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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.

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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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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.

3. Results

3.1. Behavioral analyses

Icv infusions with CST, BIC or CST + BIC supplied differentiated feeding performances of our hibernating rodent model. Hamsters infused with the small neuroactive sympathoinhibitory peptide (CST) exhibited a very evident reduction ($F_{3,12} = 6.01$; $p < 0.001$) of drinking frequencies (−83%), plus a moderate ($p < 0.05$) decrease of eating frequencies with respect to ctrs (−57%; Fig. 1a). This phenomenon appeared to be related to a notable reduction of time performing such a behavior ($F_{3,12} = 5.78$ $p < 0.01$) as pointed out by extremely fewer ($p < 0.001$) eating (−83%) plus drinking (−80%) bouts with respect to ctrs (Fig. 1b). On the other hand, despite the $GABA_AR$ antagonist BIC drove hamsters ($F_{3,12} = 5.93$; $p < 0.01$) to eat (+62%) and drink (+71%) in a greater manner than that of ctrs, when it was compared with either CST alone or the combined treatment (CST + BIC), it seemed that these latter treatments caused hamsters to extremely reduce drinking (−115% and −93%, respectively) and eating (−91% and −54%, respectively) frequencies, despite being of a lesser entity for the combined treatment of this latter behavior (Fig. 1a). However, it was evident that BIC was responsible for a moderate amount of time spent consuming food (+34%) whereas such a treatment did not modify drinking time with respect to ctrs (Fig. 1b). The effects of the other two drugs treatments accounted for a notably reduced amount of time (~−81%) spent consuming food and water with respect to ctrs. Even in this case when the greater effects of BIC (versus ctrs) were compared to CST given alone or concomitantly, it appeared that such treatments inverted the actions evoked by BIC as pointed out by the substantially reduced time spent eating (−107% and −120%, respectively) and drinking (−95% and −110%, respectively).

Regarding body weight variations it seemed that although BIC treated hamsters featured an increased body weight, at the end of the behavioral observations (day 7), CST and above all the co-treatment led to a reduction of this same effect (Fig. 2a). Specifically, a very significant reduction ($F_{3,12} = 5.98$; $p < 0.001$) of body weight (−400%) was obtained not only for CST + BIC-treated hamsters with respect to ctrs (Fig. 2b) but also for CST alone, despite being numerically lower (−250%). As far as treatment with BIC alone was concerned, hamsters displayed a very elevated increase (+150%) of body weight with respect to ctrs.

3.2. D1, D2, GhsR and LepR expression variations

The first molecular indications evoked by icv infusions of CST, BIC and CST + BIC in our rodent model pointed to differentiated expression capacities of D1 and D2 along with the major feeding-related neuroreceptors (GhsR plus LepR) with respect to ctrs. This was the case of the notably high transcript levels ($F_{3,12} = 5.99$; $p < 0.001$) of the DAergic receptor subtype D1 (+118%) being largely detected in in hypothalamus, amygdala, hippocampus and parietal cortex layers of BIC treated hamsters with respect to ctrs, while only relatively elevated transcript levels were detected in these same brain areas of both CST plus CST + BIC treated animals (~+77%; Fig. 3a). Conversely, a moderate reduction was, instead, reported for both CST and CST + BIC treatments ($F_{3,12} = 5.91$; $p < 0.01$) when they were compared to BIC

