

# Effects of adipokinetic hormone/red pigment-concentrating hormone family of peptides in olfactory bulbectomy model and posttraumatic stress disorder model of rats

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## ABSTRACT

One of the major neuropeptide groups in insects is adipokinetic hormone/red pigment-concentrating hormone (AKH/RPCH) family of peptides. AKH had improving effects on depression and anxiety in animal models and it may be a new treatment choice in these disorders. Aim of this study was to investigate effects of Anax imperator AKH (Ani-AKH), Libellula auripennis AKH (Lia-AKH) and Phormia-Terra hypertrehalosemic hormone (Pht-HrTH) on animal behavior in olfactory bulbectomy (OBX) model and in posttraumatic stress disorder (PTSD) model of Wistar-albino rats. Lia-AKH and Pht-HrTH significantly increased time spent in escape platform's quadrant compared to sham control while Lia-AKH significantly increased time spent in escape platform's quadrant compared to OBX controls in probe trial of Morris water maze (MWM). Ani-AKH, Lia-AKH and Pht-HrTH significantly decreased immobility time compared to OBX controls in forced swimming test (FST). Pht-HrTH significantly increased %open arm time compared to OBX controls in elevated plus maze (EPM) test. Ani-AKH significantly increased %open arm entry compared to sham control while Ani-AKH and Pht-HrTH significantly increased %open arm entry compared to OBX controls in EPM. In PTSD study Ani-AKH and Lia-AKH significantly decreased immobility time compared to traumatized controls in FST. In acoustic startle reflex test, Ani-AKH, Lia-AKH and Pht-HrTH significantly decreased average startle amplitude compared to non-traumatized controls in PTSD study. Metabolomic studies showed that AKH may affect glutamatergic and dopaminergic system and neurochemistry. In conclusion, AKH peptides had wide ranging effects on behavior and improved performance in OBX and PTSD models in rats.

## 1. Introduction

A variety of processes including metabolic, behavioral, developmental or reproductive are influenced or regulated by neuropeptides in insects. Two of the major neuropeptide groups include the adipokinetic and hypertrehalosaemic peptides, which belong to the AKH/RPCH

family of peptides [1]. These peptide hormones are products of neurosecretory neurons located in the corpora cardiaca, neuroendocrine glands attached to the insect brain. Adipokinetic hormones (AKHs) are metabolic neuropeptides, mediating the mobilization of energy substrates from the fat body in many insects [2]. It is suggested that adipokinetic hormones may contribute to the neuronal function in the

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**Table 1**

listing the actual sequence(s) of the adipokinetic hormone/red pigment-concentrating hormone (AKH/RPCH) family of peptides.

AKH/RPCH Family	Aminoacid sequence- Chemical Name
Ani-AKH ( <i>Anax Imperator Mauricianus</i> )	pGlu-VNFPSPW-NH <sub>2</sub> pGlu-Val-Asn-Phe-Ser-Pro-Ser-Trp-NH <sub>2</sub> 5-oxo-L-prolyl-L-valyl-L-asparaginyll-L-phenylalanyl-L-seryl-L-prolyl-L-seryl-L-Tryptophanamide
Lia-AKH ( <i>Libellula Auripennis</i> )	pGlu-VNFPSPW-NH <sub>2</sub> pGlu-Val-Asn-Phe-Thr-Pro-Ser-Trp-NH <sub>2</sub> 5-oxo-L-prolyl-L-valyl-L-asparaginyll-L-phenylalanyl-L-threonyl-L-prolyl-L-seryl-L-Tryptophanamide
Pht-HrTH Hypertrehalosemic hormone ( <i>Phormia Terrae-Novae</i> )	pGlu-LTFSPDW-NH <sub>2</sub> pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH <sub>2</sub> 5-oxo-L-prolyl-L-leucyl-L-threonyl-L-phenylalanyl-L-seryl-L-prolyl-L-alfa-aspartyl-L-Tryptophanamide

human central nervous system [3], and a novel peptidergic system was suspected in the rat central nervous system by using an antiserum to locust adipokinetic hormone I [4]. AKH receptors are structurally close to gonadotropin-releasing hormone (GnRH) and the vasopressin/oxytocin superfamily of receptors [5]. In a recent study, an improvement in the effects of oxytocin on MK-801-induced deficits in the prepulse inhibition test (PPI) and the modulation of dopaminergic and glutamatergic systems by oxytocin was shown [6].

In our recent studies, we showed that the AKH/RPCH peptide family possesses antidepressant, anxiolytic and analgesic effects; causes hyperlocomotion and exerts neuroprotective effects after chronic injections in mice [7]. Also, after acute injection, we saw the antidepressant and anxiolytic effects of the AKH/RPCH family of peptides in mice, but the effect changed according to the insect type used [8]. In another study, we showed the improving effects of these peptides on MK-801-induced memory deterioration in the active allothetic place avoidance task (AAPA) while we saw no effect on the MK-801-induced psychosis model in the prepulse inhibition test (PPI) in rats [9]. We also showed that the AKH/RPCH family of peptides causes uterus contractions similar to oxytocin in human myometrium [10].

There are some structural and functional differences among the various types of insect AKHs. In this study, we used three insect AKHs, the first of which was *Anax imperator* (Ani-AKH), which has a sequence of pGlu-Val-Asn-Phe-Ser-Pro-Ser-Trp-NH<sub>2</sub>. *Libellula auripennis* AKH (Lia-AKH) was determined as pGlu-Val-Asn-Phe-Thr-Pro-Ser-Trp-NH<sub>2</sub>, and the *Phormia-Terra* hypertrehalosemic hormone (Pht-HrTH) showed a sequence of pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH<sub>2</sub> [11] (Table 1). Studies showed that the adipokinetic hormone was responsible for both carbohydrate and lipid metabolism while the hypertrehalosemic hormone mostly played a role in carbohydrate metabolism in insects [12].

AKH improved the effects of depression and anxiety by increasing neurogenesis and brain neurotrophic factors in the central nervous system [7,8]; it also improved memory in the MK-801 induced schizophrenia model [9]. Due to the structural similarity with the mammalian hormones we hypothesize, it is possible to use AKH/RPCH as a drug or an adjuvant drug of natural origin. In this study, we investigated the effects of three insect peptides of which we have found significant behavioral effects in our previous studies [7–9]: *Anax imperator* AKH (Ani-AKH), *Libellula auripennis* AKH (Lia-AKH) and the *Phormia-Terra* hypertrehalosemic hormone (Pht-HrTH) on the olfactory bulbectomy (OBX) and post-traumatic stress disorder (PTSD) models of rats.

## 2. Materials and methods

### 2.1. Animals

Male outbred Wistar rats (Velaz, Czech Republic) weighing

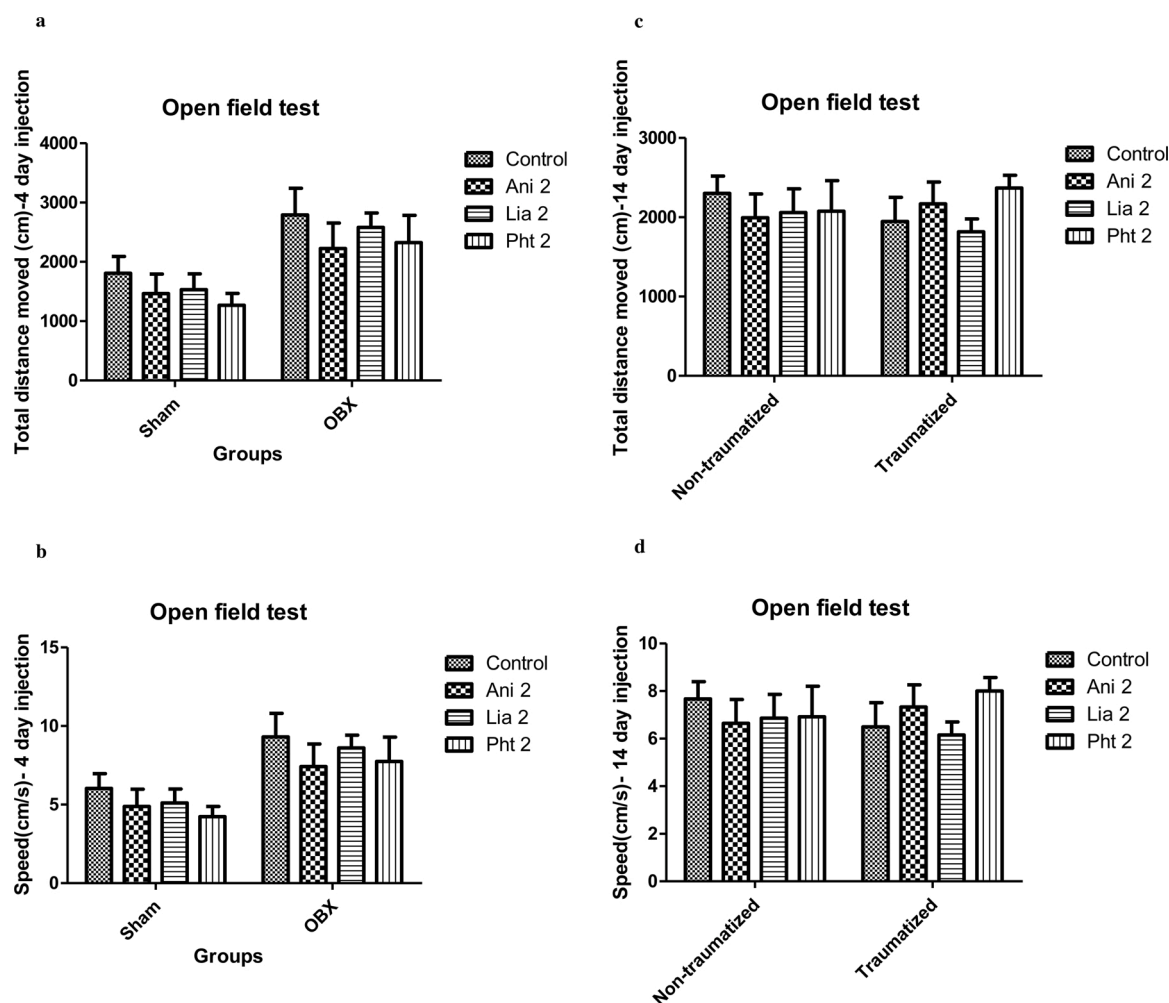
250–350 g were used for the OBX and PTSD studies. The rats were acclimatized for 7–10 days prior to testing, during which time they were weighed twice and handled four times. The rats were housed in pairs in transparent plastic cages (30 × 30 × 40 cm) in an air-conditioned animal facility with a constant temperature of 21 °C ± 2 °C and a 12/12 light/dark cycle (lights on at 7:00 a.m.). All animals received food and water ad libitum. All procedures were in accordance with Czech and European legislation regarding the treatment of laboratory animals (Directive 86/609/EEC).

### 2.2. Drugs and treatments

Ani-AKH, Lia-AKH and Pht-HrTH were purchased from TRC (Toronto/Canada). All drugs were dissolved in saline mixed with 5% DMSO. A vehicle consisting of saline with 5% DMSO was used as the control group. Three weeks after the OBX model, animals were injected with drugs, and the behavioral tests were applied on the 5th day of injection. In the OBX model, we had eight groups during the behavioral tests: Sham control, Sham Ani-AKH 2 mg/kg, Sham Lia-AKH 2 mg/kg, Sham Pht-HrTH 2 mg/kg, OBX control, OBX Ani-AKH 2 mg/kg, OBX Lia-AKH 2 mg/kg and OBX Pht-HrTH 2 mg/kg. In the OBX model, the number of animals in each group was changing between 7 and 10. In the PTSD model, one week after the second exposure to the predator odor, drug administration started and continued for three weeks. We had eight different groups: non-traumatized control (n = 9), non-traumatized Ani-AKH 2 mg/kg (n = 8), non-traumatized Lia-AKH 2 mg/kg (n = 8), non-traumatized Pht-HrTH 2 mg/kg (n = 8), traumatized control (n = 9), traumatized Ani-AKH 2 mg/kg (n = 9), traumatized Lia-AKH 2 mg/kg (n = 9) and traumatized Pht-HrTH 2 mg/kg (n = 9). Strains of the animals used in the OBX and PTSD models, doses of the drugs and administration periods of the drugs were chosen according to the previous studies [8–10,13,14].

