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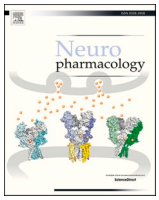

The translocator protein 18 kDa (TSPO) ligand etifoxine in an animal model of anxiety: Line- and sex-dependent effects on emotionality, stress reactivity, spine density, oxytocin receptors, steroids, and microbiome composition

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ABSTRACT

The treatment of stress-related disorders such as anxiety and depression is still challenging. One potential therapeutical option are neurosteroids. Their synthesis is promoted by ligands of the mitochondrial translocator protein 18 kDa (TSPO). We tested the TSPO ligand etifoxine (ETX) in a rat model of hyper-anxiety and depression-like behavior, i.e., in female and male HAB (high anxiety-related behavior) rats, as well as in respective low anxiety (LAB) and non-selected control (NAB) rats for behavioral, molecular, cellular, and physiological parameters. Daily acute i.p. treatment with ETX or vehicle over 5 or 9 days revealed that ETX was most effective in female HAB rats; it reduced anxiety levels (5 days) and OXT-R binding brain site-specifically (5 and 9 days), and increased spine density (5 days). The behavioral ETX effect exclusively found in female HABs was accompanied by increased 3β5α-THDOC levels, without any effect in female LABs and NABs and on other neurosteroids. In males of all breeding lines, ETX changed a total of 10 out of 23 brain steroids. Passive stress-coping during 10-min forced swimming was not affected by 9-day treatment with ETX, the resulting stress-induced plasma corticosterone levels were higher in ETX-treated NAB rats of both sexes compared with their VEH-treated groups. The fecal bacterial composition was similar but beta diversity differed between HABs and LABs and from NABs independent of sex; ETX treatment had no effect. Therefore, we propose considering the aspect of sex in treatment strategies for anxiety disorders. This is particularly important to establish better treatment regimens for women.

1. Introduction

Stress-related disorders such as anxiety and depression are among the most frequently diagnosed mental diseases worldwide and the leading cause for a high amount of disability-adjusted life-years (WHO, 2017). The treatment of such disorders is still a challenge. A rather delayed occurrence of a clinically meaningful therapeutic effect or various side effects after long-term treatment hamper the use of

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currently available established anxiolytics and antidepressants (Cheng et al., 2020; Rupprecht et al., 2009; Edinoff et al., 2021). An emergency add-on therapy to antidepressants are benzodiazepines due to their rapid onset of action especially in the early treatment phase (Furukawa et al., 2001; Ogawa et al., 2019; Olfson et al., 2015; Rupprecht et al., 2022). Benzodiazepines act as positive allosteric modulators of $GABA_A$ receptors via a specific binding site containing α subunits (Rudolph et al., 1999), thereby enhancing GABA-gated chloride currents. However, while benzodiazepines target only synaptic $GABA_A$ receptors, 3α-reduced steroids such as allopregnanolone (AlloP) can modulate both synaptic and extrasynaptic $GABA_A$ receptors. This can explain their distinct clinical profile (Meltzer-Brody and Kanes, 2020) resulting in reduced anxiety (Paul et al., 2020). Interestingly, also antidepressants, e.g., selective serotonin reuptake inhibitors, have been shown to interfere with steroidogenic enzymes, thus increasing AlloP levels (Griffin and Mellon, 1999; Schüle et al., 2006). A link between dysregulated GABAergic neurosteroids assessed in plasma or CSF and stress-related disorders has been found in patients suffering from depression and anxiety disorders. In these patients, antidepressant treatment affected the neurosteroid composition (Schüle et al., 2006; Uzunova et al., 1998; Romeo et al., 1998; Rupprecht, 2003; Ströhle et al., 2003). Based on these observations, neurosteroids currently attract considerable interest as therapeutical option in the context of stress-related disorders, especially postpartum depression or major depression (Meltzer-Brody et al., 2018; Althaus et al., 2020; Gunduz-Bruce et al., 2019; Riebel et al., 2024).

In addition to the administration of synthetic neurosteroids (Althaus et al., 2020; Gunduz-Bruce et al., 2019), enhancing endogenous steroidogenesis may constitute an attractive alternative. In fact, administration of synthetic steroids induces different steroid profiles, especially when given at hyper-physiological doses, in contrast to enhancement of endogenous steroidogenesis. In this context, the mitochondrial translocator protein 18 kDa (TSPO), which is expressed in steroidogenic tissues including the brain (Papadopoulos et al., 2006; Rupprecht et al., 2010), has been described as central for promoting steroid synthesis. TSPO is located in the outer mitochondrial membrane and mediates the transport of cholesterol to the mitochondrial matrix thereby facilitating steroidogenesis. TSPO ligands, such as XBD173 and etifoxine (ETX) can enhance this process resulting in increased steroidogenesis, such as the synthesis of the precursor pregnenolone (PREG), and the 3α-reduced neurosteroids such as AlloP enhancing GABAergic neurotransmission (Papadopoulos et al., 2018; Rupprecht et al., 2009, 2010; Nothdurfter et al., 2012a; Wolf et al., 2015; Barron et al., 2021). In human and animal studies, these TSPO ligands exert anxiolytic effects (Rupprecht et al., 2009, 2010; Nothdurfter et al., 2012a, 2012b) with a comparable anxiolytic efficacy as benzodiazepines (Nguyen et al., 2006; Vicente et al., 2020). However, the observed side effects of benzodiazepines (Rupprecht et al., 2009) have not been reported for TSPO ligand treatment so far. Moreover, TSPO ligands may also act as modulators of brain inflammation and bioenergetics (Setiawan et al., 2015).

Although TSPO became a promising target for the development of novel treatment options (Nguyen et al., 2006; Micallef et al., 2001; Rupprecht et al., 2010, 2022), further neuropharmacological studies on the effects of TSPO ligands in suitable animal models are crucial to characterize the role of TSPO in stress-related psychiatric disorders. So far, only few studies investigated the TSPO system in animal models of psychopathologies (psychosis: Fukudome et al., 2018; postpartum depression: Li et al., 2018). In this context, rats selectively bred for high (HAB) and low (LAB) anxiety-related behavior are among the best established and characterized animal models in terms of genetic and neuroendocrine factors involved in anxiety-related and depressive-like behaviors (Neumann and Landgraf, 2012; Wegener et al., 2012; Gryksa et al., 2023). The continuous selection of these rats is based on their performance in a validated anxiety test, i.e., the elevated plus-maze (EPM) (Liebsch et al., 1998; Landgraf and Wigger, 2002; Gryksa et al., 2023). Importantly, the selective breeding for extremes in innate anxiety-related behavior is consistently associated with differences in depressive-like behavior. HAB rats rather display passive stress-coping accompanied by an increased neuroendocrine response of the hypothalamo-pituitary-adrenal (HPA) axis to emotional stressors. The breeding lines also substantially differ in various social behaviors including social motivation, intermale aggression, and maternal behavior (for review see Gryksa et al., 2023). Some neurobiological mechanisms underlying those extremes in behavior of HAB versus LAB rats have been revealed in detail and include differences in the neuropeptide balance, i.e., the arginine vasopressin, oxytocin (OXT) and corticotropin-releasing factor systems (Murgatroyd et al., 2004; for review see Gryksa et al., 2023). HAB and LAB rats were successfully used as valuable animal model of psychopathology to test the potency of various anxiolytic and antidepressive compounds (Neumann and Slattery, 2016; Schmidtner et al., 2019; for review see Gryksa et al., 2023).

Here, we have studied the sex- and breeding line-dependent effects of the clinically available TSPO ligand ETX applied intraperitoneally (i.p.) in female and male rats of the HAB and LAB breeding lines complemented by rats non-selected for anxiety-related behavior (NAB). In an initial experiment, anxiety-related behavior was determined after 5 days of treatment, and the same rats were tested on the 9th day of ETX treatment for passive stress-coping (forced swim test; FST), the hormonal stress response, and OXT-R binding in selected brain regions due to the involvement of the brain OXT system in anxiety-related behavior (Jurek and Neumann, 2018). In follow-up experiments with 5-day ETX treatment, we re-evaluated the effect of on OXT-R binding and further studied potential changes in brain neurosteroid levels. Furthermore, we investigated changes in spine density after 5-day ETX treatment as the resulting changes in steroid signaling could alter dendritic complexity and spine density via $GABA_A$ receptors (Afroz et al., 2017; Wang et al., 2013). Finally, as HAB and NAB rats differ in their microbiome composition under basal conditions (Schmidtner et al., 2019) and ETX has subtle effects on the human microbiome (Manook et al., 2023), we studied whether 5-day treatment with ETX affects the fecal microbiome composition in rodents. This is of interest since (i) the gut microbiome has been related to anxiety and depressive-like symptoms (Johnson and Foster, 2018), and (ii) bacterial and mammalian TSPO show similarities in structural and functional characteristics (Chapalain et al., 2009; Isokpehi et al., 2017).

2. Materials and methods

2.1. Animals

Male (280–300 g) and virgin female (220–250 g) HAB and LAB rats (local breeding colony; for selection procedures see (Gryksa et al., 2023)) as well as NAB Wistar rats (experiment 1: Charles River Laboratories, Sulzfeld, Germany; experiment 2, 3: local breeding colony) at an average age of 15 weeks were kept under standard laboratory conditions (change of bedding once per week; 12 : 12 h light/dark cycle, lights on at 7 a.m.; RT 22 ± 2 °C, 55 ± 5 % relative humidity) in same-sex, same breeding-line groups of up to 4 rats with access to water and standard rat chow *ad libitum*. To avoid litter-effects, siblings were distributed to different groups and/or different experiments. Female rats were tested daily for their cycle stage, and experiments were only performed in females being in met-/diestrus, i.e., when female rats show the highest anxiety-related behavior (D'Souza and Sadananda, 2017), thereby allowing to reveal potential anxiolytic effects. The stage of the estrous cycle was confirmed by taking vaginal smears following the experiment on the respective days. All rats were handled carefully twice a day to familiarize them with the experimental procedures and to reduce non-specific stress responses during the experiment.

The studies were conducted in accordance with the ARRIVE guidelines and the European Communities Council Directive (86/609/EEC) and received approval by the local government. All efforts were made to minimize the number of animals used and their suffering. Number of rats

per group were between 6 and 12.

2.2. Treatment with ETX suspension

The suspension of ETX (2-ethyl-amino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride) was prepared according to (Liere et al., 2017). Briefly, ETX was dissolved in vehicle (VEH), i.e., NaCl 0.9 % containing 1 % Tween 80 (Sigma-Aldrich). The mixture was prepared freshly every morning and maintained with constant agitation. At 9 a.m., rats received an i.p. injection of ETX or VEH at a volume of 50 mg/kg/5 ml (Liere et al., 2017).

2.3. Experimental schedule

Graphical overviews of the different experimental schedules are depicted in Fig. 1.

2.3.1 Experiment 1: In the initial experiment, rats were daily treated with either VEH (5 ml) or ETX (50 mg/kg/5 ml, i.p.) at 9 a.m. for 9 consecutive days. All tests were performed 30 min after the injection. On day 5, rats were tested in the light-dark box (LDB). On day 9, rats were tested in the FST. Ten minutes after ending the FST, rats were rapidly sacrificed, and brain and trunk blood were collected for OXT-R autoradiography and corticosterone measurement, respectively.

Since in experiment 1, behavioral effects were only apparent on the 5th day of daily ETX injection, we continued with the 5-day treatment regime in experiments 2 and 3.

2.3.2 Experiment 2: Rats were daily injected with either VEH (5 ml) or ETX (50 mg/kg/5 ml, i.p.) at 9 a.m. for 5 consecutive days. Thirty minutes after the last injection, rats were rapidly sacrificed, and brains prepared for OXT-R autoradiography and steroid analysis (one hemisphere, each). Furthermore, fecal boli were collected before the first and after the last injection to determine the fecal microbiome composition.

