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Neuropeptide Y, Calcitonin Gene-Related Peptide, and Neurokinin A in brain regions of HAB rats correlate with anxiety-like behaviours

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Abstract

Anxiety disorders are pervasive psychiatric disorders causing great suffering. The high (HAB) and low (LAB) anxiety-related behaviour rats were selectively bred to investigate neurobiological correlates of anxiety. We compared the level of neuropeptides relevant for anxiety- and depression-related behaviours in selected brain regions of HAB and LAB rats.

Increased anxiety and depression-like behaviours of male and female HAB rats in the elevated plus-maze and forced swim tests were accompanied by elevated levels of neuropeptide Y (NPY) in the prefrontal (PFC), frontal (FC) and cingulate cortex (CCx), the striatum, and periaqueductal grey (PAG). Moreover, HAB rats displayed sex-dependent, elevated levels of calcitonin gene-related peptide (CGRP) in PFC, FC, CCx, hippocampus, and PAG. Higher neurokinin A (NKA) levels were detected in CCx, striatum, and PAG in HAB males and in CCx and hypothalamus in HAB females. Increased neurotensin was detected in CCx and PAG in HAB males and in hypothalamus in HAB females. Elevated corticotropin-releasing hormone (CRH) levels appeared in female HAB hypothalamus. Significant correlations were found between anxiety-like behaviour and NPY, CGRP, NKA, and neurotensin, particularly with NPY in CCx and striatum, CGRP in FC and hippocampus, and NKA in entorhinal cortex.

This is the first report of NPY, CGRP, NKA, Neurotensin, and CRH measurements in brain regions of HAB and LAB rats, which showed widespread NPY and CGRP alterations in cortical regions, with NKA and neurotensin changes localised in sub-cortical areas. The results may contribute to elucidate pathophysiological mechanisms underlying anxiety and depression and should facilitate identifying novel therapeutic targets.

Keywords

Anxiety; NPY; CGRP; neurokinin A; neurotensin; corticotropin releasing hormone; HAB LAB rats.

1. Introduction

Anxiety and fear responses evolved to promote survival by optimising responses to threats and dangers. In humans, anxiety reactions appear as a continuum, with excessive and inappropriate anxiety producing distress and impairing quality of life (Bateson et al., 2011). Anxiety disorders are the most widespread among psychiatric disorders, causing immense suffering, and imposing heavy direct and indirect costs on society (Kessler et al., 2010). Anxiety disorders include distinct conditions characterised by specific symptoms as defined by the Diagnostic and Statistical Manual of Mental Disorders, which share the common trait that the fear or anxiety is marked, persistent, and impairing (Craske et al., 2017). In spite of a variety of psychotherapeutic and as pharmacological approaches, a clinically meaningful response is elicited in about half of the patients, and relapse occurs in up to 40% patients (Craske et al., 2017). Although the conditions included have been changing from DSM-3 to DSM-4 to the current DSM-5, the description of general anxiety disorder (GAD) has remained stable. Of note, while the classical diagnostic rating instruments, e.g., Hamilton Anxiety Rating Scale includes a depression item and, conversely, Hamilton Depression Rating Scale and Montgomery Åsberg Depression Scale include questions regarding anxiety this does not per se result in a double diagnostic categorization (of particular importance in psychiatric disorders since symptoms are seldom pathognomonic and the distinction between a symptom and a diagnosis is decisive). Although patients with greater illness severity may be diagnosed as suffering from both conditions (Lamers et al 2011), GAD and major depressive disorder (MDD) have distinguished genetic and epigenetics characteristics that have been extensively documented (for GAD see (Gottschalk and Domschke, 2017); for MDD see (Wray et al., 2018). Furthermore, “anxious depression” is used clinically to describe MDD patients with high initial anxiety and a worse treatment response (Wurst et al., 2021); however, the diagnosis has not been classified as a separate entity in the DSM-5. The level of comorbidity suggests existence of certain common underpinnings of anxiety and depression but does not contradict existence of the neurobiological correlates selective for depression and separately for anxiety disorders.

To gain insight into the molecular underpinning of the disorder, and to test for novel therapeutic strategies (Murgatroyd et al., 2004; Schmidtner et al., 2019; Slattery et al., 2015; Slattery and Neumann, 2010), an animal model of anxiety was developed by selectively breeding Wistar rats for high anxiety-related behaviour (HAB) versus low anxiety-related behaviour (LAB) assessed on the elevated plus-maze (EPM). Avoidance of the open arms of the EPM expressed as low percentage of time the rat stayed on the open arms (below 10% for HAB rats) and low number of entries performed into the open arms was considered as the main selection criteria (Landgraf et al., 2007; Landgraf and Wigger, 2002; Wegener et al., 2012). Thus, two genetically and behaviourally stable lines reflecting the extremes in anxiety-related behaviour were established. In addition to the differences in EPM behaviour, the HAB compared to the LAB rats also show diminished exploration of the illuminated part of the black-white box, of the central zone of the open field, and on the modified hole

board. In addition, passive stress coping strategies resembling depressive-like behaviours characterise male and female HAB rats (Landgraf and Wigger, 2002; Schmidtner et al., 2019). Moreover, robust strain differences were found with respect to various social behaviours including social preference, aggressive and maternal behaviours (Beiderbeck et al., 2012; Bosch and Neumann, 2008; Slattery and Neumann, 2010). Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis upon stress exposure further differentiates the HAB strain, as demonstrated by much larger ACTH and corticosterone surges evoked in response to a stressful experience specifically observed in HAB rats, whereas basal levels were comparable in both lines (Landgraf et al., 1999). Other autonomic functions were found to differ between HAB and LAB rats, such as higher respiratory rates, altered respiratory patterns, and impaired autonomic modulation of heart rate, in particular in response to stress, in HAB rats, and bradycardia with hypothermia in response to motion in LAB rats (Carnevali et al., 2016).

A single nucleotide polymorphism in the vasopressin gene resulting in increased synthesis and central release of vasopressin has been identified to underlie the characteristic hyper-anxiety and HPA axis dysregulation in these animals (Keck et al., 2002; Wigger et al., 2004). Genetic differences in the neuropeptide S (NPS) system are also likely to contribute to the behavioural phenotype of HAB and LAB rats (Slattery et al., 2015). Interestingly, interactions between genetic predispositions and early environmental experiences induce opposite adult stress-coping behaviours following exposure to prenatal stress in these rat strains. Indeed, HAB rats displayed reduced anxiety-like behaviours in the EPM and hole board tests, whereas LAB rats became more anxious as a result (Bosch et al., 2006). Moreover, prenatal stress differentially influenced the expression of hypothalamic vasopressin and corticotropin-releasing hormone (CRH) in HAB versus LAB rats (Bosch et al., 2006). In summary, genetic differences mainly located on the vasopressin, NPS, and glyoxalase-I genes are likely to interact with environmental conditions to contribute to the anxiety and depression-like behavioural differences between lines (Bosch et al., 2006; Murgatroyd et al., 2004; Slattery et al., 2015).