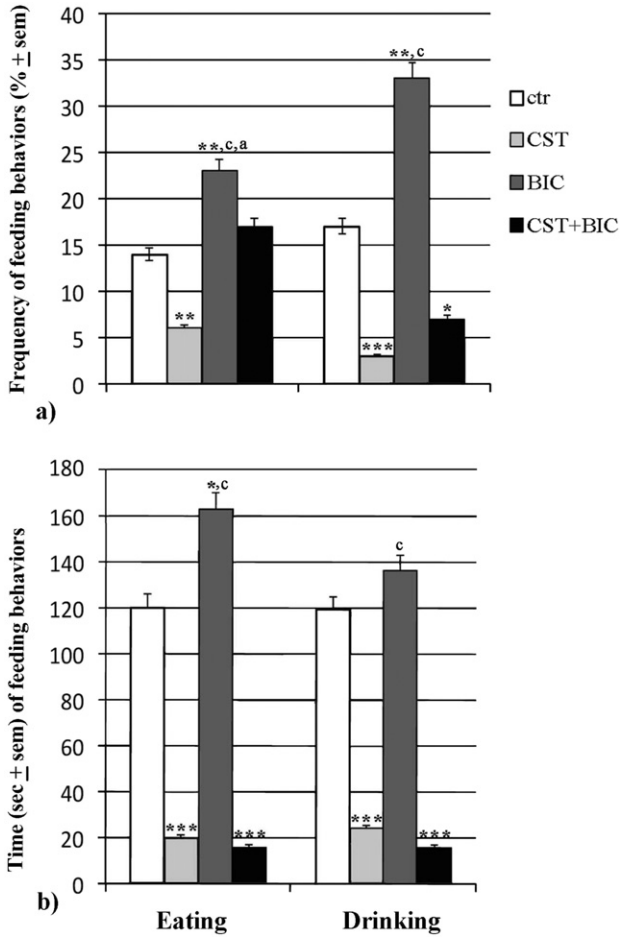

Fig. 1. Hamsters behaviors: a) frequency of eating and drinking (% ± s.e.m.); b) time (sec ± s.e.m.) spent in eating and drinking bouts induced by icv infusion (5/each treatment) with, CST (50 μM), BIC (1.96 mM) or CST + BIC versus ctrs (treatment with NaCl). The behavioral changes were determined by ANOVA plus a *post hoc* Newman–Keul's test with respect to ctrs when $p < 0.05$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Moreover significant variations of CST and CST + BIC treatments with respect to BIC treated hamsters were indicated, where necessary, with letters. [a]$p < 0.05$, [c]$p < 0.001$.

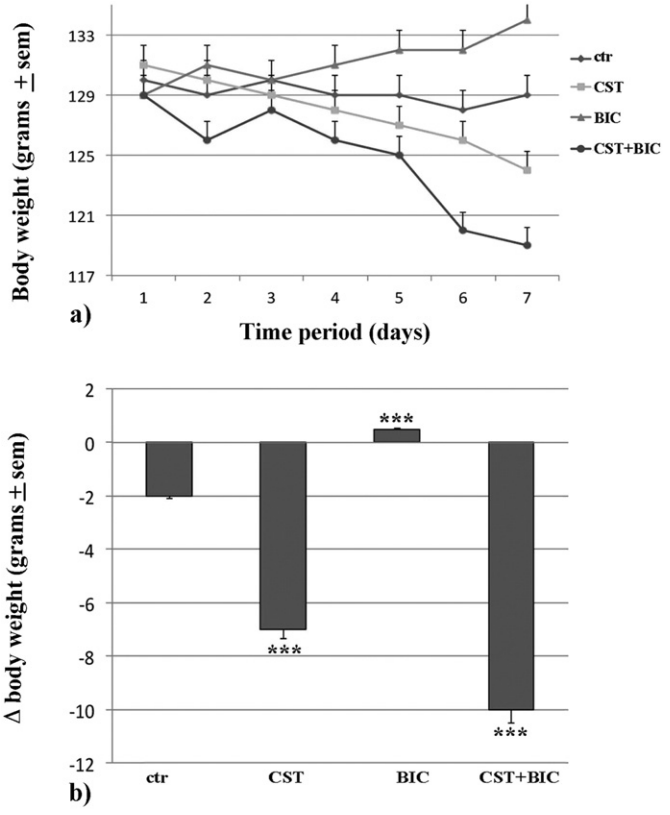

Fig. 2. Hamsters body weight variations (grams ± s.e.m.) evoked by icv infusion (5/each treatment) with NaCl, CST (50 μM), BIC (1.96 mM) or CST + BIC during the 7 days of observations (a). b) Each bar represents Δbody weight (grams ± s.e.m.). Body weight changes were determined by ANOVA plus a *post hoc* Newman–Keul's test when $p < 0.05$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