2.3.3 Experiment 3: Rats were daily injected i.p. with either VEH (5 ml) or ETX (50 mg/kg/5 ml, i.p.) for 5 consecutive days. Thirty minutes after the last injection, rats were rapidly sacrificed, and brains were prepared for Golgi-Cox staining.

2.4. Anxiety-related behavior in the light-dark box

On the 5th day of treatment, anxiety-related behavior was tested in the LDB, which creates a conflict between the rodent's natural aversion to bright areas and its exploratory behavior (Crawley and Goodwin, 1980). Briefly, the LDB consists of a lit (40 × 50 cm, 100 lux) and a dark (40 × 30 cm, 0 lux) compartment, which are connected via a small opening (7.5 × 7.5 cm) to enable the rats to move between the two compartments. Thirty min after the last treatment, rats were placed into the lit compartment of the LDB. During the 5-min test session, rats could freely roam around the compartments. The time spent in the lit box was taken as measure for anxiety-related behavior. The behavior was videotaped for later analysis using an automated video tracking system (EthoVision XT, version 12, Noldus, Wageningen, NL).

2.5. Passive stress-coping in the forced swim test

On the 9th day of treatment, passive stress-coping behavior was assessed in the adapted FST (Porsolt et al., 1977; Slattery and Cryan, 2012). Briefly, the FST tank consists of a perspex cylinder (height 50 cm, diameter 30 cm) filled with water (23 ± 1 °C) to a height of 30 cm. Thirty min after the last treatment, rats were placed into the water for 10 min, after which they were gently dried with a towel and returned to their home cage. The behavior was videotaped for later analysis performed by an experienced experimenter blind to the treatment. Active (struggling, swimming) and passive stress-coping behavior (floating) were scored using JWatcher (https://www.jwatcher.ucla.edu).

2.6. Plasma corticosterone measurement

Ten minutes after termination of the FST, rats were rapidly decapitated, trunk blood was collected in EDTA-coated tubes on ice (Sarstedt, Numbrecht, Germany), centrifuged (5000 rpm, 10 min at 4 °C), aliquoted and stored at −20 °C until the assay was performed using a commercially available ELISA kit for corticosterone (IBL International, Hamburg, Germany).

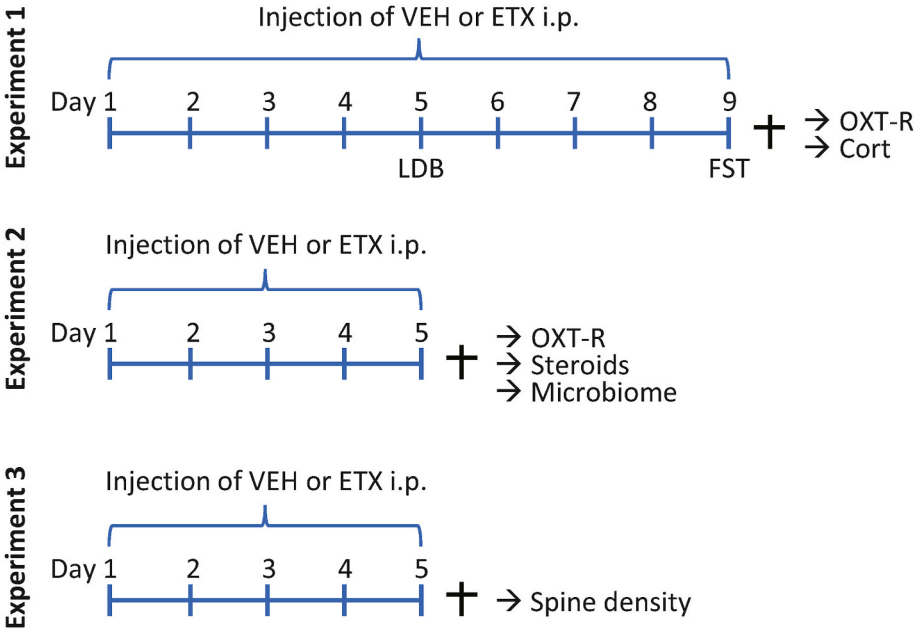

Fig. 1. Experimental schedules. All rats received daily an acute injection of etifoxine (ETX; 50 mg/kg i.p.) or vehicle (VEH). In experiment 1, the treatment lasted 9 days (experiment 1), and rats were tested for anxiety-related behavior in the light-dark box (LDB) on day 5 and passive stress-coping behavior in the forced swim test (FST) on day 9, after which brains (OXT-R binding) and blood (corticosterone) were sampled. In experiments 2 and 3, rats received the same drug treatment over 5 days; brains (experiment 2: OXT-R binding, steroids; experiment 3: spine density) and fecal boli (experiment 2: microbiome) were sampled.

2.7. OXT-R autoradiography

For analysis of OXT-R binding, receptor autoradiography was performed on two sets of brains. From experiment 1, brains were taken 10 min after termination of the FST on treatment day 9 (whole brain). From experiment 2, brains were taken after 5 days of treatment without further manipulations. In the latter, each brain was divided into two hemispheres, one being used for OXT-R binding, the other one was analyzed for steroid patterns as described below. All brains were flash-frozen in n-methylbutane and stored at −20 °C until cutting into coronal 16-µm cryosections (CM3050S, Leica Microsystem GmbH). For each brain region of interest, i.e., central amygdala (CeA), nucleus accumbens shell (NAcc shell), dorsal (dLS) and ventral lateral septum (vLS), ventral medial hypothalamus (VMH), six slices per rat were collected on SUPERFROST microscope slides and stored at −20 °C until further processing. The OXT-R autoradiography was performed for each brain region in female and male samples separately following an established protocol (Oliveira et al., 2021) using ornithin-vasotocin analogue [^{125}I]-OVTA [d (CH_2)$_5$ [Tyr (Me)^2,Thr^4,Orn^8, [^{125}I]Tyr^9-NH_2]; PerkinElmer, USA) as a tracer. Briefly, slides were thawed and left at room temperature until thoroughly dry. The tissues were fixed in 0.1 % PFA, washed 2 x in Tris (50 mM, pH 7.4), covered with tracer solution (50 mM tracer, 10 mM $MgCl_2$, 0.1 % BSA) for 60 min, washed 3 x in Tris/MgCl2 buffer for 7 min, each, followed by 30 min spinning in Tris/$MgCl_2$. Finally, slides were dipped into water and air dried before being exposed to Biomax MR films (Kodak, Cedex, France) for three days (experiment 1) or 7 days (experiment 2). All films were digitized using the EPSON Perfection V800 Scanner (Epson GmbH, Munich, Germany). For each region of interest, which were anatomically identified using a stereotaxic rat brain atlas (Paxinos and Watson, 2013), the optical density was analyzed using ImageJ (Fiji, ImageJ V. 2.1.0, http://www.imagej.net) by subtracting the background activity as previously described (Bosch and Neumann, 2008; Oliveira et al., 2021).

2.8. Steroid pattern measurements by gas chromatography tandem mass spectrometry (GC-MS/MS)

Steroids were extracted from the brain hemispheres of adult male NAB (average weight of hemispheres: 700.2 ± 94.2 mg), HAB (653.0 ± 52.4 mg), LAB rats (649.4 ± 49.0 mg), and of adult female NAB (640.2 ± 52.4 mg), HAB (642.2 ± 37.4 mg) and LAB rats (600.4 ± 54.7 mg) with 8 ml methanol. The internal standards added to the extracts for steroid quantification are shown in Table S1 (Supplementary Material). Following sonication for 5 min and centrifugation at 15,000 rpm for 5 min, the supernatant was withdrawn, and 5 ml methanol was added on the residue. After a centrifugation step at 15,000 rpm for 5 min, the supernatants were pooled and aliquots corresponding to 400 mg of rat brain were collected.

Samples were then purified and fractionated by solid-phase extraction (SPE) with the recycling procedure (Liere et al., 2009). The extracts were dissolved in 1 ml of methanol and applied to the C18 cartridge (500 mg, 6 ml; International Sorbent Technology, Norcross, USA), followed by 5 ml of methanol/H_2O (85/15). The flow-through containing the free steroids was collected and dried. After a previous reconditioning of the same cartridge with 5 ml of MeOH/H_2O (2/8), the dried samples were dissolved in methanol/H_2O (2/8) and reapplied. The cartridge was then washed with 5 ml of H_2O and 5 ml of methanol/H_2O (4/6) and unconjugated steroids were eluted with 5 ml of methanol/H_2O (9/1). The unconjugated steroid-containing fraction was then filtered and further purified and fractionated by high performance liquid chromatography (HPLC). The HPLC system was composed of a WPS-3000SL analytical autosampler and a LPG-3400SD quaternary pump coupled with a SR-3000 fraction collector (Thermo Fisher Scientific, San Jose, USA). The HPLC separation was achieved with a Lichrosorb Diol column (25 cm, 4.6 mm, 5 µm) in a thermostatic block at 30 °C. The column was equilibrated in a solvent system of 90% heptane and 10% of a mixture composed of heptane/isopropanol (85/15). Elution was performed at a flow rate of 1 ml/min, first with 90% heptane and 10% of heptane/isopropanol (85/15) for 15 min, then with a linear gradient to 100% acetone in 2 min. This mobile phase was kept constant for 13 min. Two fractions were collected from the HPLC system: 5α/β-DHPROG were eluted in the first HPLC fraction (4–17 min) and all other more polar steroids were eluted in the second fraction (17–30 min). The latter fraction was derivatized with 25 µl of heptafluorobutyric anhydride (HFBA) and 25 µl of anhydrous acetone for 60 min at 80 °C. Both fractions were dried under a stream of N_2 and resuspended in heptane for the GC-MS/MS analysis. The brain extracts were injected using an AI 1310 autosampler and analyzed by a Trace 1310 GC and a TSQ 9000 MS/MS (Thermo Fisher Scientific) using argon as the collision gas. Injection was performed in the splitless mode at 250 °C (1 min of splitless time) and the temperature of the GC oven was initially maintained at 80 °C for 1 min and ramped between 80 °C and 350 °C at 10 °C/min. The helium carrier gas flow was maintained at 1 ml/min during the analysis. The transfer line and ionization chamber temperatures were 330 °C and 200 °C, respectively. Electron impact ionization was used for mass spectrometry (MS) with ionization energy of 70 eV. GC-MS/MS signals were evaluated using the software Excalibur, release 4.1 (Thermo Fisher Scientific). Identification of steroids was supported by their retention time and according to 3 multiple reaction monitoring (MRM) transitions. Quantification was performed according to the transition giving the more abundant product ion of the targeted steroid with a previously established calibration curve.

The analytical protocol has been validated for all the targeted steroids using extracts of 200 mg from a pool of C57Bl/6J male mice brain. The validation included the limit of detection, linearity, accuracy, intra-assay, and inter-assay precision (Zhu et al., 2017; Fernandez et al., 2023). The limit of detection was determined as the lowest amount of steroids that can be measured by GC-MS/MS with a signal-to-noise ratio >3 and ranged from 0.5 to 20 pg/g. When steroid concentrations were below the limit of detection, the limit of detection was used as an arbitrary concentration for the corresponding steroid. The linearity was assessed by analyzing increasing amounts of mouse brain extracts (20, 50, 100, and 200 mg) in triplicate. The linearity was satisfactory for all the steroids with a coefficient of correlation ranging from 0.992 to 0.999. The accuracy of the assay was evaluated by determining the analytical recovery, which was defined as C/(C0 + S) × 100 (%), where C is the concentration of the steroid in the spiked brain extract (200 mg), C0 is the concentration of a steroid in the unspiked brain extract (200 mg), and S is the spiked concentration. The range of accuracy was 95–107 %. The precision of the intra-assays and inter-assays were evaluated by analyzing 5 replicates of 200 mg of brain extracts on 1 day and over 4 days, and the coefficient of variation was found around 1.1–8.7 % and 1.7–9.2 %, respectively. All steroids brain concentrations are expressed in ng/g.

