Involvement of the cocaine-amphetamine regulated transcript peptide (CART 55-102) in the modulation of rat immune cell activity

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Abstract

OBJECTIVE: Cocaine-amphetamine regulated transcript peptides (CART) belong to a neuropeptide family expressed in the central nervous system, especially in the hypothalamus, and also in peripheral tissues. The physiological functions of CART include modulation of pituitary hormone release, regulation of body weight, and the control of feeding behavior and metabolic activity. The reciprocal relationships between CART and immune system function have to be established. Therefore, in the present study we aimed to investigate the influence of CART, administered intracerebroventricularly (icv), on selected immune parameters and pituitary-adrenal axis hormone secretion in the rat. RESEARCH METHODS: In rats submitted to icv infusion of CART or artificial cerebrospinal fluid (aCSF, control) selected immune parameters: splenocyte proliferation (spontaneous and mitogen-stimulated) and peritoneal leukocyte (PTL) activity (spontaneous and phorbol myristate acetate (PMA)-stimulated) were examined 60 and 120 min after treatment. The direct effect of CART on splenocytes in culture in vitro was also examined. Concentration of adrenocorticotrophic hormone (ACTH) and corticosterone was also measured in serum of control and CART infused rats. RESULTS: Splenocytes isolated 60 min after CART infusion exhibited a decreased, albeit non-significant, ability to proliferate spontaneously and were unable to answering to the mitogenic stimulation. This effect was not seen 120 min after CART treatment, which restored splenocyte proliferation decreased by aCSF infusion. CART addition in vitro did not influence proliferation of splenocytes from control rats. Spontaneous activity of peritoneal leukocytes was not modified by CART infusion. PMA-stimulated PTL activity was significantly decreased in aCSF-infused rats 120 min after treatment and CART infusion antagonized this effect. Non-significant increase in serum cortisol after 60 min followed by a significant decrease after 120 min with no change in ACTH concentration was found. CONCLUSION: The immunomodulatory activity of icv-infused CART appears to consist in the creation of a...
short-lasting immunosuppressive internal milieu, followed by the immunostimulatory one. This first effect was most probably due to the activation of the HPA axis and/or other immunosuppressive peptides, but not through the direct action of CART on immune cells. Thus, CART appears to be short-lasting and indirect modulator of immunity.

1. INTRODUCTION

Cocaine-amphetamine regulated transcript peptides (CART) belong to a recently discovered neuropeptide family widely expressed in the central nervous system, especially in the hypothalamus, and also in peripheral tissues [1, 2, 3]. Among the physiological functions attributed to CART due to their influence on pituitary hormone release [4], are regulation of body weight via control of feeding behavior and metabolic activity [1, 2]. CART increases thermogenesis, energy expenditure and inhibits gastric emptying [1]. Several studies have identified a polymorphism of the CART gene in obese individuals and indicate that it could be involved in the development of human and experimental animal obesity [5, 6, 7]. On the other hand, it has been found that obesity is associated with an increase in the synthesis of proinflammatory cytokines in adipocytes [8, 9, 10].

Immunohistochemical studies have demonstrated the presence of CART-expressing neurons in the rat pituitary and adrenal glands as well as in the pancreas [11, 12, 13, 14]. The exact location of CART receptors is not known, although specific CART binding has been reported in the AtT20 pituitary cell line [15, 16]. Moreover, anatomical and functional evidence suggest that CART may be associated with regulation of the hypothalamo-pituitary-adrenal axis (HPA). In particular, our previous studies on rats demonstrated that intravenous (iv) and intracerebroventricular (icv) administration of CART stimulates secretion of ACTH and corticosterone [4, 17], which are among the most important and efficient endogenous anti-inflammatory factors.

To our knowledge, reciprocal relationships between CART and immune system function have not been established yet. A suppression of hypothalamic CART expression by cytokine-mediated LPS-induced immune challenge accomplished by the well-known anorectic behavior was a first published finding on this topic [18]. The aim of the present study was, therefore, to investigate the influence of CART, administered intracerebroventricularly (icv), on selected immune parameters and pituitary-adrenal axis hormone secretion in the rat.

