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CDP-Choline Activates NUCB2/Nesfatin-1 Neurons via Cholinergic Pathways

La CDP-Colina Activa las Neuronas NUCB2/Nesfatina-1 Através de Vías Colinérgicas

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SUMMARY: Nesfatin-1 is an anorexigenic peptide that, in addition to its inhibitory effect on food intake, also plays a role in cardiovascular regulation, energy homeostasis, and the regulation of stress responses. Exogenous administration of cytidine-5'-diphosphate choline (CDP-choline) produces several effects on cardiovascular and endocrine systems. A recent study has demonstrated that CDP-choline causes an increase in the peripheral levels of nesfatin-1 peptide. In this study, we aimed to investigate the activation-inducing effect of CDP-choline application on nesfatin-1 neuronsexpressed in the supraoptic nucleus (SON) of the hypothalamus and the role of the cholinergic antagonist atropine sulfate on this activation, using c-Fos immunohistochemistry as a marker of neuronal activation. The results were evaluated as the percentage of double-labeled (activated) nesfatin-1 neurons relative to all nesfatin-1 neurons. Compared to the control group, CDP-choline administration was found to activate a statistically significant number of NUCB2/nesfatin-1 neurons in the SON ($p<0.001$). It has been found that intracerebroventricular administration of atropine sulfate (ATR), a muscarinic acetylcholine receptor antagonist, prior to CDP-choline, significantly inhibits neuronal activation. These findings suggest that the effects of CDP-choline may be partially mediated through acetylcholine receptors, and that the cholinergic system plays a crucial role in regulating nesfatin-1 neurons. Furthermore, it has been proposed that CDP-choline may modulate food intake via a central mechanism at the hypothalamic level.

KEY WORDS: NUCB2/nesfatin-1; Neuronal activation; CDP-choline; SON.

INTRODUCTION

Nesfatin-1 (named as acronym for NEFA/nucleobindin2-encoded satiety- and fat-influencing protein) is an anorexigenic peptide that was first described as an endogenous protein with 82 amino acids and plays a role in the regulation food intake in rat brain nuclei such as the paraventricular nucleus (PVN), arcuate nucleus (ARC), nucleus of the solitary tract (NTS) (Oh-I *et al*., 2006). Then, subsequent studies extended this distribution to other brain nuclei in the rat and mice, such as the dorsomedial hypothalamic nucleus (DMH), ventromedial hypothalamic nucleus (VMH), lateral hypothalamic area (LH), and supraoptic nucleus (SON) (Brailoiu *et al*., 2007; Goebel-Stengel *et al*., 2011). Several reports indicate that NUCB2/Nesfatin-1 is also expressed in several organs and tissues (e.g., heart, stomach, pancreas, reproductive organs, and adipose tissue) (Goebel-Stengel & Wang, 2013). Furthermore, there is increasing evidence that nesfatin-1 has a wide range of biological effects, including the regulation of cardiovascular and reproductive functions (Ranjan *et al*., 2020), energy metabolism (Feijóo-Bandín *et al*., 2016), and the modulation of anxiety, depression, and stress (Weibert *et al*., 2019).

Transient expression of c-Fos, the protein product of the c-fos gene, is used as a marker to differentiate active neurons from inactive neurons in the central nervous system. When an acute stimulus is experimentally applied, after approximately 60-90 minutes, neuronal nuclei with c-Fos immunoreactivity are considered active neurons (Hoffman *et al*., 1993; Kovacs, 2008).

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Cytidine-5′-diphosphocholine (CDP-choline or Citicoline) is an endogenous chemical compound. CDP-choline is an endogenously synthesized mononucleotide that serves as an intermediate in the biosynthesis of membrane phosphatidylcholine (Weiss, 1995). Exogenously administered CDP-choline is hydrolyzed and dephosphorylated to produce cytidine and choline, which are subsequently used in the biosynthesis of nucleic acids, phospholipids, proteins, and acetylcholine in the brain (Lopez G-Coviella *et al.*, 1987,1995; Savci & Wurtman 1995).