2.9. Microbiome sequencing and data processing

To test for effects of ETX treatment on the microbiome, fecal boli were collected before the first treatment and on the 5th day of treatment appr. 30 min after the last injection with VEH or ETX, snap frozen using dry ice and stored at −80 °C until further analyses.

Microbial DNA was extracted from 25 mg of frozen fecal boli (−80 °C) using Lysing Matrix Y beads (MP Biomedicals, Solon, USA) and a TissueLyzer II (Qiagen, Hilden, Germany) for bead beating. Nucleic acids were purified with the MagNA Pure 96 system (Roche Diagnostics, Rotkreuz, Switzerland). Bacterial 16S rRNA gene copy numbers were quantified using qPCR on a LightCycler 480 II (Roche Diagnostics), following (Stämmler et al., 2016). Universal primers 764F and 907R, along with SYBR Green I Master mix (Roche Diagnostics), were used in the reactions. Full-length 16S rRNA genes from human fecal DNA were cloned into the pGEM-T Easy vector (Thermo Fisher Scientific, Waltham MA, USA) for standard quantification. Microbiome sequencing followed

an ISO 15189 accredited workflow. The V3-V4 regions of the 16S rRNA gene were amplified using primers 341F/815R from DNA normalized to 1E7 16S rRNA gene copies per sample. Barcoded PCR products were pooled and purified with MagSi-NGSPREP-PLUS beads (Steinbrenner Laborsysteme GmbH, Wiesenbach Germany) at a 1:1,2 DNA to bead ratio. Sequencing libraries were quantified with the Ion Library TaqMan™ Quantitation Kit, and sequencing was conducted using the Ion GeneStudio S5 Plus system (Thermo Fisher Scientific). Raw data from Torrent Suite 5.18 was processed using cutadapt 4.4 (Martin, 2011) to remove adapters and primers, followed by demultiplexing. Sequences were filtered using Trimmomatic 0.39 with a sliding window (10 bases, quality threshold of 15), and reads shorter than 250 bp were discarded. High-quality reads were processed with vsearch 2.28.1 (Rognes et al., 2016), and those with more than five expected errors were excluded. Zero-radius operational taxonomic units (zOTUs) were generated with an alpha value of 2 and a minimum read count of 5. Chimeras were removed using uchime3_denovo, and reads with ≥98% identity were aligned to zOTUs using usearch_global. Taxonomic classification was performed in R (version 4.4.0) using the IDTAXA classifier from DECIPHER (version 2.26.0) (Murali et al., 2018) and the Genome Taxonomy Database (GTDB, release 220) (Parks et al., 2018). A 98% bootstrap confidence cutoff and a threshold of 40 for taxonomic identification were applied.

2.10. Spine density

The Golgi-Cox method was used to study the morphology of neuronal dendrites and dendritic spines of rats of experiment 3, i.e., after 5 days of ETX or VEH i.p. treatment. 30 min after the last injection, brains were removed and stained with the FD Rapid Golgi Stain™ Kit (FD Neuro Technologies, Columbia, MD, US) according to an established protocol of the manufacturer (Du, 2019). Briefly, after brains were rinsed under fresh water and immersed in different impregnation solutions (A + B for 3 weeks, C for 1 week) in the dark at room temperature, they were cut into 100-μm thick coronal sections using a cryostat (Leica, Wetzlar, Germany) and mounted onto gelatinated microscope slides, which were stained and cover-slipped using permount mounting medium. The dendritic spine density was analyzed in the CeA, hippocampus, dLS, vLS, and paraventricular nucleus (PVN) using an epi-fluorescence microscope (Thunder Imaging Systems, Leica). Digital images were processed using the Leica Application Suite X (Leica) and ImageJ (Fiji). At the beginning, an overview image of every region was compiled using the spiral feature of LAS X. From that image, about 8 secondary dendrites of ≥10 μm length from at least 4 different neurons were selected for z-stack-photography. In rare cases, this condition could not be fulfilled due to unsatisfactory staining of neurons and, therefore, less images could be taken. Exposure time, contrast, intensity, and aperture of the microscope were set at optimal spine visibility and remained unchanged for every region. Spines were counted using Fiji's multi-point feature, and the dendrites' length was measured with the freehand-line tool. Spine density is expressed as number of spines per 10 μm.

2.11. Statistics

Statistical analysis was performed using SPSS (Version 26; SPSS Inc, Chicago, IL, USA) or GraphPad Prism for steroid analysis (Version 10.0; GraphPad Inc, San Diego, CA, USA). Either three-way (factors sex; breeding line; treatment), two-way or one-way analysis of variance (ANOVA), or the non-parametric Kruskal–Wallis test (if data were not normally distributed) were applied. Normality of the data sets was checked using the Shapiro–Wilk tests. When applicable, post-hoc tests were carried out using either Turkey's, Bonferroni, Fisher's LSD-test, or Mann-Whitney *U* test. The outliers were identified by using the Grubb's test. All data are presented as mean ± SEM; the significance level was set at $p \leq 0.05$.

For microbiome data, statistical analyses were conducted in R (version 4.4.0) with the Inverse Simpson index used to measure alpha diversity via the mia package (version 1.1.7). Relative abundance plots were created using microeco (version 1.6.0) (Liu et al., 2021). Linear mixed-effects models (LME), fitted with REML using lmerTest::lmer (version 3.1–3), accounted for fixed effects of treatment, sampling timepoint, breeding lines, and sex with individual animals as random effects. Statistical inference was performed using likelihood-ratio tests (lmerTest::anova), and post-hoc comparisons were performed with emmeans::emmeans (version 1.8.4–1). One-way anova was used to test for differences in alpha diversity between breeding lines in female and male animals at baseline. Differential abundance was assessed using MicrobiomeStat::LinDA (version 1.1) (Zhou et al., 2022), filtering features with <0.1% mean abundance and <1% prevalence. zOTUs with an alpha value < 0.1 were considered significant. Differences in beta diversity was analyzed by Permutational multivariate analysis of variance (PERMANOVA) with pairwiseAdonis::pairwise.adonis2 (Martinez Arbizu, 2020). P-values were adjusted using the Benjamini-Hochberg method.

3. Results

3.1. Experiment 1: effect of daily i.p. ETX on innate emotionality, corticosterone response, and OXT-R binding in female and male HAB, LAB and NAB rats

3.1.1. Anxiety-related behavior in the LDB on day 5 of treatment

Female HAB, LAB and NAB rats differed in their anxiety-related behavior depending on the treatment (two-way ANOVA, factors breeding line x treatment: $F_{2,57} = 3.71$; $p = 0.031$; Fig. 2A). The time spent in the light compartment was lower in HAB compared to LAB ($p = 0.04$) or NAB rats ($p = 0.01$); whereas LAB and NAB rats did not differ. Treatment with ETX increased the time spent in the light compartment in HAB females ($p = 0.01$), only. Regarding locomotion, female rats differed depending on the breeding line (factor breeding line: $F_{2,57} = 29.43$; $p = 0.000$; data not shown) and treatment (factor treatment: $F_{2,57} = 7.61$; $p = 0.008$), but no interaction was detected. In detail, HAB rats showed the least locomotion compared to LAB and NAB rats ($p = 0.01$), and LAB rats showed less locomotion compared with NABs ($p = 0.01$). Treatment with ETX decreased locomotion in HAB rats ($p = 0.04$), only.

Male HAB, LAB and NAB rats also differed in anxiety-related behavior (factor breeding line: $F_{2,56} = 5.32$; $p = 0.008$; Fig. 2B), but no treatment or interaction effects were detected. In detail, male HAB rats spent less time in the light compartment than LAB ($p = 0.04$) and NAB ($p = 0.01$), whereas LAB and NAB rats did not differ. With respect to locomotion, male rats differed between the breeding lines (factor breeding line: $F_{2,56} = 9.46$; $p = 0.000$) and treatments (factor treatment: $F_{2,56} = 5.10$; $p = 0.028$; data not shown), but no interaction was detected. Male HAB rats moved significant less than LABs ($p = 0.01$) and NABs ($p = 0.01$), whereas LAB and NAB rats did not differ. Treatment with ETX led to reduced locomotion in LAB males ($p = 0.01$), only.

3.1.2. Passive stress-coping behavior in the FST on day 9 of treatment

The time spent floating as a measure of passive stress-coping behavior significantly differed between the breeding lines in females (factor breeding line: $F_{2,57} = 18.83$; $p = 0.000$; Fig. 2C) and males (factor breeding line: $F_{2,56} = 25.53$; $p = 0.000$; Fig. 2D), but no treatment or interaction effect was detected. In females, post-hoc analysis revealed that VEH-treated LAB rats showed less floating compared to female HAB ($p = 0.01$) and NAB rats ($p = 0.01$), which did not differ from each other. In males, VEH-treated NAB rats showed more floating than HAB ($p = 0.01$) and LAB rats ($p = 0.01$).

3.1.3. Corticosterone response on day 9 of treatment

Plasma corticosterone levels in response to 10-min forced swimming significantly differed between breeding lines and treatments in female (factors breeding x treatment: $F_{2,57} = 4.63$; $p = 0.014$; Fig. 2E) and

