Physiology & Behavior 157 (2016) 225–230

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Catestatin and $GABA_AR$ related feeding habits rely on dopamine, ghrelin plus leptin neuroreceptor expression variations

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HIGHLIGHTS

- CST reduces feeding via D1 up-regulation.
- BIC favors the increase of body weight via D2 up-regulation.
- Anorexigenic role of CST is in part due to $GABA_AR$ modulatory signals.

ARTICLE INFO

Article history:
Received 21 November 2015
Received in revised form 20 January 2016
Accepted 9 February 2016
Available online 12 February 2016

Keywords:
Bicucullin
Hibernating hamster
Chromogranin-A derivative

ABSTRACT

Catestatin (CST), an endogenously small sympathoinhibitory peptide is capable of interfering with the major cerebral neuroreceptor-blocking site, i.e. γ-aminobutyric $acid_A$ receptor ($GABA_AR$) system especially in limbic brain areas that are involved with feeding behaviors. The $GABA_A$Rergic-related effects seem to derive from its interaction with other molecular neuroreceptors such as dopaminergic, ghrelin and leptinergic. In this context, the present study aimed to investigate probable feeding responses (eating and drinking) induced by treatment with CST and the $GABA_AR$ antagonist bicucullin (BIC) alone or simultaneously (CST + BIC) in the Syrian hibernating hamster (*Mesocricetus auratus*) model. Hamsters that received these compounds via intracerebroventricular infusions displayed notable variations of feeding and drinking bouts. In particular, an anorexigenic response was evident following treatment with CST while BIC evoked a significant increase of eating and drinking behaviors. Surprisingly when both agents were given simultaneously, a predominating anorexigenic response was detected as shown by evident CST-dependent reduction of feeding bouts. Contextually such behaviors, especially those following the combined treatment were tightly correlated with the significantly increased cerebral dopamine receptor 1 (D1) plus reduced ghrelin receptor (GhsR) and leptin receptor (LepR) transcript levels. Overall, the anorexigenic effect of CST deriving from its tight interaction with $GABA_ARs$ activity plus D1 and GhsR transcripts tends to propose these neuronal elements as pivotal factors responsible for feeding disorders.

1. Introduction

The novel small hydrophobic neuroactive sympathoinhibitory peptide catestatin (CST), deriving from the proteolytic cleavage of chromogranin A, contribute to autocrine and homeostatic mechanisms driving from the *in vitro* catecholamine release of chromaffin cells and neurons [29], while *in vivo* CST itself blocks the stimulation of both secretion and transcription functions [30]. CST by acting on nicotinic acetylcholine plus α2-adrenergic receptors is capable of evoking cardiovascular as well as metabolic effects, since it is capable of reducing lipid deposition plus increasing lipolysis and fatty acids oxidation [6]. It appears that this highly novel peptide does not bind to a specific class of neuroreceptors, and so its neuroactive responses tend to derive from interactions with other major neuroreceptor systems [15, 18, 20]. In particular, CST is capable of interfering with inhibitory GABAergic outputs [4] above all in cerebral areas involved with feeding behaviors [25]. Of the different GABAergic sites, $GABA_AR$ is considered a versatile receptor subtype due to its interaction with other neurotransmitter receptor systems and namely dopaminergic plus leptinergic (DAergic; [12]). Together, these receptor systems not only control hypothalamic feeding-related behaviors [11, 34, 38], but also grelin production [22, 24]. As far as the latter feeding-related factor is concerned, it is retained a major orexigenic gut hormone regulating nutritional homeostatic processes [26].

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http://dx.doi.org/10.1016/j.physbeh.2016.02.015
0031-9384/

In the past, DAergic projections of the midbrain ventral tegmental area (VTA) to the nucleus accumbens have been considered a major brain circuit controlling feeding rhythms via its rich interconnections with hypothalamic pathways [8]. Indications deriving from this work point to medial VTA-related DA production as a key signaling condition capable of regulating eating habits through the involvement of DA receptors (D1 and D2; [41]). Moreover, studies have demonstrated that the specific ghrelin receptor (GhsR) interacting with DAergic receptors either attenuates or stimulates food intake [41]. In a similar fashion, even the anorectic effects of leptinergic neurons tend to be correlated to VTA DA inhibitory signals reducing feeding stimuli very probably through the blocking actions of the Janus kinase/STAT neuronal pathway [35].

On the basis of the above features, it was our intention to establish the type of feeding relationship deriving from intracerebroventricular (icv) treatment of the Syrian hibernating hamster (*Mesocricetus auratus*) with two major cardio-regulative factors and namely, CST or the $GABA_A$ergic antagonist bicucullin (BIC) given alone or together (CST + BIC). The selection of this permissive hibernating rodent model was based on its capability of tolerating stressful conditions, which allowed us to study the different feeding and drinking responses, after the stereotaxically central injection of drugs, during the euthermic state [2, 32]. Contextually, the above experimental intentions were also co-related to the expression variations of the main feeding-related neuroreceptor systems and namely D1, D2, GhsR and leptin receptor (LepR) of whole limbic regions that included hypothalamus, amygdala, hippocampus and parietal cortex layers. Overall, indications deriving from these results tend to propose CST plus $GABA_A$Rs cross-talking properties as major factor(s) operating during the different eating and drinking intervals, which together with the above feeding related neuroreceptors may constitute novel regulatory actors responsible for feeding disorders.

