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Sulfated cholecystokinin-8 activates phospho-mTOR immunoreactive neurons of the paraventricular nucleus in rats

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Abstract

The serin/threonin-kinase, mammalian target of rapamycin (mTOR) was detected in the arcuate nucleus (ARC) and paraventricular nucleus of the hypothalamus (PVN) and suggested to play a role in the integration of satiety signals. Since cholecystokinin (CCK) plays a role in the short-term inhibition of food intake and induces c-Fos in PVN neurons, the aim was to determine whether intraperitoneally injected CCK-8S affects the neuronal activity in cells immunoreactive for phospho-mTOR in the PVN. *Ad libitum* fed male Sprague-Dawley rats received 6 or 10 µg/kg CCK-8S or 0.15 M NaCl ip (n=4/group). The number of c-Fosimmunoreactive (ir) neurons was assessed in the PVN, ARC and in the nucleus of the solitary tract (NTS). CCK-8S increased the number of c-Fos-ir neurons in the PVN (6 µg: 103 ± 13 vs. 10 µg: 165 ± 14 neurons/section; $p < 0.05$) compared to vehicle treated rats (4 ± 1, $p < 0.05$), but not in the ARC. CCK-8S also dose-dependently increased the number of c-Fos neurons in the NTS. Staining for phospho-mTOR and c-Fos in the PVN showed a dose-dependent increase of activated phospho-mTOR neurons (17 ± 3 vs. 38 ± 2 neurons/section; $p < 0.05$), while no activated phospho-mTOR neurons were observed in the vehicle group. Triple staining in the PVN showed activation of phospho-mTOR neurons co-localized with oxytocin, corresponding to 9.8 ± 3.6% and 19.5 ± 3.3% of oxytocin neurons

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respectively. Our observations indicate that peripheral CCK-8S activates phospho-mTOR neurons in the PVN and suggest that phospho-mTOR plays a role in the mediation of CCK-8S's anorexigenic effects.

Keywords

CCK; c-Fos oxytocin; phospho-mTOR; PVN

1. Introduction

The serin/threonin-kinase, mammalian target of rapamycin (mTOR) is known to be involved in intracellular signaling pathways regulating food intake. It has been suggested that mTOR plays a role in the integration of peptidergic satiety signals [6,7,36]. However, mTOR has been detected in various areas of the central nervous system (CNS), but can be found in particularly high concentrations in hypothalamic brain areas such as the arcuate nucleus (ARC) and the paraventricular nucleus of the hypothalamus (PVN) [7].

Neuropeptide Y (NPY) neurons in the ARC were shown to contain predominantly the active, phosphorylated form of mTOR (phospho-mTOR) [7,9]. Interestingly, an increased activity of mTOR suppresses food intake in rodents, when energy supply is sufficient [7]. Conversely, under fasting conditions phospho-mTOR activity in the ARC is decreased to promote food intake [7]. In addition, leptin and insulin also influence mTOR activity [7].

Cholecystokinin (CCK) is a satiety peptide derived from the gastrointestinal tract and exerting a short inhibitory effect on food intake after peripheral injection in rodents [11,14,16]. The pharmacological effects of CCK on food intake as well as its effects on the hypothalamic-pituitary-adrenal (HPA) axis are mediated *via* the CCK_A receptor located on vagal afferent fibers [8,27,30,33]. Peripheral injection of CCK induces neuronal activation in the PVN as assessed by quantifying c-Fos expression [10,11,18,34]. Neurons immunoreactive for several peptides, involved in the regulation of food intake [2,13,15,19,21] such as corticotropin-releasing factor (CRF), oxytocin, nesfatin-1, and cocaine- and amphetamine regulated transcript (CART) have been shown to be activated by CCK [18,24,34]. Especially, oxytocin is thought to mediate the short-term satiating effect induced by peripheral CCK [21].