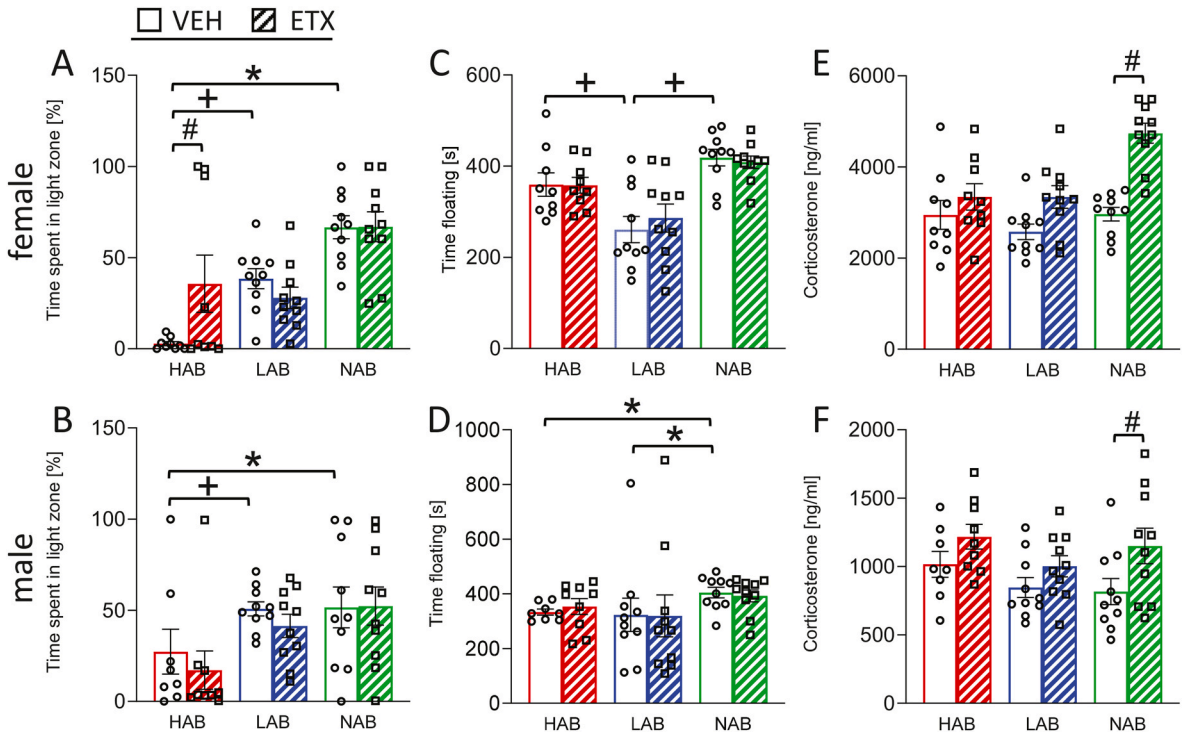

Fig. 2. Anxiety-related behavior, passive stress-coping behavior and the immediate stress response in female and male HAB, LAB and NAB rats after VEH or ETX treatment. Effects of 9-day etifoxine (ETX; 50 mg/kg i.p.) or vehicle (VEH) treatment on anxiety-related behavior on day 5 (A, B), as well as passive stress-coping behavior in the forced swim test (C, D) and the immediate stress response (E, F) on day 9 in female (top row) and male (bottom row) HAB, LAB and NAB rats. (A, B) In the VEH-treated groups, HAB rats were more anxious than LAB rats. ETX treatment decreased anxiety-related behavior in the light-dark box as reflected by time spent in the light compartment in female HAB rats, only. (C) VEH-Treated HAB and NAB females showed more floating behavior than LABs. (D) In males, only VEH-treated NABs showed more floating behavior than HAB and LAB rats. (C, D) ETX had no effect on floating duration in the forced swim test in any group. (E, F) Ten minutes after termination of the forced swim test, plasma corticosterone levels were higher in ETX-treated female and male NAB rats. Data are presented as mean ± SEM. *p < 0.05 vs. NAB; + $p < 0.05$ vs. LAB; #$p < 0.05$ vs. VEH.

between treatments in male (factor treatment: $F_{2,56} = 8.58$; $p = 0.005$; factor breeding line: $F_{2,56} = 2.02$; $p = 0.143$; Fig. 2F) NAB rats. Post-hoc analysis revealed that ETX treatment increased stress-induced corticosterone levels in female ($p = 0.01$) and male ($p = 0.02$) NAB rats, only.

3.1.4. OXT-R binding on day 9 of treatment

OXT-R binding was analyzed by receptor autoradiography in various brain regions relevant for emotionality. In the CeA of females, differences in OXT-R binding were found depending on the breeding line and treatment (factors line x treatment: $F_{2,38} = 3.63$; $p = 0.022$; Fig. 3A). Post-hoc test with Bonferroni corrections revealed lower OXT-R binding in the CeA of ETX-treated HAB females ($p = 0.023$ vs. VEH). No further differences were observed in females or males either between the breeding lines or depending on treatment in any other brain region, i.e., NAcc shell, dLS, vLS, VMH.

3.2. Experiment 2: effect of daily i.p. ETX on OXT-R, steroids and microbiome in female and male HAB, LAB and NAB rats

3.2.1. OXT-R binding on day 5 of ETX treatment

OXT-R binding was analyzed in one brain hemisphere of female and male HAB, LAB and NAB rats after 5-days of treatment with ETX or VEH in the same brain regions as in the 9-day treatment groups (see experiment 1). OXT-R binding in the dLS differed between breeding lines and treatment groups in females (factors line x treatment: $F_{2,28} = 5.32$; $p = 0.011$; Fig. 3B), but not in males. Post-hoc test with Bonferroni corrections revealed lower OXT-R binding in the dLS of ETX-treated HAB females ($p = 0.050$ vs. VEH). No other treatment effects were found in any other groups or brain regions.

3.2.2. Steroid profile on day 5 of ETX treatment

Steroids were measured in the other brain hemisphere of female and male HAB, LAB and NAB rats after 5 days of i.p. treatment with VEH or ETX 30 min after the last administration. All data and statistical comparisons are compiled in Table 1 (females) and Table 2 (males).

In female HAB rats, treatment with ETX increased brain 3β5α-THDOC levels compared to VEH. No other effect of ETX within a breeding line could be observed. In addition, we found breeding lines differences within the same treatment groups. For a comprehensive overview including breeding line differences see Table 1.

In males, treatment with ETX caused more significant changes within the same breeding line than it did in females. In HAB rats, treatment with ETX increased 5α-DHP, 3β5α-THDOC and corticosterone compared to VEH. In LAB rats, ETX treatment increased progesterone (PROG), 5α-DHP, 17α-OH AlloP and 3α5α-THDOC but decreased 3β5α-THP and 5α20β-THP compared with VEH. In NAB rats, 3α5α-THP and 5α-DHDOC were increased in ETX-versus VEH-treated rats compared with VEH. Furthermore, we found differences between breeding lines receiving the same treatment. For a detailed overview including breeding line differences see Table 2.

3.2.3. Microbiome differences across breeding lines and in response to 5-days ETX treatment

The bacterial microbiome in fecal samples from male and female HAB, LAB, and NAB rats was assessed before the first injection and after 5 days of i.p. treatment with VEH or ETX 30 min after the last administration using 16S rDNA sequencing. In males, NAB rats had significantly lower alpha diversity (Inverse Simpson index) than HAB and LAB rats ($p = 0.0001$), with no differences observed in females ($p = 0.387$, Fig. 4A). Alpha diversity remained unaffected by ETX or VEH across all

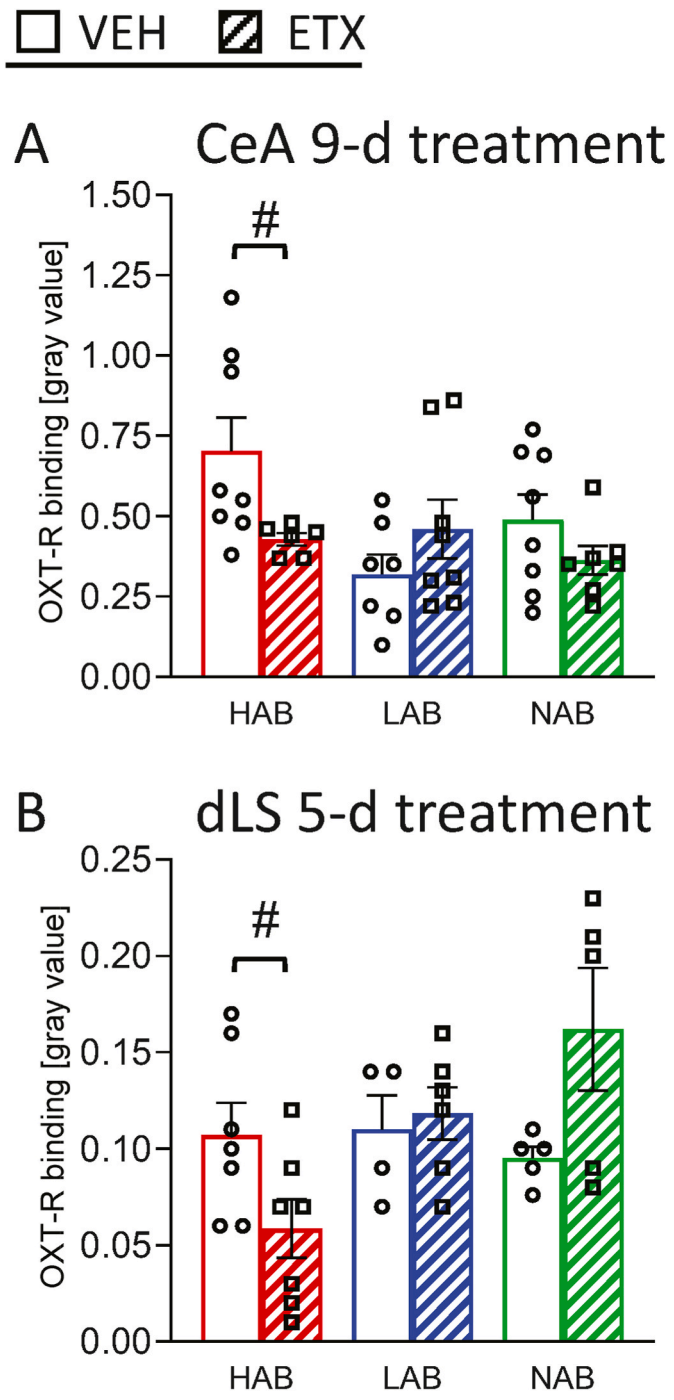

Fig. 3. OXT-R binding in female HAB, LAB and NAB rats after VEH or ETX treatment. Effects of 9-day (A) or 5-day (B) treatment with etifoxine (ETX; 50 mg/kg i.p.) or vehicle (VEH) on OXT-R binding in female HAB, LAB and NAB rats. ETX treatment decreased OXT-R binding in the CeA (9-day; A) and in the dLS (5-day; C) of female HAB rats. Data are presented as mean ± SEM. #p ≤ 0.05 vs. VEH.

breeding lines and sexes (Fig. 4C), as confirmed by a linear mixed-effects model ($F_{2,77} = 0.162$; $p = 0.850$).

Beta diversity analysis revealed significant microbiome composition differences among HAB, LAB, and NAB rats in both sexes after 5 days of treatment (PERMANOVA: HAB vs. NAB: $F = 10.66$, $p = 0.003$; HAB vs. LAB: $F = 3.18$, $p = 0.003$), though ETX did not significantly alter composition, and no sex differences were observed (Fig. 4D).

The observed beta diversity differences were associated with lower abundances of Gram-negative genera in NAB rats, including Taurinivorans (Family: Desulfovibrionaceae), an unclassified genus of UBA932 (Order: Bacteroidales), UBA4372 (Family: Bacteroidaceae), Alloprevotella, Odoribacter (Family: Marinifilaceae), and Anaerovibrio (Family: Selenomonadaceae). In contrast, HAB rats showed higher levels of Gram-positive bacteria from the class Clostridia, including Avidehalobacter (Family: UBA4997/Peptococcaceae), unclassified CAG-272 and UBA664 (Order: Oscillospirales), along with Alistipes (Family: Rikenellaceae) and Akkermansia (Family: Akkermansiaceae) (Fig. 4D). A linear mixed-effects model found no significant interaction between treatment, breeding line, and sex, indicating no detectable ETX impact on specific bacterial taxa (data not shown).

3.3. Experiment 3: effect of daily i.p. ETX on spine density in female and male HAB, LAB and NAB rats

Spine density was measured in female and male HAB, LAB and NAB rats after 5 days of i.p. treatment with either VEH or ETX using the Golgi-Cox method in brain regions relevant for emotionality.

3.3.1. Spine density in hippocampal CA1 region

In the CA1 stratum radiatum of female rats, spine density differed between breeding lines depending on the treatment (factors breeding line x treatment: $F_{2,44} = 3.27$; $p = 0.049$; Fig. 5A). Post-hoc analysis revealed a lower spine density in VEH-treated female HAB rats compared with NABs ($p = 0.02$). Treatment with ETX increased the number of spines in female HAB rats ($p = 0.03$; see representative images in Fig. 5A), but not in LAB or NAB rats. No further differences were found. In male rats, spine density differed due to the treatment ($F_{1,46} = 5.82$; $p = 0.02$; Fig. 5A), but not between the breeding lines. Post-hoc test revealed no further differences.

In the CA1 stratum oriens, no differences between breeding lines or treatment were found for either sex (data not shown).

3.3.2. Spine density in the LS

In the ventral lateral septum (vLS) of female rats, no effect of breeding line or treatment was found (Fig. 5B). Spine density in the vLS of male rats differed between the breeding lines ($F_{2,47} = 4.91$; $p = 0.012$; Fig. 5B) independent of treatment, and no interaction was detected. Post-hoc analysis revealed that in VEH-treated groups, spine density in the vLS of LAB rats was higher compared to NABs ($p = 0.01$; see representative images in Fig. 5B). No further differences between breeding lines or treatment were found (data not shown). Spine density in the dLS did not differ between breeding lines or treatment in female or male rats (data not shown).

