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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.