To date, it is unknown whether PVN neurons activated by peripheral CCK contain phospho-mTOR. Therefore, the aim of the present study was to examine whether CCK-8S injected intraperitoneally (ip) at two different doses modulates neuronal activity (c-Fos) of phospho-mTOR neurons localized in the PVN. It has been reported that a proportion of activated cells in the PVN contains oxytocin (OT) [18,34], and these neurons are important to mediate the short-term effect of CCK on food intake [21]. Therefore, we also investigated whether CCK-8S injected ip activates phospho-mTOR neurons in the PVN that contain oxytocin using triple-labeling. In addition, we investigated the effect of CCK8-S on neuronal activation in two other brain nuclei, the ARC of the hypothalamus and the brainstem nucleus of the solitary tract (NTS).

2. Methods

2.1 Animals

Male Sprague-Dawley rats (Harlan-Winkelmann Co., Borcheln, Germany) weighing 250-300 g were housed (4 rats/group) under conditions of controlled illumination (12:12 h light/dark cycle), humidity, and temperature (22 ± 2 °C) for at least 21 days prior to experiments. Animals were fed *ad libitum* with a standard rat diet (Altromin®, Lage, Germany) and tap water. All rats were accustomed to the experimental conditions for a period of 14 days by handling them daily and putting them in the position to mimic the procedure of intraperitoneal (ip) injection. The handling was carried out between 9:00 and 11:00 h during the light phase. Animal care and experimental procedures followed institutional ethic guidelines and conformed to the requirements of the state authority for animal research conduct.

2.2 Peptide

In this study we used sulphated CCK-8 (CCK-8S) which is the most commonly used form of CCK in behavioral and pharmacological studies on food intake [3,10,37]. CCK-8S (Bachem AG, Heidelberg, Germany) was dissolved in water with 1% v/v 1 N NH₄OH, aliquoted and stored at -20 °C. Immediately before starting the experiments, the peptide was diluted in vehicle solution consisting of sterile 0.15 M NaCl (Braun, Melsungen, Germany) to reach the final concentration of 6 and 10 µg/kg body weight (5.2 and 8.7 nmol/kg). Peptide solutions were kept on ice for the duration of the experiments. The doses of CCK-8S were selected based on our previous studies [18,24].

2.3 Experimental design

Ad libitum fed rats received an ip injection (final volume: 0.5 ml) of 6 and 10 µg/kg CCK-8S (n=4/group) or vehicle solution (0.15 M NaCl, n=4). Ninety min after ip injection, animals were deeply anesthetized with 100 mg/kg ketamine (Ketanest®, Curamed, Karlsruhe, Germany) and 10 mg/kg xylazine (Rompun® 2%, Bayer, Leverkusen, Germany) and heparinized with 2500 IU heparin ip (Liquemin®, Hoffmann-La Roche, Grenzach-Whylen, Germany). Transcardial perfusion was performed as detailed before [10].

2.4 Immunohistochemistry

2.4.1 Single staining for c-Fos detection in the arcuate nucleus (ARC) and paraventricular nucleus (PVN) of the hypothalamus and in the nucleus of the solitary tract (NTS) of the brainstem—Free-floating 25 µm brain sections were pre-treated with 1% w/v sodium borohydride in phosphate buffered saline (PBS) for 15 min. Subsequently, sections were incubated in a solution containing 1% w/v bovine serum albumin (BSA) and 0.3% v/v Triton X-100, and 0.05% v/v phenylhydrazine in PBS for 60 min to block unspecific antibody binding. Thereafter, the diluted primary antibody solution (rabbit anti-c-Fos, Oncogene Research Products, Boston, MA, USA; 1:3,000 in a solution of 1% w/v BSA, and 0.3% v/v Triton X-100 in PBS) was applied for 24 h at room temperature. After washing in PBS, sections were incubated with the secondary antibody solution (goat biotin-SP-conjugated anti-rabbit IgG, Jackson ImmunoResearch Laboratories Inc., West

Grove, PA, USA; 1:1,000 in 1% w/v BSA in PBS) for 12 h at room temperature. After rinsing in PBS three times, sections were incubated in avidin-biotin peroxidase complex (ABC; 1:1000; Vector Laboratories, UK) in PBS for 6 h. Subsequently, sections were rinsed in PBS three times again, and then incubated in TSATM fluorescein tyramide in amplification solution (PerkinElmer, Waltham, MA, USA) for 15 min at room temperature. After washing in PBS, sections were stained with 4', 6-diamidino-2-phenylindole (DAPI) for 15 min to counterstain cell chromatin. Brain sections were finally embedded in 8 µl anti-fading solution (100 mg/ml 1,4-Diazabicyclo [2.2.2] octane, Sigma, St. Louis, USA; in 90% v/v glycerine, 10% v/v PBS, pH 7.4) and analyzed using confocal laser scanning microscopy (cLSM 510 Meta, Carl Zeiss, Germany).