3.3.3. Spine density in the PVN

In the parvocellular part of the PVN of female rats, no differences between the breeding lines or treatments were found (Fig. 5C). Male rats differed between the breeding lines ($F_{2,47} = 4.06$; $p = 0.024$; Fig. 5C) independent of treatment, and no interaction was detected. Post-hoc analysis revealed that spine density within the parvocellular part of the PVN of male HAB ($p = 0.05$) and LAB rats ($p = 0.01$) was higher compared to NABs in VEH groups (see representative images in Fig. 5C). No further differences were found.

In the magnocellular part of the PVN of female rats, no differences between the breeding lines or treatments were found. Male rats differed between the breeding lines ($F_{2,47} = 5.06$; $p = 0.011$; data not shown) independent of treatment, and no interaction was detected. Post-hoc analysis revealed no further differences.

3.3.4. Spine density in the CeA

No differences between breeding lines or treatment were found for either sex (data not shown).

Table 1
Steroid profiles measured in one brain hemisphere of female HAB, LAB and NAB rats after 5-days of i.p. treatment with vehicle (VEH) or etifoxine (ETX). Data are presented as mean [ng/g brain weight] ± SEM.

	HAB		LAB		NAB				
	VEH	ETX	VEH	ETX	VEH	ETX	statistics	F-value	p-value
PREG	6.40 ± 0.70	7.34 ± 0.89	**4.19 ± 0.19$^{\#}$**	**4.81 ± 0.45$^{\#}$**	15.1 ± 3.97	16.4 ± 6.03	interaction	(2, 35) = 0.006	0.994
							breeding line	**(2, 35) = 7.588**	* **0.002**
							treatment	(1, 35) = 0.143	0.708
PROG	5.72 ± 1.20	5.56 ± 0.72	2.47 ± 0.82	**2.63 ± 0.65$^{\#\#}$**	6.73 ± 1.78	9.41 ± 2.42	interaction	(2, 35) = 0.587	0.562
							breeding line	**(2, 35) = 7.175**	* **0.002**
							treatment	(1, 35) = 0.571	0.455
5α-DHP	30.1 ± 3.92	**37.9 ± 2.98$^{\pounds\pounds}$**	11.7 ± 3.04	**8.81 ± 1.80$^{\#\#}$**	27.8 ± 7.56	38.4 ± 9.87	interaction	(2, 36) = 0.785	0.464
							breeding line	**(2, 36) = 11.40**	* < **0.001**
							treatment	(1, 36) = 1.253	0.271
3α5α-THP	**3.07 ± 0.43$^{\pounds\pounds}$**	**3.74 ± 0.49$^{\pounds\pounds}$**	0.88 ± 0.12	**0.93 ± 0.19$^{\#\#}$**	2.09 ± 0.45	3.50 ± 0.88	interaction	(2, 36) = 0.964	0.391
							breeding line	**(2, 36) = 14.07**	* < **0.001**
							treatment	(1, 36) = 3.163	0.084
3β5α-THP	**0.171 ± 0.022$^{\pounds\pounds}$**	**0.201 ± 0.015$^{\#\pounds\pounds}$**	**0.052 ± 0.009$^{\#\#}$**	**0.038 ± 0.006$^{\#}$**	0.136 ± 0.031	0.122 ± 0.019	interaction	(2, 35) = 0.948	0.397
							breeding line	**(2, 35) = 28.61**	* < **0.001**
							treatment	(1, 35) = 0.003	0.960
5α20β-THP	**8.62 ± 1.67$^{\pounds\pounds}$**	**6.65 ± 1.32$^{\#\pounds}$**	**2.52 ± 0.32$^{\#\#}$**	**2.06 ± 0.35$^{\#\#}$**	11.77 ± 1.71	15.86 ± 1.36	interaction	(2, 36) = 3.127	(*) 0.056
							breeding line	**(2, 36) = 41.88**	* < **0.001**
							treatment	(1, 36) = 0.289	0.594
3α5α20α-HHP	**2.06 ± 0.29$^{\#\#}$**	**1.92 ± 0.32$^{\#\#}$**	**0.65 ± 0.09$^{\#\#}$**	**0.51 ± 0.18$^{\#\#}$**	7.08 ± 0.79	7.74 ± 0.58	interaction	(2, 36) = 0.522	0.598
							breeding line	**(2, 36) = 130.2**	* < **0.001**
							treatment	(1, 36) = 0.117	0.735
3β5α20α-HHP	**0.139 ± 0.019$^{\pounds\pounds}$**	**0.176 ± 0.022$^{\pounds\pounds}$**	**0.025 ± 0.004$^{\#\#}$**	**0.029 ± 0.004$^{\#\#}$**	0.100 ± 0.018	0.134 ± 0.018	interaction	(2, 36) = 0.683	0.512
							breeding line	**(2, 36) = 35.28**	* < **0.001**
							treatment	(1, 36) = 3.637	(*) 0.065
17α-OHP	0.039 ± 0.011	0.025 ± 0.014	btl	btl	0.029 ± 0.007	0.024 ± 0.003	interaction	(1, 22) = 0.165	0.689
							breeding line	(1, 22) = 0.324	0.575
							treatment	(1, 22) = 0.937	0.344
17α-OH AlloP	0.136 ± 0.020	0.171 ± 0.026	**0.248 ± 0.083$^{\#\#}$**	0.113 ± 0.022	0.071 ± 0.021	0.088 ± 0.030	interaction	(2, 35) = 2.989	(*) 0.063
							breeding line	**(2, 35) = 3.954**	* **0.028**
							treatment	(1, 35) = 0.397	0.533
DOC	**1.84 ± 0.20$^{\#\#\pounds\pounds}$**	**2.28 ± 0.50$^{\#\#\pounds\pounds}$**	0.121 ± 0.01	0.189 ± 0.03	1.17 ± 0.11	1.05 ± 0.07	interaction	(2, 34) = 0.567	0.573
							breeding line	**(2, 34) = 46.16**	* < **0.001**
							treatment	(1, 34) = 0.739	0.396
5α-DHDOC	**0.152 ± 0.014$^{\#}$**	**0.195 ± 0.056$^{\#\#}$**	**0.217 ± 0.031$^{\#}$**	**0.357 ± 0.097$^{\#\#}$**	1.39 ± 0.43	1.65 ± 0.50	interaction	(2, 34) = 0.074	0.929
							breeding line	**(2, 34) = 13.51**	* < **0.001**
							treatment	(1, 34) = 0.402	0.531

(*continued on next page*)

Table 1 (*continued*)

	HAB		LAB		NAB				
	VEH	ETX	VEH	ETX	VEH	ETX	statistics	F-value	p-value
3α5α-THDOC	**0.035 ± 0.005$^{\#}$**	0.047 ± 0.009	**0.008 ± 0.002$^{\#\#}$**	**0.014 ± 0.003$^{\#\#}$**	0.084 ± 0.021	0.085 ± 0.022	interaction	(2, 34) = 0.089	0.915
							breeding line	**(2, 34) = 15.07**	* < **0.001**
							treatment	(1, 34) = 0.322	0.574
3β5α-THDOC	**0.029 ± 0.003$^{\#}$**	**0.099 ± 0.030$^{*£}$**	**0.017 ± 0.002$^{\#\#}$**	0.024 ± 0.004	0.101 ± 0.024	0.073 ± 0.019	**interaction**	**(2, 34) = 3.719**	* **0.035**
							breeding line	**(2, 34) = 7.014**	* **0.003**
							treatment	(1, 34) = 1.172	0.287
Corticosterone	3.15 ± 0.57	10.08 ± 2.96	**1.49 ± 0.40$^{\#}$**	**3.05 ± 0.76$^{\#}$**	10.1 ± 1.77	11.4 ± 4.22	interaction	(2, 35) = 0.911	0.412
							breeding line	**(2, 35) = 6.755**	* **0.003**
							treatment	(1, 35) = 2.955	0.094
5α-DHB	0.29 ± 0.07	1.02 ± 0.36	0.14 ± 0.04	0.33 ± 0.07	0.67 ± 0.15	1.17 ± 0.45	interaction	(2, 34) = 0.528	0.595
							breeding line	**(2, 34) = 3.507**	* **0.041**
							treatment	**(1, 34) = 4.922**	* **0.033**
20α-DHP	**15.21 ± 2.33$^{££}$**	**23.85 ± 4.70$^{\#\#££}$**	3.70 ± 0.53	3.70 ± 0.80	7.16 ± 0.89	9.30 ± 0.70	interaction	(2, 35) = 1.990	0.152
							breeding line	**(2, 35) = 26.18**	* < **0.001** $^{(*)}$
							treatment	(1, 35) = 3.728	0.062
DHEA	**0.083 ± 0.007$^{\#\#££}$**	0.062 ± 0.010	0.040 ± 0.002	0.043 ± 0.005	0.052 ± 0.005	0.053 ± 0.002	interaction	(2, 34) = 2.617	0.088
							breeding line	**(2, 34) = 14.80**	* < **0.001**
							treatment	(1, 34) = 1.029	0.318
ADIONE	0.101 ± 0.022	0.074 ± 0.016	**0.072 ± 0.006$^{\#}$**	0.093 ± 0.025	0.178 ± 0.044	0.118 ± 0.028	interaction	(2, 36) = 1.208	0.311
							breeding line	**(2, 36) = 3.854**	* **0.030**
							treatment	(1, 36) = 1.048	0.313
Testosterone	0.007 ± 0.003	0.005 ± 0.003	0.004 ± 0.001	0.005 ± 0.002	0.006 ± 0.0011	0.003 ± 0.0007	interaction	(2, 35) = 0.670	0.518
							breeding line	(2, 35) = 0.952	0.396
							treatment	(1, 35) = 0.462	0.501
5α-DHT	<0.002	0.006 ± 0.003	<0.002	<0.002	<0.002	<0.002	interaction	(2, 35) = 2.093	0.138
							breeding line	(2, 35) = 2.093	0.138
							treatment	(1, 35) = 2.150	0.152
3α5α-THT	btl	btl	btl	btl	btl	btl			
E2	0.004 ± 0.004	btl	0.007 ± 0.002	0.009 ± 0.005	0.021 ± 0.011	0.019 ± 0.009	interaction	(2, 36) = 0.127	0.881
							breeding line	(2, 36) = 0.947	0.397
							treatment	(1, 36) = 0.154	0.697

btl, below detection limit; *p < 0.05 vs. VEH same breeding line; ##p < 0.01, #p < 0.05 vs. NAB same treatment; ££p < 0.01, £p < 0.05 vs. LAB same treatment.

Table 2
Steroid profiles measured in one brain hemisphere of male HAB, LAB and NAB rats after 5-days of i.p. treatment with vehicle (VEH) or etifoxine (ETX). Data are presented as mean [ng/g brain weight] ± SEM.

