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**Abstract**

Background: The endocannabinoid (eCB) system is strongly involved in the regulation of anxiety and feeding behavior. RVD-hemopressin(α) [RVD-hp(α)], a N-terminally extended form of hemopressin, is a negative allosteric modulator of the cannabinoid (CB) 1 receptor and a positive allosteric modulator of CB2 receptor which has been recently reported to exert anxiolytic/antidepressant and anorexigenic effects after peripheral administration in rats. Pharmacological evidences reported a possible link between brain hypocretin/orexin, monoamine and eCB systems, as regards appetite and emotional behavior control. Considering this, the aim of our work was to investigated the effects of RVD-hp(α) on anxiety like behavior and food intake after central administration and related it to monoamine levels and orexin-A gene expression, in the hypothalamus.

Methods: We have studied the effects of central RVD-hp(α) (10 nmol) injection on anxiety-like behavior and feeding using different behavioral tests. Hypothalamic levels of norepinephrine (NE), dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT) and gene expression of orexin-A and proopiomelanocortin (POMC) were measured by high performance liquid chromatography (HPLC) and real-time reverse transcription polymerase chain reaction (RT-PCR) analysis, respectively.

Results: Central RVD-hp(α) administration decreased locomotion activity and stereotypies. Moreover, RVD-hp(α) treatment inhibited anxiogenic-like behavior and food intake, NE levels and orexin-A gene expression, in the hypothalamus.

Conclusion: Concluding, in the present study we demonstrated that central RVD-hp(α) induced anxiolytic and anorexigenic effects possibly related to reduced NE and orexin-A and POMC signaling, in the hypothalamus. These findings further support the central

role of the peptide in rat brain thus representing an innovative pharmacological approach for designing new anorexigenic drugs targeting eCB system.

Keywords:

RVD-hemopressin(α)

Anxiety

Orexin-A

Proopiomelanocortin

Food intake

Monoamines

Abbreviations:

CB1, cannabinoid 1; CBD, cannabidiol; DA, dopamine; eCB, endocannabinoid; Hp, Hemopressin; 5-HT, serotonin; POMC, proopiomelanocortin; NE, norepinephrine; RVD-hp(α), RVD-hemopressin(α).

## Introduction

The endocannabinoid (eCB) system plays an important role in mood disorders [1] Different studies suggested the involvement of cannabinoid receptor activation on anxiety and depression [2-4]. In addition, the eCB system is involved in the regulation of food intake, metabolism and calorie storage [5]. However, it is well known that the first generation of CB1 blockers designed to reduce food intake and body weight, such as rimonabant, was discontinued due to psychiatric disorders, such as anxiety and depression [6-9].

The discovery of hemopressin (hp), an hemoglobin α chain-derived peptide and RVD-hemopressin(α) [RVD-hp(α)], the N-terminally extended peptide of Hp, also known as PEPCAN-12, revealed a promising research field for the pharmacotherapy of obesity [10,11]. Hp and RVD-hp(α) were found to bind CB1 receptors, as antagonist/inverse agonist and negative allosteric modulator, respectively [12-14]. RVD-hp(α) has been also described as a positive allosteric modulator of CB2 receptor [15]. Recently, we have also reported the anxiolytic/antidepressent effects of RVD-hp(α), after peripheral administration in rats [16]. These results are consistent with multiple studies showing the role of cannabidiol (CBD), an allosteric modulator of CB1/CB2 receptors [17,18], as potential anxiolytic/antidepressant drug [19,20], possibly acting via the enhancement of serotonergic and glutammatergic signalling [21,22]. Additionally, CBD inhibited the hyperphagia induced by CB1 or 5-HT1A receptor agonists [23]. Pharmacological evidences also reported a cross-talk between orexinergic and eCB systems, as regards appetite and emotional behavior control [24,25]. Additionally, the orexinergic neurons activate sympathetic neurons and arousal, playing a crucial role in emotional behavioral [26]. We have previously suggested that the behavioral activities by peripheral RVD-

hp(α) could involve modulatory effects on monoaminergic signaling in the prefrontal cortex [16]. On the other hand, despite the hemopressin peptides have been reported to cross blood brain barrier [27], a full comprehension of central mechanism of action is still required. Previously, we observed strong discrepancies between central and peripheral peptide administration, with particular regards to neuropeptidergic pathways underlying feeding control [28-30]. Considering these findings, the aim of our work was to investigate the direct central effects induced by RVD-hp(α), following intracerebroventricular (*icv*) administration in the rat, with particular regards to food intake and anxiety-like behavior. The behavioral data were also related to hypothalamic NE, DA and 5-HT levels, proopiomelanocortin (POMC) and orexin-A gene expression, evaluated by high performance liquid chromatography (HPLC) analysis and real time reverse transcription polymerase chain reaction (RT-PCR), respectively.