2.4.2 Double staining for c-Fos and phospho-mTOR in the PVN of the hypothalamus—

Brain sections (25 µm) were incubated with a 1% w/v sodium borohydride solution for 15 min. After rinsing in PBS three times, sections were incubated in PBS blocking buffer (0.1 M PBS, 1% w/v BSA, 0.3% v/v Triton X-100, and 0.05% v/v phenylhydrazine, pH 7.5) for 1 h. The diluted primary antibody solution (rabbit anti-c-Fos; 1:3,000 in PBS blocking buffer without Triton X-100 and phenylhydrazine) was applied for 24 h at room temperature. After rinsing in PBS three times, sections were incubated with goat biotin-SP-conjugated anti-rabbit IgG (1:1,000) for 12 h at room temperature. After rinsing in PBS three times, sections were incubated in avidin-biotin peroxidase complex (1:1,000) in PBS for 6 h. Sections were rinsed in PBS three times again, and then incubated in TSATM fluorescein tyramide for 15 min at room temperature.

After rinsing in Tris-buffered saline three times, brain sections were incubated with the second primary antibody solution (rabbit anti-phospho-mTOR; Cell Signaling Technology, Inc., Danvers, MA, USA; 1:100 in PBS containing 1% w/v BSA) for 24 h at room temperature. After rinsing in PBS three times, sections were incubated in a PBS containing 1% w/v BSA for 60 min. Then, sections were incubated with goat biotin-SP-conjugated anti-rabbit IgG (1:1,000) for 12 h at room temperature, and after rinsing in PBS three times, sections were incubated in ABC (1:1000) in PBS for 6 h. Sections were rinsed in PBS three times again, and then incubated in TSATM tetramethyl rhodamine tyramide for 15 min at room temperature. After washing in PBS, sections were stained with DAPI and embedded in anti-fading solution as described above.

2.4.3 Triple staining for c-Fos, phospho-mTOR and oxytocin in the PVN—For triple staining against c-Fos, phospho-mTOR and oxytocin we used the protocol described above with slight modifications: The c-Fos protein was visualized with TSATM tetramethyl rhodamine tyramide and phospho-mTOR with TSATM fluorescein tyramide. After this step, sections were washed in PBS and incubated with the third primary antibody solution (monoclonal mouse anti-oxytocin; Chemicon International, 1:6,000 in 1% w/v BSA, and 0.1% v/v sodium azide in PBS) for 24 h at room temperature. After washing in PBS three times, brain sections were incubated overnight with the secondary goat anti-mouse IgG antibody, Alexa Fluor® 633 (Molecular Probes, Leiden, Netherlands; 1:300 in 1% w/v BSA). Then, sections were washed, counterstained with DAPI, and embedded with anti-fading solution.

2.5 Data assessment and statistical analysis

Quantitative assessment of c-Fos immunoreactivity was achieved by counting the number of c-Fos immunoreactive (ir) cells. Neurons with green nuclear staining were considered c-Fos-ir. Every fourth of all consecutive coronal 25 μ m sections was counted for c-Fos-ir staining bilaterally in the PVN (mm from bregma: -1.30 to -2.04), ARC (mm from bregma: -2.12 to -3.60 mm) and in the NTS (mm from bregma -13.24 to -14.30) according to the coordinates by Paxinos and Watson [23]. The other sections in the PVN were used for immunohistochemical triple staining. The investigator counting the number of c-Fos- and phospho-mTOR neurons was blinded to the treatments received by the rats. The average number of c-Fos-ir cells per section for each brain nucleus was calculated for four rats per treatment group.

