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ORIGINAL RESEARCH

Assessment of the Role of the Glutamatergic System in the Activation of Melanin-Concentrating Hormone Neurons*

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ABSTRACT

The hypothalamus is the part of the central nervous system that contains many neuropeptide and neurotransmitter systems. It functions as an integrative center for peripheral and central signals that control appetite and regulate energy homeostasis. Neurons located in various nuclei of the hypothalamus regulate feeding behavior with the many different hormones and peptides they synthesize. While some of these molecules have orexigenic effects that stimulate food intake, others have anorexigenic effects that suppress food intake. One orexigenic peptide is melanin-concentrating hormone (MCH). The mammalian melanin-concentrating hormone is a nineteen-amino acid neuropeptide produced in the lateral hypothalamic area (LHA) and incerto-hypothalamic area (IHA) in the brain and exerts its orexigenic effects through MCHR1 receptors. In our study, we investigated the relationship between the glutamatergic system and MCH neurons histologically. In the experiments, double immunohistochemical labeling with anti-MCH and anti-pCREB antibodies was performed on sections from rat brains injected with glutamate agonists and antagonists. CREB phosphorylation (pCREB) was used as a marker of neuronal activation in the experiments. In male rats, an increased proportion of MCH neurons expressing pCREB was observed in the lateral hypothalamic nucleus after injection of all three agonists. Applying the specific antagonist before injections of all three agonists led to a statistically significant decrease in the number of activated MCH neurons. This study shows that intracellular pathways that require p-CREB protein expression are used to activate MCH neurons. Additionally, for the first time in the literature, the presence of phosphorylated CREB protein in MCH neurons was determined. New information has been added to the literature on glutamatergic regulation of MCH neurons.

Key Words: Melanin-concentrating hormone (MCH). pCREB. Glutamate. Hypothalamus. Immunohistochemistry. Rat.

Glutamaterjik Sistemin Melanin Yoğunlaştırıcı Hormon Nöronlarının Aktivasyonundaki Rolünün Değerlendirilmesi

ÖZET

Hipotalamus, çok sayıda nöropeptit ve nörotransmitter sistemin yer aldığı merkezi sinir sistemi bölümüdür. İştahın kontrol edilmesi ve enerji homeostazisinin düzenlenmesini sağlayan periferal ve merkezi sinyaller için integratif bir merkez olarak fonksiyon görür. Hipotalamusun çeşitli çekirdeklerinde yerleşik nöronlar, sentezledikleri çok sayıda farklı hormon ve/veya peptit ile beslenme davranışı üzerinde düzenleme yapmaktadırlar. Bu moleküllerin bir kısmı besin alımını tetikleyici oreksijenik etki gösterirken, bir bölümü de baskılayıcı anoreksijenik etkiye sahiptirler. Oreksijenik peptitlerden biri de melanin konsantre edici hormondur (MCH). Memeli MCH'ı, beyinde lateral hipotalamik alan (LHA) ile inserto-hipotalamik alanda (IHA) üretilen on dokuz amino asitli bir nöropeptiddir ve oreksijenik etkilerini MCHR1 reseptörleri üzerinden göstermektedir. Çalışmamızda glutamaterjik sistemle MCH nöronları arasındaki ilişki histolojik olarak araştırılmıştır. Deneylerde glutamat agonisti ve antagonisti verilen sıçan beyinlerinden elde edilen kesitlere, anti-MCH ve anti-pCREB antikorlarıyla ikili immünohistokimyasal işaretlemeler yapılmıştır. Çalışmalarda CREB fosforilasyonu (pCREB) nöronal aktivasyon belirteci olarak kullanılmıştır. Erkek deneklerde, lateral hipotalamik çekirdekte her üç agonistin enjeksiyonu sonrasında pCREB'i eksprese eden MCH nöronlarının oranının arttığı gözlenmiştir. Her üç agonistin enjeksiyonlarından önce özgün antagonist uygulanması, aktive olan MCH nöron sayısında istatistiksel olarak anlamlı bir azalmaya yol açmıştır. Bu çalışma ile MCH nöronlarının aktivasyonunda p-CREB protein ekspresyonunu gerektiren hücre içi yolakların kullanıldığı gösterilmiştir. Ayrıca literatürde ilk kez MCH nöronlarında fosforile CREB proteininin varlığı belirlenmiştir. MCH nöronlarının glutamaterjik regülasyonu açısından literatüre yeni bilgiler kazandırılmıştır.

Anahtar Kelimeler: Melanin konsantre edici hormon (MCH). p-CREB. Glutamat. Hipotalamus. İmmünohistokimya. Sıçan.

