Abstract

Background: The endocannabinoid (eCB) system plays an important role in regulating emotional disorders, and is involved, directly or indirectly, in psychiatric diseases, such as anxiety and depression. Hemopressin, a hemoglobin α chain-derived peptide, and RVD-hemopressin(α), a N-terminally extended form of hemopressin, act as antagonist/inverse agonist and negative allosteric modulator of the cannabinoid 1 (CB1) receptor, respectively.

Methods: Considering the possible involvement of these peptides on emotional behaviour, the aim of our study was to investigate the behavioural effects of a single intraperitoneal (*ip*) injection of hemopressin (0.05 mg/kg) and RVD-hemopressin(α) (0.05 mg/kg), using a series of validated behavioural tests (locomotor activity/open field test, light-dark exploration test, forced swim test) in rats. Prefrontal cortex levels of norepinephrine (NE), dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT) and the gene expression of monoamine oxidase (MAO-B) and catechol-O-methyltransferase (COMT) were measured by high performance liquid chromatography (HPLC) analysis and real-time reverse transcription polymerase chain reaction (RT-PCR), respectively.

Results: Hemopressin administration induced anxiogenic and depressive behaviour, decreased monoamine steady state levels in prefrontal cortex, and increased the gene expression of the enzymes involved in their catabolism. By contrast, RVD- hemopressin(α) induced anxiolytic and antidepressive effects, increased monoamines and decreased the enzymes in prefrontal cortex.

Conclusion: In conclusion, in the present study we demonstrated behavioral effects induced by peripheral hemopressin and RVD- hemopressin(α) injections, that could involve modulatory effects on monoaminergic signaling, in the prefrontal cortex.

Keywords:

Anxiety

Depression

Hemopressin

RVD-hemopressin(α)

Monoamines

Introduction

The endocannabinoid (eCB) system is strongly involved in psychiatric diseases such as anxiety, depression, bipolar disorder, schizophrenia and suicide [1]. Different studies have demonstrated a dysfunction of eCB signaling in patients with mood disorders [2–4] and preclinical data suggest that the direct or indirect stimulation of cannabinoid (CB) receptors exert anxiolytic and antidepressant actions [5–8]. On the other hand, the eCB system modulates serotonin (5-HT), norepinephrine (NE) and dopamine (DA) neurotransmission [9], which could represent an attractive and novel approach in the treatment of depression and other mood disorders. However, the failure of clinical trials involving endocannabinoid modulators has dampened the initial enthusiasms [10]. Hemopressin (Hp), a hemoglobin α chain-derived peptide which plays an antagonist/inverse agonist role on CB1 receptors [11–12] points to a new pharmacological approach in the treatment of mood disorders. Recent studies have reported the anxiogenic effects of Hp, after central and peripheral administration in rats tested with the elevated plus maze. The authors suggested either a direct involvement of Transient Receptor Potential Vanilloid Type (TRPV1) channel or increased levels of anandamide, but excluding CB1 receptor activation [13]. Recently, a N-terminally extended peptide of Hp, RVD-hemopressin(α) [RVD-hp(α)], also known as PEPCAN-12 (Fig. 1), was found to bind CB1 receptors as a negative allosteric modulator [14–15]. The aim of our work was to further elucidate the role of Hp and RVD-hp(α) in emotional disorders, using a series of validated behavioural tests (locomotor activity/open field test, light-dark exploration test, forced swim test). The behavioural data were also related to NE, DA and 5-HT, levels and to the gene expression of monoamine oxidase (MAO-B) and catechol-O-methyltransferase (COMT), evaluated by

high performance liquid chromatography (HPLC) analysis and real-time reverse transcription polymerase chain reaction (RT-PCR), respectively, in rat prefrontal cortex.

Materials and methods

Peptide synthesis and characterization

Hp and RVD-hp(α) have been obtained in our laboratory by Fmoc-solid phase peptide synthesis (Fmoc-SPPS) strategy on 2-CTC (2-chlorotrityl chloride) resin, as previously reported [16-19]. Chromatographic purification was performed by RP-HPLC semi-preparative C18 column (eluent: ACN/H_2O gradient, 5-95% over 32 min) at a flow gradient of 4 mL/min. RVD-hp(α) was characterized by ^{1}H NMR spectra on 300MHz Varian Inova spectrometer (Varian Inc., Palo Alto, CA) and mass spectra on Thermo Scientific Q Exactive (Thermo Fisher Scientific, San Jose, CA), in, the positive mode, capillary temperature 220°C, spray voltage 2.3 kV, and sheath gas 5 units.