CDP-choline alters membrane metabolism (Lopez G-Coviella *et al*., 1987) and increases central cholinergic transmission (Savci *et al*., 2002; 2003), resulting in various physiological and pharmacological effects on body functions. It is also used as a drug for the treatment of brain trauma and stroke as a neuroprotective molecule (Xu *et al*., 2018). It has a beneficial effect on neurodegenerative diseases such as Alzheimer's disease (Bonda *et al*., 2011). Previous reports revealed the effects of CDP-choline on the cardiovascular and endocrine systems (Savci *et al*., 2002; 2003). Furthermore, in our previous study, c-Fos expression was observed in oxytocin and vasopressin neurons following CDP-choline administration, demonstrating their activation (Eyigor *et al*., 2012). CDP-choline has also been implicated in the regulation of food intake behavior. A recent study reported that CDP-choline administration increases peripheral nesfatin-1 peptide levels (Usta *et al*., 2019).

There are no reports in the literature showing the effects of the cholinergic system on NUCB2/nesfatin-1 neurons, and it is also unknown whether CDP-choline directly affects nesfatin-1 neurons. Therefore, in this study, we aimed to demonstrate the activation of nesfatin-1 neurons following CDP-choline administration and the effect of the cholinergic antagonist atropine sulfate on this activation using c-Fos immunohistochemistry as the marker of neuronal activation.

MATERIALS AND METHODS

Animals

Adult male Wistar Albino rats (250–300 g) were used in the study. Before the experiment, the rats were kept in three groups in a temperature and light-controlled environment (12:12 h light–dark cycle with lights on at 7:00 am) with food and water freely available (ad libitum). All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Experimental Ethical Committee of Bursa Uludag University (approval date 20/11/2007, no. 2007–12).

Surgical procedures

The surgical procedures were performed under sevofluorane (3 % in 100 % O_2) anesthesia. The animals were kept on a heating pad, and the rectal temperature was monitored and maintained at 37 °C during surgery. Rats were cannulated via the left jugular vein for CDP-choline administration, and intracerebroventricular (ICV) cannulation for atropine sulfate treatment was performed as previously described. (Savci *et al*., 2003). Animals were anesthetized with sevoflurane (3–5 % in 100 % O_2) and placed in a stereotaxic apparatus. A 2-cm midline scalp incision was made to expose the skull, and the fascia and blood were carefully removed. Using the stereotaxic coordinates (1.5 mm lateral to the midline, 1.0 mm posterior to the bregma; Paxinos & Watson, 2007), a burr hole was drilled, and a stainless-steel cannula (21–22 gauge hypodermic tubing) was directed through the hole toward the lateral ventricle. The cannula was lowered 4.5 mm below the skull surface and fixed to the skull with dental acrylic cement. Following surgery, rats were allowed to recover and were treated with appropriate analgesics and antibiotics before being housed individually. No signs of pain or distress were observed during the recovery period.

Experimental groups

All experiments were conducted 24 h after the animals were cannulated. CDP-choline (1.0 g/kg; Sigma-Aldrich Co., Deisenhofen, Germany) was intravenously administered to the CDP-choline group. The control group received saline (1.0 ml/kg), whereas the antagonist group received intracerebroventricular injections of atropine sulfate (10 μg/5 μl saline) 15 min prior to the administration of intravenous CDP-choline (1.0 g/kg).

Tissue preparation

Ninety minutes after the last injections, the animals were sacrificed by perfusion fixation. After deep anesthesia, the thoracic cavity was opened, and a catheter was transcardially inserted into the aorta and clamped. First, saline (0.9 % NaCl) was perfused through the vascular system using a perfusion pump to clear the blood. Subsequently, 400 ml of 4 % paraformaldehyde solution prepared in 0.13 M Sorenson's phosphate buffer was delivered to each subject as a fixative. The collected brain tissues were post-fixed in 4 % paraformaldehyde at +4 °C overnight. After fixation, 40-μm-thick coronal brain sections were cut in five series using a vibratome. The sections were washed in 0.05 M Tris–HCl buffer to remove fixative residues and stored in cryoprotectant at -20 °C.

