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Short communication

Effects of cocaine- and amphetamine-regulated transcript peptide, leptin and orexins on hypothalamic serotonin release

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Abstract

We have studied the effects of cocaine- and amphetamine-regulated transcript (CART) peptide-(55–102), leptin, orexin-A and orexin-B on basal and depolarization (K^+ 15 mM)-induced serotonin (5-hydroxytryptamine, 5-HT) release from rat hypothalamic neuronal endings (synaptosomes) in vitro. We have found that leptin and CART peptide-(55–102) have no effect on 5-HT release, while orexin-A and orexin-B inhibit depolarization-stimulated serotonin release. We can conclude that leptin and CART peptide-(55–102), which play a physiological role as feeding inhibitors, do not acutely affect 5-HT release from hypothalamic synaptosomes; on the other hand, feeding induced by orexin-A and orexin-B could be partially explained by decreased 5-HT release.

Keywords: CART (cocaine- and amphetamine-regulated transcript); Leptin; Orexin; Fenfluramine; Hypothalamus; 5-HT (5-hydroxytryptamine, serotonin)

1. Introduction

Feeding behavior is finely tuned by a complex interplay of classical neurotransmitter, neuropeptide and hormonal network, and the hypothalamus plays a pivotal role in such a cross-talk signaling (Kalra et al., 1999). Serotonin (5-hydroxytryptamine, 5-HT) has long been implicated in the neural processes that regulate food intake, and compounds that stimulate 5-HT release, inhibit its presynaptic reuptake or directly stimulate postsynaptic 5-HT receptors have anorectic effects (Leibowitz and Alexander, 1998). Among the endogenous peptides which are involved in appetite regulatory pathways in the hypothalamus, leptin, a polypeptide hormone secreted by adipocytes and transported through the blood–brain barrier (Zhang et al., 1994; Banks et al., 1996), and cocaine- and amphetamine-regulated transcript (CART) peptide, mainly expressed in the paraventricular nucleus of the hypothalamus (Kristensen et al., 1998; Lambert et al., 1998), have been shown to inhibit feeding behavior. On the contrary, orexin-A and orexin-B, produced in neurons of the lateral hypothalamic area, have been demonstrated to stimulate food intake (Sakurai et al., 1998).

In order to investigate the possible mediators of appetite regulatory pathways in the hypothalamus, we have studied the effects of CART peptide-(55–102), leptin, orexin-A and orexin-B (in the dose range 1–100 nM) on 5-HT release from rat hypothalamic neuronal endings (synaptosomes) in vitro.

2. Materials and methods

2.1. Animals

Male adult Wistar rats (200–220 g) were housed in a thermoregulated environment (23 ± 1 °C), with automatic control of light/darkness cycle. Food and water were available ad libitum.

2.2. Drugs

Rat CART peptide-(55–102), 0.1 mg, was purchased from Sigma-Aldrich, Italy. Rat orexin-A, 0.5 mg, was purchased from Alexis Biochemicals, USA. Mouse orexin-B, 0.5 mg, was purchased from American Peptide, USA.

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Recombinant human leptin, 1 mg, was a kind gift of A.F. Parlow, National Hormone and Pituitary Program, USA. Fenfluramine, 1 g, was purchased from Sigma-Aldrich. [^{3}H]serotonin (15.1 mCi/mg, 1 mCi pack size), was purchased from Amersham Pharmacia Biotech, Italy.