Quantitative assessment of c-Fos-ir and phospho-mTOR staining was achieved by counting the total number of c-Fos-ir and double labeled c-Fos and phospho-mTOR neurons in the PVN. Thereafter, the percentage of c-Fos neurons immunopositive for phospho-mTOR -ir (4 rats /group) was calculated. Additionally, the percentage of oxytocin neurons which was colocalized with c-Fos and phospho-mTOR were calculated (3 rats/group). All data are presented as means $\pm$ SEM and were analyzed by ANOVA or Kruskal-Wallis One Way Analysis of Variance on Ranks. Differences between groups were evaluated by the Student-Newman-Keuls or with Fisher LSD *post hoc* test, and $p < 0.05$ considered significant.

3. Results

CCK-8S injected ip at both doses (6 and 10 μ g/kg) induced a dose-dependent 26- and 41-times increase in the number of c-Fos-ir neurons in the PVN (mean $\pm$ SEM: 103 ± 13 , $p < 0.05$, and 165 ± 14 neurons/section, $p < 0.05$) compared to vehicle-treated animals (4 ± 1 neurons/section, Figs. 1 and 4). In contrast, CCK-8S at both doses had no effect on the number of c-Fos-ir neurons in the arcuate nucleus of the hypothalamus (1 ± 1 and 3 ± 1 neurons/section; Figs. 1 and 2) compared to the vehicle rats (3 ± 1 neurons/section, $p > 0.05$; Figs. 1 and 2). In the NTS we observed that the number of c-Fos positive neurons (Figs. 1 and 3) was also dose-dependently increased after treatment with CCK-8S (51 ± 2 vs. 155 ± 11 neurons/section, $p < 0.05$) compared to vehicle treated rats (2 ± 1 neurons/section; $p < 0.05$). Staining against phospho-mTOR showed that no phospho-mTOR neurons were localized in the NTS (data not shown).

In the PVN there was no significant difference in the number of phospho-mTOR neurons between the experimental groups (Fig. 6A), although the quantity of the phosphomTOR immunoreactive neurons in the CCK-8S-treated groups was slightly increased (255 ± 30 , and 229 ± 46 neurons/section) compared to the vehicle-treated groups (197 ± 24 neurons/section) without reaching statistical significance ($p > 0.05$). Phospho-mTOR-ir neurons were localized throughout the rostrocaudal extent of the PVN. It is to note that most of the phospho-mTOR-ir neurons were observed in the magnocellular part of the PVN where most of the oxytocin immunopositive neurons are localized. In the vehicle group $48 \pm 5\%$ of the oxytocin neurons in the PVN were immunoreactive for phospho-mTOR. A moderate increase was observed in the groups treated with CCK-8S: $62 \pm 11\%$ (6 μ g CCK-8S)

respectively $64 \pm 6\%$ ($10 \mu\text{g}$ CCK-8S) of the oxytocin neurons were also immunoreactive for phospho-mTOR without reaching statistical significance ($p > 0.05$).

Double staining for c-Fos and phospho-mTOR showed a significant dose-dependent neuronal activation of the phospho-mTOR-ir cells as indicated by $17 \pm 2\%$ / section (total count: 17 ± 3) of the c-Fos positive cells being immunoreactive for phospho-mTOR after the injection of $6 \mu\text{g/kg}$ CCK-8S and $24 \pm 1\%$ / section (total count: 38 ± 2) after the injection $10 \mu\text{g/kg}$ CCK-8S (Figs. 4, 5 and 6B). No activated phospho-mTOR neurons were observed in the vehicle group (Figs. 4 and 6B). Additional triple staining for c-Fos, phospho-mTOR, and oxytocin showed that many activated neurons, particularly in the magnocellular part, contained oxytocin and phospho-mTOR in the cytoplasm (Fig. 7). Quantification indicated that $9.8 \pm 3.6\%$ of the oxytocin neurons were colocalized with c-Fos and phospho-mTOR after ip injection of $6 \mu\text{g}$ CCK-8S, and after injection of $10 \mu\text{g}$ CCK-8S, $19.5 \pm 3.3\%$ of the oxytocin neurons were colocalized with c-Fos and phospho-mTOR.