Immunohistochemistry

During immunohistochemistry, all the washing steps were carried out in Tris-HCl buffer throughout the procedure. All antibodies were prepared in blocking buffer, which consisted of normal horse serum (10 %), Triton X-100 (0.2 %), and sodium azide (0.1%) in Tris-HCl buffer. The sections were stained for c-Fos and NUCB2/nesfatin-1 immunoreactivity using the double immunohistochemical method. The free-floating sections were incubated with rabbit anti-c-Fos antibody (dilution 1:20000, Chemicon, Billerica, MA, USA) overnight at room temperature. Then sections were incubated with biotin-SP-conjugated donkey anti-rabbit IgG (1:300, Jackson Immunoresearch Labs, West Grove, PA) for 2 h. Nickel-intensified diaminobenzidine (DAB-consisting of 2 g nickel ammonium sulfate, 12.5 mg DAB, and 1.3 µL hydrogen peroxide) solution was used to achieve a black end product visible in the nucleus (Eyigor *et al*., 2010). After this first labeling, the sections were washed and incubated with rabbit anti-NUCB2/nesfatin-1 antibody (1:40,000, Phoenix Pharmaceuticals, H-003–22-Lot:01759, Burlingame, CA, USA) overnight at room temperature. The above protocol was used. The reaction was visualized in a DAB solution (12.5 mg DAB, 1.3 µl hydrogen peroxide in 25 ml Tris–HCl buffer). Finally, sections were washed, mounted, dried, and cover-slipped using DPX.

Cell counting and statistical analysis

Following double immunostaining, sections were analyzed using a BX-50 light microscope (Olympus Corp., Tokyo, Japan), and images were captured using a digital camera (Olympus DP71 CCD color camera, 1.5 million pixels) and transferred to a computer. Sections in the hypothalamic area, including SON (bregma between -0.48 mm and -1.44 mm), were evaluated for the presence of immunopositive NUCB2/nesfatin-1 neurons (Paxinos & Watson, 2007). The ratio of the number of c-Fos-positive NUCB2/nesfatin-1 neurons to the number of all neurons with NUCB2/nesfatin-1 immunoreaction was calculated for each animal. The percentage of activated (c-Fos-positive) neurons in these neurons was calculated, and the percentages were reported as the mean ± standard error for each animal. The differences between groups were first assessed using the Kruskal–Wallis test, and post hoc comparisons were performed with the Mann–Whitney U test to identify significant group differences. Statistical significance was considered at $p < 0.05$, and data analysis was performed using IBM SPSS Statistics version 23.0 (Armonk, NY).

RESULTS

We did not observe any adverse reaction or mortality caused by the atropine or CDP-choline injections during the experiments. Microscopic analysis showed that NUCB2/nesfatin-1-expressing neurons were localized in the supraoptic nucleus of the hypothalamus. As a result of dual immunohistochemistry, Double-labeled neurons with cytoplasmic (brown) and nuclear (blue–black) staining were considered as activated neurons (Fig. 1). The percentage of c-Fos-positive NUCB2/nesfatin-1 neurons in the supraoptic nucleus was determined to be 6.66 ± 1.82% in the saline group, 82.56 ± 3.11% in the CDP-choline group, and 19.22 ± 5.10% in the ATR Sulfate + CDP Choline group (Fig.1, Fig. 2 A-C). Compared to the control group (Fig. 1 A1-A2), the data showed that CDP-choline administration significantly increased the activation of NUCB2/nesfatin-1 neurons in the SON (Fig. 1 B1-B2, $p<0.001$). Atropine sulfate (ATR) administration prior to CDP-choline caused a significant decrease in the number of activated neurons (Fig. 1 C1-C2, $p<0.001$). Additionally, it was statistically demonstrated that there was no significant difference in the percentage of activated neurons between the control and ATR Sulfate + CDP Choline groups ($p = 0.057$). Data are summarized in Table I.

DISCUSSION

This study provides new evidence that systemic administration of cytidine-5¢-diphosphate choline (CDP-choline) induces significant activation in NUCB2/nesfatin-1 expressing neurons in the supraoptic nucleus (SON) of the hypothalamus. This activation, characterized by a significant increase in c-Fos immunoreactivity, was markedly attenuated by the intracerebroventricular administration of the muscarinic acetylcholine receptor antagonist atropine sulfate. These findings suggest that the excitatory effects of CDP-choline on NUCB2/nesfatin-1 neurons occur via muscarinic receptor-mediated cholinergic mechanisms.