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Food intake is a complex physiological behavior regulated by coordinated central and peripheral signaling mechanisms, in which neuropeptidergic circuits within the central nervous system play a major role[1]. Over the past decade, numerous neuropeptides and neural pathways that modulate feeding behavior and energy expenditure have been identified. Melanin-concentrating hormone (MCH) is a potent orexigenic neuropeptide expressed predominantly in the lateral hypothalamus and zona incerta[2,3]. Its orexigenic effects are primarily mediated by the MCH receptor 1 (MCHR1), with prominent signaling in the nucleus accumbens[4].

MCH neurons located in the lateral hypothalamic area project not only to hypothalamic nuclei involved in energy homeostasis but also to brain regions implicated in sleep–wake regulation and arousal[5,6]. Experimental studies demonstrate that central administration of MCH induces hyperphagia and increases body weight[7,8]. In addition, MCH expression appears to be sensitive to metabolic state; for example, MCH mRNA levels are elevated in leptin-deficient ob/ob mice and decrease following leptin administration[9]. Together, these observations support MCH as a key component of the hypothalamic network integrating metabolic signals with motivated feeding.

Evidence also indicates that MCH neuronal activity is modulated by classical neurotransmitters[10]. Electrophysiological and pharmacological studies have shown that MCH neurons respond to both glutamatergic and GABAergic stimulation[5]. However, histomorphological evidence defining intracellular signaling correlates of glutamatergic activation in MCH neurons remains limited. Moreover, studies directly demonstrating the expression of ionotropic glutamate receptor subunits in MCH neurons are scarce, whereas prior work has documented receptor mechanisms related to inhibitory neurotransmission, including GABAergic receptors[11]. This gap limits our understanding of how excitatory synaptic inputs engage intracellular pathways that may regulate MCH neuron function.

CREB phosphorylation (p-CREB) is a widely used marker of activity-dependent intracellular signaling and can provide insight into transcriptionally relevant neuronal activation states[12]. Accordingly, the present study aimed to evaluate MCH neuron activation in response to glutamatergic agonists using p-CREB immunohistochemistry and to determine whether this response is attenuated by antagonists targeting distinct glutamate receptor subtypes. By addressing whether glutamate-dependent activation of MCH neurons engages CREB-related intracellular signaling, this work seeks to provide new insights into the glutamatergic regulation of MCH neurons.

Materials and Methods

Experimental Animals and Tissues

In this study, sections obtained from brain tissues from our previously approved project studies were used. The use of these sections in our study was found to be ethically appropriate by the Bursa Uludağ University Local Ethics Committee for Animal Experiments, with decision number 2023-05/06 dated 21.03.2023.

Brain tissues from male Sprague Dawley rats weighing 250-300 g, exposed to glutamate agonist (kainic acid, AMPA, and NMDA) and antagonist (CNQX and NMDA) injections in our previous project, were used in this project. 50 µm-thick coronal sections were obtained in 5 series from the injected subjects, which were perfusion-fixed and sacrificed using a vibratome. These sections were stored at -20°C in cryoprotective material.

General Rules Followed in Immunohistochemical Procedures

All immunohistochemical procedures were performed on sections floating in glass vials. Incubation and washing of the sections were performed with appropriate agitation using an orbital shaker. Between the main steps in the protocols (except between blocking non-specific binding and primary antibody incubation), the sections were washed with 0.05 M Tris-HCl buffer (pH: 7.6). 10% blocking serum prepared in Tris-HCl buffer was used as the blocking buffer to block non-specific binding and dilute all antibodies. The specificity of the immunohistochemical stainings performed with the antibodies used in the study (Table I) was determined by control studies. In control studies, control staining was performed simultaneously with the main immunohistochemical staining, and normal blocking serum was used instead of the primary antibody step in this staining protocol. When the control stains were evaluated, no marking was observed. This result was considered as a control of the specific labeling of the antibody.

Table I. Antibodies used in the study.

Antibody	Rabbit anti-MCH	Rabbit anti-p-CREB	Biyotin-conjugated donkey anti-rabbit IgG
Manufacturer	Abcam	Cell Signaling	Merck
Catalog No.	AB274415	9198S	AP182B
Dilution	1:60 000	1:2000	1:1000
Incubation period	Overnight	Overnight	2 h
Incubation temp. ºC	Room temp.	Room temp.	Room temp.