4. Discussion

In the present study, we showed for the first time that peripheral injection of CCK-8S induces a dose-dependent activation of phospho-mTOR immunoreactive neurons in the paraventricular nucleus of the hypothalamus, mostly in the magnocellular part, and a proportion of these activated phospho-mTOR neurons was also oxytocin-immunoreactive.

Many studies performed during the past decades showed that peripheral injection of the peptide hormone CCK-8S leads to neuronal activation of distinct autonomic brain nuclei [8,10,12,17,18,22,24,34]. These findings indicate that peripheral CCK-8S has multiple interactions and affects a very complex neuronal network. In this neuronal network the PVN plays an important role in the activation of the hypothalamic pituitary axis (HPA) and in the regulation of food intake.

It is known that peripheral injection of CCK-8S results in a significant increase of the quantity of c-Fos positive neurons in the PVN [10,12,18,24,34,35]. Studies investigating the neuropeptidergic phenotype of these activated PVN neurons after injection of CCK-8S showed the activation of oxytocin, CRF, nesfatin-1 and CART neurons [18,24,34]. These neuropeptides feature an inhibitory effect on food intake in rodents and intracerebroventricular (icv) injection induces an inhibition of food intake [1,2,13,15,20,31]. Furthermore, an oxytocin receptor antagonist applied icv curtailed the inhibitory effect of systemically injected CCK-8S on food intake in rodents [21]. This discovery is of particular importance, since it underlines the relevance of the oxytocinergic pathway for the action of peripheral CCK-8S.

In the present study, we observed that CCK-8S causes a dose-dependent activation of phospho-mTOR neurons in the PVN. The serin/threonin-kinase mTOR is involved in the intracellular signaling pathways influencing food intake. It was suggested that mTOR plays a major role in the integration of satiety signals [6,7,36]. Based on our data, phospho-mTOR may be involved in the mediation of the anorexigenic effects of peripheral CCK.

In particular, neurons of the ARC were reported to have reduced phospho-mTOR activity after fasting compared to *ad libitum* feeding conditions [7]. Phospho-mTOR co-localizes with 90% of neurons containing the orexigenic neuropeptide Y (NPY) and with a lower percentage of other neuropeptides in the ARC like cocaine- and amphetamine-regulated transcript (CART)/pro-opiomelanocortin (POMC) [7] or nesfatin-1 [9] suggesting the modulation of these neurons by phospho-mTOR.

Phospho-mTOR neurons are distributed throughout the CNS and with a high concentration in the ARC and in the PVN of the hypothalamus [7]. Studies by Cota *et al.* showed no significant change in the phospho-mTOR concentration in the PVN after fasting *versus ad libitum* feeding [7]. In the present study, we did not observe differences in the number of phospho-mTOR neurons in the PVN after peripheral injection of CCK-8S or vehicle solution, although a trend towards an increased number of phospho-mTOR neurons in the PVN was observed after injection of CCK-8S without reaching statistical significance compared to vehicle. An explanation for the relatively high and steady quantity of phosphomTOR neurons might be the constant integration of metabolic and other information in this hypothalamic brain nucleus which makes it difficult to assess a possible increase by means of immunohistochemistry.

We observed co-localization of oxytocin and phospho-mTOR in the magnocellular part of the PVN with a total of ~48% of the oxytocin neurons in the PVN being colocalized with phospho-mTOR. In addition, a slight increase of 14 or 16% respectively was found in the CCK-8S treated animals compared to the vehicle group, and a proportion of these neurons were activated by peripheral injection of CCK-8S. This observation suggests that phosphomTOR might play a role in the modulation of oxytocin signaling after injection of CCK-8S. Peripheral CCK-8S activates noradrenergic neurons in the nucleus of the solitary tract (NTS), as well as in other brain nuclei [4,25]. It is assumed that oxytocin neurons in the PVN are innervated by noradrenergic projections of the A2-cell group of the NTS [25]. This is supported by the observation that after injection of CCK a proportion of the catecholaminergic NTS neurons are activated [26]. In addition, it is well established by tracing studies that catecholaminergic neurons of the NTS project to neurons in the PVN [28,29]. Furthermore, an inhibition of the noradrenergic transmission in the PVN results in an inhibition of oxytocin neurons, which are activated by CCK-8S [32]. These findings underline the relevance of oxytocin neurons in the mediation of CCK-8S's effects. Therefore, it would be possible that a catecholaminergic transmission to oxytocin neurons can influence the mTOR activity in the PVN. Recently, in a different context, it has been reported that noradrenalin leads to an increase of the neuronal monocarboxylate transporter MCT2 expression, which is partially mediated through an activation of the mTOR/S6K pathway [5]. However, future studies are warranted to investigate whether CCK-8S is able to indirectly influence mTOR activity *via* catecholaminergic transmission.