### 2.3. OBX model of rats

The rats were anesthetized with isoflurane (no. B306, Abbot Laboratories, Queen-Borough, UK) in a preparation chamber (3% of isoflurane) and then placed in a stereotactic apparatus (no. 430005-GR-GP/K, TSE systems, Germany). The level of isoflurane anesthesia during the operation was kept at 2%. For local anesthesia, 0.5 ml of 1% Mesocaine (Zentiva, Czech Republic) was applied before an incision. The eyes were treated with Vidisic ocular gel (Bausch + Lomb, Canada). The incision was made in the scalp above the olfactory bulbs. Two 2-mm burr holes were made with a microdrill (Dremel, USA), 8 mm AP and ±2 mm ML from bregma. The olfactory bulbs were removed with a blunt hypodermic needle connected to a water pump [15]. The hemorrhage was stopped using silver nitrate stick, and the incision was sutured using absorbent material. The sutures were treated with the local antibiotic Framykoin (Zentiva, Czech Republic). Sham-operated control rats underwent the same procedure of the OBX procedure without removal of the olfactory bulbs. After surgery, 2.5 ml of sterile water was administered intraperitoneally (i.p.), and the analgesic Nurofen was added into the drinking water. The injections and experiments were performed after a postoperative period of three weeks. A vehicle consisting of saline with 5% DMSO administered to sham control rats. Behavioral tests started on the fifth day of the injections. The animals were subjected to the following tests: openfield (OF) (day 5), Morris water maze (MWM) (days 6–11), elevated plus maze (EPM) (day 12) and forced swimming (FST) (days 13–14) to evaluate the effects of AKH on locomotion, memory, anxiety and depression in the OBX model. After the termination of behavioral procedures, the brains were extracted, and the lesions were inspected. Animals with remains of olfactory tissue were evaluated as partial OBX animals. Removing of the olfactory bulb causes loss of sensory input, disturbs serotonergic neurons in raphe nucleus and also causes neuronal degeneration in habenular nucleus which causes depression like behavioral effects. OBX results in degeneration of many



**Fig. 1.** a-b illustrates (a) the total distance moved (cm) (b) speed (cm/s) data after intraperitoneally (i.p.) administration of 4 days in sham control (n = 8), sham Ani-AKH (2 mg/kg) (n = 8), sham Lia-AKH (2 mg/kg) (n = 9), sham Pht-HrTH (2 mg/kg) (n = 10), OBX control (n = 9), OBX Ani-AKH (2 mg/kg) (n = 8), OBX Lia-AKH (2 mg/kg) (n = 8), OBX Pht-HrTH (2 mg/kg) (n = 7) in the open field test in the OBX model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean. c-d illustrates (c) the total distance moved (cm) (d) speed (cm/s) data after intraperitoneally (i.p.) administration of 14 days in non-traumatized control (n = 9), non-traumatized Ani-AKH (2 mg/kg) (n = 8), non-traumatized Lia-AKH (2 mg/kg) (n = 8), non-traumatized Pht-HrTH (2 mg/kg) (n = 8), traumatized control (n = 9), traumatized Ani-AKH (2 mg/kg) (n = 9), traumatized Lia-AKH (2 mg/kg) (n = 9), traumatized Pht-HrTH (2 mg/kg) (n = 9) in the open field test in the PTSD model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean.

areas such as the cortex, hippocampus, amygdala, locus coeruleus, and dorsal raphe nuclei, and produces enlargement of the lateral and third ventricles. The degenerative changes in the amygdala, hippocampus, and cortex induce in turn alterations in memory and behavior as well as cognitive deficits. OBX causes increase in locomotion, increases immobility time in FST test and disturbs memory in MWM test in rodents.

#### 2.4. Morris water maze test

The Morris water maze consisted of a circular pool (150-cm diameter) that was filled with water (25 °C). Small black pieces of plastic were placed on the surface of the water to obscure the platform [16]. The pool was located in a dimly lit, soundproof test room with a number of extra-maze visual cues, including a white-and-black poster on the wall, a halogen lamp, a camera and the experimenter. The maze was divided into four quadrants. Three equally spaced points around the edge of the pool were used as the release positions. The order of the release positions was varied systematically throughout the experiment. An escape platform was located in one quadrant, 1 cm above the water surface during the familiarization session and 1 cm below the water surface during the other sessions. Video tracking was conducted with a video camera focused on the full diameter of the pool. Navigation

parameters were analyzed by using the EthoVision 3.1 video analysis system (Noldus, Amsterdam, Netherlands). The rats were trained in the Morris water maze over five daily sessions (familiarization session, S1, S2, S3, S4). The five sessions were performed on consecutive days between 9:00 and 12:00. During the acquisition phase of the experiments, each rat participated in three trials per day [17]. For each daily trial, each rat was taken from the home cage and placed into the water maze at one of three randomly determined locations with its head facing the center of the water maze. A trial was started when the rat was released from one of the three randomly chosen start positions. After the rat found and climbed onto the platform, the trial was stopped, and the escape latency was recorded. The maximum trial length was 60 s. If the rat had not climbed onto the platform in 60 s, the experimenter guided the rat by hand to the platform, and an escape latency of 60 s was recorded. The inter-trial time was 60 s. During this time, the rat was kept on the escape platform before starting the next trial. The rat was then placed in the pool again, but at a different location, and the next trial began upon its release. Normally, the escape latency declines during acquisition as the animal learns the location of the hidden platform. At the end of the third trial, the rat was returned to its cage. Twenty-four hours after the last acquisition session, a “probe trial” was used to assess each rat’s spatial retention of the location of the hidden platform.

**Table 2**  
listing the behavioral tests and effect of drug vs condition (OBX, PTSD).

Tests	OBX	PTSD
Open field MWM	No significant effect of drugs Lia-AKH and Pht-HrTH improved spatial learning compared to sham control. All drugs improved spatial memory compared to sham control. Lia-AKH and Pht-HrTH improved spatial memory compared to OBX control. Lia-AKH and Pht-HrTH increased speed of the animals compared to OBX control.	No significant effect of drugs –
FST	All drugs had antidepressant effect compared to OBX control	Ani-AKH and Lia-AKH had antidepressant effect compared to OBX control
EPM	Ani-AKH had anxiolytic effect compared to sham control while Lia-AKH and Pht-HrTH had anxiolytic effect compared to OBX control	No significant effect of drugs
Exploratory activity	–	No significant effect of drugs
Startle reflex	–	All drugs decreased the average startle amplitude compared to non-traumatized control group which supports their anxiolytic effect
Weight	Pht-HrTH decreased weight gain compared to sham control	Ani-AKH and Pht-HrTH decreased weight gain compared to non-traumatized control while Ani-AKH and Pht-HrTH increased weight gain compared to traumatized control

During this trial, the platform was removed from the maze, and each rat was allowed to search the pool for 60 s before being removed. It was determined that during this trial, animals should spend more time swimming in the quadrant that previously contained the hidden platform than in the other three quadrants.

## 2.5. PTSD model of rats

The PTSD model of rats was applied according to Zoladz et al. [14]. In the PTSD model, rats were exposed to 31 days of psychosocial stress, including acute and chronic components. The acute component included a two-hour predator odor exposure (immobilization during predator odor exposure) on days 1 and 11, and the chronic component included social stress to be applied for 31 days. Cat fur was used as predator odor. All traumatized rats were isolated in different cages. A small portion of cat fur was placed in each cage of the traumatized rats for two hours. Nontraumatized rats were nonimmobilized in their own cages. During social stress, animals were placed two per cage, and every day, these paired animals were changed. One week after the second exposure to predator odor, drug administration started and continued for three weeks. During the last week of drug administration, the open field test, the forced swimming test (FST), the elevated plus-maze test (EPM), the exploratory activity test and the acoustic startle reflex (ASR) test were applied for the evaluation of behavioral parameters of animals. The body weight of the animals were also measured at the beginning and ending of the injections.

## 2.6. Open field test

The rats' spontaneous activity in a new environment was observed in the open field (OF) test for five minutes. The OF (50 × 50 cm) was dimly illuminated by red light, which is considered to be less distressing for

rodents. Activity was detected by evenly spaced infrared light beams. Beam interruptions caused by movements of the animal were registered by the software (EthoVision, Noldus, Netherlands). The software analyzed the total distance moved and the speed of the animals during a five-minute test period.

## 2.7. Elevated plus maze test (EPM)

The apparatus consisted of two open arms (45 × 10 cm) crossed at right angles with two opposed arms of the same length. Two of the opposed arms were enclosed by walls 40 cm high, except for the central platform where the arms crossed. The whole apparatus was elevated 50 cm above the floor. At the beginning of each experiment, each rat was placed on the central platform facing the closed arm. Time spent in open arms, closed arms and in central platform, as well as the distance moved were recorded during a five-minute test session by a camera positioned above the maze and analyzed by the software (EthoVision, Noldus, Netherlands).

## 2.8. Forced swimming test (FST)

The procedure described by Porsolt et al. [18] was used. The rats were placed individually in plexiglass cylinders (40 cm in height, 18 cm in diameter) filled with water (25 °C) up to 15 cm. A 15-minute pre-swimming period was followed 24 h later by a five-minute test period during which the total immobility time was recorded. Rats were considered immobile when they made no further attempts to escape, except for necessary movements to keep their heads above water. The absence of hind leg movement was recorded as immobility by stopwatch cumulation by a single observer during the exposures. The water in the cylinders was changed before every trial. All experiments were performed between 10:00 and 12:00 a.m.

## 2.9. Exploratory activity test

The apparatus consisted of a polyvinyl chloride apparatus covered with Plexiglas and subdivided into two identical square exploration units (50 × 50 cm) with small interpassing partitions [19]. A partition divided the apparatus in half lengthwise. To familiarize the animals with the apparatus, each subject was placed in one half of the apparatus approximately two hours before testing. After two hours, the same rat was exposed to both the familiar and novel environments. The subject was then observed under a red light for five minutes. Parameters recorded were the number of units entered to the novel side, total number of entries to both sides and the time spent in the novel side.

## 2.10. Acoustic startle reflex test (ASR)

Rats were placed inside a small Plexiglas box (19 × 10 × 10 cm), which was inside a larger startle monitor cabinet (Hamilton-Kinder; San Diego, CA; 36 × 28 × 50 cm). The small Plexiglas box within this cabinet contained a sensory transducer on which rats were placed at the beginning of the trial. The sensory transducer was connected to a computer (Startle Monitor computer program; Hamilton-Kinder; San Diego, CA), which recorded rats' startle responses by measuring the maximum amount of force (in N) that rats exerted on the sensory transducer for a period of 250 ms after the presentation of each auditory stimulus. To control the differences in body weight, the sensitivity of the sensory transducer was adjusted prior to each trial via a vernier adjustment with a sensitivity range of zero to seven arbitrary units. The startle trial began with a five-minute acclimation period, followed by the presentation of 24 bursts of white noise (50 ms each), eight from each of the three auditory intensities (90, 100 and 110 dB). The noise bursts were presented in sequential order (i.e., eight bursts at 90 dB, followed by eight bursts at 100 dB, followed by eight bursts at 110 dB), and the time between each noise burst varied pseudorandomly between 25 and

55 seconds. Upon the commencement of the first noise burst, the startle apparatus provided uninterrupted background white noise (57 dB).

## 2.11. Liquid chromatography-mass spectrometry (LC-MS)

### 2.11.1. Targeted analysis

**2.11.1.1. Reagents.** Dopamine (DA), 5-hydroxy tryptamine (5-HT),  $\gamma$ -aminobutyric acid (GABA) and glutamate (Glu) were purchased from Sigma-Aldrich (USA). LC-MS grade acetonitrile and methanol were obtained from Honeywell-Research Chemicals (France). High-purity water was provided by a Milli-Q system Aqual Elga Flex3. LC-MS grade formic acid was purchased from Sigma-Aldrich (USA).