Table I. c-Fos expression in NUCB2/Nesfatin-1 neurons in the supraoptic (SON).

Hypothalamic area	Groups	Mean number of NUCB2/nesfatin-1 neurons counted per animal	Mean number of NUCB2/nesfatin-1 neurons expressing c-Fos per animal	Mean percentage of NUCB2/nesfatin-1 neurons expressing c-Fos per animal
SON	Control	2017±49,22	114,8±27,67	6,66±1,82
	CDP-Choline	1910,20±142,91	1579,80±144,79	82,56±3,11
	ATR Sulphate+CDP Choline	1416,66±137,93	283,66±88,07	19,22±5,10

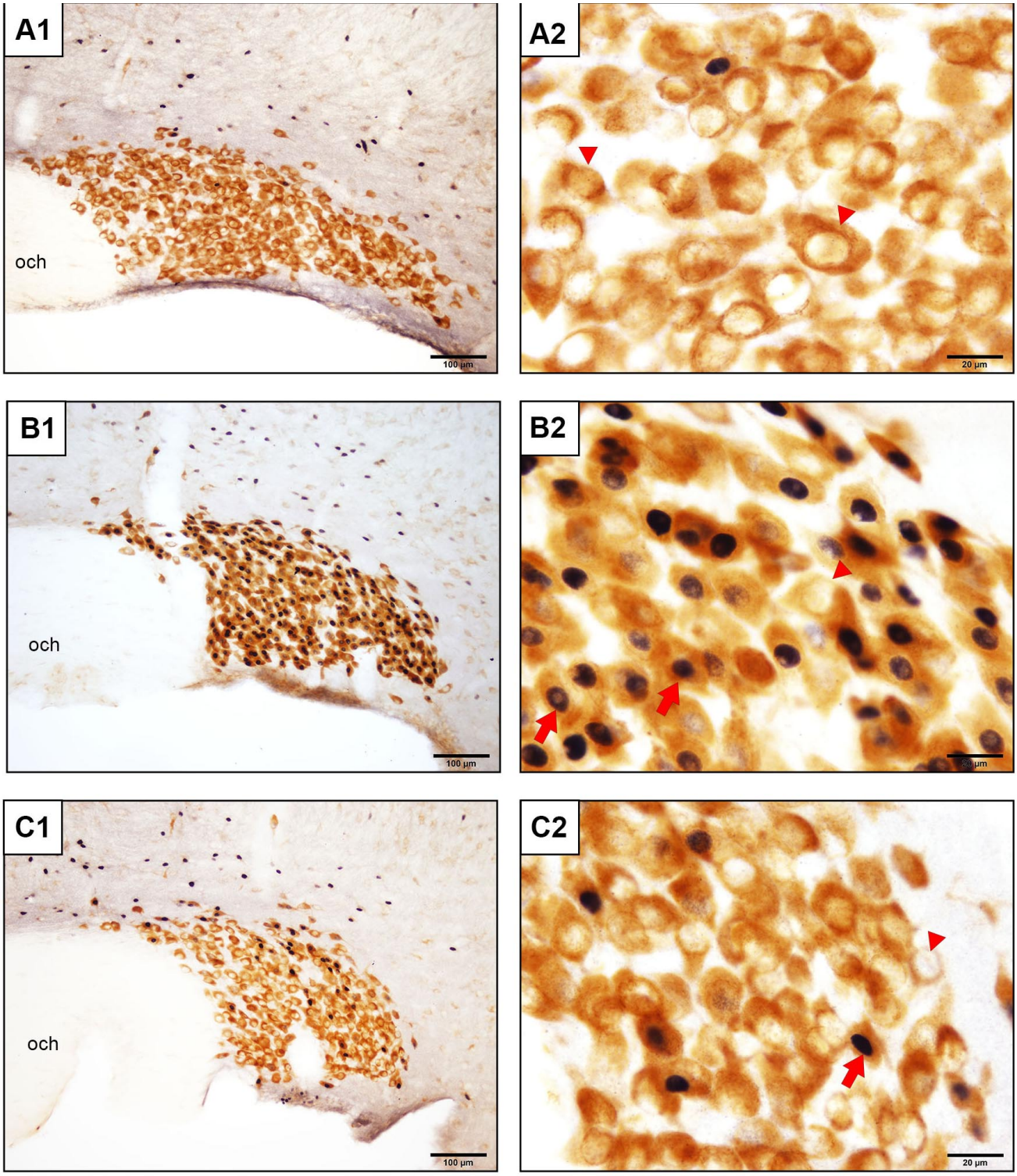


Fig. 1. Representative images demonstrate the effects of CDP-Choline and atropine sulfate on NUCB2/nesfatin-1 neurons in the SON. A1-A2 results from the saline group. B1-B2 findings CDP-choline group. C1-C2 results for the ATR Sulfate+CDP Choline group. The cytoplasm of the NUCB2/nesfatin-1-positive neurons is labeled in brown color (red arrowhead, DAB). Double-labeled NUCB2/nesfatin-1 neurons expressing c-Fos are also marked by red arrow. Scale bar: 100 µm for A1, B1, C1 micrographs; 20 µm for A2, B2, C2 micrographs. och: optic chiasm.

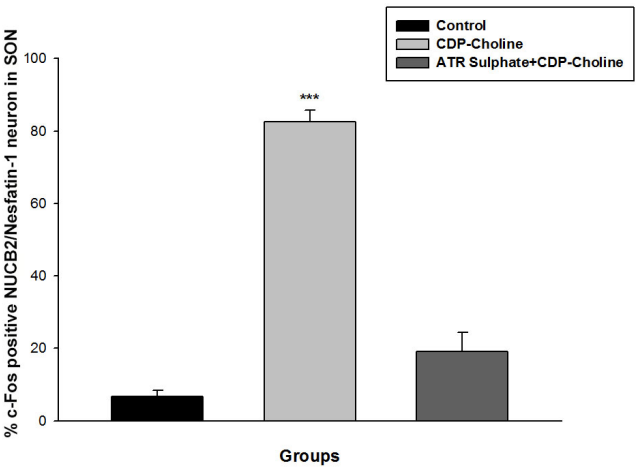


Fig. 2. Comparison of the percentages of c-Fos-positive NUCB2/nesfatin-1 within groups. Data are presented as mean ± standard error of the mean (n = 5/group, *** $p < 0.001$).

It is well established that CDP-choline (citicoline) is rapidly metabolized to cytidine and choline, both of which are absorbed into cells and utilized for phosphatidylcholine resynthesis and acetylcholine production (Lopez-G-Coviella *et al*., 1987; Weiss, 1995). It is previously reported that intravenously injected CDP-choline markedly increases choline levels in both plasma and brain