2. MATERIALS AND METHODS

2.1. Animals and surgery

Twenty-eight male Wistar-Kyoto rats (240–260 g) were maintained under controlled conditions (14 h L: 10 h D, lights on at 06:00, temperature 23 ± 1°C) with free access to food and water. All animal procedures were in accordance with the Guiding Principles for the Care and Use of Research Animals and were approved by the First Warsaw Ethics Committee for Experiments on Animals.

Animals were anesthetized by intramuscular (im) injection of ketamine at a dose of 100 mg/kg and subjected to surgery to implant icv cannulae, as described previously [4]. Briefly, the stainless-steel 23-gauge guide cannula was implanted into the third cerebroventricle (0.8 mm posterior and 7.0 mm ventral to the bregma at the midline) according to the atlas of Paxinos and Watson [19]. The external end of the cannula was closed by a removable stainless-steel plug. The placement of the icv cannulae was verified at autopsy by an injection of methylene blue dye. The brain was inspected for complete spread of the dye in the third ventricle.

After surgery, the rats were transferred to individual cages with food and water freely available. During a 7-day recovery period the animals were handled daily to minimize any stress associated with handling on the day of experiment.

2.2 Experiment

On the day of the experiment, 2 h before CART administration, each stainless-steel guide cannula was opened and its patency confirmed. Freely moving rats (14 animals) were infused icv with rat CART (55-102): 5 μg in 5 μl of vehicle (artificial cerebrospinal fluid, aCSF). A second group of 14 rats was infused with the same volume of the vehicle alone (control group). Infusion (1 μl/min) was performed with an automatic pump (CMA/100; Sweden) through an inner cannula inserted into the guide cannula. After the infusion, rats were transferred to their home cages with free access to food and water. Sixty and 120 min after infusion, 7 rats from each group were decapitated and trunk blood was collected in plastic tubes containing protease inhibitor (1000 IU aprotinine per ml of blood). Peritoneal leukocytes (PTLs) and spleens were isolated and used immediately for in vitro studies. The time from removal of the animals from their cages to decapitation was approximately 2 min.

2.3 Reagents

Rat CART (55-102) was obtained from Bachem (Germany). Phytohemagglutinin (PHA), 2-mercaptoethanol (2-ME), phorbol myristate acetate (PMA) and dichlorodihydrofluorescin diacetate (CM-H2DCFDA) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibiotic-antimycotic solution was purchased from GibcoBRL, heparin from Biochemie (Vienna, Austria) and [3H]-thymidine ([6-3H]dThd, 40 MBq/ml) from UVVVR (Prague, Czech Republic). Phosphate buffered saline (PBS) and Eagle's minimal essential medium (MEM) were obtained from Biomed (Lublin, Poland) and other buffers and reagents were prepared using chemicals purchased from POCH (Gliwice, Poland). Ketamine was from Vetoquinol-Biowet (Gor-

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zow Wlkp, Poland) and aprotinine from Polfa-Kutno (Kutno, Poland).

The RIA kit for measuring serum corticosterone (CS) concentration was from ICN Biomedicals Inc. (Solon, OH, USA) and that for adrenocorticotropic hormone (ACTH) from Phoenix Pharmaceuticals (Burlingame, CA, USA).

2.4 Isolation of peritoneal leukocytes (PTLs) and measurement of activity

Peritoneal leukocytes were obtained, as described previously [20], by flushing the peritoneal cavity with sterile Eagle's minimal essential medium (MEM) supplemented with heparin (1 μg/ml), pooled within groups, then counted using a hemocytometer and collected by centrifugation (10 min, 300 g, 4°C). The sedimented cells were diluted to 2 × 10^6 cells/ml in PBS containing glucose (90 mg/100 ml) and EDTA (20 mg/100 ml), and then intracellular reactive oxygen species (ROS) level was analyzed as a measure of their activity. For this purpose, PTLs were incubated for 45 min at 37°C with the oxidation-sensitive dye CM-H_2DCFDA at a concentration of 4 μg/ml [21], alone or together with the activator phorbol myristate acetate (PMA, 4 μg per 10^6 cells). Fluorescence was measured using a VICTOR-3 counter (PerkinElmer) and the stimulation index (fluorescence in PMA-stimulated cells divided by basal level) was calculated.