## Materials and methods

*Peptide synthesis and characterization*

RVD-hp(α) has been obtained in our laboratory by Fmoc-solid phase peptide synthesis (Fmoc-SPPS) strategy on 2-CTC (2-chlorotrityl chloride) resin, following the procedure reported by Mollica and colleagues [31-32]. Fmoc-Lys(Boc)-OH, Fmoc-Hys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(tBu)-OH and Fmoc-Ser(tBu)-OH were used as orthogonally protected building blocks for coupling reactions involving HATU/TBTU, and basic cocktail solution for Fmoc removal [33].

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 $^1H$ 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 (200-250 g) were housed in plexiglas cages (2 animals per cage; 40 cm × 25 cm × 15 cm) and maintained under standard laboratory conditions (22 ± 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. RVD-hp (α) was synthesized in our laboratories by using solid phase synthesis techniques and was diluted in saline at concentrations 10 nmol, as previously reported [16]. The following experimental groups were designed: Sham-operated (SHAM), saline-treated (Vehicle) and RVD-hp(α)-treated [RVD-hp(α)]. The initial group size was n=8 for behavior studies and n=6 for feeding behavior, hypothalamic monoamine levels and orexin-A gene expression after RVD-hp (α) administration in lateral ventricle.

*Stereotaxic surgery*

Rats were anesthetized by intraperitoneal injection with ketamine-xilazine (50 and 5 mg/kg, respectively) and placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA). A stainless steel guide cannula (21 G) was inserted stereotaxically to a depth of 3.5 mm in a position 1.5 mm to the left and 0.8 mm caudal to bregma, as previously reported [34]. The cannula was secured in position with dental acrylic (Formatray, Salerno, Italy) and the animal was kept warm during recovery. During surgery, rats were injected subcutaneously with 1 ml of sterile saline solution and 1 ml

of 5% glucose solution (Galenica Senese, Siena, Italy) and intraperitoneally with amoxicillin (20 mg $kg^{-1}$) (Farmalabor, Milano, Italy).

*Microinjection*

Animals received unilateral microinjections of vehicle (saline) or drugs (1 ul) into lateral ventricle before being submitted to the behavioral test, by connecting the cannula with a 5 µl syringe (Hamilton, Switzerland). After behavioral test the rats were sacrificed, as previously reported [34].

*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 10:00 and 12:00 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* 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, duration of stereotypic behavior (self-grooming and scratching), time spent in movements and resting time. 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 [35].

*Open field test*: To evaluate anxiety-like behavior, 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). Immediately after the injection, each animal was monitored for 10 min. In the open field test, the distance traveled and time spent into the center were recorded [36].

*Elevated plus maze:* The apparatus consisted of two open arms (50 × 10 cm) without side walls, perpendicular to two enclosed arms (50 × 10 × 40 cm) with a central platform common to all arms (10 × 10 cm). The maze was elevated to a height of 50 cm above floor level and rats were individually placed in the centre of the maze facing an open arm. The time spent on open arms, the latency to first exit and the number of transitions between the arms were recorded during a 10 min test period [34].

*Food intake*

24 h after RVD-hemopressin(α) (10 nmol) administration, food intake were evaluated, as previously reported [37]. After completion of feeding and anxiety-like behavioral test, the animals were sacrificed by $CO_2$ inhalation (100 % $CO_2$ at a flow rate of 20 % of the chamber volume per minute).

*Hypothalamic monoamine extraction and high performance liquid chromatography (HPLC) determination*

Immediately after sacrifice, brains were rapidly removed and individual hypothalami dissected and subjected to biogenic amine extractive procedures [38]. Briefly, tissues were homogenized in ice bath for 2 min with Potter-Elvehjem homogenizer in 1 ml of