2.3. Hypothalamic synaptosomes

Hypothalamic synaptosomes were prepared as previously described (Brunetti et al., 1999a). Briefly, male Wistar rats (200–250 g) were sacrificed by decapitation, the hypothalami quickly dissected, homogenized in 0.32 M saccharose and centrifuged, first at $1000 \times g$ for 5 min, and then at $12{,}000 \times g$ for 20 min, to isolate neuronal endings from cell nuclei and glia. Then, the synaptosome suspension was incubated at 37 °C, under O_2/CO_2 95%/5%, pH 7.2–7.4, in Krebs–Ringer buffer (mM: NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 5, Tris–HCl 10, glucose 10, ascorbic acid 1), with 0.05 μM [^{3}H]serotonin, for 15 min, to allow synaptosomal reuptake of the labeled amine, substituting for the endogenous 5-HT pool. Then, synaptosomes were layered onto 0.8 μm Millipore filters, placed into 37 °C water-jacketed superfusion chambers (18 different chambers for each experiment), and perfused with the above buffer (0.6 ml/min), following two experimental protocols. First, after 30-min perfusion with buffer, to allow stable release (equilibration period), perfusate was collected in 1-min fractions, and after the first two fractions (basal release), the peptides to be tested were added to the perfusion buffer for 5 min (stimulus), followed by 7 min with Krebs buffer alone (return to basal). Serotonin release was calculated as the means ± S.E.M of the percentage of [^{3}H] recovered in the stimulus and return to basal fractions (a total of 12 fractions, 1 min each) compared to total loaded [^{3}H]. A second set of experiments was run to evaluate the effects of the peptides on neurotransmitter release induced by a mild depolarizing stimulus. After a 30-min equilibration perfusion with buffer alone, a 23-min perfusion with graded concentrations of the peptides was started, where in the final 3 min, K^+ concentrations in the perfusion buffer were elevated to 15 mM (after removal of equimolar concentrations of Na^+). Perfusate was collected in 1-min fractions, a time–response curve relative to the percentage of [^{3}H] recovered in each fraction compared to total loaded [^{3}H] was plotted, and serotonin release was calculated as the area under the time–response curve (AUC) corresponding to 3-min depolarization + 4 min return to basal period in Krebs–Ringer buffer (a total of seven fractions, 1 min each). Since 5-HT undergoes rapid catabolism inside synaptosomes, the perfusate was added with 100 μl protective solution (1.5% EDTA, 1% ascorbic acid, 0.001% unlabelled 5-HT), and a column chromatography was performed to separate 5-HT from its metabolites. Briefly, the perfusate was filtered through a column containing a resin (Biorex 70), which specifically binds serotonin at neutral pH in phosphate buffer, but not its metabolites. After washing at pH 7.5 with phosphate buffer and bidistilled water, serotonin was released from the resin by elution through acidic solution containing formic acid 1 N and hydrochloric acid 1 N (85:15). Finally, the amount of released [^{3}H] in perfusate fractions was detected by liquid scintillation scanning, through a beta-counter, which can give an indication of changed release of 5-HT.

2.4. Analysis of data

Data represent the group means ± S.E.M. of three to five experiments performed in triplicate. Treatment and control group means were compared by the analysis of variance (ANOVA) followed by Student–Newman–Keul's multiple comparison test (GraphPad Prism 2.00 software).

3. Results

Basal 5-HT release was not modified by either peptide tested (means ± S.E.M. of the percentage of [^{3}H]serotonin recovered in the stimulus and return to basal fractions compared to total loaded [^{3}H]. CART: control 0.89 ± 0.03; 1 nM, 0.86 ± 0.02; 10 nM, 0.94 ± 0.02; 100 nM, 0.88 ± 0.02. Leptin: control 0.91 ± 0.01; 1 nM, 0.92 ± 0.02; 10 nM, 0.93 ± 0.03; 100 nM, 0.92 ± 0.01. Orexin-A: control 1.38 ± 0.02; 1 nM, 1.38 ± 0.03; 10 nM, 1.40 ± 0.03; 100 nM, 1.41 ± 0.03. Orexin-B: control 1.05 ± 0.02; 1 nM,