2.5 Splenocyte isolation and culture in vitro

After flushing the peritoneal cavity, spleens were isolated aseptically, pooled within groups and immediately used to prepare in vitro splenocyte cultures according to a previously described method [20]. Briefly, spleens were homogenized in MEM containing L-glutamine and NaHCO_3 and the homogenate filtered through nylon mesh to remove tissue debris. After centrifugation (8 min, 310 g, 4°C), the cells were resuspended in lysis buffer (0.17 M Tris, 0.16 M NH_4Cl) and held at 4°C for 15 min to lyse contaminating erythrocytes. Erythrocyte-free splenocytes were counted and tested for viability (standard trypan blue exclusion test). Final suspensions were prepared using cells with viability of ≥ 90% and adjusted to 2.5 × 10^6 cells/ml with RPMI medium supplemented with glutamine, heat-inactivated fetal calf serum (10%), antibiotic-antimycotic (1%) and 2-mercaptoethanol (50 μM). Splenocyte cultures (2.5 × 10^6 cells/well) were prepared in 96-well tissue culture plates (Falcon) in the presence of serial dilutions of T-cell mitogen PHA (0.5, 1.0, 2.0 μg/well) or B-cell mitogen LPS (0.5, 1.0, 2.0 μg/well). Control cultures consisted of cells incubated with culture medium alone (spontaneous proliferation). To examine the possible direct effect of CART on rat splenocytes in vitro, additional cultures, prepared using cells isolated from control rats, 60 and 120 min after infusion with aCSF, were incubated with CART (0.5 μg per well), alone or plus mitogens. The culture plates were incubated for 72 h at 37°C in a fully humidified, 5% CO_2 atmosphere. Prior to harvesting with a semi-automatic cell harvester (Skatron), the cells were pulsed for 18 h with 1 μCi/well of [3H]-thymidine. Incorporation of tritiated thymidine was measured by liquid scintillation spectrometry (Beckman) and expressed in both cpm and as the stimulation index, SI (cpm in mitogen-stimulated culture divided by cpm in control culture).

2.6 Serum hormone assay

Serum was prepared from blood taken from the rats after decapitation and the concentrations of ACTH and corticosterone (CS) were measured using RIA kits. All measurements were made in one assay. Intra-assay coefficients for CS and ACTH were 6.6% and 6.5%, respectively. The limit of detection for CS was 25 ng/ml and that for ACTH was 10 pg/ml.

2.7 Statistical analysis

All results were expressed as means ± SD. Statistical evaluation of the differences between groups was performed on original data using the Kruskal-Wallis rank test followed by the Mann-Whitney U test. Leukocyte activity measurements were analyzed by ANOVA followed by the Student-Neuman-Keuls test. Results were considered statistically significant when p < 0.05.

3. RESULTS

3.1. Splenocyte proliferation

Spontaneous proliferation of splenocytes obtained from control rats was similar both at 60 and 120 min after aCSF infusion, and comparable with that of splenocytes isolated 60 min after CART infusion (a decrease in proliferation seen in this group was non-significant). However, the level of spontaneous proliferation observed in splenocytes from rats 120 min after CART infusion was significantly higher than that seen in cells from both control (aCSF 120, p < 0.01) and 60 min earlier CART-infused animals (p < 0.001, Fig. 1a).