In vivo studies

Male adult Sprague-Dawley rats (200-230 g) were housed in plexiglas cages (3 animals per cage; 40 cm $\times$ 25 cm $\times$ 15 cm) and maintained under standard laboratory conditions (22 $\pm$ 1 °C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00–19:00 h), with free access to tap water and food, in accordance with the European Community ethical regulations (EU Directive 2010/63/EU) on the care of animals for scientific research. The project (n. 880/2015) was approved by Italian Ministry of Health. Hp and RVD-hp(α) were diluted in saline and dosages were selected on the basis of previous studies [13,15]. After 2-week acclimation, rats were randomized into 3 groups of 6 animals for each test and a single dose injected intraperitoneally (*ip*) during the light

phase as follows: (1) vehicle (0.2 ml saline); (2) RVD-hp(α) (0.05 mg/kg); (3) Hp (0.05 mg/kg). 10 nmol. All solutions were prepared freshly before use.

Behavioral test

The animals were brought into the experimental room 30 min prior to the test in order for them to acclimate to the environment, and were kept in the testing chamber for 5 min prior to each test. All treatments were administered at 09:00 am, and the experiments performed between 1000 and 1200 am. Each test session was recorded by a video camera connected to a computer; a single video frame was acquired with a highly accurate, programmable, monochrome frame grabber board (Data TranslationTM, type DT3153). The intelligent software Smart version 2.5 (Panlab, sl Bioresearch and Technology, Barcelona, Spain) was used for data processing. The apparatuses were purchased from 2 Biological Instruments (Besozzo VA, Italy). At the end of each test, the animals were returned to their home cage, and the apparatus was cleaned with 75% ethanol and dried before the next procedure.

Locomotor activity/open field test: Locomotor activity was recorded in the home cage over 10 min. The activity monitor consisted of a black and white video camera, mounted in the top-center of the cage. Locomotor activity was assessed as horizontal activity, vertical activity, time spent in movements (s) and resting time (s). Resting time was considered when animal's movements were below a threshold set by comparing the score of resting rated manually with the score from the automated device in preliminary studies [20].

To evaluate anxiety-like behaviour, each animal was placed in an open field box (60 × 60 × 60 cm) made of clear Plexiglas with a white laminated sheet of paper marked into sixteen squares (15 × 15 cm). Each animal was monitored for 10 min. In the open field

test, the distance travelled and time spent into the center of the observation chamber were recorded [21].

Light-dark exploration test: The light-dark box test assesses bright-space related anxiety and consists of two compartments (30 × 80 × 60 cm, each), dark and light ones, separated by a wall pierced with an open door (15 height × 10 cm width). The dark compartment has opaque black walls, while the light compartment is transparent to light. Rats were placed in the black compartment, and time spent by the animal in the light compartment, latency of first exit from dark compartment, and number of transitions between compartments were recorded during a 10 min interval.

Forced swim test: The forced swim test was based on the original version of the Porsolt forced swim test for mice [22] with modifications. Rats were forced to swim individually in a glass cylinder (50 cm high × 23 cm in diameter), filled with water to a height of 30 cm. The temperature of the water was adjusted to 28 ± 1 °C to avoid severe hypothermia [23]. The total time that rats spent immobile was measured. Immobility was determined when the rat was only marking movements necessary to keep its head above the water and maintained a stationary posture. In this posture forelimbs of the rat are motionless and directed forward, the tail is directed outward and the hind legs are in limited motion. No animals showed difficulty in swimming or in staying afloat. On day 1, rats were placed in water to swim for a single trial of 15 min, and immobility was recorded during the last 4 min of the trial. On day 2 the mice were placed in water through a series of four trials of 6 min each and immobility was recorded during the last 4 min of each trial. Each trial was followed by an 8 min rest period when the animals were dried with towels and returned to their cage. Finally, rats were sacrificed, as previously reported [24].

Prefrontal cortex monoamine extraction and high performance liquid chromatography (HPLC) determination

Immediately after sacrifice, brains were rapidly removed and prefrontal cortex were dissected and subjected to biogenic amine extractive procedures, as previously reported [25]. Thereafter, samples were analyzed by HPLC coupled to electrochemical detection consisting of ESA Coulochem III detector equipped with ESA 5014B analytical cell (selected potentials: electrode 1: -150 mV; electrode 2: +300 mV), as previously reported [26]. Monoamine levels were expressed as ng/mg wet tissue.

RNA extraction, reverse transcription and real-time reverse transcription polymerase chain reaction (real-time RT PCR)

Immediately after sacrifice, prefrontal cortex was rapidly removed, dissected and stored in RNAlater solution (Ambion, Austin, TX) at -20 °C until further processed. Total RNA was extracted from the prefrontal cortex using TRI Reagent (Sigma-Aldrich, St. Louis, MO), as previously reported [27].