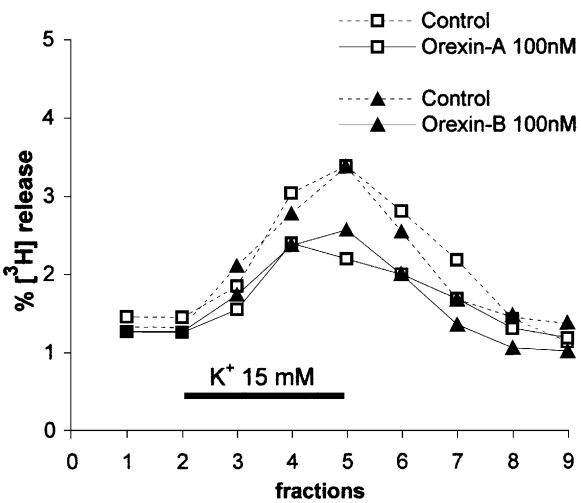

Fig. 1. Time–response curve relative to the effects of orexin-A and orexin-B (100 nM) on depolarization-induced 5-HT release. The curves represent the percentage of [^{3}H] recovered in each 1-min perfusion fraction, corresponding to 5-HT release, compared to total loaded [^{3}H]. After a 30-min equilibration perfusion with buffer alone, a 23-min perfusion with graded concentrations of the peptides was started, where in the final 3 min (fractions 2–5), K^+ concentrations in the perfusion buffer were elevated to 15 mM (after removal of equimolar concentrations of Na^+). Then, Krebs–Ringer buffer perfusion was restarted for 4 min (fractions 6–9) to record a return to basal. Controls followed the same protocol, but without peptides in perfusion buffer.

1.02 ± 0.01; 10 nM, 1.04 ± 0.04; 100 nM, 1.03 ± 0.01), while fenfluramine, a well-known 5-HT stimulating drug, was found to increase hypothalamic 5-HT release (means ± S.E.M. of the percentage of [^{3}H]5-HT recovered in the stimulus and return to basal fractions compared to total loaded [^{3}H]. Fenfluramine: control 1.04 ± 0.03; 1 nM, 1.02 ± 0.01; 10 nM, 1.13 ± 0.05; 100 nM, 1.96 ± 0.08; 0.5 μM, 2.65 ± 0.07 *; 1 μM, 3.94 ± 0.09 *; ANOVA $P < 0.0001$, * $P < 0.01$ vs. control).

We have further tested the possible role of the above peptides in potentiating or inhibiting the stimulated release of 5-HT by perfusing the synaptosomes with graded concentrations of CART peptide-(55–102), leptin, orexin-A and orexin-B, 20 min before and during a 3-min depolarizing stimulus, which was induced by raising the K^+ concentration in the Krebs–Ringer perfusion buffer to 15 mM. Both CART peptide-(55–102) and leptin did not modify depolarization-stimulated 5-HT release (means ± S.E.M. of the area under the time–response curve (AUC) of the percentage of [^{3}H] recovered in the 3-min depolarization + 6-min return to basal fractions. CART: control 6.25 ± 0.09; 1 nM, 6.18 ± 0.13; 10 nM, 6.22 ± 0.13; 100 nM, 6.24 ± 0.12. Leptin: control 5.91 ± 0.09; 1 nM, 5.80 ± 0.09; 10 nM, 5.92 ± 0.10; 100 nM, 5.86 ± 0.07), while orexin-A and orexin-B were able to inhibit K^+ (15 mM)-induced 5-HT release (Figs. 1 and 2), with IC_{80}s of 4.25 and 0.41 nM, respectively.

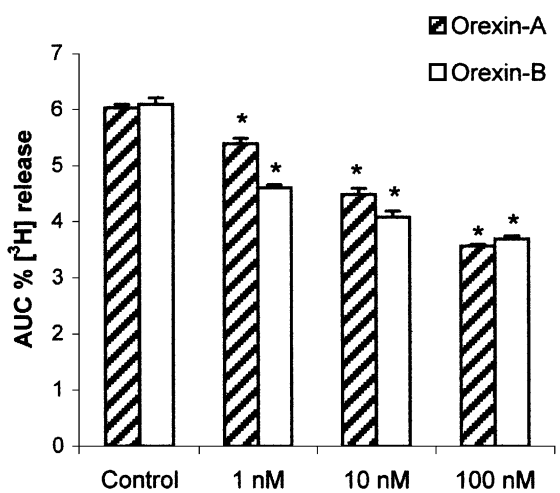

Fig. 2. Areas under the time–response curves (AUCs) relative to the effects of orexin-A and orexin-B (1–100 nM) on depolarization-induced 5-HT release. After a 30-min equilibration perfusion with buffer alone, a 23-min perfusion with graded concentrations of the peptides was started, where in the final 3 min, K^+ concentrations in the perfusion buffer were elevated to 15 mM (after removal of equimolar concentrations of Na^+). Then, Krebs–Ringer buffer perfusion was restarted for 4 min to record a return to basal. Controls followed the same protocol, but without peptides in perfusion buffer. AUCs were calculated corresponding to 3-min depolarization + 4-min return to basal periods (a total of seven fractions, 1 min each). Each column represents the mean ± S.E.M. of three to five experiments performed in triplicate; ANOVA, $P < 0.0001$; * $P < 0.001$ vs. controls.