regions, including the lateral cerebral ventricle and hypothalamus (Savci *et al*., 2003). The elevation in brain choline levels enhances acetylcholine synthesis and release, thereby stimulating central cholinergic transmission (Blusztajn & Wurtman, 1983). In line with these observations, it has been reported that central choline administration can increase plasma oxytocin levels, a response that can be abolished by pretreatment with atropine sulfate, a muscarinic cholinergic receptor antagonist (Savci *et al*., 1996). Moreover, we have demonstrated that intracerebroventricularly injected CDP-choline increases plasma vasopressin levels through activation of central cholinergic transmission, and that intravenous CDP-choline at a 250 mg/kg dose produces a similar elevation in plasma vasopressin (Cavun *et al*., 2004; Eyigor *et al*., 2012). In those studies, the dose- and time-dependent profile of intravenous CDP-choline on plasma vasopressin levels was clearly identified.

In mammals, the hypothalamus regulates cardiovascular function, thermoregulation, defensive and aggressive behaviors, water and food intake, circadian biological rhythms, and digestive processes. Considering the previously demonstrated effects of CDP-choline on oxytocin and vasopressin release, it is plausible to suggest that this compound may also influence food intake through NUCB2/nesfatin-1 neurons. It is therefore plausible to suggest that the cholinergic system may play a role in some of these functions. This finding aligns with previous biochemical data, which show that CDP-choline administration increases circulating nesfatin-1 peptide levels (Usta *et al*., 2019).