One μg of total RNA extracted from each sample in a 20 μl reaction volume was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reactions were incubated in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) initially at 25°C for 10 min, then at 37°C for 120 min, and finally at 85°C for 5 s. Gene expression was determined by quantitative real-time PCR using TaqMan probe-based chemistry (Applied Biosystems, Foster City, CA, USA). PCR primers and TaqMan probes were obtained from Applied Biosystems (Assays-on-Demand Gene Expression Products, Rn005676203_m1 for MAO-B gene, Rn01404927_m1 for COMT gene) β-actin (Applied Biosystems, Foster City, CA, USA,

Part No. 4352340E) was used as the housekeeping gene. Data were calculated as previously reported [28-29].

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). All data were collected from each of the animals used in the experimental procedure and means ± SEM were determined for each experimental group and analyzed by unpaired *t* test (two-tailed *p* value) and two way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test. As for gene expression analysis, 1.00 (calibrator sample) was considered the theoretical mean for the comparison. Statistical significance was accepted at *p* < 0.05. As regards to the animals randomized for each experimental group, the number was calculated on the basis of the "Resource Equation" [30].

Results

Exploration behavioural analysis

As shown in Fig.1, i.p. RVD-hp(α) (0.05 mg/kg) did not modify locomotor activity with respect to vehicle injected animals. On the other hand, *ip* Hp (0.05 mg/kg) injection induced a significant decrease of locomotor activity compared to controls. Two-way ANOVA showed significant differences in horizontal (**p* <0.005 *vs.* vehicle) and vertical activity (**p*<0.005 *vs.* vehicle) with respect to controls (Fig 1 A and B). Hp group also showed a significant decrease in time spent in moviments compared to control group (Fig. 1 C; ** *p* <0.005 *vs.* vehicle).

Anxiety-like behaviour

In the open field test, *ip* RVD-hp(α) (0.05 mg/kg) injection induced a significant increase of travelled distance and time spent in the central zone with respect to controls

(Fig. 2 A; ** p <0.005 and ** p <0.005 *vs.* vehicle, respectively). Similarly, in light-dark box, *ip* RVD-hp(α) (0.05 mg/kg) injection decreased the anxiety-related behavior. RVD-hp(α) treatment increased time spent in the light area and the number of total transitions (Fig. 2 B; ** p <0.005 and ** p <0.005 *vs.* vehicle, respectively), as well as decreased latencies to emerge from enclosed dark compartment in the light-dark box (** p <0.005 *vs.* vehicle). Hp (0.05 mg/kg) injection induced opposite effects with respect to RVD-hp(α), in all anxiety-related behavior tests (** p <0.005 *vs.* vehicle)

Behavioural despair

Fig 3 shows the total immobility time in the forced swim test. RVD-hp(α) (0.05 mg/kg) injection induced a significant decrease of total immobility in days 1 and 2 as compared to controls (Fig. 3 A and B; ** p <0.005 *vs.* vehicle, ** p <0.005 *vs.* vehicle, respectively). Hp (0.05 mg/kg) injection induced opposite effects with respect to RVD-hp(α), in forced swim test in both days (** p <0.005 *vs.* vehicle).

Prefrontal cortex monoamine levels

Fig 4 shows an increase in NE, DA and 5-HT levels in prefrontal cortex in rats treated with RVD-hp(α), as compared to controls (** p <0.005 *vs.* vehicle). On the other hand, Hp administration decreased monoamine levels in prefrontal cortex (** p <0.005 *vs.* vehicle).

MAO-B and COMT gene expression

Fig. 5 shows a significant decrease in prefrontal cortex MAO-B, (** p <0.005 *vs.* vehicle) and COMT (* p <0.05 *vs.* vehicle) gene expressions in RVD-hp(α) group compared to control. On the other hand, Hp (0.05 mg/kg) injection increased MAO-B and COMT gene expression (* p <0.05 and ** p <0.005 *vs.* vehicle).