**2.11.1.2. Sample preparation for targeted LC-MS/MS analysis.** Whole rat brains were weighed directly after the sacrifice and deeply frozen ( $-84^{\circ}\text{C}$ ) immediately. After thawing, ice cold methanol was added to each sample (for 1 g of tissue, 4 mL of liquid), and each sample was homogenized and vortex-mixed (one minute) before undergoing centrifugation ( $18,000 \times g$  for 10 min at  $0^{\circ}\text{C}$ ). The supernatant was transferred and evaporated to dryness by speedvac (Hanil Modul 4080C). The dry residue was reconstituted in 100  $\mu\text{L}$  of Methanol, and the aliquot of 10  $\mu\text{L}$  was injected into the LC-MS system for analysis.

**2.11.1.3. LC-MS/MS conditions.** LC-MS/MS was run on an AB SCIEX QTRAP 6500 spectrometer equipped with an ESI ion source and a Thermo Scientific Ultimate 3000 HPLC system with an autosampler. The analytes were separated on a Phenomenex Kinetex C18 column ( $2.1 \times 50 \text{ mm}$ ,  $1.7 \mu\text{m}$ ) used at  $30^{\circ}\text{C}$ . The mobile phase consisting of 0.1

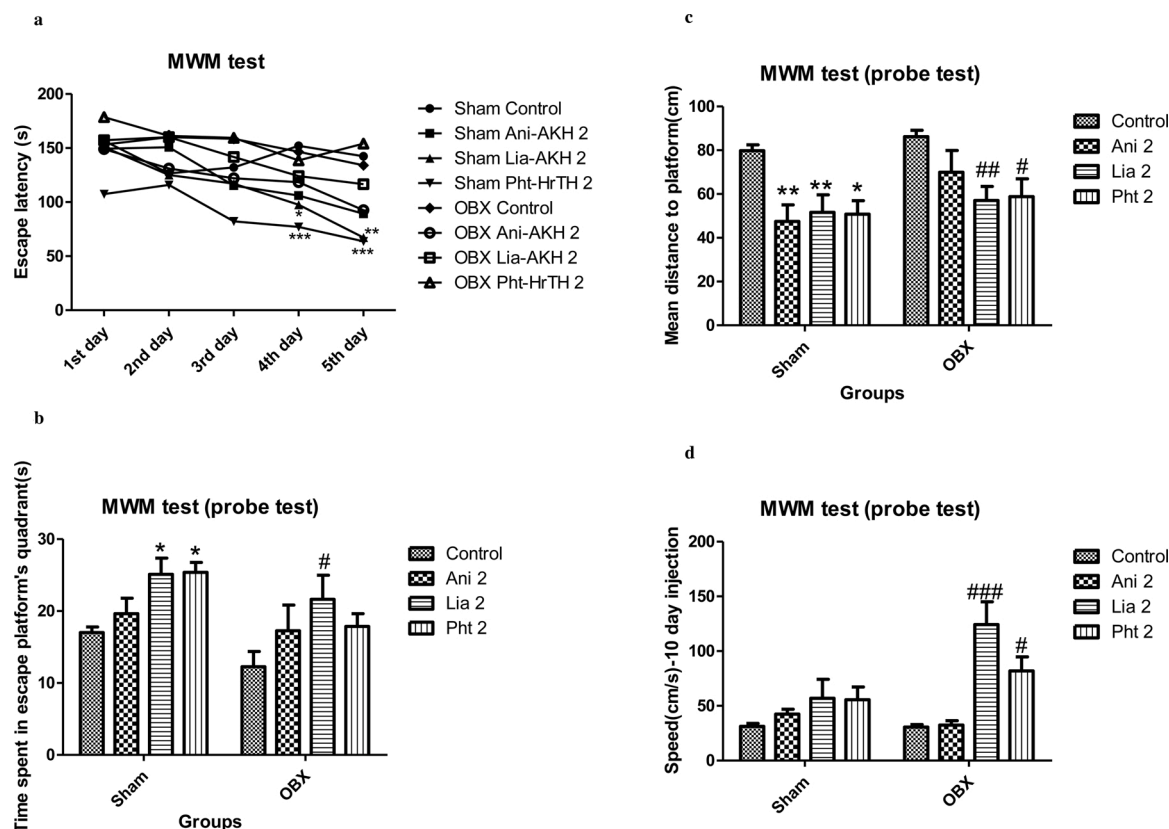
% formic acid in water (Solvent A) and Methanol (Solvent B) was used with a gradient elution: 0–1.5 min, 2% B; 7 min, 98 % B; 8.5 min, 98 % B; 10 min, 2% B, 11.5 min and 2% B at a flowrate of 0.37 mL/min. MS acquisition of 5-HT, GABA, Glu and DA was performed in electrospray positive ionization multiple reaction monitoring (MRM) mode.

### 2.11.2. Untargeted analysis

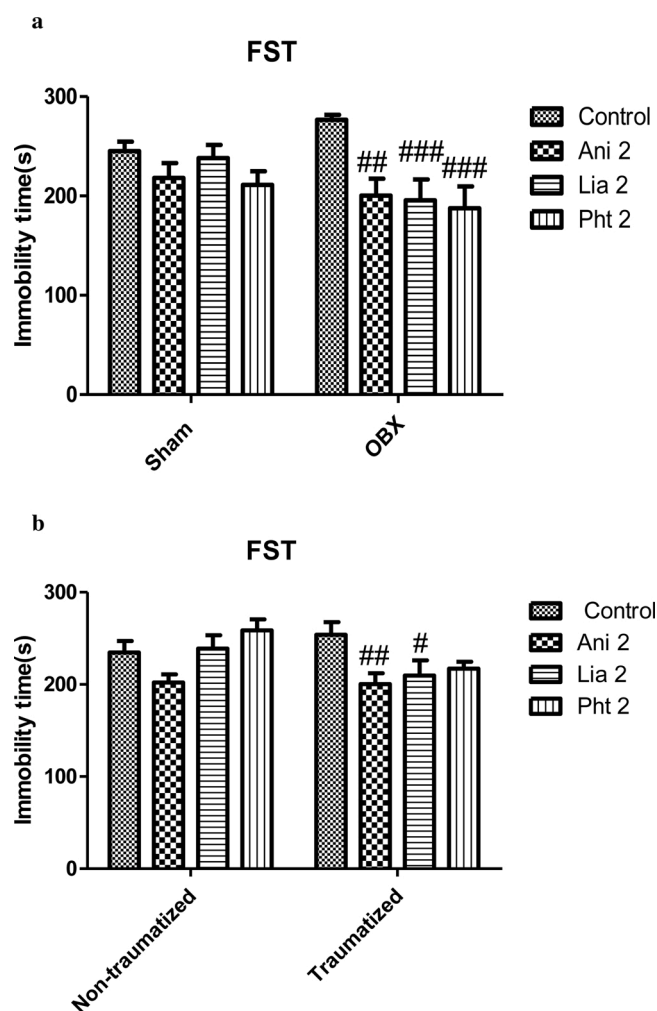
**2.11.2.1. Reagents.** LC-MS grade acetonitrile and methanol were obtained from Honeywell-Research Chemicals (France). High-purity water was provided by a Milli-Q system Aqual Elga Flex3. LC-MS grade formic acid was purchased from Sigma-Aldrich (USA).

**2.11.2.2. Sample preparation for untargeted LC-MS/MS analysis.** Whole rat brains were weighed directly after the sacrifice and deeply frozen ( $-84^{\circ}\text{C}$ ) immediately. After thawing, ice cold methanol was added to each sample (for 1 g of tissue, 4 mL of liquid), and each sample was homogenized and vortex-mixed (one minute) before undergoing centrifugation ( $18,000 \times g$  for 10 min at  $0^{\circ}\text{C}$ ). The supernatant was transferred and evaporated to dryness with a SpeedVac (Hanil Modul 4080C). The dry residue was reconstituted in 100  $\mu\text{L}$  of Methanol, and the aliquot of 10  $\mu\text{L}$  was injected into the LC-MS system for analysis.

**2.11.2.3. LC-MS/MS conditions.** LC-MS/MS was run on an AB SCIEX TripleTOF 5600 spectrometer equipped with an ESI ion source and a Thermo Scientific Ultimate 3000 HPLC system with an autosampler. The analytes were separated on a Phenomenex Kinetex C18 column ( $3 \times 150 \text{ mm}$ ,  $2.6 \mu\text{m}$ ) used at  $30^{\circ}\text{C}$ . The mobile phase consisting of 0.1 % formic acid in water (Solvent A) and Methanol (Solvent B) was used



**Fig. 2.** illustrates (a) escape latency (s) (b) time spent in escape platform's quadrant (s) (c) mean distance to platform (cm) (d) speed (cm/s) data after intraperitoneally (i.p.) administration of 10 days in sham control (n = 8), sham Ani-AKH (2 mg/kg) (n = 8), sham Lia-AKH (2 mg/kg) (n = 9), sham Pht-HrTH (2 mg/kg) (n = 10), OBX control (n = 8), OBX Ani-AKH (2 mg/kg) (n = 8), OBX Lia-AKH (2 mg/kg) (n = 8), OBX Pht-HrTH (2 mg/kg) (n = 7) in the MWM test in the OBX model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean. (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 compared with the sham control group; #p < 0.05, ##p < 0.01, ###p < 0.001 compared with the OBX control group).



**Fig. 3.** a illustrates immobility time (s) data after intraperitoneally (i.p.) administration of 13 days in sham control (n = 8), sham Ani-AKH (2 mg/kg) (n = 8), sham Lia-AKH (2 mg/kg) (n = 9), sham Pht-HrTH (2 mg/kg) (n = 10), OBX control (n = 8), OBX Ani-AKH (2 mg/kg) (n = 8), OBX Lia-AKH (2 mg/kg) (n = 8), OBX Pht-HrTH (2 mg/kg) (n = 7) in the FST test in the OBX model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean. (## $p$  < 0.01, ### $p$  < 0.001 compared with the OBX control group). b illustrates immobility time (s) data after intraperitoneally (i.p.) administration of 16 days in non-traumatized control (n = 9), non-traumatized Ani-AKH (2 mg/kg) (n = 8), non-traumatized Lia-AKH (2 mg/kg) (n = 8), non-traumatized Pht-HrTH (2 mg/kg) (n = 8), traumatized control (n = 9), traumatized Ani-AKH (2 mg/kg) (n = 9), traumatized Lia-AKH (2 mg/kg) (n = 9), traumatized Pht-HrTH (2 mg/kg) (n = 9) in the FST in the PTSD model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean (# $p$  < 0.05, ## $p$  < 0.01 compared with the traumatized control group).

with a gradient elution: 0–4.5 min, 20 % B; 17.5 min, 99 % B; 25.5 min, 99 % B; 27 min, 20 % B and 30 min, 20 % B at a flowrate of 0.5 mL/min. MS acquisition was performed in electrospray positive ionization.

## 2.12. Statistical evaluation

The results of the behavioral tests and weight gain were evaluated by a two-way ANOVA followed by Bonferroni's post hoc test when significant differences were detected. Results of neurotransmitters were evaluated by a one-way ANOVA followed by Tukey's post hoc test when significant differences were found. The data were expressed as mean values  $\pm$  SEM. The differences were considered statistically significant when the alpha value was equal to or below 0.05. The results of untargeted analysis were evaluated by the software called

Metaboanalyst.

## 3. Results

### 3.1. Effects of Ani-AKH, Lia-AKH and Pht-HrTH on locomotion in the open field test in the OBX and PTSD models

There were significant effects of OBX [ $F(1, 59) = 16.07$ ;  $p = 0.0002$ ] while there were no significant effects of drugs [ $F(3, 59) = 0.92$ ;  $p = 0.43$ ] and interaction [ $F(3, 59) = 0.08$ ;  $p = 0.96$ ] on the total distance moved in the open field test in OBX study. Bonferroni post hoc tests showed no significant differences among groups (Fig. 1a, Table 2).

There were significant effects of OBX [ $F(1, 59) = 16.08$ ;  $p = 0.0002$ ] while there were no significant effects of drugs [ $F(3, 59) = 0.92$ ;  $p = 0.43$ ] and interaction [ $F(3, 59) = 0.08$ ;  $p = 0.96$ ] on the speed in the open field test in OBX study. Bonferroni post hoc tests showed no significant differences among groups (Fig. 1b, Table 2).