In addition to vasopressin, NPS and CRH, other neuropeptides participate in the regulation of anxiety- and depressive-like behaviours. A critical role for neuropeptide Y (NPY) has been established in the control of functions relevant for brain disorders e.g. stress responses, mood, sleep and circadian rhythm, energy homeostasis, and memory function. NPY levels were consistently reduced in models of depression, chronic stress, and post-traumatic stress disorder (Thorsell and Mathé, 2017). Another peptide of relevance in fear and anxiety is calcitonin gene-related peptide (CGRP). CGRP is a 37-amino acid peptide deriving from the calcitonin gene which also encodes for calcitonin and its carboxyl-terminal flanking peptide katacalcin. CGRP is widely distributed in the brain, within the hypothalamus, amygdala, hippocampus, basal ganglia, playing roles in vasodilation, nociception and energy metabolism (Angelucci et al., 2019; Neugebauer et al., 2020), contributes to stress responses, and anxiety- and depressive-like behaviours (Jiao et al., 2013). Previous studies also indicated that neurokinin A (NKA) plays a role in the regulation of depressive-and anxiety-like

behaviours (Bilkei-Gorzo et al., 2002; Husum et al., 2008, 2001; Mathé et al., 1998; Ribeiro and De Lima, 2002). NKA-positive neurons have been identified in the hypothalamus, basal ganglia and midbrain (Otsuka and Yoshioka, 1993). Likewise, accumulating evidence supports the involvement of neurotensin in anxiety and depression (Ellenbroek et al., 2016; Li et al., 2021; Normandeau et al., 2018). Summarising, the brain levels and the role of NPY, CGRP, NKA, neurotensin, and CRH have previously not been investigated in the context of distinct HAB and LAB behaviours. In particular, the focus was set on cortical regions, including the prefrontal cortex (PFC), frontal cortex (FC), cingulate cortex (CCx), entorhinal cortex (ECx), and subcortical areas, including the dorsal striatum (ST), nucleus accumbens (NAc), hippocampus, periaqueductal grey (PAG), hypothalamus, and amygdala, due to their relevance in mediating cognitive, emotional, and autonomic responses related to anxiety (Shin and Liberzon, 2010).

Consequently, male and female rats were used in this study, since anxiety and depression have a different prevalence by gender (Kokras and Dalla, 2014). The overriding aims were to for the first time: i. map expression of NPY, CGRP, NKA, neurotensin, and CRH in brain areas relevant for psychiatric disorders in HAB and LAB rat strains, ii. explore possible differences in peptide levels between the two strains, and iii. correlate behaviours indicative of anxiety and depression to the neuropeptides measured. This will facilitate hypothesis generation regarding pathophysiology of GAD and MDD and indicate possible strategies for development of novel treatments.

2. Experimental procedures

2.1 Experimental design

The HAB and LAB strains were selectively bred from Wistar rats (Landgraf et al., 2007; Landgraf and Wigger, 2002; Wegener et al., 2012) using the degree of anxiety-related behaviour displayed on the EPM at the age of 10 weeks as the main selection criterion.

HAB and LAB rats were tested on the EPM at the age of 9 weeks and, one week later, in the forced swim test (FST). After an additional week, rats were euthanised, brains were harvested, and neuropeptide levels measured in selected brain punches. The experimental design is displayed in Supplementary Figure S1.

2.2 Animals

Male (n=16) and female (n=12) HAB and LAB rats (between 250 and 290 g body weight) from the colony maintained at the University of Regensburg were used. The rats were housed 3-5/cage at constant room temperature of 21±1°C and maintained on a 12:12h light/dark cycle with freely available food and water. The Ethical Committee for Protection of Animals approved the study (approval number: 54-2531.2-16/08) and all procedures were conducted in accordance with the European Communities Council Directive of 24

November 1986. Efforts were performed to reduce the number of animals and minimise their suffering. This study is compliant with the ARRIVE guidelines (du Sert et al., 2020).

2.3 Behaviours

Rats were tested on the EPM as previously reported (Slattery et al., 2015). Briefly, rats were placed in a plus-shaped maze consisting of two open arms surrounded by a 0.5 cm high rim and two closed arms surrounded by 40 cm high walls linked by a neutral zone. Rat behaviour was recorded by a camera located above the maze for a 5-min testing period. The anxiety-related behaviours, including the time spent in open or closed arms and the latency to enter open arms were subsequently assessed by an observer blind to the strain.

The FST was performed as previously reported (Carboni et al., 2018). A 15-min pre-test was carried out 24 h before the test. Rats were placed in a water tank that does not allow escape; behaviour was camera-recorded for 5 min and subsequently scored by an observer blind to the strain. Three distinct behaviours were scored as previously reported (Cryan et al., 2002; Schmidtner et al., 2019): struggling, with vertical climbing movements of the forepaws; swimming, with horizontal movements; and floating, characterised by an immobile upright posture, in which the rat only moves to keep its head above the water surface.

2.4 Peptide levels

One week after the last behavioural test rats were decapitated, the brains were removed, quickly frozen and stored at -80°C; the regions of interest were isolated by microdissection (Slattery et al., 2015). The following brain regions were collected: prefrontal cortex (PFC), frontal cortex (FC), cingulate cortex (CCx), entorhinal cortex (ECx), dorsal striatum (ST), nucleus accumbens (NAc), hippocampus, periaqueductal grey (PAG), hypothalamus, and amygdala.

Brain tissues were homogenized, twice extracted by boiling in acetic acid and then water and centrifuged for 20 min. at 1600g. Supernatants were lyophilized, reconstituted in phosphate buffer, and finally subjected to radioimmunoassay (RIA) analysis for NPY-like (NPY-LI) immunoreactivity (Peninsula Laboratories, BMA Biomedicals, Switzerland), CGRP-LI (Peninsula Laboratories), NKA-LI (antibody K12, a generous gift from professor E. Theodorsson, Sweden), neurotensin-LI (Peninsula Laboratories), and CRH-LI (antibody a generous gift from Dr P. Lowry, UK) as described previously (Ellenbroek et al., 2016; Husum et al., 2008; Husum and Mathé, 2002; Wörtwein et al., 2006). Briefly, the assay was run in duplicates as follows: 25 µl standards prepared in the same buffer as samples or samples were incubated at 4°C for 48 h with 100 µl of antibody. Following this incubation, 100 µl of ^{125}I-labelled peptide was added and the solution was incubated for an additional 24 h. Free and antibody-bound peptides were separated by addition of a sheep anti-rabbit antibody-coated Sepharose suspension. Samples were left for 30 min at room temperature; the reaction was then blocked with 1 ml distilled water. After centrifugation at 3000 × g for 20 min at 4°C, the pellets were

counted in a γ counter. Lower detection limits were: 1.28 pmol/L for NPY, CGPR, and NKA; 1.9 pmol/L for neurotensin; 3.9 pmol/L for CRH. Inter-assay coefficients of variations were 5-7%. Sample duplicates were averaged before statistical analysis.

2.5 Statistical analysis

The data are presented as the mean values ± SEM. The results were analysed using a 2-way ANOVA approach with sex (males and females) and line (HAB and LAB) as factors of interest. The analyses were followed by Planned Comparisons of the predicted means to compare the mean of the HAB and LAB groups within each sex. The data were log- or square root-transformed, where appropriate, in order to stabilize the variance and satisfy the parametric assumptions. Multivariate analysis was carried out with Principal Components Analysis (PCA) with the objective of explaining most variability with a reduced number of components identified in unsupervised manner. Correlation analysis was based on Pearson's product moment correlation coefficient. All analyses were performed using the InVivoStat v4.1.0 software (Bate and Clark, 2014; Clark et al., 2012). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 Behaviour

3.1.1 Elevated plus-maze

The EPM test measures anxiety-like behaviours which are displayed as preference for closed spaces, perceived as safer compared to open areas. Marked behavioural differences between HAB and LAB animals were found [percentage of time spent in open arms: line $F_{(1,24)}$=305.11, p<0.0001; sex $F_{(1,24)}$=0.00, p=0.89; interaction $F_{(1,24)}$=0.17, p=0.68; percentage of open arms entries: line $F_{(1,24)}$=20.06, p=0.0002; sex $F_{(1,24)}$=3.80, p=0.063; interaction $F_{(1,24)}$=0.99, p=0.33;latency to open arms entry: line $F_{(1,24)}$=18.78, p=0.0002; sex $F_{(1,24)}$=0.81, p=0.38; interaction $F_{(1,24)}$=1.00, p=0.33]. As indicator of their elevated anxiety-related behaviour, HAB male and female rats spent significantly less time in the open arms compared to LAB rats and performed less entries into the open arms (Fig. 1). Moreover, HAB rats had significantly lower scores of entries into closed arms indicating a reduced level of locomotion [line $F_{(1,24)}$=79.28, p<0.0001; sex $F_{(1,24)}$=4.16, p=0.053; interaction $F_{(1,24)}$=0.59, p=0.45] (not shown).