4. Discussion

It is well established that the hypothalamus and its serotonergic neural afferents from the dorsal raphe nucleus in the brain stem play a key role in regulating feeding behavior, as evidenced by the anorectic effect of fenfluramine and dexfenfluramine, both in rodents and in humans (Davis and Faulds, 1996; Leibowitz and Alexander, 1998). In particular, direct microinjection of serotonergic agents in hypothalamic paraventricular and ventromedial nuclei inhibit food intake (Leibowitz et al., 1990). We have confirmed that fenfluramine is able to stimulate 5-HT release from hypothalamic synaptosomes, which validates this in vitro system for studying the neurochemistry of agents that modulate feeding behavior.

More recently, a number of peptides have been shown to be involved in either stimulating or inhibiting food intake in the hypothalamus. Leptin, a polypeptide hormone secreted by peripheral adipocytes (Zhang et al., 1994), is delivered to the brain through a saturable transport system localized on endothelial cells (Banks et al., 1996) and leptin receptors are found in the rat hypothalamus (Håkansson et al., 1998). Leptin is believed to negatively regulate adipose store repletion by modulating several neuropeptide and neurotransmitter pathways in the hypothalamus. It stimulates anorectic mediators, such as corticotropin releasing hormone (CRH), pro-opiomelanocortin, and prostaglandin E_2 and $F_{2\alpha}$ (Thornton et al., 1997; Brunetti et al., 1999b), and inhibits neuropeptide Y, galanin, melanin concentrating hormone, dopamine and norepinephrine, which are thought to be involved in stimulating food intake (Sahu, 1998; Brunetti et al., 1999a). Recent reports have focused on a possible role of 5-HT in mediating the anorexia induced by leptin. In mice, diencefalic 5-HT turnover is increased following peripheral or intracerebroventricular leptin treatment (Calapai et al., 1999), and 5-HT transporter binding sites in the frontal cortex of the rat are decreased after chronic intracerebroventricular infusion of leptin (Charnay et al., 2000). Our findings show that leptin does not modify basal and depolarization-stimulated 5-HT release, which seems to exclude an involvement of the serotonergic pathway in the acute hypothalamic effect of leptin.

Several translation products of CART have been isolated in the hypothalamus (Koylu et al., 1997), of which CART peptide-(55–102) plays an anorectic role, as demonstrated by intracerebroventricular administration or antibody neutralization of the endogenous peptide (Kristensen et al., 1998; Lambert et al., 1998). Possible mediators of the anorectic effects of CART peptide in the hypothalamus include the stimulated secretion of CRH and thyrotropin releasing hormone (TRH) (Stanley et al., 2001) and the inhibition of dopamine release (Brunetti et al., 2000). Similar to leptin, our results show that the hypothalamic serotonin release is not affected by an acute CART peptide-(55–102) treatment.

Orexin-A and orexin-B, and their precursor peptide preproorexin, have been identified in the lateral hypothalamus, where they are thought to play a physiological role in stimulating food intake (Sakurai et al., 1998). This effect could be mediated by increased release of neuropeptide Y (Jain et al., 2000) or inhibited release of TRH (Mitsuma et al., 1999) in the hypothalamus. It has also been reported that orexin-A excites the dorsal raphe nucleus serotonergic neurons maintained in vitro (Brown et al., 2001). Our findings, showing that both orexin-A and orexin-B inhibit the hypothalamic release of 5-HT (Figs. 1 and 2), suggest that the feeding stimulating effects of the orexins could be mediated in part by an acute reduction of the anorectic serotonergic inputs to the hypothalamus.

These preliminary data provide evidence that, though in the limited setting of an in vitro superfusion system, leptin and CART peptide-(55–102), which play a physiological role as feeding inhibitors, do not acutely affect 5-HT release from hypothalamic synaptosomes; on the other hand, feeding induced by orexin-A and orexin-B could be partially explained by decreased 5-HT release in the hypothalamus.

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