In conclusion, in the present study we show that peripheral CCK-8S induces a dose-dependent neuronal activation of phospho-mTOR neurons in the PVN. Apart from that, we observed that a proportion of these activated phospho-mTOR neurons are co-localized with the neuropeptide oxytocin. The results of the present study suggest that the effects of

CCK-8S on the HPA axis and on food intake may, at least partially, be mediated by phospho-mTOR neurons in the PVN.

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Research Highlights

Sulphated cholecystokinin-8 activates c-Fos in the paraventricular nucleus in rats > these activated cells are phospho-mTOR immunoreactive neurons > a proportion of activated phospho-mTOR neurons are co-localized with oxytocin > suggest that phospho-mTOR may play a role in the mediation of CCK-8S's anorexigenic effects

Fig. 1.

Effects of intraperitoneally injected CCK-8S on the number of Fos-ir neurons in the arcuate nucleus (ARC), paraventricular nucleus (PVN) of the hypothalamus and in the nucleus of the solitary tract of the brainstem (NTS). CCK-8S (6 and 10 µg/kg) injected ip induced neuronal activation of PVN and NTS neurons. No effect on c-Fos immunoreactivity was observed in the ARC. Data are mean $\pm$ SEM of the number of rats indicated in parentheses. #: $p < 0.05$ vs. vehicle; * $p < 0.05$ vs. 6 µg CCK-8S and vs. vehicle.

Fig. 2.

In the ARC, no effects were observed on the number of activated neurons (green staining) at both doses of CCK-8S. The white outer line delineates the area of the ARC. Scale bar represents 100 µm. 3V: third brain ventricle.

Fig. 3.
CCK-8S injected ip dose-dependently increases the number of c-Fos immunopositive neurons (green staining) in the nucleus of the solitary tract (NTS). Scale bar represents 100 μm . AP: area postrema.

Fig. 4. CCK-8S injected intraperitoneally activates phospho-mTOR positive neurons of the PVN. Rats were injected with CCK-8S (6 or 10 µg/kg) or vehicle and 90 min later, brains were processed for immunostaining for c-Fos (green staining) and phospho-mTOR (red staining). Scale bar represents 100 µm. 3V: third brain ventricle.

Fig. 5. Magnification of c-Fos positive neurons co-localized with phospho-mTOR in the PVN after CCK-8S injection (the area of the magnification is indicated in Figure 4). The white scale bar represents 10 μ m.

Fig. 6.

Number of phospho-mTOR immunoreactive neurons in all treatment groups in the PVN (A) as well as number of double labeled neurons (c-Fos/phospho-mTOR) in the PVN (B).

Double staining showed that CCK-8S significantly and dose-dependently increased the number of c-Fos positive phospho-mTOR neurons in the PVN. Data are expressed as mean $\pm$ SEM of the number of rats indicated in parentheses. #: $p < 0.05$ vs. vehicle; *: $p < 0.05$ vs. 6 µg CCK-8S and vs. vehicle.

Fig. 7.

Triple staining against c-Fos, phospho-mTOR and oxytocin in the PVN after ip injection of 6 μg/kg CCK-8S. CCK-8S induces neuronal activity in oxytocin neurons which are also immunoreactive for phospho-mTOR. The insert shows a magnification of c-Fos positive phospho-mTOR neurons co-localizing with oxytocin (asterisk) in the PVN. Scale bar represents 100 μm, and in the insert 10 μm.