There were no significant effects of drugs [ $F(3, 61) = 0.38$ ;  $p = 0.76$ ], PTSD [ $F(1, 61) = 0.02$ ;  $p = 0.86$ ] and interaction [ $F(3, 61) = 0.69$ ;  $p = 0.56$ ] on the total distance moved in the open field test in the PTSD study (Fig. 1c, Table 2). Also, there were no significant effects of drugs [ $F(3, 61) = 0.37$ ;  $p = 0.77$ ], PTSD [ $F(1, 61) = 0.002$ ;  $p = 0.96$ ] and interaction [ $F(3, 61) = 0.72$ ;  $p = 0.53$ ] on speed in the open field test in the PTSD study (Fig. 1d, Table 2).

### 3.2. Effects of Ani-AKH, Lia-AKH and Pht-HrTH on learning and memory in the Morris water maze test in the OBX model

There were significant effects of drugs [ $F(7, 290) = 11.95$ ;  $p < 0.0001$ ] and sessions [ $F(4, 290) = 10.74$ ;  $p < 0.0001$ ] while there were no significant effects of interaction [ $F(28, 290) = 0.78$ ;  $p = 0.77$ ] on escape latency during five days of training sessions of the MWM test in the OBX study. Bonferroni post hoc tests showed that Lia-AKH 2 mg/kg ( $p < 0.05$  and  $p < 0.01$ ; respectively) and Pht-HrTH 2 mg/kg ( $p < 0.001$ ) significantly decreased escape latency compared to sham control in the fourth and fifth days of the MWM test in the OBX study (Fig. 2a, Table 2).

There were significant effects of drugs [ $F(3, 58) = 5.45$ ;  $p = 0.002$ ] and OBX [ $F(1, 58) = 7.58$ ;  $p = 0.007$ ] while there were no significant effects of interaction [ $F(3, 58) = 0.45$ ;  $p = 0.71$ ] on time spent in the escape platform's quadrant in the probe trial of the MWM test in the OBX study. Bonferroni post hoc tests showed that Lia-AKH 2 mg/kg ( $p < 0.05$ ) and Pht-HrTH 2 mg/kg ( $p < 0.05$ ) significantly increased time spent in the escape platform's quadrant compared to the sham control group while Lia-AKH 2 mg/kg ( $p < 0.05$ ) significantly increased time spent in the escape platform's quadrant compared to the OBX control in the probe trial of the MWM test in the OBX study (Fig. 2b, Table 2).

There were significant effects of drugs [ $F(3, 58) = 7.67$ ;  $p = 0.002$ ] and OBX [ $F(1, 58) = 4.67$ ;  $p = 0.03$ ] while there were no significant effects of interaction [ $F(3, 58) = 0.65$ ;  $p = 0.58$ ] on mean distance to the platform in the probe trial of the MWM test in the OBX study. Bonferroni post hoc tests showed that Ani-AKH 2 mg/kg ( $p < 0.01$ ), Lia-AKH 2 mg/kg ( $p < 0.01$ ) and Pht-HrTH 2 mg/kg ( $p < 0.05$ ) significantly decreased mean distance to the platform compared to the sham control group while Lia-AKH 2 mg/kg ( $p < 0.01$ ) and Pht-HrTH 2 mg/kg ( $p < 0.05$ ) significantly decreased mean distance to the platform compared to the OBX control in the probe trial of the MWM test in the OBX study (Fig. 2c, Table 2).

There were significant effects of drugs [ $F(3, 58) = 10.94$ ;  $p < 0.0001$ ], OBX [ $F(1, 58) = 6.02$ ;  $p = 0.01$ ] and interaction [ $F(3, 58) = 4.24$ ;  $p = 0.008$ ] on speed in the probe trial of the MWM test in the OBX study. Bonferroni post hoc tests showed that Lia-AKH 2 mg/kg ( $p < 0.001$ ) and Pht-HrTH 2 mg/kg ( $p < 0.05$ ) significantly increased the speed of the animals compared to the OBX control in the probe trial of the MWM test in the OBX study (Fig. 2d, Table 2).

### 3.3. Effects of Ani-AKH, Lia-AKH and Pht-HrTH on depression in the FST in the OBX and PTSD models

There were significant effects of drugs [ $F(3, 58) = 6.23$ ;  $p = 0.001$ ] while there were no significant effects of OBX [ $F(1, 58) = 1.48$ ;  $p = 0.22$ ] and interaction [ $F(3, 58) = 2.13$ ;  $p = 0.10$ ] on immobility time in the forced swimming test in the OBX study. Bonferroni post hoc tests showed that Ani-AKH 2 mg/kg ( $p < 0.01$ ), Lia-AKH 2 mg/kg ( $p < 0.001$ ) and Pht-HrTH 2 mg/kg ( $p < 0.001$ ) significantly decreased immobility time compared to the OBX control group in the OBX study (Fig. 3a, Table 2).

There were significant effects of drugs [ $F(3, 61) = 4.63$ ;  $p = 0.005$ ] while there were no significant effects of PTSD [ $F(1, 61) = 2.26$ ;  $p = 0.13$ ] and interaction [ $F(3, 61) = 2.40$ ;  $p = 0.07$ ] on immobility time in the forced swimming test in the PTSD study. Bonferroni post hoc tests showed that Ani-AKH 2 mg/kg ( $p < 0.01$ ) and Lia-AKH 2 mg/kg ( $p < 0.05$ ) significantly decreased immobility time compared to the traumatized control group in the PTSD study (Fig. 3b, Table 2).

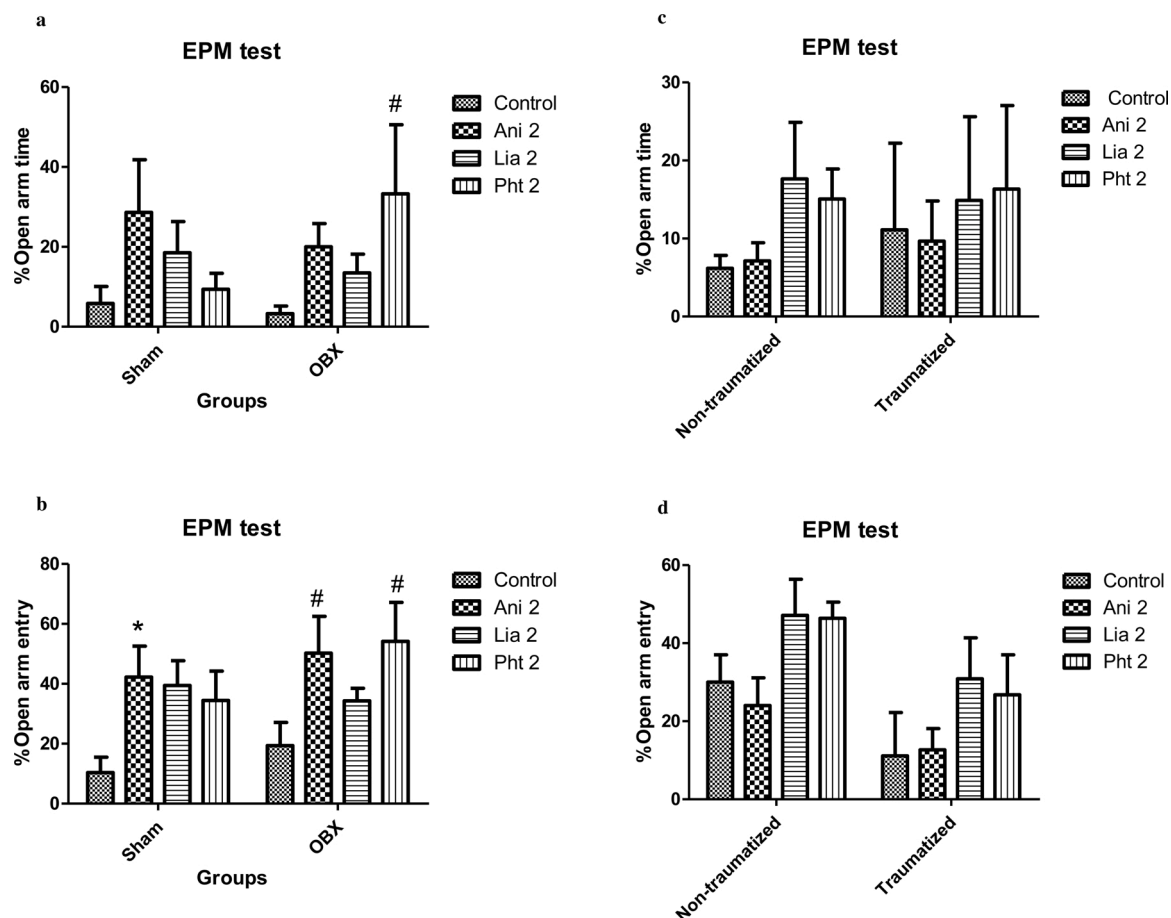
### 3.4. Effects of Ani-AKH, Lia-AKH and Pht-HrTH on anxiety in the EPM test in the OBX and PTSD models

There were no significant effects of drugs [ $F(3, 59) = 2.21$ ;  $p = 0.09$ ], OBX [ $F(1, 59) = 0.10$ ;  $p = 0.74$ ] and interaction [ $F(3, 59) = 1.55$ ;

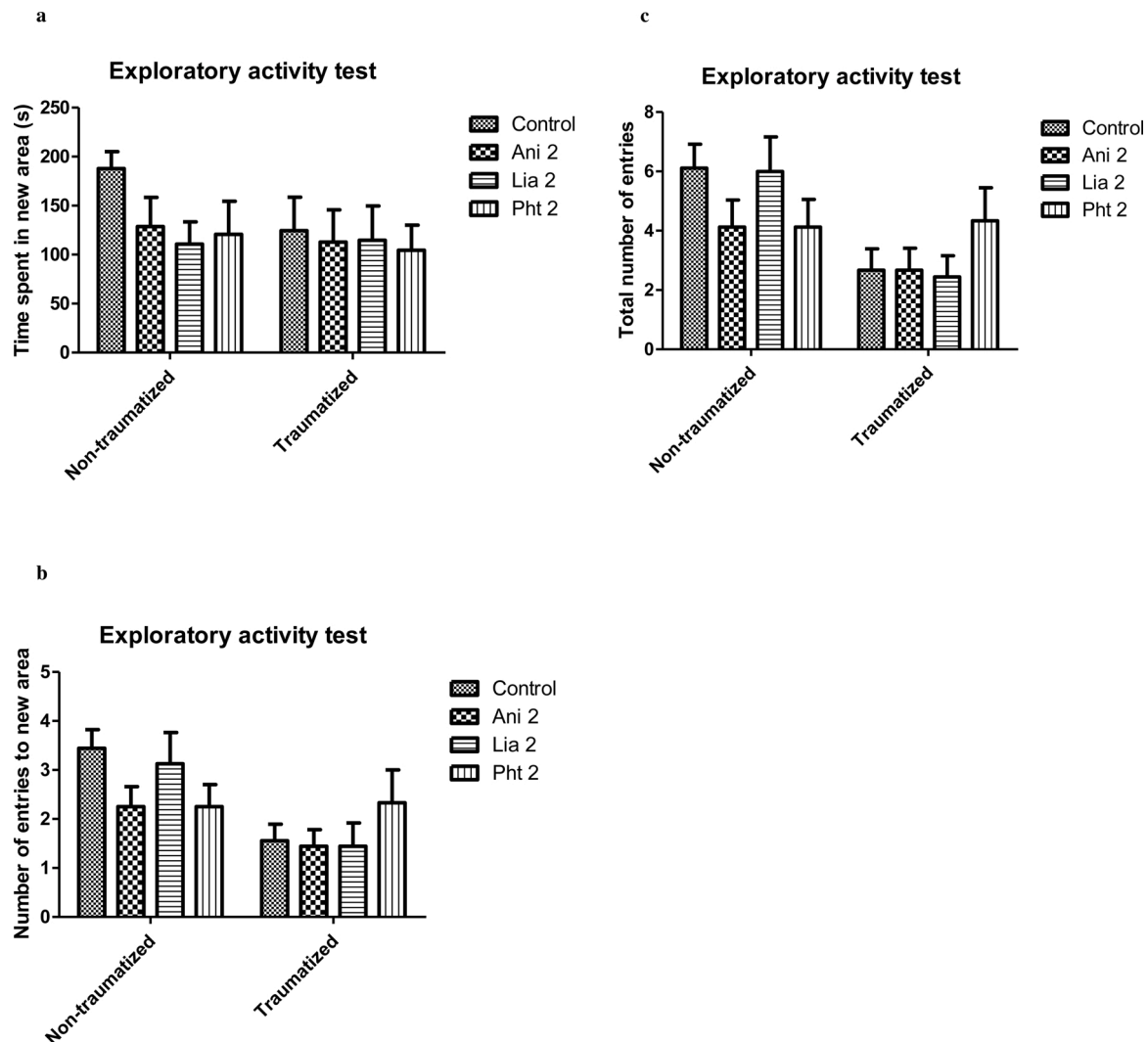
$p = 0.21$ ] on %open arm time in the EPM test in the OBX study. Bonferroni post hoc tests showed that Pht-HrTH 2 mg/kg ( $p < 0.05$ ) significantly increased %open arm time compared to the OBX control group in the OBX study (Fig. 4a, Table 2).