Mitogen-stimulated splenocyte proliferation was examined over a wide concentration range of both T-cell (PHA)- and B-cell (LPS)-specific mitogens. Since the proliferative response was clearly mitogen dose-dependent, results concerning the effect of only one PHA and LPS concentration (2 and 0.5 μg/well, respectively) are shown in Fig. 1b. The proliferation response to both PHA and LPS was comparable and strongly treatment- and time-dependent (Fig. 1b). Both T- and B-cells isolated 60 min after infusion from control aCSF rats showed marked proliferation while those from CART-infused animals failed to respond to mitogenic stimulation. In splenocytes collected from rats 120 min after infusion, the relationship was reversed: those from the aCSF-treated group were non-responsive to mitogenic stimulation while a significant proliferation response was seen in cells from the CART-infused group.
Neither spontaneous nor mitogen-stimulated proliferation of splenocytes from control, aCSF-infused rats was modified by incubation in vitro with CART at a dose corresponding to that administered to rats in vivo (Fig. 2).

3.2. Peritoneal leukocyte activity
Spontaneous activity of PTLs was similar across groups, with the exception of the less active cells retrieved from rats 120 min after aCSF infusion ($p < 0.001$, Fig. 3a).

PTLs obtained 60 min after infusion with either aCSF or CART responded to PMA-stimulation in a similar manner, and this response was comparable with that of cells collected after 120 min from the aCSF-infused group. A significant ($p < 0.01$) increase in PMA-stimulated activity was found in PTLs obtained from rats infused with CART 120 min earlier (Fig. 3b).

3.3. Serum hormone concentration
Peripheral blood levels of CS were modified only slightly by the treatments. A tendency to increase (vs aCSF infused control rats) was seen in both CART receiving groups. However, the blood CS concentration was decreased significantly ($p < 0.01$) at 120 min after CART infusion compared with 60 min after this treatment (Fig. 4a). No effect on ACTH blood concentration was observed (Fig. 4b).

4. DISCUSSION
Numerous studies have shown that neuropeptides involved in the control of feeding behavior also possess other biological activities, including immunomodulation. For example, leptin, a protein secreted by adipocytes, protects T lymphocytes from apoptosis, regulates T cell proliferation and activation, influences $T_H 1$ response and also modulates cytokine production...
Moreover, neuropeptide Y (NPY) whose function opposes that of leptin, seems to be a bimodal modulator of immune cells, acting as a negative regulator of T_{H1} lymphocytes and an activator of antigen-presenting cells (APC) [23]. On the other hand CART, another agent with an important role in appetite regulation [1], to our knowledge has not yet been linked with any immunomodulatory activity. This possibility was even not taken into consideration in various experiments conducted on CART-knockout mice [25]. On the other hand, previously we have found that in rats both icv and systemic CART infusion increased plasma levels of several hormones, including prolactin (PRL) and growth hormone (GH), as well as ACTH and corticosterone, seen 60 and 120 min after treatment [4,17]. This suggested a possible involvement of CART in the indirect, hormone-mediated, immunomodulation. In the present study, using the same experimental protocol, some immune parameters were examined in CART-infused rats.

Splenocytes isolated from rats infused with CART 60 min earlier exhibited a substantial decrease (in comparison with those from control aCSF-infused animals) in their ability to proliferate, not only spontaneously but also after stimulation with both T-cell- (PHA) and B-cell-specific (LPS) mitogens. This effect was neither seen in cultures of splenocytes obtained from rats infused with CART 120 min previously (Fig. 1a and b) nor in PTLs in response to stimulation with PMA in vitro (Fig. 3b), suggesting that the inhibitory effect of icv-infused exogenous CART on splenocytes was short-lasting and most probably indirect one. This last supposition is supported by the results obtained with splenocytes from control aCSF-infused rats, cultured in vitro with CART alone or together with either of the two mitogens (Fig. 2). In these cells, neither spontaneous nor mitogen-stimulated proliferation was modified by the presence of CART added to the culture medium. To our knowledge the CART receptor identification has been largely unsuccessful [16], and the data indicating the presence of CART receptors within the immune system are lacking. In the in vitro testing protocol used, splenocytes obtained from aCSF-infused rats appeared unable to recognize the CART message, being most probably devoid of specific receptors for this neuropeptide. Rather, the immunosuppressive effect of CART ob-