0.05 N perchloric acid containing 0.004% sodium EDTA and 0.010% sodium bisulfite. The homogenate was 5 fold diluted in chromatographic mobile phase and centrifuged at 4,500 x *g* for 10 min. The supernatant was filtered on 0.45 µm PTFE sterile filters (Whatman) and directly injected for HPLC. Neurotransmitter recovery was satisfactory (≥90%) and reproducible, with percentage relative standard deviation ≤10%. The HPLC apparatus consisting of a Jasco (Tokyo, Japan) PU-2080 chromatographic pump and an ESA (Chelmsford, MA, USA) Coulochem III coulometric detector, equipped with micro dialysis cell (ESA-5014b) porous graphite working electrode and solid state palladium reference electrode. The analytical cell was set at −0.150 V, for detector 1 and at +0.300 V, for detector 2, with a range of 100 nA. The chromatograms were monitored at the analytical detector 2. Integration was performed by Jasco Borwin Chromatography software, version 1.5. The chromatographic separation was performed by isocratic elution on Phenomenex Kinetex reverse phase column (C18, 150 mm×4.6 mm i.d., 2.6 µm). The mobile phase was (10:90, v/v) acetonitrile and 75 mM pH 3.00 phosphate buffer containing octanesulfonic acid 1.8 mM, EDTA 30 µM and triethylamine 0.015% v/v. Flow rate was 0.6 ml/min and the samples were manually injected through a 20 µl loop. Neurotransmitter peaks were identified by comparison with the retention time of pure standard. Neurotransmitter concentrations in the samples were calculated by linear regression curve (y = bx + m) obtained with standard. Neither internal nor external standard were necessary for neurotransmitter quantification, in the hypothalamus homogenate, and all tests performed for method validation yielded results in accordance to limits indicated in official guidelines for applicability in laboratory trials. The standard stock solutions of DA, NE and 5-HT at 2 mg/ml were prepared in bidistilled water containing 0.004% EDTA and 0.010% sodium bisulfite. The stock

solutions were stored at 4°C. Work solutions (1.25-20.00 ng/ml) were obtained daily progressively diluting stock solutions in mobile phase.

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

Immediately after sacrifice, hypothalami were rapidly removed, dissected and stored in RNAlater solution (Ambion, Austin, TX) at −20 ◦C until further processed. Total RNA was extracted from the hypothalamus using TRI Reagent (Sigma-Aldrich, St. Louis, MO), as previously reported [37]. Contaminating DNA was removed using 2 units of RNase-free DNase 1 (DNA-free kit, Ambion, Austin, TX). The RNA solution was quantified at 260 nm by spectrophotometer reading (BioPhotometer, Eppendorf, Hamburg, Germany) and its purity was assessed by the ratio at 260 and 280 nm readings. The quality of the extracted RNA samples was also determined by electrophoresis through agarose gels and staining with ethidium bromide, under UV light. 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, Rn00565995_m1 for orexin-A gene; Rn00595020_m1 for POMC gene,) β-actin (Applied Biosystems, Foster City, CA, USA, Part No. 4352340E) was used as the housekeeping gene. The real-time PCR was carried out in triplicate for each cDNA sample in relation to each of the

investigated genes. Data were elaborated with the Sequence Detection System (SDS) software version 2.3 (Applied Biosystems, Foster City, CA, USA). Gene expression was relatively quantified by the comparative $2^{-\Delta\Delta Ct}$ method [39].

*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" N=(E+T)/T (10≤E≤20) [40], where E, N and T represent the numbers of degrees of freedom in an ANOVA, animals and treatment, respectively.

**Results**

*Exploration behavioral analysis*

As shown in Fig. 2, *icv* RVD-hp(α) (10 nmol) injection induced a significant decrease of locomotor activity compared to SHAM and vehicle control groups. Two-way ANOVA showed significant differences in horizontal, vertical activity, time spent in movements and stereotypic behavior respect to controls (**$p$<0.005 *vs.* SHAM and vehicle)

*Anxiety-like behavior*

In the open field test, *icv* RVD-hp(α) injection decreased the anxiety-related behavior, as evidenced by a significant increase of travelled distance and time spent in the central zone respect to controls (Fig. 3A, $^{**}p<0.005$ *vs.* SHAM and vehicle). Similarly, in elevated plus maze test *icv* RVD-hp(α) injection increased the time spent in the open arms and number of total transitions, and decreased the latencies to emerge from the central zone (Fig. 3B, $^{**}p<0.005$ *vs.* SHAM and vehicle).

*Food intake evaluation*

Fig. 4 shows food intake (g) in rats fed a standard diet. Vehicle or peptide were administered by *icv* injection, during the light phase, at 9:00 a.m.. Food intake was evaluated 24 h after treatment in each group of rats. Values represent the means ± SEM. Compared to SHAM and vehicle control groups, RVD-hemopressin(α) significantly inhibited food intake ($^{***}p<0.001$ *vs.* SHAM and vehicle).

*Hypothalamic monoamine levels*

Fig. 5 shows decreased NE levels in the hypothalamus after RVD-hp(α) (10 nmol) injection, compared to SHAM and vehicle control groups ($^{**}p<0.005$ *vs.* SHAM and vehicle). On the other hand, we did not observe any alteration as regards DA and 5-HT levels, following peptide administration.