3.1.2 Forced swim test

In the FST depressive-like behaviour is reflected by passive stress coping, namely shorter time swimming and longer time floating in an immobile posture. HAB rats spent significantly more time floating [line $F_{(1,22)}$=21.54, p=0.0001; sex $F_{(1,22)}$=0.05, p=0.83; interaction $F_{(1,22)}$=2.36, p=0.14] and shorter time swimming [line $F_{(1,22)}$=13.95, p=0.0011; sex $F_{(1,22)}$=0.34, p=0.57; interaction $F_{(1,22)}$=1.32, p=0.26] and struggling [line $F_{(1,22)}$=

4.52, p=0.045; sex $F_{(1,22)}$=0.00, p=0.89; interaction $F_{(1,22)}$=0.78, p=0.39], i.e. active stress coping, than the LAB rats (Fig. 2). The differences between HAB and LAB lines in both active and passive behaviours were more pronounced in males compared to females (Fig. 2). HAB females showed a similar pattern with less struggling and swimming, but only longer floating time reached significance (Fig. 2).

3.2 Neuropeptides

We first carried out a multivariate analysis including all neuropeptide levels measured in all brain regions. Principal Components Analysis showed that a strong separation based on line (HAB/LAB) was achieved by Principal Component 1 and Principal Component 2 (Fig. 3). Variables providing stronger contribution to the Principal Component 1 were cortical levels of NPY and CGRP (Supplementary Table 1). These findings encouraged us to undertake specific comparisons between lines within brain areas for each neuropeptide.

3.2.1 NPY

The highest levels were measured in hypothalamus, followed by PAG in both males and females (Fig. 4A and B). NPY-LI levels differed between the HAB and LAB lines in the PFC [factor line: $F_{(1,23)}$=11.93, p=0.0022; sex $F_{(1,23)}$=0.87, p=0.36; interaction $F_{(1,23)}$=0.01, p=0.92]; FC [line $F_{(1,24)}$=18.4, p=0.0003; sex $F_{(1,24)}$=1.51, p=0.23; interaction $F_{(1,24)}$=0.00, p=0.95]; CCx [line $F_{(1,24)}$=38.49, p<0.0001; sex $F_{(1,24)}$=0.14, p=0.71; interaction $F_{(1,24)}$=1.20, p=0.28]; ECx [line $F_{(1,22)}$=6.27, p=0.020; sex $F_{(1,22)}$=0.13, p=0.72; interaction $F_{(1,22)}$=0.08, p=0.78]; ST [line $F_{(1,24)}$=24.36, p<0.0001; sex $F_{(1,24)}$=0.02, p=0.89; interaction $F_{(1,24)}$=0.27, p=0.61]; PAG [line $F_{(1,23)}$=4.55, p=0.044; sex $F_{(1,23)}$=1.16, p=0.29; interaction $F_{(1,23)}$=0.29, p=0.60]. Analysis by sex showed that in HAB males, NPY levels were higher in all brain regions studied with significant differences in the PFC (p=0.019), FC (p=0.0027), CCx (p=0.0006), ST (p=0.0003), and PAG (p=0.047) (Fig. 4A). In HAB females, the mean NPY-LI were also higher in all regions, with significant differences in PFC (p=0.027), FC (p=0.010), CCx (p<0.0001), and ST (p=0.0078) (Fig. 4B).

3.2.2 CGRP

Significant differences between HAB and LAB were detected in the PFC [line factor $F_{(1,23)}$=13.6, p=0.0012; sex $F_{(1,23)}$=2.04, p=0.17; interaction $F_{(1,23)}$=0.42, p=0.52]; FC [line $F_{(1,24)}$=30.44, p<0.0001; sex $F_{(1,24)}$=0.00, p=0.95; interaction $F_{(1,24)}$=0.11, p=0.74]; CCx [line $F_{(1,24)}$=8.65, p=0.0071; sex $F_{(1,24)}$=0.05, p=0.82; interaction $F_{(1,24)}$=0.02, p=0.89]; hippocampus [line $F_{(1,24)}$=23.11, p<0.0001; sex $F_{(1,24)}$=0.59, p=0.45; interaction $F_{(1,24)}$=0.21, p=0.65]; PAG [line $F_{(1,23)}$=10.75, p=0.0033; sex $F_{(1,23)}$=6.12, p=0.021; interaction $F_{(1,23)}$=0.38, p=0.55]; amygdala [line $F_{(1,24)}$=0.72, p=0.40; sex $F_{(1,24)}$=4.21, p=0.051; interaction $F_{(1,24)}$=0.14, p=0.71]; and hypothalamus [line $F_{(1,24)}$=0.09, p=0.77; sex $F_{(1,24)}$=4.24, p=0.051; interaction $F_{(1,24)}$=1.42, p=0.25] (Fig. 5). In males, the mean CGRP-LI was higher in HAB compared to LAB in PFC (p=0.031), FC (p=0.005), CCx (p=0.041), hippocampus

(p=0.0005), and PAG (p=0.048) (Fig 5A). In females, CGRP-LI was higher in HAB rats in PFC (p=0.0082), FC (p=0.0008), hippocampus (p=0.0087), and PAG (p=0.019), with a trend in CCx (p=0.054) (Fig. 5B).

3.2.3 NKA

Comparable levels of NKA-LI were detected in brain regions of rats of both sexes. Areas showing higher NKA levels were hypothalamus and PAG, followed by St and NAc (Fig. 6).

Strain-related differences were revealed in the CCx [line $F_{(1,24)}$=12.97, p=0.0014; sex $F_{(1,24)}$=0.01, p=0.93; interaction $F_{(1,24)}$=0.03, p=0.86]; ECx [line $F_{(1,23)}$=6.23, p=0.020; sex $F_{(1,23)}$=0.55, p=0.47; interaction $F_{(1,23)}$=0.01, p=0.92]; ST [line $F_{(1,24)}$=7.38, p=0.012; sex $F_{(1,24)}$=0.60, p=0.45; interaction $F_{(1,24)}$=0.07, p=0.79]; PAG [line $F_{(1,22)}$=9.14, p=0.006; sex $F_{(1,22)}$=0.39, p=0.54; interaction $F_{(1,22)}$=0.14, p=0.71]; hypothalamus [line $F_{(1,20)}$=9.56, p=0.0058; sex $F_{(1,20)}$=1.89, p=0.18; interaction $F_{(1,20)}$=3.0, p=0.10]. In the analysis by sex, in males, higher NKA levels were observed in HAB rats in CCx (p=0.0077), ST (p=0.031), and PAG (p=0.012) (Fig. 6A). In contrast, in HAB females increased NKA amounts were detected in CCx (p=0.034) and in hypothalamus (p=0.0071) (Fig. 6B). Of note, NKA levels were higher in PAG and hypothalamus in the HAB strain of both males and females, although not always reaching the statistical level of significance (Fig. 6).

3.2.4 Neurotensin

Neurotensin-LI brain distribution was similar in male and female rats, with higher levels in PAG and hypothalamus compared to cortical areas (Fig. 7).

Significant differences were detected between HAB and LAB in the CCx [line $F_{(1,24)}$=7.97, p=0.0094; sex $F_{(1,24)}$=0.00, p=0.98; interaction $F_{(1,24)}$=2.95, p=0.099]; hippocampus [line $F_{(1,24)}$=4.59, p=0.042; sex $F_{(1,24)}$=0.42, p=0.52; interaction $F_{(1,24)}$=0.34, p=0.57]; PAG [line $F_{(1,22)}$=9.75, p=0.005; sex $F_{(1,22)}$=0.08, p=0.78; interaction $F_{(1,22)}$=0.15, p=0.71]; hypothalamus line [$F_{(1,21)}$=14.03, p=0.0012; sex $F_{(1,21)}$=2.67, p=0.12; interaction $F_{(1,21)}$=12.03, p=0.0023]. In the analysis by sex, in males, higher neurotensin-LI in the HAB strain was detected in CCx (p=0.0019) and PAG (p=0.0098) (Fig. 7A). In contrast, almost two-fold higher neurotensin levels were found in hypothalamus (p=0.0002) of female HAB rats (Fig. 7B).