2. Materials and methods

2.1. Animals, stereotaxic surgery and microinjections

For this study, Syrian golden hamsters (n = 23; 10 month-old; Charles River, Italy) with free access to food and water were entrained at room temperature (25 °C), under a 14 h light/10 h dark cycle in order to allow them to adapt to their new conditions before surgery. Subsequently hamsters, anesthetized intraperitoneally with urethane (1.3–1.4 g/kg i.p.; Sigma Chemical Co., St. Louis, MO, USA), were placed in a Stoelting stereotaxic instrument and the stainless steel guide cannula (CMA/Microdialysis AB, Stockholm, Sweden) was stereotaxically implanted unilaterally directed toward and 1 mm above the icv space (coordinates relative to lambda: AP +6 mm, ML +2.5 mm and DV −4 mm) according to the hamster stereotaxic atlas [33]. The cannula was fixed to the skull with acrylic dental cement and animals were allowed 7 days to recover before behavioral studies.

Starting at day 7, infusions were carried out through an inner cannula (33 G) that extended 1 mm beyond the tip of the guide cannula, which was connected to a Hamilton micro-syringe (1 μl) by polyethylene tubing. Animals were divided into four groups so that they were infused with drugs in a same manner as previously described by us and others: the first received 1 μl of CST (50 μM; n = 5; [17, 32]). The second group received a dose of 1 μl of BIC (1.96 mM; n = 5; [43]); the third group was infused with 1 μl of the combined treatment (CST + BIC; n = 5) and the forth group that represents the control group (ctr; n = 8) received 1 μl of saline solution (NaCl 0.9%). Treatment was conducted every morning (at 9:00) for 7 days over a 60 s period plus a further 60 s time-interval in which the solution was allowed to diffuse from the cannula. The effects of the different drug treatments were compared with respect to ctrs. Animal maintenance and experimental procedures were carried out in compliance with the ethical provisions for Care and Use of Laboratory Animals reported in the legislative law n°116 (27-01-1992) and authorized by the National Committee of the Italian Ministry of Health.

2.2. Behavioral analyses

In the present study, all experiments were performed between 9:00 and 15:00, with hamsters handled 3 min each day prior to behavioral testing. Hamsters were allowed a thirty-minute interval after drug infusion before being checked for the most common feeding behaviors during a 20 min interval, 3 times a day for 7 days. The following feeding parameters were evaluated: eating, drinking plus body weight variations [21, 32]. All behaviors were recorded by a webcam placed perpendicularly at 60 cm above the cage floor as previously described [2]. After which, all animals were sacrificed, whole limbic areas were removed and then stored at −80 °C for further investigations. At the end of the study, some ctr animals (n = 3) received 1 μl of 1% methylene blue solution in order to verify that icv injections were conducted correctly.

2.3. RNA extraction, reverse transcription and real time PCR

For this study the expression patterns of D1, D2, GhsR and LepR, which resulted to be strongly connected with feeding behaviors [26, 34, 41] were evaluated. Total RNA was extracted from hypothalamus, amygdala, hippocampus and parietal cortex layers of treated and ctr hamsters, using TRI-Reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. Contaminating genomic DNA was removed by treatment with DNase (Ambion, Life Technologies) and RNA concentration was measured with a NanoDrop spectrophotometer [1]. 1 μg RNA was then reverse transcribed with High Capacity cDNA Reverse Transcriptase (Life Technologies). Real time PCR for D1, D2, GhsR and LepR, was carried out on Applied Biosystem 7500 Real Time System using SYBR Select Master Mix assay (Applied Biosystem, Courtaboeuf). Gene-specific primers were designed, accordingly to GenBank published sequences using Primer Express software version 3.0 (Applied Biosystems): D1 forward primer 5′-GGGATTTCTC CTTTCGCATTC-3′; D1 reverse primer 5′-CCAGGAGAGTGGACAGGA TGA-3′; D2 forward primer 5′-AAGCGCCGAGTCACTGTCA-3′; D2 reverse primer 5′-GTGGGCAGGAGATGGTGAAG-3′; GhsR forward primer 5′-GCTGGAGCCTAACGTCACTAGAG-3′; GhsR reverse primer 5′-CGTC CGTCAGAGAGTCATTGC-3′; LepR forward primer 5′-GGGCAGAGCAAG CACATACTG-3′; LepR reverse primer 5′-CAAGGGAAGCACCAATGGAA-3′. The primers pairs of the housekeeping β-actin gene, utilized as a reference endogenous control, were: forward 5′-TATCGGCAATGAGC GGTTCC-3′; reverse 5′-AGCACTGTGTTGGCATAGAGG-3′. The amount of target cDNA was calculated by comparative threshold (Ct) method and expressed applying $2^{-\Delta\Delta Ct}$ method [28] using β-actin gene as a reference endogenous control. All experiments were carried out in triplicates.

2.4. Statistical analysis

Behavioral performances of hamsters treated with CST, BIC and CST + BIC were evaluated using a Etholog 2.2 program [37] and the different behaviors (value ± s.e.m.) were compared to ctrs using ANOVA followed by a *post hoc* multiple range Newman-Keul's test when p-value <0.05. *p < 0.05, **p < 0.01, ***p < 0.001. At the same time the effects of CST plus CST + BIC were compared to effects of BIC $^{a}p < 0.05$, $^{c}p < 0.001$. mRNA levels (arbitrary units ± s.e.m.) of hamsters treated with CST, BIC and CST + BIC were also determined by ANOVA followed by a *post hoc* multiple range Newman-Keul's test when p-value <0.05. $^{a,*}p < 0.05$, **p < 0.01, $^{c,***}p < 0.001$, by using statistical software GraphPad Prism Software, version 5.0.