Following endogenous and exogenous stimulation, the c-Fos gene, an Immediate-Early gene (IEG), causes c-Fos protein expression in the cytoplasm. The c-Fos protein then moves from the cytoplasm to the nucleus, binding to specific regions in genes and triggering gene expression (Lara Aparicio *et al*., 2022). Therefore, c-Fos immunoreactivity is widely accepted as a reliable marker of neuronal activation, reflecting the functional state of neurons in response to physiological or pharmacological stimuli. In our previous study, CDP-choline administration was shown to activate neurons expressing vasopressin and oxytocin in the SON, as evidenced by increased c-Fos expression. This neuronal activation was dose-dependent, showing a proportional increase in c-Fos-positive neurons with increasing CDP-choline dose (Eyigor *et al*., 2012). It has been reported that 35 % of neurons expressing NUCB2/nesfatin-1 in the SON are also positive for oxytocin and 28 % for vasopressin (Kohno *et al*., 2008). Nesfatin-1 is a newly identified peptide with regulatory effects on food and water intake, energy expenditure, cardiovascular and gastrointestinal functions, anxiety and depression, and reproductive functions (Schalla & Stengel, 2018). This recently discovered satiety molecule, nesfatin-1, is known to be expressed in various brain areas involved in feeding and metabolic regulation, including the hypothalamic paraventricular nucleus, supraoptic nucleus, lateral hypothalamic area, and nucleus of the solitary tract (Brailoiu *et al*., 2007; Goebel-Stengel *et al*., 2011), Intracerebroventricular (i.c.v.) administration of nesfatin-1 and its precursor NEFA/nucleobindin-2 (NUCB2) has been reported to suppress food intake, while i.c.v. administration of anti-nesfatin-1 antibody increases food intake (Oh-I *et al*., 2006). Studies have shown an increase in c-Fos-expressing neurons in the SON and PVN during refeeding after fasting (Kocoglu *et al*., 2022; Kohno *et al*., 2008). The immunohistochemical study highlighted the responsiveness of c-Fos/nesfatin-1 neurons in the PVN, SON, ARC, and NTS to stress and metabolic stimuli (Weibert *et al*., 2019).

Uridine and CDP-choline are metabolically interconnected through the Kennedy pathway, where uridine-derived cytidine triphosphate (CTP) is required for the synthesis of CDP-choline, a key intermediate in phosphatidylcholine and acetylcholine biosynthesis (Kennedy & Weiss,1956). Consistent with this biochemical relationship, our current study has demonstrated that uridine and its nucleotides modulate the activation of NUCB2/nesfatin-1 neurons (Hasanoglu Akbulut *et al*., 2025). This study also demonstrates for the first time in the literature that CDP-choline increasesnesfatin neuron activation. This

study did not evaluate the effect of CDP-choline on serum nesfatin-1 levels. Future studies evaluating the effect of CDP-choline on changes in serum nesfatin-1 levels following peripheral administration of CDP-choline may reveal its importance in controlling nutrition and body weight.