3.2.5 CRH

CRH-LI levels did not differ between the sexes except for PAG levels, which were higher in females [line $F_{(1,22)}$=0.10, p=0.76; sex $F_{(1,22)}$=6.12, p=0.022; interaction $F_{(1,22)}$=1.83, p=0.19). The highest levels were measured in hypothalamus (Fig. 8), where a HAB-LAB difference was also found [line $F_{(1,24)}$=4.69, p=0.041; sex $F_{(1,24)}$=0.96, p=0.34; interaction $F_{(1,24)}$=3.09, p=0.091]. No significant differences between HAB and LAB were found in males, thus confirming previous results with mRNA levels (Wigger et al., 2004) (Fig. 8A). In females, higher CRH-LI was detected in hypothalamus of HAB rats (p= 0.0058) (Fig. 8B).

3.3 Behaviour-neuropeptide correlations

Next, we investigated whether regional neuropeptide levels correlated to anxiety- or depression-related behaviour. To this aim, we performed pairwise correlation analyses between behavioural parameters and peptide levels. Both males and females were included in the analysis. Percentage of time spent in open arms and time spent in floating behaviour were selected as indicative results of the EPM and FST tests, respectively. A strong negative correlation was found between percentage of time spent in open arms and floating time ($r=-0.700$, $p<0.0001$) (Table 1) indicating that high anxiety levels are associated with depression-like behaviour.

Correlations were also tested between behavioural scores and neuropeptide levels in all brain areas. Significant negative correlations were observed between the percentage of time spent in open arms and NPY, CGRP, NKA, neurotensin, and CRH levels (Table 1). Among those, the most significant pointed to the importance of NPY levels in the CCx ($r=-0.653$, $p=0.0002$) and ST ($r=-0.667$, $p=0.0001$); CGRP levels in FC ($r=-0.644$; $p=0.0002$) and hippocampus ($r=-0.681$, $p<0.0001$), and NKA levels in ECx ($r=-0.567$, $p=0.002$) (Table 1). As regards floating time, weaker positive correlations were identified with NPY, CGRP, and NKA levels, with the strongest impact played by CCx NKA levels ($r=0.581$, $p=0.0018$) (Table 1).

4. Discussion

This is the first report of NPY-LI, CGRP-LI, NKA-LI, neurotensin-LI, and CRH-LI in cortical (PFC, FC, CCx, ECx) and sub-cortical (hippocampus, ST, NAc, amygdala, PAG, hypothalamus) brain regions of rats selected for extremes in trait anxiety, i.e., HAB rats and their low anxiety behaviour controls LAB. The most seminal findings were significantly higher neuropeptide levels of NPY, CGRP, NKA, and neurotensin (and CRH in hypothalamus) in the brain regions of the HAB compared to LAB rats. The largest differences were in the levels of NPY and CGRP, independent of the brain region studied. On the other hand, line-specific differences in NKA and neurotensin levels were more brain region-dependent. Of note, CRH levels showed higher levels detected only in the hypothalamus of female HAB compared with male LAB rats.

Although the overall design was identical for both sexes, specific changes were detected in each sex, i.e., higher neuropeptide levels were consistently observed in females. For example, in female rats higher CGRP levels were measured in the PAG, amygdala and hypothalamus, and higher CRH levels were detected in the PAG. Therefore, elevated neuropeptide levels define the more anxiety-vulnerable HAB strain, that is HAB vs. LAB, and the more anxiety-susceptible female sex. Whereas robust behavioural differences between HAB and LAB rats were found in the EPM test in both sexes, increased depressive-like behaviour of HAB rats assessed in the FST was less evident in females than in males. Behavioural differences between males and females have been previously reported in rats. In the EPM, female rats have been reported to make greater percent open arm entries than males, differently from HAB/LAB rats, with no differences in distance moved

or in percent time spent on the open arms (Kokras and Dalla, 2014; Simpson et al., 2012). In the FST, higher levels of immobility has been described in female rats than males (Kokras et al., 2015; Kokras and Dalla, 2014), a finding that was not reproduced in HAB/LAB rats.

With regard to peptides, specific changes were detected for each peptide measured. Thus, NPY was increased in HAB rats exhibiting high anxiety-related behaviours in regions involved in the regulation of anxiety and fear, such as PAG, CCx, PFC, and FC. The finding of increased NPY was in contrast to the decreased NPY expression found in animal models of depression and in post-traumatic stress disorder (Cohen et al., 2012; Husum et al., 2008, 2002). In addition, NPY neuron ablation in mouse NAc caused increased anxiety-like behaviours, whereas DREADD-mediated activation of the same neurons reversed the effect (Yamada et al., 2020). Along the same line, mice lacking NPY display an anxiogenic-like phenotype (Bannon et al., 2000). Interestingly, while transgenic NPY over-expressing rats showed attenuated sensitivity to stress-induced anxiogenesis, their baseline behaviour was not anxiolytic (Thorsell et al., 2000), suggesting that adaptations may develop as a consequence of high NPY levels. Of note, NPY was increased in hypothalamus and in the frontal cortex of rats both normally reared and rats subjected to maternal separation if they were housed in cages with a grid floor (Husum et al., 2008, 2002, 2001; Husum and Mathé, 2002). Moreover, rats exposed to immobilization stress also show increased NPY expression in amygdala (Thorsell et al., 1999). Consequently, the increase or decrease in NPY expression depends on regions examined and the experimental conditions. Whether elevated NPY is inherent to the HAB model with specific vasopressin changes or generally reflects fear and anxiety traits in other anxiety models with different endophenotype cannot be answered at this time. Translationally reversed, NPY is reduced in cerebrospinal fluid of MDD patients, in bipolar patients at risk for suicide, and in post-traumatic stress disorder patients. On the other hand, NPY was elevated in cerebrospinal fluid of patients with impulsive aggression (Coccaro et al., 2012) and in MDD patients who were exposed to early childhood trauma (Soleimani et al., 2014) and preclinically in rats exposed to physical stress early in life (Husum et al., 2008, 2002). In genetic and environmental animal models of depression, our previous studies revealed lower NPY levels in relevant brain regions and consistently with these results administration of efficacious antidepressant treatments reversed the decrease (Husum et al., 2001; Husum and Mathé, 2002; Jiménez-Vasquez et al., 2000; Mathé et al., 1998; Wörtwein et al., 2006). In agreement with the preclinical findings, lower NPY levels have been reported in depressed patients (Caberlotto and Hurd, 2001; Heilig et al., 2004) and intranasal NPY insufflation can reduce depression symptom severity (Mathé et al., 2020). The increased NPY levels observed in the present study could reflect a compensatory mechanism which developed as an attempt to counteract excessive anxiety- and depression-like behaviours. Since NPY receptors were not investigated in the anxiety model, the functional significance of the results remains to be explored. In agreement with this hypothesis, recent experiments performed in transgenic mice exhibiting entopic NPY overexpression in GABAergic neurons demonstrated that no general change in anxiety-like and fear learning behaviours were revealed, in contrast

with the expected anxiolysis. In addition, a small but statistically significant decrease was observed in the time spent in open arms of the EPM. The mild anxiety-like phenotype was attributed to a decreased NPY receptors responsiveness to the ligand (Corder et al., 2020). Moreover, vector-mediated adult-onset hippocampal NPY overexpression in mice conferred a depressive-like phenotype (Lin et al., 2010), thus further supporting the notion that adaptations are developed to high NPY expression, which may bring about unanticipated behavioural responses. Our heuristic hypothesis is that the NPY system is altered and impaired in both anxiety and depression.