There were significant effects of drugs [ $F(3, 59) = 4.86$ ;  $p = 0.004$ ] while there were no significant effects of OBX [ $F(1, 59) = 1.45$ ;  $p = 0.23$ ] and interaction [ $F(3, 59) = 0.61$ ;  $p = 0.61$ ] on %open arm entry in the EPM test in the OBX study. Bonferroni post hoc tests showed that Ani-AKH 2 mg/kg ( $p < 0.05$ ) significantly increased %open arm entry compared to the sham control group while Ani-AKH 2 mg/kg ( $p < 0.05$ ) and Pht-HrTH 2 mg/kg ( $p < 0.05$ ) significantly increased %open arm entry compared to the OBX control group in the OBX study (Fig. 4b, Table 2).

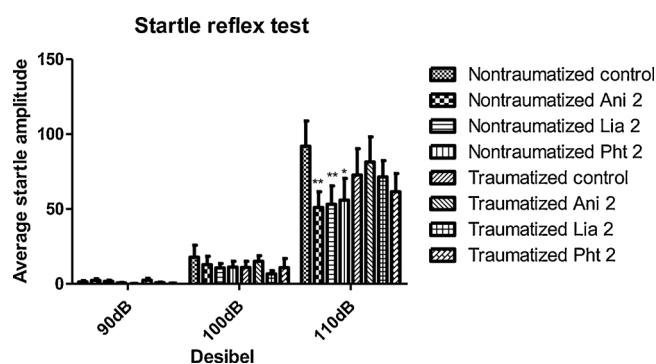
There were no significant effects of drugs [ $F(3, 61) = 0.61$ ;  $p = 0.60$ ], PTSD [ $F(1, 61) = 0.07$ ;  $p = 0.78$ ] and interaction [ $F(3, 61) = 0.08$ ;  $p = 0.96$ ] on %open arm time in the elevated plus maze test in the PTSD study (Fig. 4c, Table 2). There were significant effects of drugs [ $F(3, 61) = 3.08$ ;  $p = 0.03$ ] and PTSD [ $F(1, 61) = 7.40$ ;  $p = 0.0085$ ] while there were no significant effects of interaction [ $F(3, 61) = 0.09$ ;  $p = 0.96$ ] on %open arm entry in the elevated plus maze test in the PTSD study. Bonferroni post hoc tests showed no significant differences among the groups. There was a partial effect of Lia-AKH 2 mg/kg and Pht-HrTH 2 mg/kg on %open arm entry, but it did not reach a significant level (Fig. 4d, Table 2).



**Fig. 4.** a-b illustrates (a)%open arm time (b)%open arm entry after intraperitoneally (i.p.) administration of 11 days in sham control ( $n = 8$ ), sham Ani-AKH (2 mg/kg) ( $n = 8$ ), sham Lia-AKH (2 mg/kg) ( $n = 10$ ), sham Pht-HrTH (2 mg/kg) ( $n = 9$ ), OBX control ( $n = 9$ ), OBX Ani-AKH (2 mg/kg) ( $n = 8$ ), OBX Lia-AKH (2 mg/kg) ( $n = 8$ ), OBX Pht-HrTH (2 mg/kg) ( $n = 7$ ) in the EPM test in the OBX model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean. (\* $p < 0.05$  compared with the sham control group; # $p < 0.05$  compared with the OBX control group). c-d illustrates (c)%open arm time (d)%open arm entry after intraperitoneally (i.p.) administration of 17 days in non-traumatized control ( $n = 9$ ), non-traumatized Ani-AKH (2 mg/kg) ( $n = 8$ ), non-traumatized Lia-AKH (2 mg/kg) ( $n = 8$ ), non-traumatized Pht-HrTH (2 mg/kg) ( $n = 8$ ), traumatized control ( $n = 9$ ), traumatized Ani-AKH (2 mg/kg) ( $n = 9$ ), traumatized Lia-AKH (2 mg/kg) ( $n = 9$ ), traumatized Pht-HrTH (2 mg/kg) ( $n = 9$ ) in the EPM test in the PTSD model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean.



**Fig. 5.** illustrates (a)time spent in new area (s) (b)number of entries to new area (c)total number of entries after intraperitoneally (i.p.) administration of 18 days in non-traumatized control (n = 9), non-traumatized Ani-AKH (2 mg/kg) (n = 8), non-traumatized Lia-AKH (2 mg/kg) (n = 8), non-traumatized Pht-HrTH (2 mg/kg) (n = 8), traumatized control (n = 9), traumatized Ani-AKH (2 mg/kg) (n = 9), traumatized Lia-AKH (2 mg/kg) (n = 9), traumatized Pht-HrTH (2 mg/kg) (n = 9) in the exploratory activity test in the PTSD model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean.

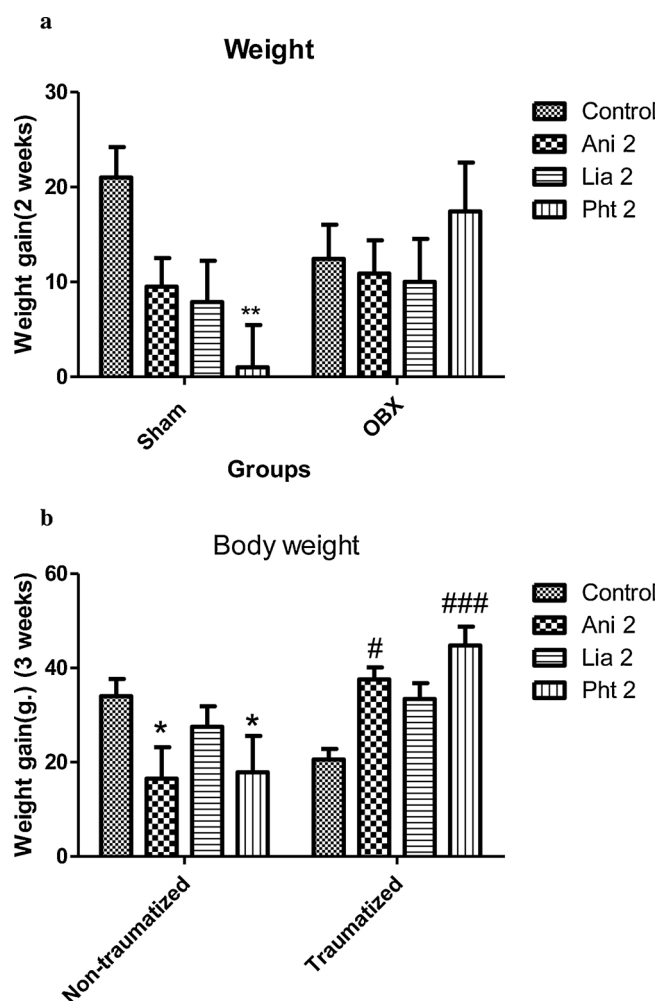


**Fig. 6.** illustrates average startle amplitude data after intraperitoneally (i.p.) administration of 20 days at 90, 100 and 110 dB in non-traumatized control (n = 9), non-traumatized Ani-AKH (2 mg/kg) (n = 8), non-traumatized Lia-AKH (2 mg/kg) (n = 8), non-traumatized Pht-HrTH (2 mg/kg) (n = 8), traumatized control (n = 9), traumatized Ani-AKH (2 mg/kg) (n = 9), traumatized Lia-AKH (2 mg/kg) (n = 9), traumatized Pht-HrTH (2 mg/kg) (n = 9) in the ASR test in the PTSD model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean (\* $p$  < 0.05, \*\* $p$  < 0.01 compared with the nontraumatized control group).

### 3.5. Effects of Ani-AKH, Lia-AKH and Pht-HrTH on exploratory activity in the PTSD model

There were no significant effects of drugs [ $F(3, 61) = 1.00$ ;  $p = 0.39$ ], PTSD [ $F(1, 61) = 1.18$ ;  $p = 0.28$ ] and interaction [ $F(3, 61) = 0.47$ ;  $p = 0.70$ ] on time spent in new areas in the exploratory activity test in the PTSD study (Fig. 5a, Table 2). There were significant effects of PTSD [ $F(1, 61) = 10.17$ ;  $p = 0.002$ ] while there were no significant effects of drugs [ $F(3, 61) = 0.66$ ;  $p = 0.57$ ] and interaction [ $F(3, 61) = 1.80$ ;  $p = 0.15$ ] on the number of entries to new areas in the exploratory activity test in the PTSD study (Fig. 5b, Table 2). Bonferroni post hoc tests showed no significant differences among the groups. There was a partial effect of Pht-HrTH 2 mg/kg on the number of entries to new areas, but it did not reach a significant level.

There were significant effects of PTSD [ $F(1, 61) = 10.55$ ;  $p = 0.0019$ ] while there were no significant effects of drugs [ $F(3, 61) = 0.49$ ;  $p = 0.68$ ] and interaction [ $F(3, 61) = 1.99$ ;  $p = 0.12$ ] on the total number of entries in the exploratory activity test in the PTSD study (Fig. 5c, Table 2). Bonferroni post hoc tests showed no significant differences among the groups. There was a partial effect of Pht-HrTH 2 mg/kg on the total number of entries, but it did not reach a significant level.



**Fig. 7.** **a** illustrates weight gain (g.) data after intraperitoneally (i.p.) administration of 14 days in sham control (n = 8), sham Ani-AKH (2 mg/kg) (n = 8), sham Lia-AKH (2 mg/kg) (n = 9), sham Pht-HrTH (2 mg/kg) (n = 10), OBX control (n = 9), OBX Ani-AKH (2 mg/kg) (n = 8), OBX Lia-AKH (2 mg/kg) (n = 8), OBX Pht-HrTH (2 mg/kg) (n = 7) in the OBX model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean. (\*\*p < 0.05 compared with the sham control group). **b** illustrates weight gain (g.) data after intraperitoneally (i.p.) administration of 21 days in non-traumatized control (n = 9), non-traumatized Ani-AKH (2 mg/kg) (n = 8), non-traumatized Lia-AKH (2 mg/kg) (n = 8), non-traumatized Pht-HrTH (2 mg/kg) (n = 8), traumatized control (n = 9), traumatized Ani-AKH (2 mg/kg) (n = 9), traumatized Lia-AKH (2 mg/kg) (n = 9), traumatized Pht-HrTH (2 mg/kg) (n = 9) in the PTSD model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean (\*p < 0.05 compared with the non-traumatized control group; #p < 0.05, ###p < 0.001 compared with traumatized control group).

### 3.6. Effects of Ani-AKH, Lia-AKH and Pht-HrTH on startle amplitude in the startle reflex test in the PTSD model

There were no significant effects of drugs [ $F(7, 177) = 1.20$ ;  $p = 0.30$ ] and interaction [ $F(14, 177) = 0.85$ ;  $p = 0.61$ ] while there were significant effects of decibels [ $F(2, 177) = 130.7$ ;  $p < 0.0001$ ] on the average startle amplitude in the startle reflex test in the PTSD study. Post hoc Bonferroni tests showed that Ani-AKH 2 mg/kg ( $p < 0.01$ ), Lia-AKH 2 mg/kg ( $p < 0.01$ ) and Pht-HrTH 2 mg/kg ( $p < 0.05$ ) significantly decreased the average startle amplitude compared to the non-traumatized control group in the PTSD study (Fig. 6, Table 2).