	HAB		LAB		NAB				
	VEH	ETX	VEH	ETX	VEH	ETX	statistics	F-value	p-value
PREG	3.56 ± 0.64	5.25 ± 0.63	2.89 ± 0.19	4.65 ± 0.87	4.42 ± 0.56	4.92 ± 1.12	interaction	(2, 35) = 0.699	0.504
							breeding line	(2, 35) = 0.616	0.546
							treatment	(1, 35) = 0.242	0.626
PROG	0.66 ± 0.15	1.40 ± 0.32	0.32 ± 0.10	**1.96 ± 0.68****	0.78 ± 0.14	1.02 ± 0.15	interaction	(2, 32) = 2.132	0.135
							breeding line	(2, 32) = 0.237	0.790
							treatment	**(1, 32) = 9.300**	*** 0.005**
5α-DHP	1.66 ± 0.27	**3.76 ± 0.67***	0.92 ± 0.12	**3.29 ± 1.17***	0.68 ± 0.20	1.73 ± 0.48	interaction	(2, 32) = 0.525	0.596
							breeding line	(2, 32) = 2.475	0.100
							treatment	**(1, 32) = 11.64**	*** 0.002**
3α5α-THP	0.14 ± 0.02	**0.21 ± 0.03$^{\#\#}$**	0.12 ± 0.01	**0.15 ± 0.03$^{\#\#}$**	0.20 ± 0.04	**0.40 ± 0.06****	interaction	(2, 34) = 2.923	$^{(*)}$ 0.067
							breeding line	**(2, 34) = 10.57**	*** < 0.001**
							treatment	**(1, 34) = 11.87**	*** 0.002**
3β5α-THP	**0.014 ± 0.001$^{£}$**	0.019 ± 0.002	**0.022 ± 0.002$^{\#\#}$**	**0.015 ± 0.003***	0.011 ± 0.002	0.015 ± 0.003	**interaction**	**(2, 33) = 4.186**	*** 0.024**
							breeding line	(2, 33) = 3.116	$^{(*)}$ 0.058
							treatment	(1, 33) = 0.217	0.645
5α20β-THP	**0.038 ± 0.004$^{\#\#}$**	**0.057 ± 0.016$^{\#\#}$**	**0.056 ± 0.018$^{\#\#}$**	**0.122 ± 0.022$^{\#\#}$***	0.391 ± 0.102	0.579 ± 0.129	interaction	(2, 34) = 0.852	0.436
							breeding line	**(2, 34) = 25.64**	*** < 0.001**
							treatment	(1, 34) = 2.908	0.097
3α5α20α-HHP	**0.238 ± 0.058$^{\#\#}$**	**0.361 ± 0.091$^{\#\#}$**	**0.221 ± 0.01$^{\#}$**	**0.230 ± 0.03$^{\#}$**	0.014 ± 0.003	0.042 ± 0.013	interaction	(2, 34) = 0.845	0.438
							breeding line	**(2, 34) = 17.18**	*** < 0.001**
							treatment	(1, 34) = 1.910	0.176
3β5α20α-HHP	**0.021 ± 0.003$^{\#\#££}$**	**0.023 ± 0.004$^{\#\#}$**	0.005 ± 0.002	**0.017 ± 0.006$^{\#}$**	0.005 ± 0.001	0.005 ± 0.001	interaction	(2, 35) = 1.639	0.209
							breeding line	**(2, 35) = 12.55**	*** < 0.001**
							treatment	(1, 35) = 2.709	0.109
17α-OHP	0.081 ± 0.021	0.037 ± 0.014	0.088 ± 0.039	0.146 ± 0.074	0.063 ± 0.008	0.100 ± 0.011	interaction	(2, 34) = 1.006	0.376
							breeding line	(2, 34) = 1.200	0.314
							treatment	(1, 34) = 0.291	0.593
17α-OH AlloP	0.004 ± 0.0008	0.003 ± 0.0007	0.002 ± 0.001	**0.006 ± 0.002***	0.003 ± 0.0006	0.005 ± 0.0005	interaction	(2, 34) = 2.301	0.116
							breeding line	(2, 34) = 0.151	0.861
							treatment	**(1, 34) = 4.883**	*** 0.034**
DOC	**1.74 ± 0.04$^{\#££}$**	**2.37 ± 0.29**$^{\#\#££}$**	**0.224 ± 0.02$^{\#\#}$**	**0.457 ± 0.10$^{\#}$**	0.161 ± 0.01	0.139 ± 0.02	**interaction**	**(2, 35) = 3.512**	*** 0.041**
							breeding line	**(2, 35) = 72.38**	*** < 0.001**
							treatment	**(1, 35) = 4.512**	*** 0.041**
5α-DHDOC	0.178 ± 0.047	0.444 ± 0.072	0.144 ± 0.027	**0.747 ± 0.247$^{\#\#}$**	0.156 ± 0.029	**0.519 ± 0.165***	interaction	(2, 34) = 0.874	0.426
							breeding line	(2, 34) = 0.588	0.561
							treatment	**(1, 34) = 13.85**	*** 0.001**
3α5α-THDOC	**0.030 ± 0.004$^{£}$**	**0.057 ± 0.007**$^{££}$**	0.006 ± 0.0001	**0.014 ± 0.003***	0.023 ± 0.001	0.045 ± 0.010	interaction	(2, 34) = 1.560	0.225
							breeding line	**(2, 34) = 19.09**	*** < 0.001**
							treatment	**(1, 34) = 15.93**	*** < 0.001**
3β5α-THDOC	0.029 ± 0.006	**0.059 ± 0.010$^{*£}$**	0.012 ± 0.005	0.030 ± 0.012	0.020 ± 0.004	0.036 ± 0.008	interaction	(2, 34) = 0.461	0.635
							breeding line	**(2, 34) = 4.476**	*** 0.019**
							treatment	**(1, 34) = 10.52**	*** 0.003**
Corticosterone	**14.09 ± 2.64$^{\#££}$**	**24.47 ± 2.92**$^{\#\#££}$**	3.89 ± 0.90	9.27 ± 2.62	5.02 ± 0.68	6.25 ± 1.07	interaction	(2, 33) = 2.121	0.136
							breeding line	**(2, 33) = 25.11**	*** < 0.001**
							treatment	**(1, 33) = 10.00**	*** 0.003**
5α-DHB	0.44 ± 0.12	0.68 ± 0.10	0.27 ± 0.04	0.64 ± 0.20	0.56 ± 0.14	0.99 ± 0.29	interaction	(2, 35) = 0.165	0.849
							breeding line	(2, 35) = 1.757	0.188
							treatment	**(1, 35) = 6.131**	*** 0.018**
20α-DHP	**0.085 ± 0.02$^{\#}$**	**0.092 ± 0.02$^{\#}$**	0.044 ± 0.01	0.046 ± 0.01	0.020 ± 0.01	0.039 ± 0.004	interaction	(2, 33) = 0.166	0.848
							breeding line	**(2, 33) = 8.531**	*** 0.001**
							treatment	(1, 33) = 0.543	0.467
DHEA	0.051 ± 0.007	**0.054 ± 0.005$^{\#\#}$**	0.089 ± 0.007	**0.056 ± 0.013$^{\#\#}$**	0.258 ± 0.053	0.461 ± 0.154	interaction	(2, 35) = 1.606	0.215

(*continued on next page*)

Table 2 (*continued*)

	HAB		LAB		NAB				
	VEH	ETX	VEH	ETX	VEH	ETX	statistics	F-value	p-value
							breeding line	**(2, 35) = 12.67**	* **< 0.001**
							treatment	(1, 35) = 1.220	0.277
ADIONE	0.341 ± 0.062	0.249 ± 0.031	0.165 ± 0.026	0.191 ± 0.062	0.173 ± 0.006	0.315 ± 0.051	interaction	(2, 32) = 3.027	(*) 0.063
							breeding line	(2, 32) = 3.181	(*) 0.055
							treatment	(1, 32) = 0.426	0.519
Testosterone	0.396 ± 0.071	0.475 ± 0.089	0.391 ± 0.087	0.700 ± 0.243	0.296 ± 0.041	0.628 ± 0.183	interaction	(2, 34) = 0.456	0.638
							breeding line	(2, 34) = 0.321	0.728
							treatment	**(1, 34) = 4.120**	* **0.050**
5α-DHT	**0.010 ± 0.002**[£]	**0.022 ± 0.005**[#£]	0.068 ± 0.017	0.101 ± 0.023	0.049 ± 0.004	0.080 ± 0.013	interaction	(2, 33) = 0.347	0.709
							breeding line	**(2, 33) = 13.32**	* **< 0.001**
							treatment	**(1, 33) = 4.944**	* **0.033**
3α5α-THT	0.058 ± 0.007	0.050 ± 0.010	0.043 ± 0.011	0.070 ± 0.025	0.035 ± 0.008	0.085 ± 0.014	interaction	(2, 34) = 2.099	0.138
							breeding line	(2, 34) = 0.101	0.905
							treatment	(1, 34) = 4.107	(*) 0.051
E2	0.008 ± 0.001	0.007 ± 0.002	0.009 ± 0.003	0.006 ± 0.003	0.004 ± 0.001	0.006 ± 0.002	interaction	(2, 35) = 0.699	0.504
							breeding line	(2, 35) = 0.616	0.546
							treatment	(1, 35) = 0.242	0.626

**p < 0.01, *p < 0.05 vs. VEH same breeding line; ##p < 0.01, #p < 0.05 vs. NAB same treatment; ££p < 0.01, £p < 0.05 vs. LAB same treatment.

4. Discussion

The present study was performed to test for the effectiveness of daily acute i.p. treatment with the TSPO ligand ETX over 5 or 9 days on anxiety-related and stress-coping behaviors and related neurobiological mechanisms. To mimic the clinical situation, we employed a rat model of hyper-anxiety and depression-like behavior, i.e., female and male HAB rats, with LAB and NAB rats as controls. Overall, ETX was most effective in female HAB rats as it reduced anxiety levels in conjunction with brain site-specific reduction of OXT-R binding, e.g., in the CeA and dLS, increased spine density in the CA1 stratum radiatum, and increased brain 3β5α-THDOC levels. Other ETX effects included (i) alterations in various brain steroid levels, but only in male rats, and (ii) higher stress-induced plasma corticosterone levels, but only in male and female NAB rats without affecting the fecal microbiome composition.

4.1. ETX decreased anxiety-related behavior in female HAB rats

We confirmed the robust differences in anxiety-related behavior between HAB, LAB and NAB rats, independent of sex (for review see Gryksa et al., 2023). Importantly, we could reveal a breeding line- and sex-specific anxiolytic effect after 5 days of daily i.p. injection with ETX, which was exclusively visible in female HAB rats (Fig. 2A). These observations seem to contrast with earlier studies, in which an anxiolytic effect of ETX was also described in male rats or mice; however, these studies were conducted in animals without a psychopathological phenotype and, furthermore, did not include female subjects (Ugale et al., 2007; Verleye et al., 2005; Verleye and Gillardin, 2004). Consequently, it would be crucial to translate our findings to human studies, as our results may impact on the clinical benefit of ETX treatment, the first TSPO ligand with an anxiolytic effect in a clinical trial (Nguyen et al., 2006). In support of our findings of sex-specific ETX effects, a recent PET imaging study in healthy humans demonstrated higher brain TSPO levels in women (Tuisku et al., 2019). More evidence from human patients is required to prove the presumed sex difference in TSPO expression and potential differences in treatment efficacies.

Our results are especially interesting given that the anxiolytic effect of ETX was only apparent in females of the highly anxious HAB rats. In preclinical studies, sex as a biological variable remains generally underrated (Beery and Zucker, 2011; Kokras and Dalla, 2017), and a sex bias is reported with a predominant number of male animals included (Beery and Zucker, 2011; Will et al., 2017). In women, the occurrence, onset, and severity of anxiety disorders might depend on hormonal fluctuations throughout the estrous cycle (Basso et al., 2011; Kundakovic and Rocks, 2022), but including the estrous cycle as a co-factor in behavioral studies remains ambiguous (Lovick and Zangrossi, 2021). During proestrus, AlloP levels are high (Palumbo et al., 1995; Schmidt et al., 1994), and females show reduced anxiety-related behavior, whereas this is the opposite in the met-/diestrus phase (Silva et al., 2016; D'Souza and Sadananda, 2017). In the present study, all female rats were in met-/diestrus on the day of behavioral testing, thereby circumventing potential interfering effects of the estrous cycle.