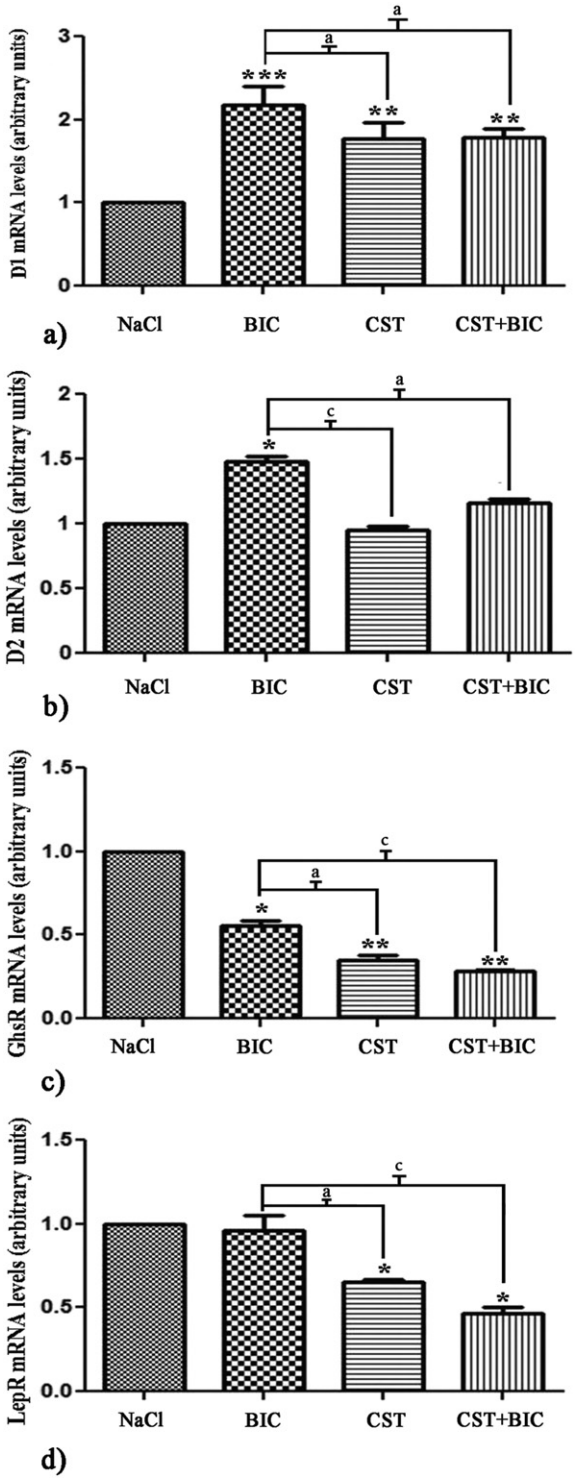

Fig. 3. D1 (a), D2 (b), GhsR (c) and LepR (d) mRNA expression levels (represented as arbitrary units ± s.e.m.) in hamsters treated with CST (50 μM), BIC (1.96 mM) and CST + BIC were compared to ctr (NaCl) or BIC using ANOVA plus a *post hoc* Newman–Keul's test when $p < 0.05$ as indicated for statistical description of Fig. 1.

treated hamsters (−40%). As far as the other dopamine receptor subtype (D2) was concerned, only a moderate up-regulation was reported for BIC with respect to ctrs (+48%; Fig. 3b) while the other two treatments did not cause significant variations. For this same receptor subtype it seemed that a notably down-regulatory trend was detected for CST (−86%) while only a moderate reduction was reported for CST + BIC (−53%).

It was worthy to note that a rather evident down-regulatory trend ($F_{3,12} = 5.94$; $p < 0.01$) of GhsR expression characterized all treatments, as displayed by negative percentage values registered in all whole limbic areas of CST + BIC (−80%), CST (−70%) and BIC (−44%) treated hamsters with respect to ctrs (Fig. 3c). This similar down-regulatory trend was also detected for hypothalamus, amygdala, hippocampus and parietal cortex layers of CST + BIC (−74%) and CST (−40%) with respect to BIC treatment. Contextually, even the notably reduced LepR transcript levels ($F_{3,12} = 5.92$; $p < 0.01$), continued to be reduced despite being of a moderate nature, this time for CST + BIC (−53%) and CST (−34%) treatments with respect to ctrs (Fig. 3d). Interestingly, a similar reducing trend still continued to characterize the effects of both treatments when compared to BIC treated hamsters as pointed out by substantially (−81%) and moderately (−38%) decreased expression levels, respectively, of LepR mRNAs in hypothalamus, amygdala, hippocampus and parietal cortex layers.

4. Discussion

The principal finding of this study regarded eating and drinking variations being caused by CST and $GABA_A$ergic system evoking, via cross-talking mechanisms, altered expression capacities of the main feeding-related neuroreceptors (D1, D2, GhsR and LepR). This diminished eating effect is particularly evident for the anti-obesity role of CST, in which other studies reported its capacity of reducing lipid deposition plus increasing lipolysis and fatty acids oxidation thereby leading mice to fewer eating and drinking bouts with consequent decreased body weight [6]. A feature that is in good agreement with diminished body weight detected during euthermic conditions of our rodent model very likely due, in this case, to the concomitant down regulation of ORX2R in the main hypothalamic feeding center (lateral hypothalamic area) and to its up-regulated levels in the anorexigenic sites (ventromedial and supraoptic nuclei) of the same brain region [32]. In addition the feeding effects evoked by CST tend to go in the same direction of the very evident up-regulation of D1 neuroreceptors of the brain areas considered in the present study. Such a relationship appears to be strongly supported by the high expression levels of D1 accounting for elevated intracellular levels of cAMP in the central amygdalar nucleus, which by favoring an anorexigenic effect via modulation of protein kinase A [7, 39] tend to reduce frequency and duration of feeding intervals in hamsters [32].

The results of the present study tend to also demonstrate that the above behaviors for CST-treated hamsters appear to be also related with down-regulatory trends of GhsR and LepR mRNA levels. In the case of the former receptor subtype this should not be so surprising since previous studies provided inhibitory feeding behaviors occurring mainly via diminished GhsR levels [13]. On the other hand, while the down-regulation of the latter factor seems to represent a contrasting feature in view of its anorexigenic role [14], it may very well be that hamsters treated with CST favor reductions of circulating catecholamines thus leading to reduced LepR transcriptional abilities as a consequence of elevated leptin levels [6].