### 3.7. Effects of Ani-AKH, Lia-AKH and Pht-HrTH on weight gain after chronic injections in the OBX and PTSD models

There were significant effects of interaction [ $F(3, 59) = 3.18$ ;  $p = 0.03$ ] while there were no significant effects of OBX [ $F(1, 59) = 0.96$ ;  $p = 0.32$ ] and drugs [ $F(3, 59) = 1.64$ ;  $p = 0.18$ ] on weight gain after two weeks of drug administration in the OBX study. Bonferroni post hoc tests showed that Pht-HrTH 2 mg/kg ( $p < 0.01$ ) significantly decreased weight gain compared to the sham control group in the OBX study (Fig. 7a, Table 2).

There were significant effects of PTSD [ $F(1, 61) = 10.09$ ;  $p = 0.002$ ] and interaction [ $F(3, 61) = 8.16$ ;  $p = 0.0001$ ] while there were no significant effects of drugs [ $F(3, 61) = 0.47$ ;  $p = 0.70$ ] on weight gain during three weeks of drug administration in the PTSD study. Post hoc Bonferroni tests showed that Ani-AKH 2 mg/kg ( $p < 0.05$ ) and Pht-HrTH 2 mg/kg ( $p < 0.05$ ) significantly decreased weight gain compared to the non-traumatized control group while Ani-AKH 2 mg/kg ( $p < 0.05$ ) and Pht-HrTH 2 mg/kg ( $p < 0.001$ ) significantly increased weight gain compared to the traumatized control group in the PTSD study (Fig. 7b, Table 2).

### 3.8. Effects of Ani-AKH, Lia-AKH and Pht-HrTH on neurotransmitter levels in the brain after chronic injections in the OBX and PTSD models

In the OBX study, there were no significant differences among groups when the effects of the hormones on dopamine levels in the brains of rats after two weeks of chronic injections were evaluated [ $F(7, 28) = 1.18$ ;  $p = 0.34$ ; Fig. 8a]. There were significant differences between the groups when the effects of hormones on GABA levels in the brains of rats after two weeks of chronic injections were evaluated [ $F(7, 28) = 4.99$ ;  $p = 0.0009$ ]. GABA levels were significantly decreased in the Ani-AKH OBX group ( $p < 0.05$ ) compared to the OBX group (Fig. 8b). There were significant differences among the groups on glutamate levels in the brains of rats after two weeks of chronic injections were evaluated [ $F(7, 28) = 2.65$ ;  $p = 0.03$ ] although there were no significant effects of the hormones (Fig. 8c). There were significant differences among the groups when the effects of the hormones on 5-HT levels in the brains of rats after two weeks of chronic injections were evaluated [ $F(7, 28) = 5.25$ ;  $p = 0.0006$ ]. 5-HT levels significantly decreased in the OBX control group ( $p < 0.05$ ) compared to the sham control group (Fig. 8d).

In the PTSD study, there were significant differences between the groups when the effects of drugs on dopamine levels in the brains of rats after three weeks of chronic injections were evaluated [ $F(7, 32) = 180.6$ ;  $p < 0.0001$ ]. There was a significant increase in dopamine levels in Ani-AKH ( $p < 0.001$ ), Lia-AKH ( $p < 0.001$ ), Pht-HrTH ( $p < 0.0019$ ) and the non-traumatized control group ( $p < 0.001$ ) compared to the traumatized control group. Ani-AKH ( $p < 0.05$ ) and Lia-AKH ( $p < 0.001$ ) significantly decreased dopamine levels compared to the non-traumatized control group while Pht-HrTH significantly increased dopamine levels compared to the non-traumatized control group ( $p < 0.001$ ) (Fig. 9a). There were significant differences among the groups when the effects of drugs on GABA levels in the brains of rats after three weeks of chronic injections were evaluated [ $F(7, 32) = 14.34$ ;  $p < 0.0001$ ].

GABA levels were significantly decreased in the traumatized Lia-AKH ( $p < 0.05$ ) and the non-traumatized control group ( $p < 0.001$ ) compared to the traumatized control group (Fig. 9b). There were significant differences among the groups when the effects of drugs on glutamate levels in the brains of rats after three weeks of chronic injections were evaluated [ $F(7, 32) = 14.43$ ;  $p < 0.0001$ ]. Glutamate levels significantly increased in the non-traumatized control group ( $p < 0.05$ ) compared to the traumatized control group, and this effect was also increased by Lia-AKH ( $p < 0.05$ ) compared to the non-traumatized control group (Fig. 9c). There were significant differences among the groups when the effects of drugs on 5-HT levels in the brains of rats after three weeks of chronic injections were evaluated [ $F(7, 32) = 9.48$ ;  $p < 0.0001$ ]. 5-HT levels significantly decreased in the

traumatized Lia-AKH ( $p < 0.05$ ) and the non-traumatized control group ( $p < 0.001$ ) compared to the traumatized control group (Fig. 9d).

### 3.9. Effects of Ani-AKH, Lia-AKH and Pht-HrTH on metabolomic pathways in the brain after chronic injections in the OBX and PTSD models

In the OBX study, there were significant differences between the sham control and the OBX control in pathways steroid hormone biosynthesis ( $p = 0.06$ ), amino sugar and nucleotide sugar metabolism ( $p = 0.06$ ) and steroid biosynthesis ( $p = 0.08$ ) [Fig. 10a]. There were significant differences between the OBX control group and the OBX Ani-AKH 2 mg/kg group in pathways biosynthesis of unsaturated fatty acids ( $p = 0.02$ ), fatty acid biosynthesis ( $p = 0.03$ ), phenylalanine metabolism ( $p = 0.03$ ), sphingolipid metabolism ( $p = 0.03$ ), phenylalanine, tyrosine and tryptophan biosynthesis ( $p = 0.04$ ), amino sugar and nucleotide sugar metabolism ( $p = 0.04$ ) [Fig. 10b]. There were significant differences between the OBX control group and the OBX Lia-AKH 2 mg/kg group in pathways terpenoid backbone biosynthesis ( $p = 0.02$ ), phenylalanine metabolism ( $p = 0.04$ ), sphingolipid metabolism ( $p = 0.04$ ), phenylalanine, tyrosine and tryptophan biosynthesis ( $p = 0.06$ ) and biosynthesis of unsaturated fatty acids ( $p = 0.07$ ) [Fig. 10c]. There were significant differences between the OBX control group and the OBX Pht-HrTH 2 mg/kg group in pathways biosynthesis of unsaturated fatty acids ( $p = 0.03$ ), pyrimidine metabolism ( $p = 0.03$ ), retinol metabolism ( $p = 0.03$ ), terpenoid backbone synthesis ( $p = 0.04$ ) and fatty acid biosynthesis ( $p = 0.04$ ) [Fig. 10d].

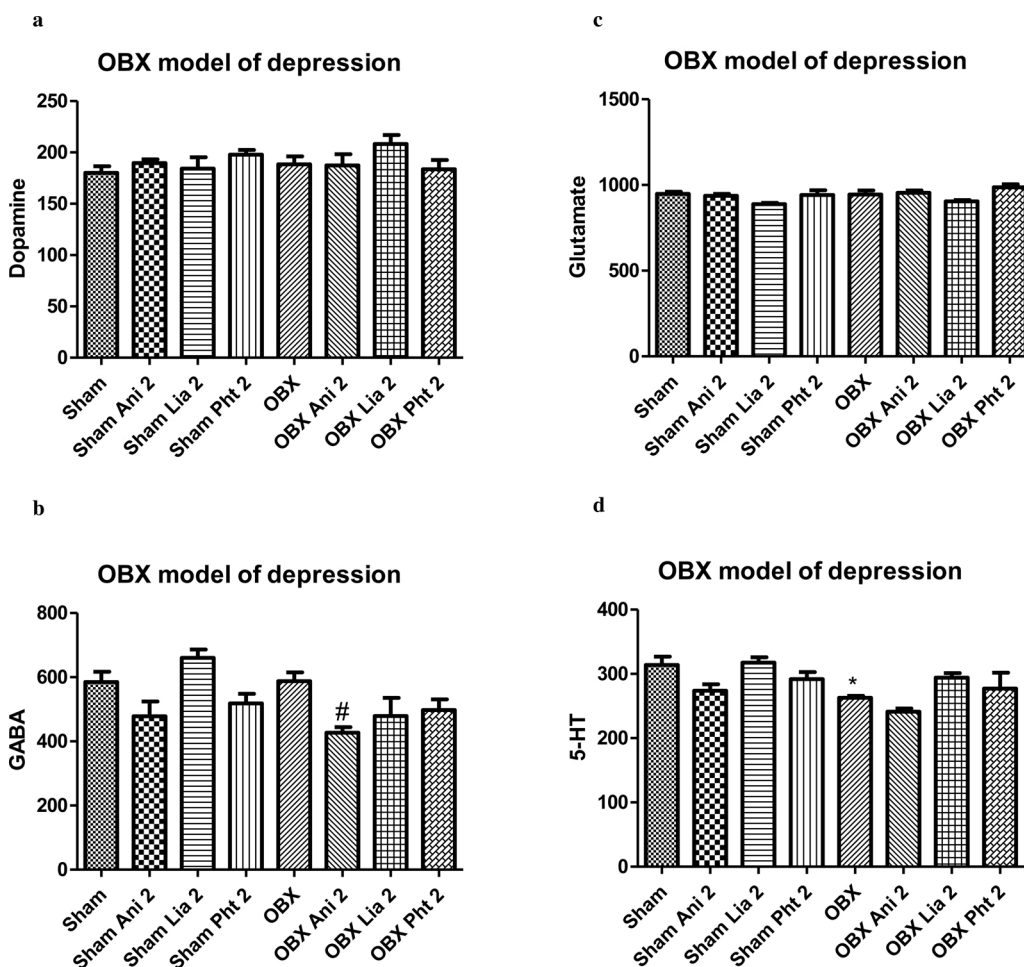
In the PTSD study, there were significant differences between the non-traumatized control and the traumatized control groups in pathways steroid hormone biosynthesis ( $p = 0.009$ ), steroid biosynthesis

( $p = 0.015$ ), arachidonic acid metabolism ( $p = 0.02$ ), retinol metabolism ( $p = 0.02$ ) and biosynthesis of unsaturated fatty acids ( $p = 0.03$ ) [Fig. 11a]. There were significant differences between the traumatized control and the traumatized Ani-AKH 2 mg/kg groups in pathways steroid hormone biosynthesis ( $p = 0.008$ ), steroid biosynthesis ( $p = 0.01$ ), biosynthesis of unsaturated fatty acids ( $p = 0.01$ ), arachidonic acid metabolism ( $p = 0.02$ ) and ascorbate and aldarate metabolisms ( $p = 0.02$ ) [Fig. 11b]. There were significant differences between the traumatized control and the traumatized Lia-AKH 2 mg/kg groups in pathways steroid hormone biosynthesis ( $p = 0.01$ ), arginine and proline metabolism ( $p = 0.02$ ), arachidonic acid metabolism ( $p = 0.03$ ) and sphingolipids metabolism ( $p = 0.04$ ) [Fig. 11c]. There were significant differences between the traumatized control and the traumatized Pht-HrTH 2 mg/kg in pathways steroid hormone biosynthesis ( $p = 0.009$ ), steroid biosynthesis ( $p = 0.01$ ), arachidonic acid metabolism ( $p = 0.01$ ), retinol metabolism ( $p = 0.01$ ), cysteine and methionine metabolism ( $p = 0.02$ ) [Fig. 11d].

## 4. Discussion

The aim of the paper is to study the behavioral, neurochemical and metabolomic impacts of three hormones from the group of adipokinetic hormones in the animal models of affective disorders. Our hypothesis is based on the fact that these hormones may represent a new class of natural drugs due to their structural similarity to human peptide hormones like oxytocin and vasopressin. Specifically, we studied the effects of Anax imperator AKH (Ani-AKH), Libellula auripennis AKH (Lia-AKH) and Phormia-Terra hypertrehalosemic hormones (Pht-HrTH).

