). Consequently, GAD patients have been found to have elevated cortisol levels (Lenze et al., 2011). Again, CHF rats were equally selective for this parameter as reported by Mousovich-Neto et al. (2015). These authors showed increased neuroendocrine responses (i.e., increased serum corticosterone) in CHF rats comparted to control animals.

Benzodiazepines are among the most commonly prescribed and effective medications for GAD (Gomez et al., 2018). For this reason, many animal models of anxiety are pharmacologically validated based on this class of drugs. Thus, in one of our studies (Cavaliere et al., 2020), systemic injections of the benzodiazepine midazolam (0·25, 0·5, 0·75, and 1·0 mg/kg, i.p.) selectively increased open-arm exploration in CHF rats exposed for 5 min to the elevated plus maze test. Interestingly, however, this anxiolytic-like profile was only observed at the lowest dose tested (0·25 mg/kg) in CLF rats. This observation is consistent with previous findings that anxiolytic-like effects of benzodiazepines and mainly serotonergic anxiolytics appear to depend on the animals' level of anxiety before testing (Blanchard et al., 2001). Accordingly, in another study, systemic (0·5 mg/kg, i.p.) and intra-infralimbic cortex (5 nmol/ml) injections of the preferential 5-HT_{2A} receptor antagonist ketanserin induced anxiolytic-like effects in CHF rats but anxiogenic-like effects in CLF rats in the elevated plus maze (León et al., 2017).

Another interesting finding that is also consistent with characteristics of GAD in humans refers to alcohol consumption. Alcohol is known for its "anxiolytic" properties in humans and animals. So, since GAD has also been associated with alcohol abuse in clinical and nonclinical populations (Kushner et al., 1990; Smith & Randall, 2012), this association was also recently investigated in the CHF rat line (Bezerra-Krounis et al., 2020). As expected, CHF animals consumed more alcohol than CLF and control animals, which opens the possibility of using this model to better understand the comorbidity between GAD and alcohol abuse.

It is important to mention that anxiety and depression are independent disorders, although, clinical studies have shown that there is a high level of comorbidity between them (Groen et al., 2020), with a co-occurrence rate of 90% (Gorman (1996). Results from our breeding line indicated that CHF animals from the fourth generation did not differ from control animals, as measured by the forced swimming test (Dias et al., 2009). However, more recent results, employing CHF animal from the 26th and 27th generation indicated a depressive like behavior when compared to control animals (Goulart et al., 2021). Further studies may explore this type of relationship and whether antidepressant drugs are capable of reversing the depressive and the anxiety like effects in these animals.

At the other extreme from animals with high trait levels, CLF animals exhibited a delayed response to haloperidol at lower doses, needing higher doses to reach similar levels of catatonia as control randomly bred animals. Moreover, methylphenidate increased freezing response and motor activity among CLF rats when compared to control animals (Lages et al., 2021b). Since haloperidol and methylphenidate are dopamine-related molecular targets, it is possible that the CLF line of rats might represent an

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animal model of hyperactivity and attention disorders. This hypothesis is currently under investigation in our laboratory.

Finally, other studies developed by our group but not discussed in the scope of this review (for details, see Dias et al., 2014; Lages et al., 2023; Léon et al., 2020) also indicate functional and structural changes in neural circuits underlying anxiety in the CHF rat line, which seems to indicate that the phenotyping process that strengthened this trait anxiety was also expressed in terms of a great capacity for neural plasticity.

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