Discussion

The eCB system is involved in the regulation of mood, specifically in anxiety and depression [1]. In the present study we show that Hp, a CB1 inverse agonist [11–12] and RVD-Hp(α), a CB1 negative allosteric modulator [14–15], are able to modulate emotional behaviors, in rats. In confirming the anxiogenic effect of a single injection of Hp (0.05 mg/kg), described by Fogaça and colleagues [13], we also found a decrease in locomotor activity in the open field test. In addition, we observed that Hp increases behavioural despair in the forced swim test. Actually, this effect could be mediated by TRPV1 channel, as described by Fogaça [13]. In fact, emotional behavior can be modulated by eCB system and endovanilloid systems interaction in prefrontal cortex [31]. Reports on the role of the TRPV1 receptor in the regulation of anxiety and depression are limited. TRPV1 receptor agonists induced depressive-like behaviours [21–33], while TRPV1 receptor antagonist induced antidepressant and anxiolytic-like behaviours [34]. In addition, different agonists or antagonists of TRPV1 receptor, inhibited or promoted the spontaneous physical (locomotor) activity in the open field test [35–37]. On the other hand, CB1 inverse agonists, such as rimonabant, are also able to induce anxiogenic and depressant behaviors, in animals and humans [8,38–39]. By contrast, we observed that a single injection of RVD-Hp (α) (0.05 mg/kg) did not modify the locomotor activity, but induced anxiolytic and antidepressant effects. It was shown that cannabidiol, a negative allosteric modulator of the CB1 receptors [40], displayed anxiolytic and antidepressant effects [41–42], without changing locomotor activity in the open field test [43]. Cannabidiol enhanced both serotonergic and glutamate cortical signalling through a 5-HT1A receptor-dependent mechanism [44]. On the other hand, different researches show that eCB modulate monoamine

transmission in prefrontal cortex [45]. In particular, CB1 agonist WIN 55,212-2 increased 5-HT [10,46] and NE signaling [47], in the cortex, and stimulated DA neurotransmission in neurodegenerative and neuropsychiatric disorders [48–49]. DA, NE and 5-HT play a well-established role in locomotion and emotional behavior [50–53]. In this work, we also evaluated monoamine levels in prefrontal cortex, and we observed an increase of DA, NE and 5-HT levels induced by RVD-hp(α) and an decrease of NE, DA and 5-HT by Hp. These effects could be related to the observed modulatory effects on emotional behavior by these peptides. In addition, we observed a decrease of MAO-B and COMT gene expression after RVD-hp(α) treatment and increased levels after Hp. MAO and COMT are the main enzymes involved in the catabolism of the catecholamines and 5-HT. Fišar and collaborators [54] reported that agonists of CB1 receptors inhibited MAO activity in mitochondrial fractions isolated from pig brain cortex , and concluded that cannabinoids can inhibit MAO activity, modulating in this way monoaminergic systems. Decreased MAO activity may be involved in some effects of cannabinoids on serotonergic, noradrenergic, and dopaminergic neurotransmission [9,54], however no further data is available. Hemopressin and its peptidic derivatives show several physiological effects, e.g., antinociception, hypophagy, and hypotension [55]. It is still a matter of debate whether those peptides do really modulate the eCB system. However the present study suggest that anxiolytic and antidepressive effects of RVD-hp(α) depend on CB1 receptor activity.

In conclusion, in the present study we demonstrated behavioral activities by peripheral RVD-hp(α) and Hp, also involving modulatory effects on monoaminergic signaling in

the prefrontal cortex. These peptides could represent a new perspective in the development of anxiolytic and antidepressive drugs.

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Legends to figures

Fig. 1: Chemical structure of hemopressin peptides.

Fig. 2. Locomotor activity in rats treated with a single *ip* administration of RVD-hp(α) and Hp (0.05 mg/kg). Compared to vehicle and RVD-hp(α), Hp significantly decreased locomotor activity. Horizontal activity (A), vertical activity (B) and movements were recorded for 10 min. Values represent the means ± SEM. (**$p < 0.005$ *vs.* vehicle).

Fig.3. Analysis of anxiety-related behaviour in rats treated with a single *ip* administration of RVD-hp(α) and Hp (0.05 mg/kg). Compared to vehicle, RVD-hp(α) decreased and Hp increased the levels of anxiety-like behaviour in open field (panel A) and light-dark box (panel B). Values represent the means ± SEM. (**$p < 0.005$ *vs.* vehicle).

Fig. 4. Behavioral despair measured in the forced swim test in rats treated with a single *ip* administration of RVD-hp(α) and Hp (0.05 mg/kg). Compared to vehicle, RVD-hp(α) decreased and Hp increased behavioral despair in days 1 and 2 (panel A and B). Values represent the means ± SEM. (**$p < 0.005$ *vs.* vehicle).

Fig.5. Norepinephrine (NE), dopamine (DA) and serotonin (5-hydrohytryptamine, 5-HT) steady state levels (ng/mg wet tissue) in prefrontal cortex tissue are expressed as mean ± SEM. Compared to vehicle, RVD-hp(α) significantly increased NE, DA and 5-HT, while Hp decreased the monoamine levels in prefrontal cortex (**$p < 0.005$ *vs.* vehicle).

Fig. 6. Relative gene expression of MAO-B and COMT in prefrontal cortex tissue as determined by real-time RT-PCR. Data were calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to β-actin mRNA levels, and expressed as relative to control (calibrator sample, defined as 1.00). Values represent the means $\pm$ SEM (*p <0.05 and ** p <0.05 *vs.* control).