Our results show that all of the hormones exerted antidepressant-like



**Fig. 8.** illustrates a) Dopamine b) GABA c) Glutamate d) 5-HT levels in the brain of rats after intraperitoneally (i.p.) administration of 14 days in sham control ( $n = 5$ ), sham Ani-AKH (2 mg/kg) ( $n = 4$ ), sham Lia-AKH (2 mg/kg) ( $n = 4$ ), sham Pht-HrTH (2 mg/kg) ( $n = 5$ ), OBX control ( $n = 5$ ), OBX Ani-AKH (2 mg/kg) ( $n = 5$ ), OBX Lia-AKH (2 mg/kg) ( $n = 4$ ), OBX Pht-HrTH (2 mg/kg) ( $n = 4$ ) in the OBX model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean (\* $p < 0.05$ , \*\*\* $p < 0.001$  compared with the sham control group; # $p < 0.05$ , ### $p < 0.001$  compared with OBX control group).

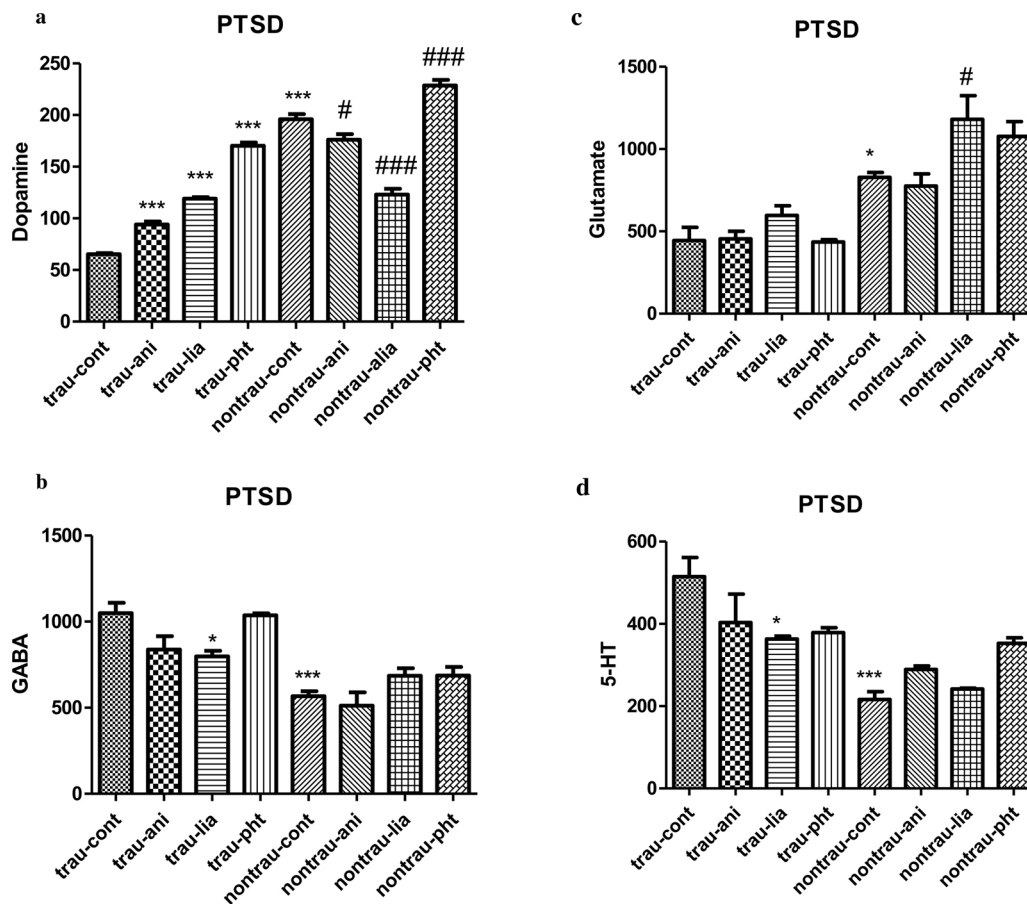


Fig. 9. illustrates a) Dopamine b) GABA c) Glutamate d) 5-HT levels in the brain of rats after intraperitoneally (i.p.) administration of 21 days in traumatized control (n = 9), traumatized Ani-AKH (2 mg/kg) (n = 9), traumatized Lia-AKH (2 mg/kg) (n = 9), traumatized Pht-HrTH (2 mg/kg) (n = 9), non-traumatized control (n = 9), non-traumatized Ani-AKH (2 mg/kg) (n = 8), non-traumatized Lia-AKH (2 mg/kg) (n = 8), non-traumatized Pht-HrTH (2 mg/kg) (n = 8) in the PTSD model of Wistar-Albino rats. The data are indicated as the means  $\pm$  standard error of the mean (\* $p < 0.05$ , \*\*\* $p < 0.001$  compared with the traumatized control group; # $p < 0.05$ , ### $p < 0.001$  compared with non-traumatized control group).

effects both in the OBX and in the PTSD studies since all of the hormones seem to have ameliorating effects in the FST because they decreased the immobility time significantly. It was only in the PTSD study that the effect of Pht-HrTH was partial, which may be due to some nonspecific effects such as experimental conditions. This result was in harmony with our previous studies in which we showed antidepressant effects of the same hormones by increasing neurogenesis and neurotrophic factors [7, 8].

In the OBX study, both Ani-AKH and Pht-HrTH showed anxiolytic effects compared to OBX control animals while Ani-AKH also showed anxiolytic effects compared to the sham control group in the EPM test. This result was also in accordance with our previous studies, which showed the anxiolytic effects of these hormones [7,8]. In the PTSD study, Lia-AKH and Pht-HrTH were more effective compared to Ani-AKH in the EPM test. They increased %open arm time and %open arm entry significantly in both traumatized and non-traumatized animals, but they did not reach a significant level. In the PTSD study, in the exploratory activity test, most effective hormone seemed to be Pht-HrTH because it increased the number of entries to new areas and the total number of entries in large quantities in traumatized animals although it was not significant. Higher doses of Pht-HrTH may be studied in anxiety and locomotion models in future studies.

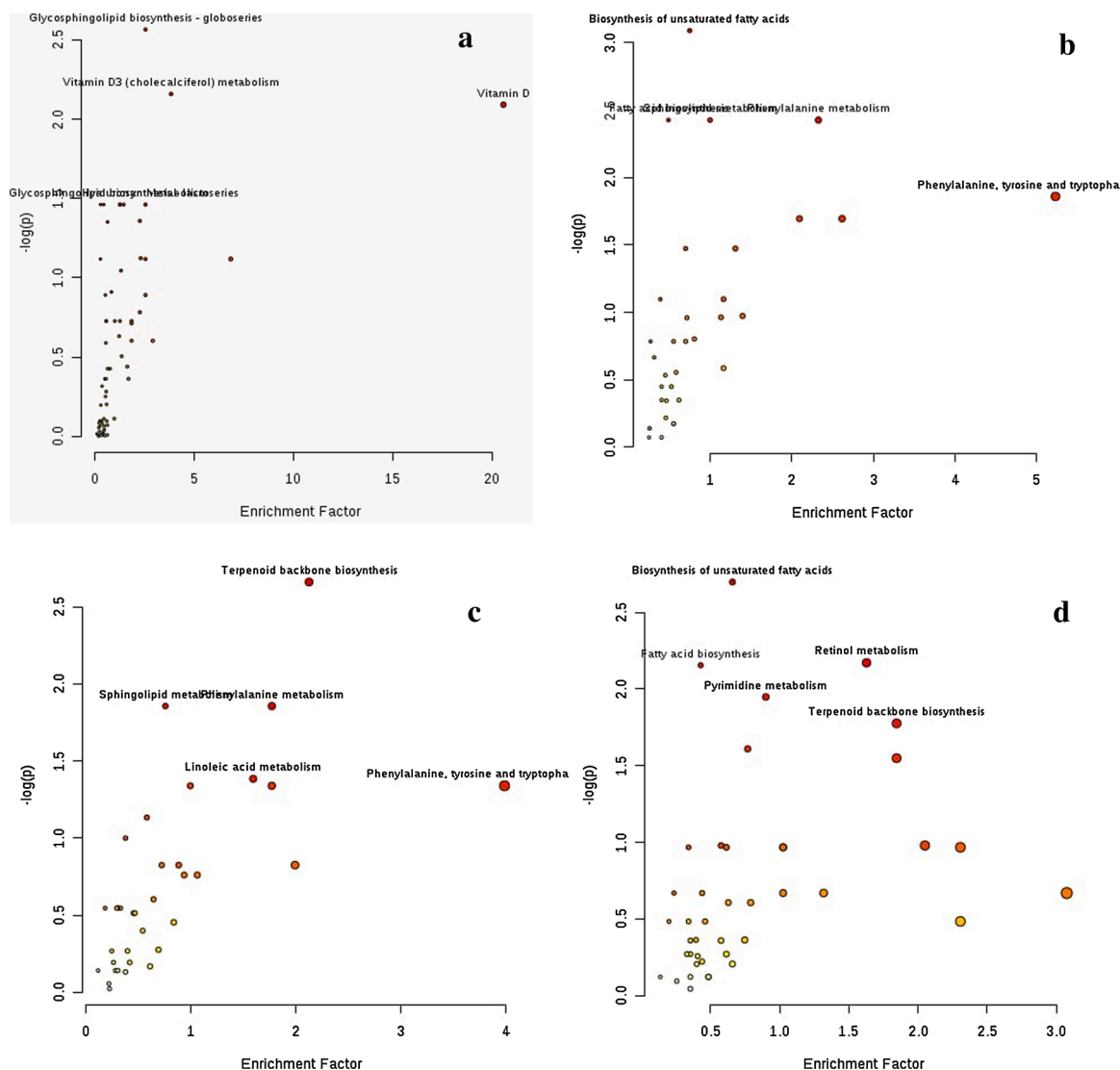
In the acoustic startle reflex (ASR) test, traumatized control animals showed less startle amplitude compared to non-traumatized control animals, which was unexpected. The reason could be that non-traumatized animals were exposed to acute stress from behavioral tests during a one-week period, therefore showing more anxiety in the startle reflex test compared to traumatized animals, which were accustomed to stress exposure during a one-month period. In the ASR test, all of the hormones decreased average startle amplitude in non-traumatized animals, which may support their anxiolytic effect while there were

some partial effects of Lia-AKH and Pht-HrTH in traumatized animals.

In this study, the OBX animals exerted a higher locomotion compared to the sham animals. In the OF test, the OBX animals exerted a higher total distance moved and a greater speed compared to the sham animals. There was no effect of all three hormones on locomotion, both in the OBX and in the sham animals. In the open field test, decreased locomotion in traumatized animals in the PTSD model was partially increased by Ani-AKH and Pht-HrTH although it did not reach a significant level. In our previous studies, after two weeks of chronic injections, we observed locomotion-enhancing effects of the same hormones at the same dose [7] in naive mice. The reason why we could not achieve a significant increasing effect in locomotion could be due to the strain differences of the experimental animals, the experimental conditions and the experimental models used.

In the PTSD study, we had seen partial effects of the PTSD model in behavioral tests. One of the reasons could be that we could not use cat exposure as the acute stress, which was in the protocol of Zoladz et al. [14], but we tried cat odor, which was used in many PTSD models [20]. Locomotion of the animals decreased partially in the traumatized control animals compared to the non-traumatized control animals in the open field test; immobility time partially increased in the FST in the traumatized animals; %open arm entry partially decreased in the traumatized animals in the EPM test; exploratory activity and body weight of the traumatized animals also decreased notably although it did not reach a significant level.

All three hormones showed beneficial effects on spatial memory compared to both the sham and the OBX control animals in the MWM test. However, there were also some differences in the effects of hormones on MWM. Lia-AKH and Pht-HrTH showed improved effects on both spatial learning and memory compared to the sham control while Ani-AKH showed improved effect on spatial memory in the probe test.