We have previously found in clinical and preclinical studies that CGRP plays a role in anxiety (Husum et al., 2002; Mathé et al., 1994; Wegener et al., 2012; Wörtwein et al., 2006). Therefore, we measured it in the current study. CGRP levels were higher in HAB compared to LAB rats in cortical regions, the hippocampus and PAG. Indeed, intracerebroventricular CGRP administration evoked behaviours suggestive of fear and anxiety responses (Kovács et al., 1999; Poore and Helmstetter, 1996). In experiments aimed at investigating CGRP-induced light-aversive behaviour, intracerebroventricular CGRP administration did not increase anxiogenic behaviour in the open field assay. However, an increased anxiety component could not be ruled out, since a significant increase in freezing behaviour induced by predator odour-evoked freezing was observed (Kaiser et al., 2012; Recober et al., 2009). Likewise, when locally infused in the bed nucleus of the stria terminalis in rats, CGRP induced neural activation in anxiety-related brain regions and produced an anxiogenic behavioural profile (Sink et al., 2011). In the Flinders Sensitive Line (FSL) - a genetic rat model of depression (Wegener et al., 2012) we detected elevated CGRP levels in comparison to the Flinders resistant line (FRL) in brain regions involved in regulating emotional responses (Wörtwein et al., 2006). This effect was increased by exposure to early-life maternal deprivation (Wörtwein et al., 2006). However, maternal deprivation per se led to reduced CGRP levels in hippocampus of Wistar rats (Husum et al., 2002). Along similar lines, CGRP level was increased in the cerebrospinal fluid and hippocampus of rats showing a depressive-like phenotype after exposure to middle cerebral artery occlusion followed by chronic unpredictable mild stress in the model of post-stroke depression (Shao et al., 2015). Jiao et al. (2013) demonstrated that gestational environmental conditions which increased depressive-like behaviours in off-springs, also increased CGRP expression in the hippocampus through epigenetic alterations of promoter methylation. Finding that central infusion of a CGRP antagonist reduced depressive-like behaviours is in line with that result (Jiao et al., 2013). In depressed patients, significantly elevated CGRP levels were reported in cerebrospinal fluid and skin sweat patches (Cizza et al., 2008; Mathé et al., 1994). Thus, a tentative conclusion is that CGRP is a marker of stress, anxiety, and depression. However, as far as depressive-like behaviours are concerned, divergent data are also available, suggesting the possibility that CGRP increase may represent an adaptive response. Indeed, Hashikawa-Hobara et al. (2015) reported lower CGRP levels in the hippocampus in mice displaying depression-like behaviours after chronic stress exposure. Interestingly, CGRP treatment could prevent the development of depression-like behaviours, in case the administration preceded stress

exposure, while it was ineffective when infused afterwards (Hashikawa-Hobara et al., 2015). In agreement with these results, central CGRP administration decreased depression-like behaviours in the FST in two distinct mice strains (Schorscher-Petcu et al., 2009). Overall, these findings suggest that CGRP can be a protective agent against depression development as a consequence of exposure to stressful situation. Thus, the possibility that higher CGRP levels observed in HAB rats developed to counterbalance the depressive-like phenotype cannot be excluded. Therefore, we are suggesting that CGRP is a marker of stress and anxiety, with the possibility that increased CGRP may play a role in counteracting depression-like behaviours.

We found that NKA-LI was predominantly localised in hypothalamus and PAG, with higher levels in the HAB strain, which were significantly different in PAG in males and in hypothalamus in females. Published results have indicated that NKA is anxiogenic and depressogenic. In fact, central NKA administration in mice induced anxiogenic-like responses (Teixeira et al., 1996) and blocked the anxiolytic action of diazepam (Ribeiro and De Lima, 2002). Mutant mice lacking the *Tac1* gene coding for the NKA precursor display decreased anxiety- and depression-like behaviour in a variety of tests, thus suggesting a critical role for this gene in modulating emotional responses (Bilkei-Gorzo et al., 2002). Since both NKA and Substance P derive from the same precursor peptide, discriminating the specific contribution caused by NKA deletion to the phenotype is not possible. In genetic models of depressive behaviours in rats, the Fawn-Hooded and the FSL strains, higher NKA levels have been reported in cortical and hypothalamic regions, as well as in the PAG, whereas lower levels were observed in the ST (Husum et al., 2001; Mathé et al., 1998). In a gene-environment interaction model, i.e., exposure of FSL rats to postnatal maternal deprivation, the increase in NKA levels in cortical regions and in the PAG were further augmented (Husum et al., 2008). Chronic lithium normalised NKA levels (Husum et al., 2001), while electroconvulsive treatment increased NKA levels in cortical regions (Mathé, 1999). NKA acts by binding and activating its cognate receptor NK2 and NK2 antagonists have repeatedly shown an anxiolytic and antidepressant profile in animal models (Griebel et al., 2001; Louis et al., 2008; Steinberg et al., 2001). Overall, these findings agree with our conclusion that NKA increase observed in the HAB strain contributes to the anxious and depressive-like phenotype.

Another peptide that we investigated is neurotensin. In HAB rats, neurotensin levels were elevated in CCx and PAG in males and in hypothalamus in females. Neurotensin has been implicated in anxiogenic responses elicited by chronic stress exposure (Li et al., 2021; Normandeau et al., 2018). However neurotensin displays anxiolytic properties when injected in the ventral pallidum (Ollmann et al., 2015), but exerts no effect on anxiety when infused into amygdala (László et al., 2010). In addition, both agonists and antagonists of the neurotensin receptor NT1 are endowed with anxiolytic properties (Griebel et al., 2001). In humans, plasma neurotensin levels positively correlate with stress, anxiety, and depressiveness in obese women (Wölk et al., 2021). Overall, these findings suggest a role for neurotensin in anxiety responses, which are modulated by brain regions and context (Li et al., 2021; Normandeau et al., 2018; Ollmann et al., 2015). Similar contrasts

are reported regarding neurotensin role in depression. Our earlier studies demonstrated that neurotensin levels were higher in the FSL model of depression and that maternal separation further increased neurotensin in the NAc, hippocampus, and ECx (Ellenbroek et al., 2016). Elevated neurotensin levels were also observed in the Fawn-Hooded depression model (Mathé et al., 1998). On the other hand, neurotensin administration or treatment with NT1 agonists induce an antidepressant profile (Carey et al., 2017).

The last peptide we investigated was CRH. The only difference found was higher CRH-LI level in the hypothalamus of female HAB rats. These results partly confirm our previous data of higher CRH mRNA levels in male HAB compared to LAB rats (Bosch et al., 2006). The continuous selection of HAB and LAB rats for trait anxiety might consequently lead to the manifestation of adaptations in the CRH system, in addition to those in the vasopressin system (Keck et al., 2002). In similarity to the results presented here, there were no CRH mRNA differences in brain regions of the depressed FSL compared to their control strain (Zambello et al., 2008).

Our findings indicate distinct differences in neuropeptide levels between HAB and LAB rats in various brain regions. . In particular, our results highlight that a prominent differential role on anxiety-like behaviours is exerted by neuropeptides in hypothalamus, as also underlined by significant time percentage spent in open arms and neuropeptide levels associations, which is especially evident in females. The hypothalamus is reported to be a critical structure in the neurobiological modulation of fear conditioning and extinction, thus acting as a crucial determinant in anxiety-related disorders (Fischer, 2021). In addition to confirming previous data about the critical relevance of hypothalamic HPA axis hormones in anxiety-like behaviours, the present findings demonstrate that NKA and neurotensin may also provide a contribution which was not formerly recognised. Moreover, association findings showed that all investigated neuropeptides appear to contribute to triggering anxiety-like behaviours, with NPY expressed in cortical regions exerting a remarkable role. Line-related difference in depressive-like behaviours were instead associated mainly to sub-cortical structures, with NPY, CGRP, and NKA as major participants. Assessment of regional neuropeptide levels largely ignores dynamic processes of neuropeptide synthesis and neuronal release, as intracellular and extracellular peptide levels cannot be distinguished (Jurek and Neumann, 2018).