4.2. Passive stress-coping was unaltered after 9-day ETX treatment

HAB and LAB rats are used as an excellent model of comorbidity of anxiety and depression disorders (Craske et al., 2017; Gryksa et al., 2023). In the present study, we confirmed higher levels of passive stress-coping, i.e., reminiscent of depressive-like behavior (Slattery and Cryan, 2012), in VEH-treated HAB compared to LAB rats during the forced swim test specifically in female rats (Slattery and Neumann, 2010; Gryksa et al., 2023). However, we could not find any effect on floating following 9-day ETX treatment either in HAB, LAB or NAB rats. This contrasts with recent studies suggesting an antidepressive effect of other TSPO ligands like XBD173 (Shang et al., 2020b; Barron et al., 2021), YL-IPA08 (Ren et al., 2020), or even the antagonistic ligand

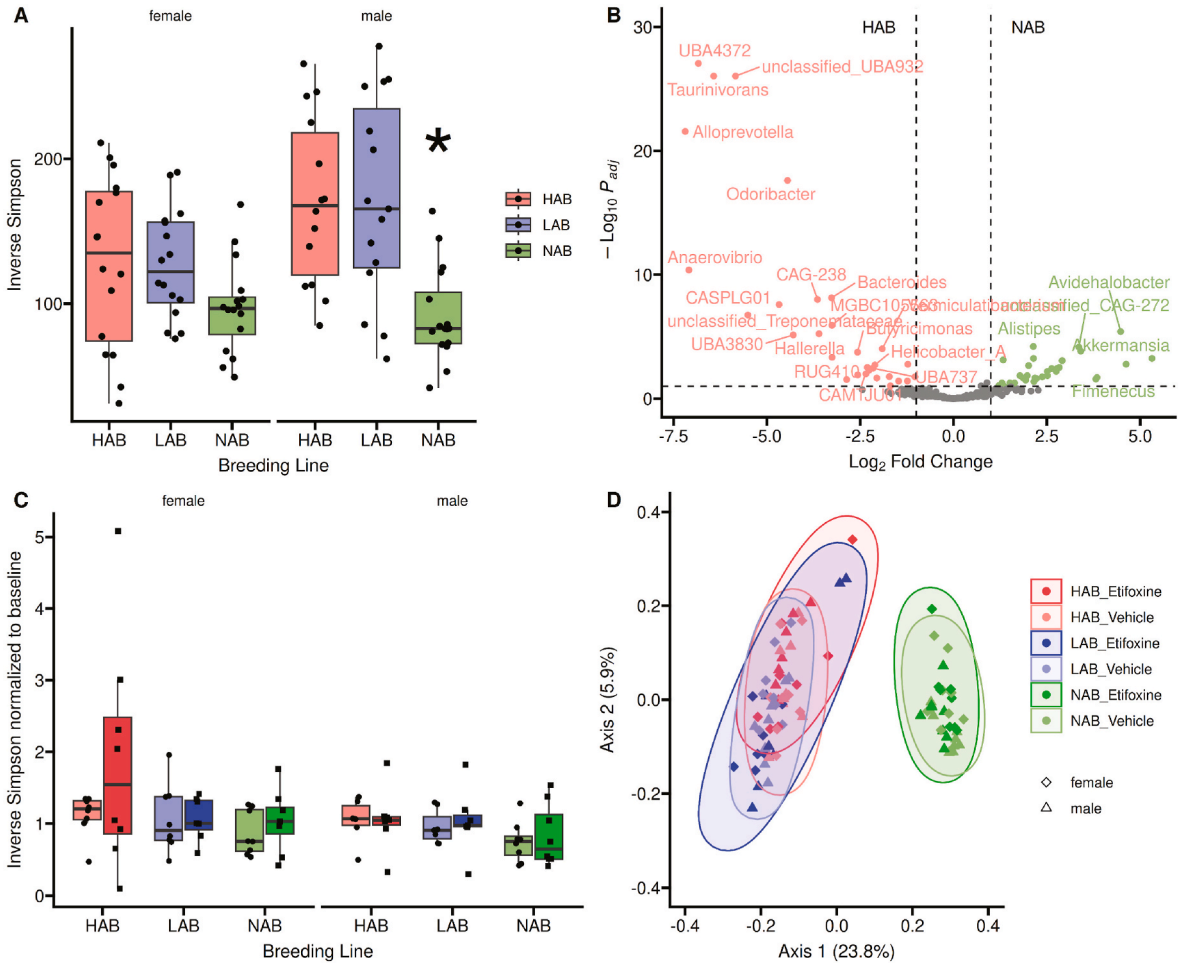

Fig. 4. Bacterial diversity and composition in female HAB, LAB, and NAB rats at baseline and after VEH or ETX treatment. (A) NAB rats displayed significantly lower bacterial alpha diversity (Inverse Simpson Index) compared to LAB and HAB breeding lines in both male and female animals at baseline. (B) Differential abundance analysis, using linear mixed-effects models, identified genus-level differences between NAB (green) and HAB (red) rats. Dashed lines on the volcano plot indicate cutoffs for Log2 fold changes (± 1) and p-value thresholds (α = 0.1). (C) Bacterial alpha diversity (Inverse Simpson Index, normalized to baseline) remained unchanged in response to ETX or VEH treatment in male and female HAB, LAB, and NAB rats. (D) Beta diversity analysis, represented by Principal Coordinates Analysis (PCoA) of Bray–Curtis dissimilarities, revealed distinct clustering of breeding lines. No compositional changes due to treatment or sex (males as triangles, females as diamonds) were observed. Ellipses indicate 95% confidence intervals around group centroids. Data are presented as mean ± SEM. *p < 0.05 vs. NAB. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ONO-2952 (Nozaki et al., 2020). As such, the putative antidepressant potential of TSPO ligands in rodents appears to depend on the model employed, and the dose and time of administration. So far, no clinical data are available on potential treatment effects of TSPO ligands in depressed patients (Rupprecht et al., 2022). A first clinical study addressing this question is currently under way (Brunner et al., 2024).

4.3. HPA axis responsiveness increased after 9-day ETX treatment in female and male NAB rats

A potential role of a dysregulated HPA axis in anxiety and depression disorders has been studied in humans and animal models alike with varying outcomes (Domin and Śmiałowska, 2024). In this context, HAB and LAB rats with their distinct behavioral phenotype also differ in their responsiveness of the HPA axis with elevated responses to acute and chronic stress in HAB rats (Landgraf et al., 1999; Gryksa et al., 2023). However, ETX treatment resulted in a more pronounced FST-induced rise in plasma corticosterone levels only in female and male NAB, but not HAB or LAB, rats compared to their VEH controls (Fig. 2E and F). As we took blood samples only after stressor exposure, we cannot exclude the possibility that ETX might already have caused differences in basal corticosterone concentrations between treatment groups. Also, given the present protocol, it is not surprising that HAB and LAB rats appear to be similar; differences were only detectable under mild stress conditions (Landgraf et al., 1999; Gryksa et al., 2023). Thus, 10 min of forced swimming might result in a neuroendocrine ceiling response. In humans, 5 days of ETX treatment on cortisol responses to an acute stressor (exposure to the Trier Social Stress Test) had no effect on the cortisol response itself (Bahr et al., 2021). It was concluded that the therapeutic effects of ETX might be restricted to stronger or even pathological stress responses in humans. This is in line with the role of TSPO as a rate-limiting factor in the initial steroidogenesis (Jaremko et al., 2014); alterations in the expression and/or activity of TSPO can impact on the synthesis of glucocorticoids (Rupprecht et al., 2009, 2010; Nothdurfter et al., 2012a) thereby affecting the physiological response to stressors.

4.4. ETX altered brain steroid profiles

The TSPO ligand ETX indirectly impacts on steroidogenesis as revealed in male rats (Verleye et al., 2005; Liere et al., 2017). Interestingly, a sex-dependent expression of anxiolytic brain steroids (Giatti et al., 2020) with, e.g., higher AlloP levels in the hippocampus and cerebral cortex in female rats was described. In human patients, blood PREG levels are reduced in males with generalized anxiety disorder

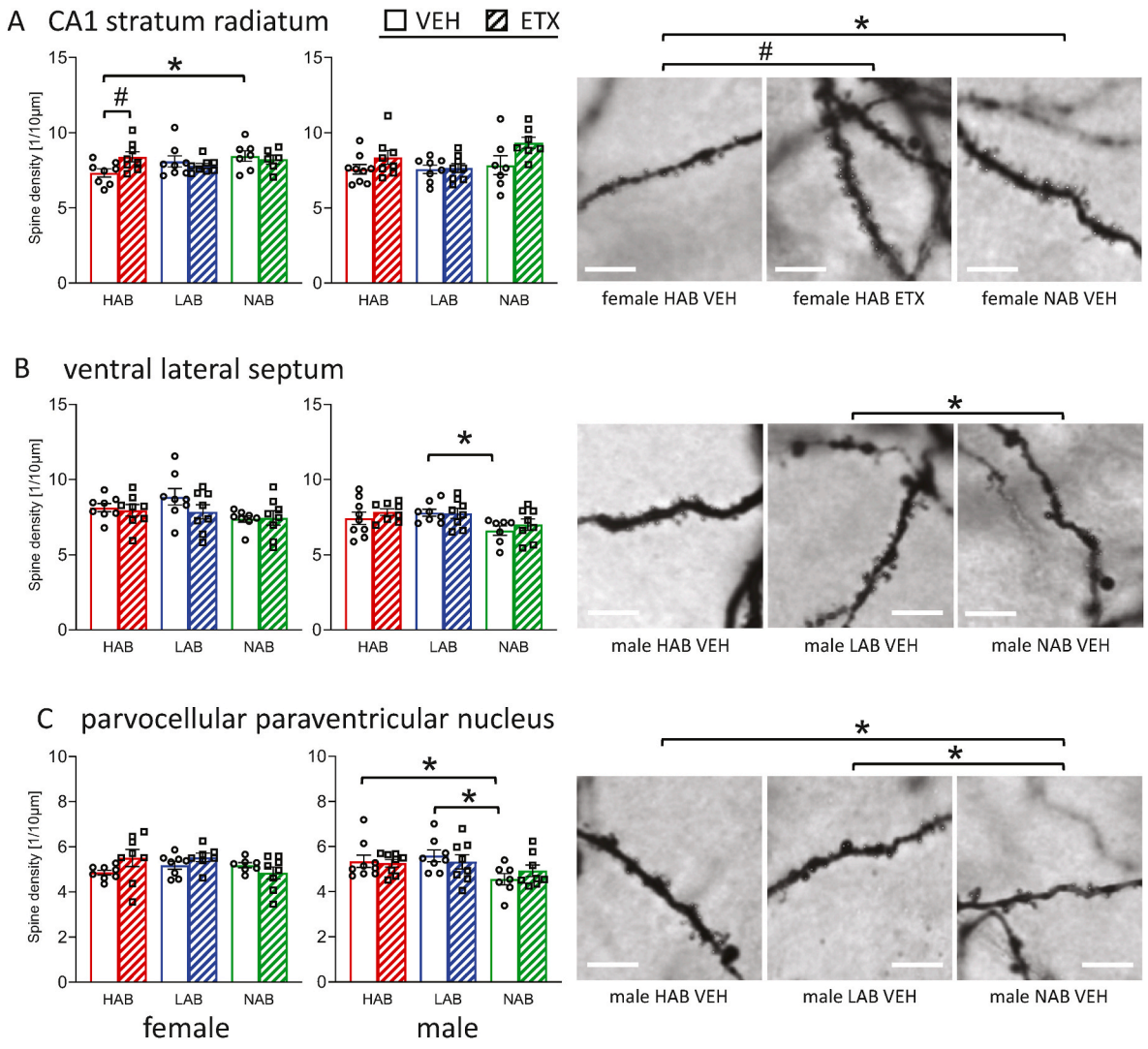

Fig. 5. Spine density in CA1 stratum radiatum, vLS, and parvocellular PVN in female and male HAB, LAB and NAB rats after VEH or ETX treatment**.** Effects of 5-day treatment with etifoxine (ETX; 50 mg/kg i.p.) or vehicle (VEH) on spine density in CA1 stratum radiatum (A), vLS (B), and parvocellular PVN (C) in female (left graph) and male (right graph) HAB, LAB and NAB rats as well as representative images of the differing groups (scale bar = 20 µm). (A) In the CA1 stratum radiatum, VEH-treated female HAB rats had lower spine density than NABs. ETX treatment increased the number of spines of female HAB rats, only. No such effects were seen in males. (B) In the vLS, female rats did not differ independent of the treatment. In males, VEH-treated LAB rats had higher spine density compared to NABs; ETX had no further effects. (C) In the parvocellular PVN, female rats did not differ independent of the treatment. In males, VEH-treated HAB and LAB rats had higher spine density compared to NABs; ETX had no further effects. Data are presented as mean ± SEM. *$p < 0.05$ vs. NAB; #$p < 0.05$ vs. VEH.