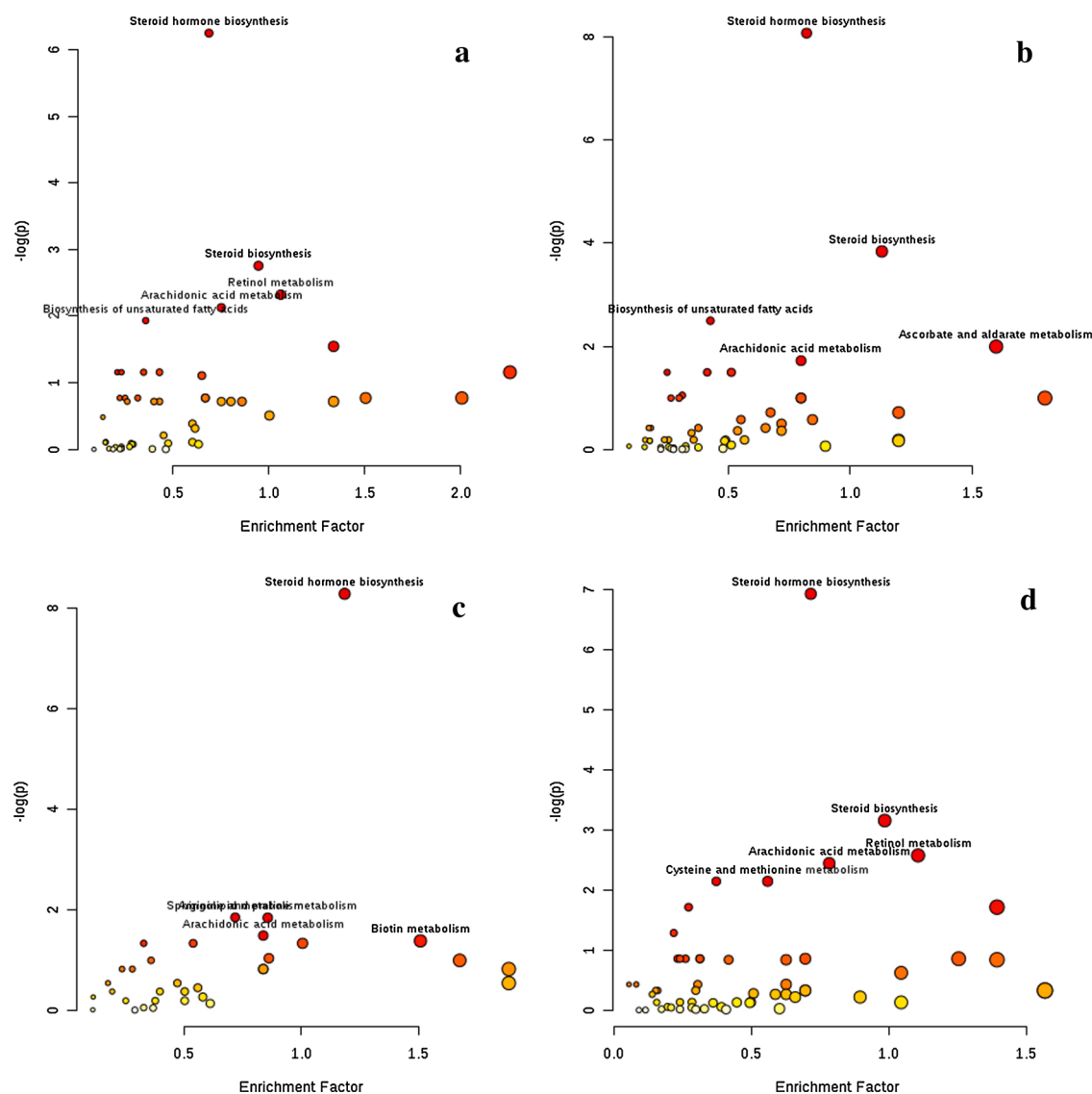
**Fig. 10.** illustrates the peak to pathways graph of comparisons (a)sham control-OBX control (b)OBX control-OBX Ani-AKH 2 mg/kg (c)OBX control-OBX Lia-AKH 2 mg/kg (d)OBX control-OBX Pht-HrTH 2 mg/kg. Color range from yellow to red shows increase in significance of the pathways in comparisons. Red pathways are the most significantly found pathways in the comparisons. In X axis we use the enrichment factor. In Y axis we use the  $-\log_{10}(p)$  to know the intensity. The enrichment analysis/factor is to test whether any functional metabolites/genes from the user selected library are significantly enriched among the currently highlighted nodes within the network. p-value is the probability of obtaining test results at least as extreme as the results actually observed, under the assumption that the null hypothesis is correct.

Lia-AKH and Pht-HrTH also reversed disturbed spatial memory in the OBX control animals while Ani-AKH had no reversal effect. Both Lia-AKH and Pht-HrTH significantly increased the speed of the animals compared to the OBX control animals in the probe trial of the MWM test, which supported our previous results [5]. All of these improved memory effects were correlated with our previous study in which we showed the improved effects of these hormones on memory in the MK-801 induced schizophrenia model [7].

In addition to the behavioral changes, it was also possible to prove the effect on weight, which is probably also influenced by the impact of the hormones on metabolism. Pht-HrTH significantly decreased weight gain in two weeks compared to the sham control group in the OBX study. In the PTSD study, we had a partial decrease in weight gain in traumatized animals compared to non-traumatized control animals, an effect that was significantly reversed by Ani-AKH and Pht-HrTH, which may support the anti-stress effect of these hormones. Conversely, Ani-AKH and Pht-HrTH decreased weight gain significantly after three weeks of injections in non-traumatized animals. This decrease in weight gain could be due to lipid and sugar mobilizing effects of these

hormones, which was also shown in some previous studies [21]. It is known that adipokinetic and hypertrehalosemic peptides control fat, carbohydrate and protein metabolism in insects. These hormones directly affect the mobilization of carbohydrates and lipids and/or the utilization of such substrates by flight muscles [11]. It was also shown that these insect peptides of the adipokinetic hormone family cause lipid mobilization in humans [21].

The second area studied is neurochemical and metabolomics. Our results in the model used show complex neurochemical and metabolomic changes. PTSD can be understood as a complex and systemic disease although we do not yet have a complete understanding of metabolomic and neurochemical processes. Preclinical and clinical studies point to changes in lipid metabolism, phospholipids, glycerophospholipids, bile acids and energy-related pathways in plasma [22]. Overall, the findings point to the role of inflammation and changes in energy metabolism. Other neurobiological features of PTSD are changes in noradrenergic, serotonergic, glutamatergic and GABA-ergic neurotransmitter systems and related changes in the HPA axis. Some studies also point to the role of metabolites produced by the GUT microbiota,



**Fig. 11.** illustrates the peak to pathways graph of comparisons (a) nontraumatized control-traumatized control (b) traumatized control-traumatized Ani-AKH 2 mg/kg (c) traumatized control- traumatized Lia-AKH 2 mg/kg (d) traumatized control- traumatized Pht-HrTH 2 mg/kg. Color range from yellow to red shows increase in significance of the pathways in comparisons. Red pathways are the most significantly found pathways in the comparisons. In X axis we use the enrichment factor. In Y axis we use the  $-\log_{10}(p)$  to know the intensity. The enrichment analysis/factor is to test whether any functional metabolites/genes from the user selected library are significantly enriched among the currently highlighted nodes within the network. p-value is the probability of obtaining test results at least as extreme as the results actually observed, under the assumption that the null hypothesis is correct.

including neuroactive metabolites such as hippurate, phenylpropionate and fermentation products of tyrosine and tryptophan. The production of these metabolites may be related to stress. In addition, the study showed elevated plasma levels of fatty acids, phospholipids and bile acids in long-term stressed mice [23]. These metabolites are associated with several biological processes, such as neuro-inflammation, oxidative stress, cell membrane dynamics, immune reaction or aging.

After two weeks of injections, there were no significant differences of brain dopamine and GABA levels between the sham control and the OBX control animals. There were also no effects of hormones on dopamine levels in all groups while Ani-AKH 2 mg/kg significantly decreased GABA levels in the brain of the OBX control animals. Since we observed the best anxiolytic effects with Ani-AKH in our study, this controversial effect could be due to some non-specific effects such as laboratory conditions. There were no effects of hormones on glutamate levels of the brain after two weeks of injections. 5-HT levels were significantly decreased in the OBX animals, which supports that our OBX model worked in this study. Contrarily, all three hormones had no significant

effects on 5-HT levels. After three weeks of drug administration, AKH significantly increased dopamine levels in traumatized animals while Ani-AKH and Lia-AKH significantly decreased dopamine levels in non-traumatized animals and only Pht-HrTH significantly increased dopamine levels in non-traumatized animals. GABA levels were significantly decreased in the brain of non-traumatized control animals compared to traumatized animals. The reason for this could be that one week of behavioral tests caused stress in non-traumatized animals while traumatized animals were accustomed to the everyday applied social stress. Oppositely, glutamate levels increased in the brains of non-traumatized animals, which is correlated with GABA results because glutamate has the opposite effects of GABA. Again, 5-HT levels decreased in non-traumatized animals, which can be explained with the same hypothesis of GABA results. Interestingly, AKH caused a partial decrease in 5-HT levels in traumatized animals while it caused a partial increase in non-traumatized animals.

According to our findings AKH is metabolized to smaller peptides in the blood circulation and pyroglutamic acid (pGlu) and dipeptides

including pyroglutamic acid may pass blood-brain barrier and may be responsible for the behavioral effects in our study. In our previous study, we showed that the AKH/RPCH family of peptides reversed memory impairments in MK-801 induced schizophrenia models, which may support that these peptides affect NMDA receptors. In a recent study [24], it was shown that acute and chronic pretreatments resulting in cell cholesterol depletion profoundly diminished NMDAR responses and increased NMDAR desensitization in cultured rat cerebellar granule cells. The lipid mobilization effect of AKH/RPCH family peptides may cause the deactivation of NMDA receptors, and this can explain some anxiolytic and antidepressant effects of AKH hormones because NMDA receptor antagonists had antidepressant and anxiolytic effects in previous studies [25,26].

There are studies investigating the relationship of cortisone and vitamin D<sub>3</sub> with depression and anxiety [27,28]. Aberrant sphingolipid metabolism has been observed in some cases of depression, specifically alterations in ceramide concentrations [29]. According to our untargeted analysis, steroid hormone biosynthesis like cortisone, steroid synthesis like vitamin D<sub>3</sub> and some sphingolipids seem to cause metabolic pathway changes in OBX and PTSD control animals. Metabolomic analysis showed that Ani-AKH, Lia-AKH and Pht-HrTH may increase fatty acid biosynthesis in the tissue, possibly by their shown lipid mobilization effects [21]. Ani-AKH, Lia-AKH and Pht-HrTH may affect steroid and steroid hormone biosynthesis, which may be related to their antidepressant effects in OBX and traumatized animals. Ani-AKH, Lia-AKH and Pht-HrTH may also affect sphingolipid metabolism, which may cause some antidepressant effects in OBX and traumatized animal models.

In this context, neuropharmacological research focuses on new ways of influencing the neurobiological substrate of PTSD. The results of oxytocin administration confirm the possibility of influencing PTSD with specific neuropeptides. Oxytocin is a similar peptide molecule composed of nine amino acids, which was reported to reduce fear [30] and produce antidepressant-like effects in animal models of depression [31]. Further, it may be involved in the pathophysiology of depression in humans [32]. The central and peripheral effects of AKH seem to be comparable to oxytocin in this study and in previous studies [7–10].

## 5. Conclusion

This study reports that AKH/RPCH family peptides may demonstrate improved effects on animal behavior in the OBX and the PTSD models in rats although effects varied depending on the test applied and the type of insect hormone used. The effects of these hormones on neurotransmitter systems and neurochemical changes in the brain should be researched in future studies to explain the behavioral effect mechanisms of AKH. Similarly, it is necessary to understand the relationship between the structure of individual hormones and the differences in their pharmacological effects

## CRediT authorship contribution statement

**Oguz Mutlu:** Conceptualization, Methodology, Software, Writing - original draft, Writing - review & editing. **Omer Kurtas:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Lenka Kleteckova:** Methodology, Software, Data curation, Writing - original draft. **Nikola Pinterova:** Methodology, Software, Data curation, Writing - original draft. **Kristina Holubová:** Methodology, Software, Data curation, Writing - original draft. **Jiří Horáček:** Visualization, Investigation, Supervision. **Cyril Hoschl:** Visualization, Investigation, Supervision. **Ibrahim Uygün:** Software, Validation, Writing - review & editing. **Daniel Bermejo Rodriguez:** Investigation, Methodology, Software. **David Kacer:** Investigation, Methodology, Software. **Francko Muhametaj:** Investigation, Methodology, Software. **Karel Vales:** Conceptualization, Methodology, Software, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

None.

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