Cytidine-5′-diphosphate choline (CDP-choline), administered orally, intravenously, or intracerebroventricularly, rapidly hydrolyzes into cytidine and choline, which then enter the systemic circulation and are reused for intracellular resynthesis of CDP-choline (Lopez G-Coviella *et al*., 1987,1995; Savci *et al*., 2003). Intravenous CDP-choline administration significantly increases plasma choline levels (≈20-fold) and increases choline concentrations in the lateral cerebral ventricle and hypothalamus by 3 to 7-fold (Lopez G-Coviella *et al*., 1987,1995; Savci *et al*., 2003). The resulting extracellular choline is taken up by cholinergic neurons, where it is converted to acetylcholine or acts directly on cholinergic receptors. Therefore, choline can exert presynaptic effects by increasing acetylcholine synthesis and release, or postsynaptic effects through direct receptor activation. Although the possibility of direct activation of cholinergic receptors by choline cannot be excluded, previous work demonstrated that choline can act as a direct agonist at neuronal nicotinic (Ki = 0.88–1.12 mM) and muscarinic (Ki = 2.51 mM) receptors (Ulus *et al*., 1988). Considering that extracellular brain choline levels are about 4–6 μM, the higher CDP-choline doses used in this study (1 g/kg) compared with previous ones (0.1–0.25 g/kg) (Savci *et al*., 2003) likely produced choline concentrations sufficient to activate central cholinergic receptors directly. Consistent with this, CDP-choline administration did not induce c-Fos expression in cholinergic neurons (unpublished observation). In line with this interpretation, the results of the present study showed that intracerebroventricular administration of atropine sulfate, a muscarinic acetylcholine receptor antagonist, markedly suppressed the activation of NUCB2/nesfatin-1 neurons in the supraoptic nucleus (SON) induced by systemic CDP-choline administration.

CONCLUSION

This study showed that CDP-choline effectively increased the number of activated nesfatin-1 neurons in the SON. The results also suggested that CDP-choline exerts this effect partly through acetylcholine receptors, since the pre-injection of muscarinic acetylcholine receptor antagonist atropine sulfate significantly blocked the neuronal activation. It is currently not known, if CDP-choline directly affects nesfatin-1 neurons, since no report is available in the literature demonstrating the expression of acetylcholine receptors by the nesfatin-1 neurons. It is thought that this effect might also be regulated by interneurons that can receive CDP-choline signal and, in turn, convey this signal to nesfatin-1 neurons using a different neurotransmitter system. Nevertheless, the results of this study strongly suggest that the cholinergic system plays a crucial role in regulating nesfatin-1 neurons. It is also plausible to suggest that CDP-choline can modulate food intake at the level of the hypothalamus through a central mechanism.

AKBULUT, N. H.; TOPAL, G.; COSKUN, N. C.; KAFA, I. M.; SAVCI, V. & EYIGOR, O. La CDP-colina activa las neuronas NUCB2/nesfatina-1 a través de vías colinérgicas. *Int. J. Morphol., 44(4)*:1509-1515, 2026.

RESUMEN: La nesfatina-1 es un péptido anorexigénico que, además de su efecto inhibidor sobre la ingesta de alimentos, desempeña un papel en la regulación cardiovascular, la homeostasis energética y la regulación de las respuestas al estrés. La administración exógena de citidina-5'-difosfato colina (CDP-colina) produce diversos efectos en los sistemas cardiovascular y endocrino. Un estudio reciente ha demostrado que la CDP-colina provoca un aumento en los niveles periféricos del péptido nesfatina-1. En este estudio, nos propusimos investigar el efecto de la CDP-colina en la activación de las neuronas que expresan nesfatina-1 en el núcleo supraóptico (NSO) del hipotálamo, así como el papel del sulfato de atropina —un antagonista colinérgico— en dicha activación, utilizando la inmunohistoquímica de c-Fos como marcador de activación neuronal. Los resultados se evaluaron como el porcentaje de neuronas con nesfatina-1 que presentaban doble marcaje (activadas) con respecto al total de neuronas con nesfatina-1. En comparación con el grupo control, se observó que la administración de CDP-colina activa un número estadísticamente significativo de neuronas NUCB2/nesfatina-1 en el NSO ($p < 0,001$). Se halló que la administración intracerebroventricular de sulfato de atropina (ATR) —un antagonista de los receptores muscarínicos de acetilcolina—, previa a la administración de CDP-colina, inhibe significativamente la activación neuronal. Estos hallazgos sugieren que los efectos de la CDP-colina podrían estar mediados, en parte, a través de los receptores de acetilcolina, y que el sistema colinérgico desempeña un papel crucial en la regulación de las neuronas que expresan nesfatina-1. Además, se ha propuesto que la CDP-colina podría modular la ingesta de alimentos mediante un mecanismo central a nivel hipotalámico.

PALABRAS CLAVE: NUCB2/nesfatina-1; Activación neuronal; CDP-colina; NSO.

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