Similarly, even the greater blocking GABAergic effects, over ctrs, tend to follow the above trend thereby supporting enhancing CST-dependent actions especially in the case of increased body weight occurring contextually to the up-regulated D2 subtype mRNA levels. This aspect seems to be strongly supported not only by high D2 levels accounting for greater food intake and thus increased body weight as indicated by pro-obesity plus metabolic disorders of the Korean National dietary guidelines [40], but also from stimulated feeding behavior in free-feeding rats by icv BIC treatment of the notable satiety center-ventromedial hypothalamic nucleus containing dense GABAergic inputs [10]. Indeed, from a molecular point of view, D2-like receptors supply a 10- to 100-fold greater affinity for DA than D1-like family [7] that turn out to be a determining factor for the consumption of high food quantities, in a similar manner to that evoked by ghrelin-treated animals [44].

In this context, the greater feeding events characterizing hamsters treated with both ghrelin plus leptin along with elevated D2 mRNAs tend to further strengthen the major role played by this DAergic receptor subtype, as pointed out by its reduced levels being responsible for the consumption of less food [9, 36]. In addition the lack of appetite, in these animals may be due to elevated concentrations of D1 subtype, which recently has shown to be tightly correlated to notable reductions of feeding behaviors in rats and consequently a very evident loss of body weight [7, 39].

It was particularly interesting to observe that still greater CST effects prevailed when hamsters were also treated with BIC as displayed by very great reduced feeding and drinking behaviors plus body weight. Indeed, a reduction of these appetitive behaviors, aside being associated with an up-regulation of D1 mRNA levels seem to also rely on the down-regulatory trend of GhsR transcripts. As far as the altered GABAergic-dependent feeding and drinking behaviors occurring in presence of CST are concerned, they seem to behave in a similar manner to the direct cardio-sympathetic effects of hypothalamic neurons [20]. In this particular case, treatment of such a diencephalic brain station with BIC and the sympathoinhibitory peptide accounted for a synergic reduction of arterial blood pressure [20, 27], which is in line also with the recent potent synergic effects of CST and PACAP on barosensitivity and chemosensitivity events [19]. At the same time, GABAergic effects on CST treated animals may be achieved by other neuronal systems such as cholinergic fibers [31], as suggested by their activation within mesolimbic sites leading to modified feeding and drinking behaviors [3]. In this same context even reduced orexigenic GhsR expression levels may be exerting similar effects together with hypothalamic neuropeptide Y neurons thereby accounting for reduced feeding stimuli [23] as observed in our rodent model.

5. Conclusions

The data of this study provided us with interesting indications regarding the blocking effects.

of CST and $GABA_A$ergic systems on feeding and drinking behaviors. In particular the anti-obesity role of CST detected not only when given alone but also co-infused with BIC may have accomplished such behavioral events via activation of DAergic neuronal fibers. Additionally, the simultaneous up-regulation of D1 and reduced orexigenic GhsR mRNA levels being related to reduced feeding behaviors very probably via the inhibition of the protein kinase A as previously reported in transgenic mice, in which the selective inhibition of this protein promoted a hypophagic condition [45]. Conversely, the surprisingly reduced mRNA levels of the anorexigenic LepR may be evoked, at least in part, by the increased production of adipocyte-related leptin, which by exerting a prevailing CSTergic brake on α-adrenergic signals account for the desensitization of LepRs. At the same time, the blocking effects of inhibitory $GABA_A$ergic currents resulted to be strongly linked with the up-regulation of D2 that in turn constitutes a major factor favoring food intake [9, 36]. In this case the predominating actions of CST over $GABA_A$Rs in the combined treatment may very well be due to the inhibitory activity of this sympathoinhibitory peptide on the $GABA_A$Rergic site that by promoting an anorexigenic effect leads hamsters to eat less [5], which is in line with hypothalamic melanocortin promoting apolipoprotein E-dependent sympathoinhibitory effects suppressing food intake in mice and rats [5, 16, 42]. We are still at the beginning but the present indications proposing CST and $GABA_A$Rs activities, as major novel neuronal signals operating during feeding bouts may constitute therapeutic alternatives for treating feeding disorders.

Author contributions

Designed all experimental steps: Canonaco and Mele.
Conducted experiments: Iachetta and Mele.
Wrote the entire manuscript and handled all analytical calculations: Canonaco, Iachetta, and Mele.
Contributed to the writing of the manuscript: Alò, Avolio, Carelli, and Laforgia.
Performed behavioral data analysis: Alò, Fazzari, Laforgia, and Mele.

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