Limitations of this study are that we did not obtain data on release of the peptides in vivo. Moreover, we did not investigate the peptide receptors. Also, the possibility that false positive results may be comprised within the high number of variables assessed cannot be discarded. Strong points are that, to the best of our knowledge, this is the first report regarding NPY, CGRP, NKA, neurotensin, and CRH levels in brain regions of HAB and LAB rat strains and the first evidence of marked differences in peptide levels between the HAB LAB strains.

In conclusion, this is the first demonstration of distinct expression of NPY, CGRP, NKA, and neurotensin levels in selected brain regions of rats selected for high anxiety (- a genetic rat model of anxiety) versus low anxiety. The direction of changes and the regions affected, in particular for NPY, were different from those found in genetic and environmental models of depression and post-traumatic brain disorder. The pattern of neuropeptide changes together with previous findings of decreased NPY in depression and increased CGRP and NKA following a variety of stressors indicate that we have identified some distinct biological correlates of anxiety- and depression-like behaviours, which might be useful as diagnostic markers and may facilitate novel therapeutic approaches.

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Figure legends

Figure 1: Behaviours observed in the elevated plus-maze test in male and female rats belonging to HAB and LAB lines. The percentage of time spent in open arms is displayed (A), as well as the percentage of entries in open arms (B). *: p<0.05; **: p<0.01; ***: p<0.001.

Figure 2: Behaviours observed in the forces swim test in rats belonging to HAB and LAB strains. Time (s) spent in struggling, swimming, or floating behaviours is displayed. A: male rats; B: female rats. *: p<0.05; **: p<0.01; ***: p<0.001.

Figure 3: PCA of neuropeptide levels in brain regions. A clear separation by HAB or LAB line is observed by adopting an unsupervised approach.

Figure 4: NPY-LI observed in brain regions of HAB and LAB rats expressed as pmol/tissue weight. A: male rats; B: female rats. Pfc: pre-frontal cortex; Fc: frontal cortex; Ccx: cingulate cortex; Ecx: entorhinal cortex; St: dorsal striatum; NAc: nucleus accumbens; Hc: hippocampus; Pag: periaqueductal grey; Ht: hypothalamus; Am: amygdala. *: p<0.05; **: p<0.01; ***: p<0.001.

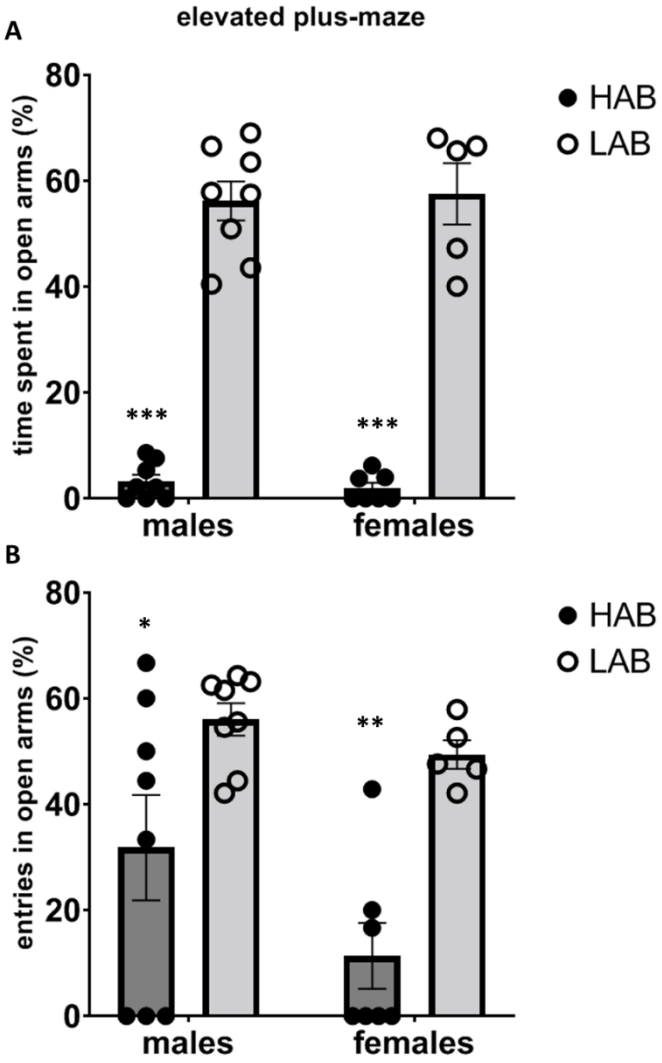
Figure 5: CGRP-LI observed in brain regions of HAB and LAB rats expressed as pmol/tissue weight. A: male rats; B: female rats. Pfc: pre-frontal cortex; Fc: frontal cortex; Ccx: cingulate cortex; Ecx: entorhinal cortex; St: dorsal striatum; NAc: nucleus accumbens; Hc: hippocampus; Pag: periaqueductal grey; Ht: hypothalamus; Am: amygdala. *: p<0.05; **: p<0.01; ***: p<0.001, #: p=0.054.

Figure 6: NKA-LI observed in brain regions of HAB and LAB rats expressed as pmol/tissue weight. A: male rats; B: female rats. Pfc: pre-frontal cortex; Fc: frontal cortex; Ccx: cingulate cortex; Ecx: entorhinal cortex; St: dorsal striatum; NAc: nucleus accumbens; Hc: hippocampus; Pag: periaqueductal grey; Ht: hypothalamus; Am: amygdala. *: p<0.05; **: p<0.01.

Figure 7: Neurotensin-LI observed in brain regions of HAB and LAB rats expressed as pmol/tissue weight. A: male rats; B: female rats. Pfc: pre-frontal cortex; Fc: frontal cortex; Ccx: cingulate cortex; Ecx: entorhinal cortex; St: dorsal striatum; NAc: nucleus accumbens; Hc: hippocampus; Pag: periaqueductal grey; Ht: hypothalamus; Am: amygdala. **: p<0.01; ***: p<0.001.

Figure 8: CRH-LI observed in brain regions of HAB and LAB rats expressed as pmol/tissue weight. A: male rats; B: female rats. Pfc: pre-frontal cortex; Fc: frontal cortex; Ccx: cingulate cortex; Ecx: entorhinal cortex; St: dorsal striatum; NAc: nucleus accumbens; Hc: hippocampus; Pag: periaqueductal grey; Ht: hypothalamus; Am: amygdala. **: p<0.01.