*POMC and orexin-A gene expression*

RVD-hp(α) injection inhibited hypothalamic POMC and orexin-A gene expressions (Fig. 6), compared to SHAM and vehicle control groups ($^{**}p<0.005$ *vs.* SHAM and vehicle).

## Discussion

The eCB system is well known to modulate anxiety-like and feeding behavior [1,5,41]. In the present study we have shown that RVD-hp(α), a CB1/CB2 allosteric modulator [13-15], is able to modulate emotional and feeding behavior. In confirming the anxiolytic and anorexigenic effects, previously described by our research group [11,16], we also observed a decrease in locomotor activity after central administration. The contrasting results with our recent published studies could be possibly related to the different route of administration, thus further supporting the hypothesis of multiple central and peripheral mechanisms underlying behavioral peptide effects *in vivo,* including the possible modulation of adipokines and gut-derived hormones, both involved in metabolic and behavioral pathways [28,29,38]. Additionally, we observed that RVD-hp(α) decreased the stereotypic behavior, such as self-grooming and scratching, which are considered useful index of anxiety behavior, in animal models [42]. We also observed a decrease in food intake after central RVD-hp(α) administration, possibly related to reduced hypothalamic NE levels and POMC gene expression. This is consistent with previous findings suggesting that POMC-derived peptide β-endorphin and NE are key modulators of appetite and anxiety behavior induced by CB1 agonists [43,44]. We also found decreased orexin-A gene expression in the hypothalamus. Multiple studies suggested a possible cross-talk between orexinergic and eCB systems in rat hypothalamus [24,25,45], which could be involved in appetite, reward, sleep/wake cycle and nociception [25]. Central administration of orexin-A stimulates food consumption, while orexin receptor antagonist (SB334867), reduced feeding [46,47]. On the other hand, orexin-A injection into the brain increased arousal,

locomotor activity and stereotypies, in rats [48-51]. Moreover, Flores and collaborators [25] have found orexin and CB1 receptors co-expressed in the hypothalamus.

In the present study, we also found that *icv* RVD-hp(α) injection decreased NE, and did not affect DA and 5-HT levels in the hypothalamus. The central noradrenergic system is involved in the control of arousal [52,53], exploration behavior [54,55] and food intake [56]. Soya and collaborators suggested that it could mediate orexin-induced behavioral effects [26]. These studies could clarify our findings of anxiolytic and anorexigenic effects observed after RVD-hp(α) peripheral and central administration, in rats. In fact, interference on hypocretinergic transmission might be useful in the control of appetite and other disorders associated with obesity, such as anxiety, probably mediated by CB1 receptors.

In conclusion, in the present study we demonstrated that central RVD-hp(α) administration induced anxiolytic and anorexigenic effects possibly mediated by reduced NE and orexin-A signaling, in the hypothalamus. These findings further support the central role of RVD-hp(α) in rat brain, and could represent a perspective in the pharmaceutical design of new anorexigenic drugs.

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**Legends to figures**

**Fig. 1.** Chemical structure of RVD-hp(α).

**Fig. 2.** Locomotor activity in rats treated with a single *icv* RVD-hp(α) administration (10 nmol). Compared to SHAM and vehicle, RVD-hp(α) significantly decreased locomotor and stereotypic activity. Horizontal activity (A), vertical activity (B), movements (C) and stereotypic movements (D) were recorded for 10 min. Data are expressed as means ± SEM. (**$p$<0.005 *vs.* vehicle).

**Fig.3.** Analysis of anxiety-related behavior in rats treated with a single *icv* RVD-hp(α) administration (10 nmol). Compared to SHAM and vehicle, RVD-hp(α) decreased levels of anxiety-like behavior in open field (A) and elevated plus maze test (B). Data are expressed as means ± SEM. (**$p$<0.005 *vs.* SHAM and vehicle).

**Fig. 4.** 24 h food intake following *icv* RVD-hp(α) administration (10 nmol). RVD-hp(α) significantly inhibited food intake with respect to SHAM and vehicle. Data are expressed as means ± SEM. (***$p$ <0.001 *vs.* SHAM and vehicle).

**Fig. 5.** Norepinephrine (NE), dopamine (DA) and serotonin (5-hydrohytryptamine, 5-HT) hypothalamic levels (ng/mg wet tissue), following *icv* RVD-hp(α) administration (10 nmol). Data are expressed as means ± SEM. Compared to SHAM and vehicle, RVD-hp(α) significantly decreased NE levels in the hypothalamus (**$p$<0.005 *vs.* SHAM and vehicle).

**Fig. 6.** Relative gene expression orexin-A and POMC in the hypothalamus A after *icv* RVD-hp(α) administration (10 nmol), 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 ± SEM. (**$p$<0.005 *vs.* SHAM and vehicle).