![Fig. 3a](image1.png)

**Fig. 3a.** Activity of peritoneal leukocytes (PTL) isolated after 60 and 120 min of treatment from aCSF- and CART-infused rats: a) spontaneous activity, expressed in arbitrary fluorescence units; and b) PMA-stimulated activity, expressed as the stimulation index (SI). Statistically significant differences: **p < 0.01, ***p < 0.001.

![Fig. 3b](image2.png)

**Fig. 3b.** Activity of peritoneal leukocytes (PTL) isolated after 60 and 120 min of treatment from aCSF- and CART-infused rats: a) spontaneous activity, expressed in arbitrary fluorescence units; and b) PMA-stimulated activity, expressed as the stimulation index (SI). Statistically significant differences: **p < 0.01, ***p < 0.001.

![Fig. 4](image3.png)

**Fig. 4.** Peripheral blood levels of: a) corticosterone (CORT) and b) ACTH in aCSF- and CART-infused rats. Statistically significant difference: **p < 0.01.
CART and aCSF infusion, or if, more probably, its level is difficult to decide whether it is really not affected by icv infusion. Nevertheless, as icv infusion failed to respond to mitogen stimulation although they still exhibited normal spontaneous proliferation (Fig. 1a and b). Moreover, these features were not modified by CART addition to the cultures in vitro, again supporting the notion of an indirect immunosuppressive effect of CART, not obviously connected with corticosterone level, which was not elevated in the serum of this group of rats (Fig. 4a).

Internal milieu, created in some way 120 min after aCSF injection, appears to be more immunosuppressive, causing also a low PTL activity, both spontaneous and PMA-stimulated (Fig. 3a and b). On the other hand, the effect of stress on CART expression in several male rat brain structures has been demonstrated [26]. It is therefore plausible to suggest that the symptoms of immunosuppression observed in our experiment 120 min after aCSF infusion (inability of splenocytes to be stimulated by mitogens, low activity of PTLs), not accompanied by the increase in serum corticosterone concentration, should resulted (at least partly) from the feedback effect of infusion-induced stress on the endogenous CART synthesis. It seems worthwhile to verify these possibilities in the future experiments.

Contrary to the effect of aCSF, the treatment with CART 120 minutes earlier caused a restoration of both spontaneous and mitogen-stimulated splenocyte proliferation (Fig. 1a and b) along with PMA-induced activation of PTLs (Fig. 3b). In the present experiment the serum level of other hormones with the well-known immunomodulatory (mainly stimulatory) potential, i.e. PRL and GH [27, 28] was not measured. However, previously [4] we demonstrated a highly significant and long-lasting (up to 240 min after icv infusion) increase in serum level of GH. It is therefore admissible that in the experimental protocol used a short-lasting (up to 60 min) immunosuppressive internal milieu created by CART icv infusion was followed by immunostimulatory events, one, supposedly due to the important increase in GH concentration.

In this rat experimental model, we failed to detect any significant changes in serum ACTH concentration with either of the treatments tested. However, it is difficult to decide whether it is really not affected by icv CART and aCSF infusion, or if, more probably, its level changes too rapidly for a response to be observed after a 60 min time interval [17].

To conclude, in the experimental model used, the immunomodulatory effect of CART infused icv seems to be represented by the creation of a short-lasting (up to 60 min) immunosuppressive internal milieu, most probably via activation of the HPA axis and/or other immunosuppressive molecules and does not involve the direct action on immune cells. Subsequently, after 120 min, the immunostimulatory hormones secreted under exogenous CART influence should overcome this short-lasting immunosuppression. A precise mechanism of this CART activity requires further examination, particularly in relation to its involvement in the regulation of feeding behavior, perturbations of which are connected with serious immune dysfunctions.

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