Figure 1


A
elevated plus-maze
time spent in open arms (%)
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males
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entries in open arms (%)
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60
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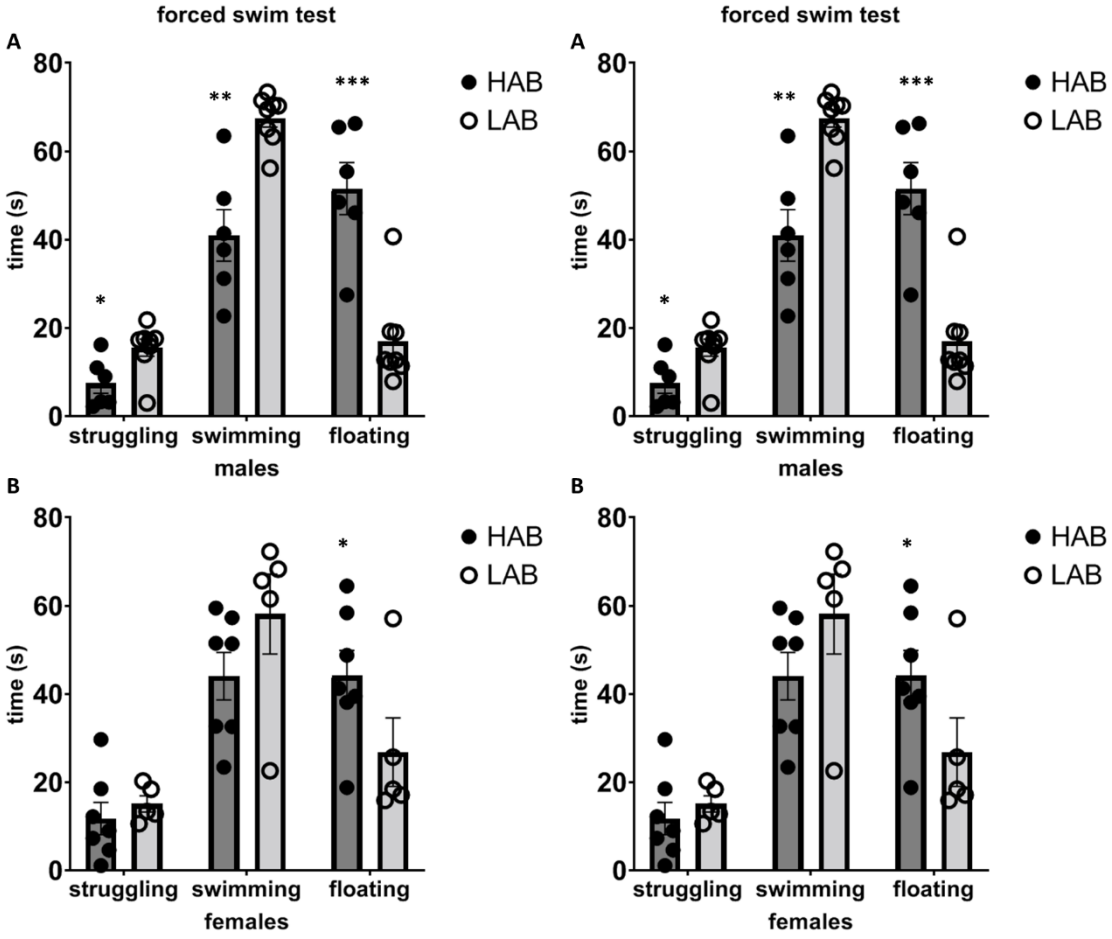
males
females
HAB
LAB

Figure 2


forced swim test
A
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time (s)
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HAB
LAB
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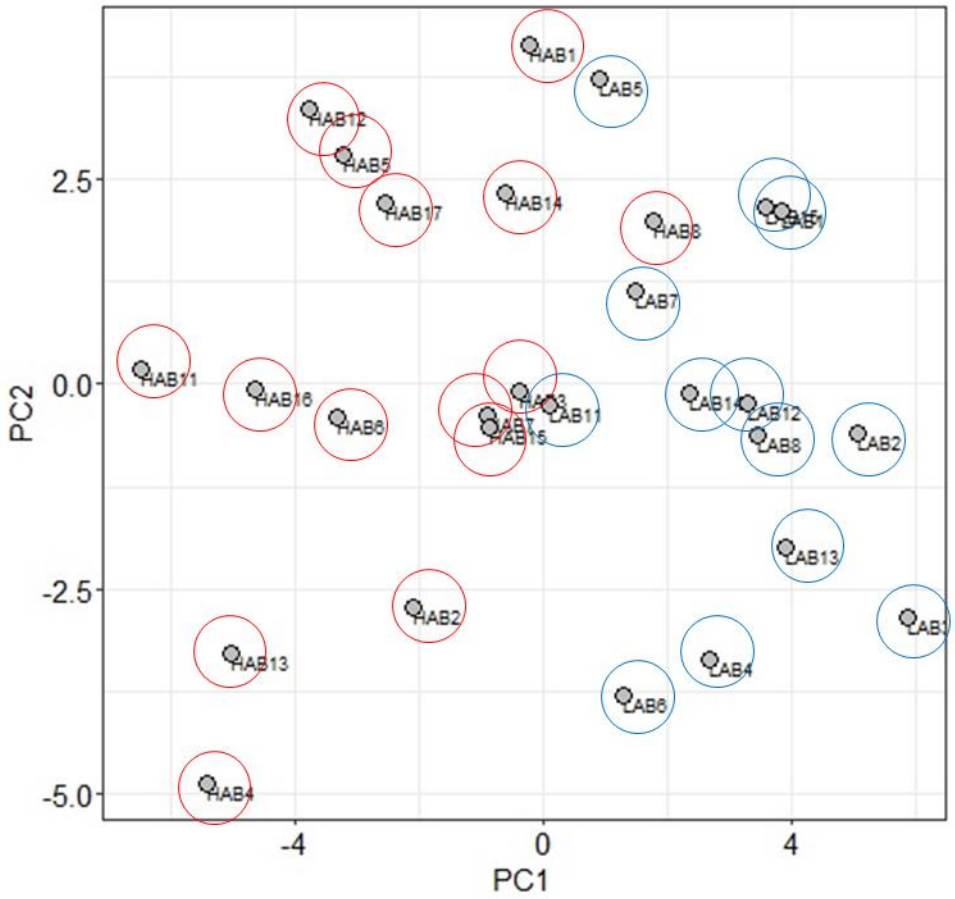
swimming
floating
males
B
females
forced swim test
A
B

Figure 3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HAB5
HAB17
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HAB8
LAB7
HAB11
HAB16
HAB6
LAB11
LAB14
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LAB8
LAB2
LAB13
HAB2
HAB13
LAB4
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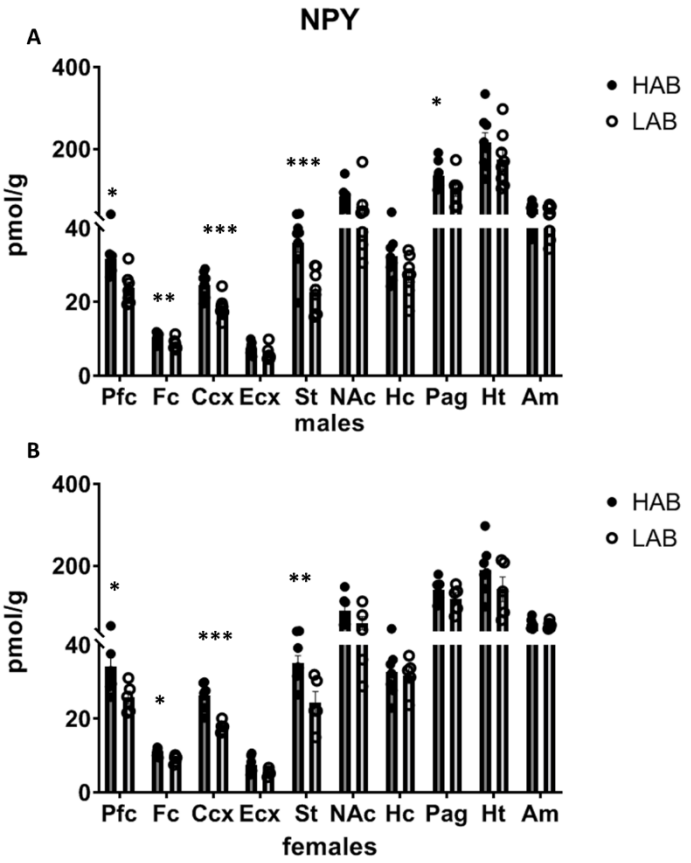
PC1