(Semeniuk et al., 2001) and social phobia (Heydari and Le Mellédo, 2002). In female patients with mixed anxiety-depression disorder, PREG sulfate is higher indicating an imbalance of steroids (Bicíková et al., 2000). In our study, we found the highest levels of AlloP in female brains of HABs and low levels of PREG in LABs. As downregulation and/or imbalance of steroids contributes to the development of anxiety disorders (Bicíková et al., 2000; Schüle et al., 2014; Nothdurfter et al., 2012; Rupprecht et al., 2010), the combination of higher levels of steroids in relevant brain regions in females, combined with the imbalance of steroids in the animal model of hyper-anxiety could explain why a behavioral effect of ETX was only observed in females. In favor of this hypothesis, and in line with the isolated treatment effects in female HABs, Bahr et al. (2021) proposed that the therapeutic effect of ETX might be limited to patients suffering from psychiatric diseases with disrupted steroid systems. Thus, treatment options targeting the steroid system may need to be carefully adapted to sex differences.

The lack of ETX impact on steroidogenesis in females might be caused by their high PROG levels due to the diestrus stage we controlled for. However, the breeding lines differed from each other (see Table 1). For example, steroids involved in the stress pathway were higher (DOC) or tended to be higher (PROG, corticosterone) in the more prone HAB rats. Hence, the data on female brain steroidogenesis suggest a slightly higher activity of the HPA axis in female HAB rats confirming their higher HPA axis reactivity (Gryksa et al., 2023).

A higher HPA axis reactivity in male HABs rats (Gryksa et al., 2023) was reflected by higher concentrations of DOC, and its metabolites 3α5α-THDOC and 3β5α-THDOC, and higher plasma corticosterone levels. Effects of ETX on steroidogenesis were breeding line-specific in male rats. Five-day treatment increased PROG and corticosterone, but also of 3β5α-THDOC (functional AlloP antagonist). As such, steroids involved in the HPA axis were affected, which was reflected by the increased stress response in male, but also female, NAB rats. Furthermore, 5α-DHP (potential PROG receptor ligand) and 5α-DHDOC concentrations were also increased, as well as the $GABA_AR$ agonists 3α5α-THP (AlloP), suggesting the potential to decrease anxiety-related behavior. However, ETX can induce steroidogenesis not only by binding to TSPO, especially adrenal steroidogenesis and 3α-reduced GABAergic steroids, but also directly by binding to $GABA_AR$ (Poisbeau

et al., 2018). Whether TSPO is required for steroidogenesis in rodents and humans is still in debate (Liere et al., 2023; Selvaraj and Tu, 2016; do Rego et al., 2015; Riebel et al., 2024).

4.5. Altered OXT-R binding after 5- and 9-day ETX treatment in female HAB rats

The brain OXT system exerts robust anxiolytic effects within distinct brain regions (Jurek and Neumann, 2018). To study potential effects of ETX treatment on emotionality via modulation of the OXT system, we first examined OXT-R binding in various brain regions after 9-day ETX treatment. OXT-R binding was significantly decreased in the CeA of ETX-treated HAB females, only (Fig. 3). OXT signaling in the CeA promotes anxiolysis and reduces fear responses via GABA modulation (Knobloch et al., 2012; Craske et al., 2017; Yoon and Kim, 2020; Bale et al., 2001; Viviani et al., 2011). Interestingly, reduced OXT-R binding after 9 days of ETX paralleled the anxiolytic effect of ETX in female HAB rats. Hence, activation of TSPO, but also of $GABA_AR$, by 9 days of ETX treatment might be associated - directly or rather indirectly - with altered OXT-R binding as a consequence of increased local OXT release and availability (Peters et al., 2014; Huang et al., 2014).

The sex-specific effect of ETX was also confirmed in female HAB rats after 5-days of treatment, however, OXT-R binding was found to be decreased in the dLS, a brain region also relevant for anxiety-related behavior. In fact, septal OXT-R+ neurons might induce anxiety by stimulating the amygdala through disinhibition via GABA-ergic projections (Huang et al., 2021). Conversely, downregulation of OXT-R in female HABs could attenuate the anxiety-inducing effect of OXT in this region. The underlying mechanisms of the altered OXT-R binding after ETX treatment are still unknown. As they were only apparent in females, an involvement of gonadal steroids is in debate. ETX induces steroid synthesis (reviewed by Papadopoulos et al., 2006), e.g., the production of precursors of gonadal steroids like estrogen, which influence OXT-R expression in different brain regions (Choleris et al., 2003; Young et al., 1998; Jurek and Neumann, 2018). The underlying mechanism involves the action of estrogen receptors as transcription factors for the OXT-R gene promoter (Sharma et al., 2012). However, OXT-R expression is independent of estradiol at least in the CeA (Bale and Dorsa, 1995).

4.6. Hippocampal spine density increased after 5-day of ETX treatment in female HAB rats

Different to the benzodiazepine diazepam, which impairs the structural plasticity of dendritic spines (Shi et al., 2022), TSPO ligands my alter dendritic complexity and spine density indirectly by increasing steroid signaling (Afroz et al., 2017; Wang et al., 2013; Papadopoulos et al., 2018; Rupprecht et al., 2009, 2010). Hence, we tested for the effects of 5-day ETX treatment on distinct brain regions relevant for emotionality. First, in VEH-treated controls spine density within the hippocampal stratum radiatum was significantly lower in female HAB compared to LAB or NAB rats (Fig. 5A), whereas males did not differ from each other. These findings confirm previous studies demonstrating lower hippocampal spine density in depression (von Bohlen und Halbach, 2009; Duman and Duman, 2015; Boku et al., 2018), an emotional state that is reflected in our HAB animal model (Wegener et al., 2012; Neumann and Slattery, 2016). Interestingly, 5-days treatment with ETX increased spine density in female HAB rats up to a level comparable to those in LAB and NAB rats (Fig. 5A). This suggests that ETX has the potential to improve dendritic complexity and spine density in animals with pathological anxiety- and depression-related parameters and is in favor of meaningful effects of ETX in stress-related disorders.

We did not detect any alterations in spine density due to ETX treatment in any other brain region investigated. However, we found general differences between the lines in VEH-treated controls. Spine density was higher in the vLS of male LAB rats compared to NABs (Fig. 5B), and in the parvocellular PVN of male HAB and LAB rats compared to NABs (Fig. 5C). However, there is no information available of potential differences in spine density in one of these regions with respect to anxiety-related or depressive-like behavior.

4.7. Etifoxine treatment did not alter the fecal microbiome

Since ETX treatment reduced anxiety-related behavior in female HAB rats, we aimed to study whether this would be an effect of ETX on TSPO of the gut microbiome based on previous findings. In fact, TSPO of bacteria and mammals show similarities in structural and functional characteristics (Chapalain et al., 2009; Isokpehi et al., 2017), and ETX has subtle effects on the human microbiome (Manook et al., 2023). Furthermore, the composition of the gut microbiome and, consequently, the gut-brain axis pathway has been linked to anxiety and depressive-like symptoms as studied in rodents and humans (Johnson and Foster, 2018; Spichak et al., 2021; Olavarría-Ramírez et al., 2023). As gut microbiota are thought to be key components of regulating anxiety and depression (Cryan et al., 2019), and the high anxious HAB rats differ in their microbiome composition from NAB rats (Schmidtner et al., 2019), a potential link to ETX-induced changes in anxiety-related behavior was plausible.

Microbiome sequencing revealed distinct basal bacterial compositions between the breeding lines, with NAB rats exhibiting lower microbial diversity than HAB and LAB rats. HAB rats showed a reduced abundance of bacteria of the class Clostridia, consistent with findings by Schmidtner et al. (2019). Differences in microbial composition may influence anxiety through the production of short-chain fatty acids (SCFAs) and hydrogen sulfide (H_2S). The enrichment of the sulfate-reducing bacterium *Taurinivorans* in HAB rats likely increases H_2S levels, promoting neuroinflammation and potentially contributing to behavioral and neurobiological differences between HAB and NAB rats (Gadalla and Snyder, 2010; Zuhra et al., 2020). In contrast, higher proportions of SCFA-producing bacteria, such as members of the Clostridia class including the family Oscilloscpiraceae, have anti-inflammatory effects that support gut barrier integrity and reduce systemic inflammation (Furusawa et al., 2013). ETX did not significantly influence the microbiome composition even when there were divergent baseline microbiota structures as described above.

The absence of significant ETX-induced changes in microbiome diversity or composition is consistent with recent findings (Manook et al., 2023), where short-term ETX treatment had a subtle but not substantial effect on the microbiome in healthy men. This indicates that the behavioral and neurochemical effects of ETX may operate independently of a direct impact on the gut microbial composition.

4.8. Summary and outlook

The disproportion in the inclusion of females versus males can directly affect the development of sufficient drug treatments (Check Hayden, 2010). Consequently, women might show a weaker response to treatments that have mainly been developed in men (Lovick and Zangrossi, 2021). Hence, sex differences regarding psychiatric diseases have become increasingly apparent in recent years. For example, women have a two times higher risk to suffer from anxiety disorders than men (Altemus, 2006; Bandelow and Michaelis, 2015). This sex difference might, at least partly, be due to a greater sensibility to traumatic and stressful life experiences in women (Kogler et al., 2015; Goldstein et al., 2010). Yet, it remains unclear whether the sex differences are causal for the development of such disorders or if they are a consequence of the pathology (Giatti et al., 2019).

Our study in an animal model for anxiety disorders demonstrates sex-specific effects of ETX with an anxiolytic effect only in female HAB rats accompanied by alterations in OXT-R binding and neuroplasticity within relevant brain regions, and brain steroid levels. With those findings, we extent previous knowledge on a potential role of TSPO

ligands in psychopathological disorders (Ugale et al., 2007; Verleye et al., 2005; Verleye and Gillardin, 2004). Thus, given the marked sex-dependent effects in terms of ETX and breeding lines, we propose to consider the aspect of sex in treatment strategies for anxiety disorders.

CRediT authorship contribution statement

Lilith Fischer: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Bjarne Paschke:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Franziska Gareis:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Michael Schumacher:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Philippe Liere:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Andreas Hiergeist:** Writing – review & editing, Visualization, Investigation, Formal analysis. **André Gessner:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Rainer Rupprecht:** Writing – review & editing, Methodology. **Inga D. Neumann:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Oliver J. Bosch:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare no conflict of interest. R.R. has received consultancy honoraria from SAGE/Biogen and GABA Therapeutics.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2024.110282.

Data availability

Data will be made available on request.

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