Figure 4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females

Figure 5

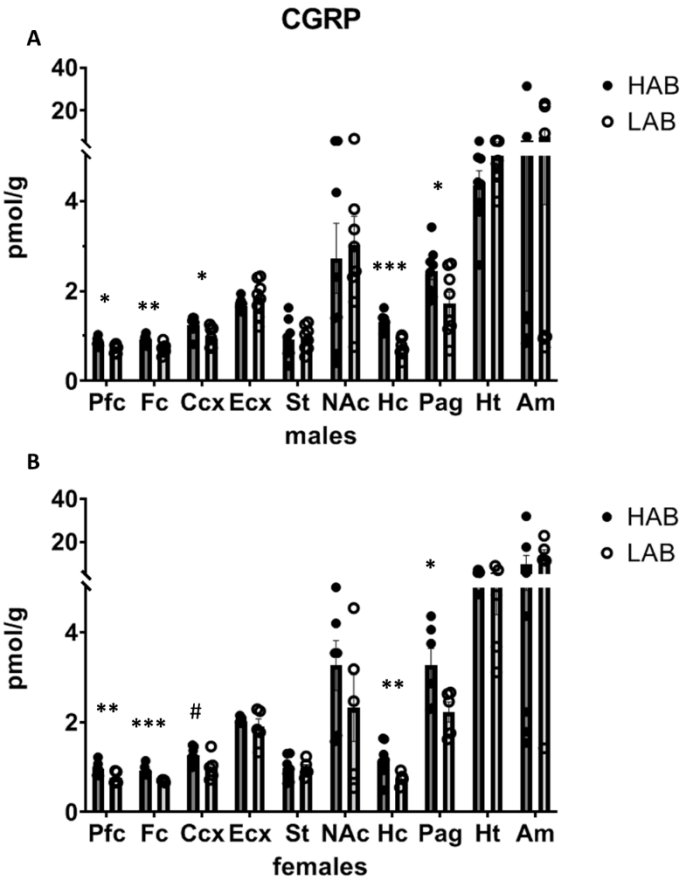
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Figure 6

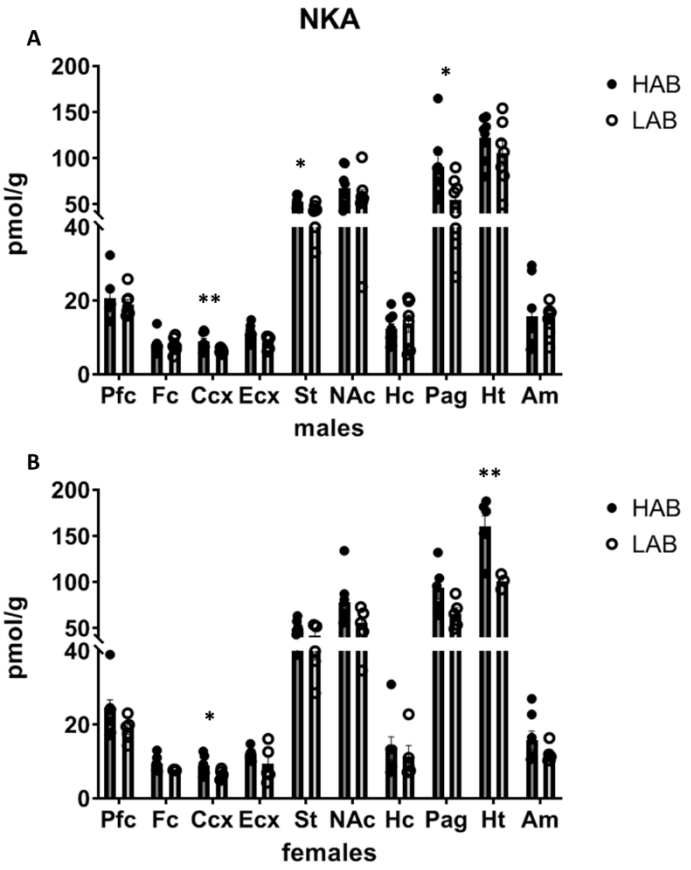
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Figure 7

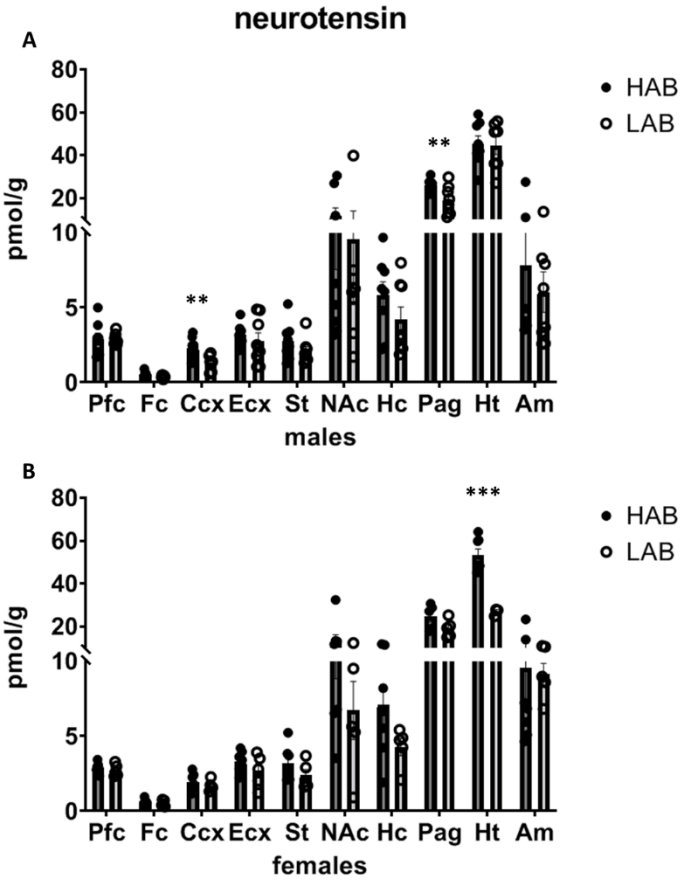
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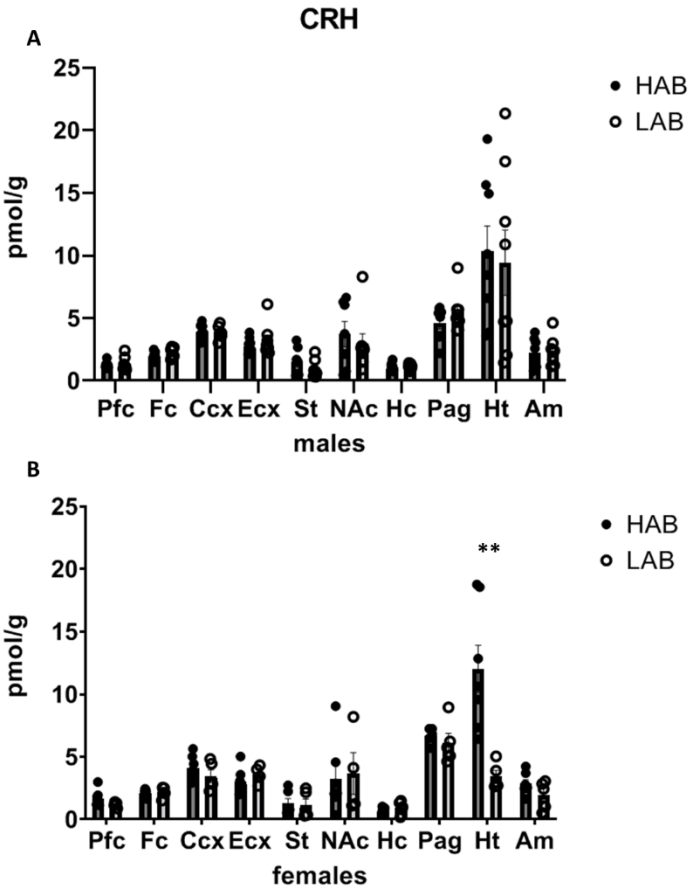
Figure 8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