Experimental Groups

In the current study, the experimental groups aimed to determine the activation responses of MCH neurons to glutamatergic agonists and whether this activation is suppressed by the use of antagonists of different glutamate receptors are as follows;

Effects of kainic acid and its antagonist-CNQX:

Group I (Control group): Intraperitoneal (ip) saline injection (300 µl/subject). (n=5)

Group II (Kainic acid group): Intraperitoneal kainic acid injection (2.5 mg/kg, 300 µl/subject). (n=5)

Group III (CNQX group): CNQX (1 mg/kg, ip, 300 µl/subject), an AMPA-kainate receptor antagonist, injected 15 minutes before the kainic acid injection. (n=5)

Effects of AMPA and its antagonist CNQX:

Group I (Control group): Intraperitoneal (ip) saline injection (750 µl/subject). (n=5)

Group II (AMPA group): Intraperitoneal cainic acid injection (5 mg/kg, 750 µl/subject). (n=5)

Group III (CNQX group): AMPA-cainate receptor antagonist CNQX injection (1 mg/kg, ip, 300 µl/subject) 15 minutes before AMPA injection. (n=5)

Effects of NMDA and its antagonist MK801:

Group I (Control group): Intraperitoneal (ip) saline injection (2 ml/subject). (n=5)

Group II (NMDA group): Intraperitoneal injection of cainic acid (100 mg/kg, 2 ml/subject). (n=5)

Group III (MK801 group): Injection of MK801 (1 mg/kg, ip, 300 µl/subject), an NMDA-cainate receptor antagonist, 15 minutes before NMDA injection. (n=5)

The dosages of the chemicals used in the study were determined based on our previously published studies with the same chemicals[13-14].

Immunohistochemical Labeling

All immunohistochemical procedures followed the procedures described in our previously published studies[15-16]. Briefly, before immunohistochemical application, sections were brought from -20°C to room temperature, and the solution temperature was allowed to reach ambient temperature. Sections then removed from the cryoprotectant were washed in Tris-HCl buffer for 3x10 min and incubated with 10% normal horse serum for 2 hours to mask the background staining. In the primary antibody application, sections were incubated with primary antibodies at the dilutions and durations determined in the optimization phase (Table I). After washing with Tris-HCl buffer, sections were incubated for 2 hours with biotin-conjugated secondary antibodies that recognize the primary antibody used. Sections were then purified from secondary antibodies by washing in Tris-HCl buffer were incubated for 1 hour with avidin-biotin complex (ABC Elite Kit) solution. After washing, the substrate chromogen solution diaminobenzidine (DAB) was applied to the sections, washed again with Tris-HCl buffer, and the sections were placed on slides and dried. The dried sections were covered with covering material and made ready for examination.

Microscopic Analysis

Examination of immunoperoxidase-stained sections was performed by analyzing images captured on a computer screen with a digital camera and a 40X objective on an Olympus BX-50 photomicroscope.

The coordinates of the sections prepared as a result of the labeling performed within the scope of the studies were determined according to the rat brain atlas[17]. Counts were performed in the lateral hypothalamic area where MCH neurons are localized. Five equally spaced sections containing the lateral hypothalamic area were analyzed for each experimental animal, and all neurons that were immunopositive for MCH in the double-labeled sections were counted. The number of neurons in which pCREB immunoreactivity was determined in their nuclei (double-labeled) was also recorded. For each subject, the ratio of double-labeled neurons to all neurons showing MCH labeling (=total of both paired-labeled and MCH-only labeled neurons) was calculated.

IBM SPSS 20 (Armonk, NY) was used to analyze the obtained data. SigmaPlot 10.0 (Sigma-Aldrich, St. Louis, MO) was used to create the graphs. The normality of quantitative data was assessed using the Shapiro-Wilk test. Descriptive statistics are presented as mean ± standard deviation. Significant differences between groups were analyzed using One-Way Analysis of Variance (ANOVA). When significant differences were identified, Tukey's post hoc test was used for multiple comparisons. Statistical significance was set at $p < 0.05$.

Results

Evaluation of Immunohistochemical Labeling

In dual immunolabeling performed using immunohistochemical techniques, MCH, known to be expressed in the cytoplasm, was stained using the diaminobenzidine (DAB) chromogen, and the signals of the resulting complex (antigen-antibody-enzyme-substrate) were identified as brown under a light microscope. With the application of nickel ammonium sulfate-enriched DAB (Ni-DAB), the nuclear protein (p-CREB) was visualized by the resulting immunoreaction signal. The result of this reaction was observed in shades ranging from dark blue to black under a light microscope. Neurons with labeling in both the nucleus (dark blue-black staining) and the

cytoplasm (brown staining) were considered to be dually labeled.

Expression of pCREB in MCH Neurons Stimulated with Glutamate Agonists

When the data were analyzed using One-Way Analysis of Variance (ANOVA) and significant differences were identified between groups ($p<0.001$). Data showed that following kainic acid injection, 72.87±1.35% of MCH neurons located in the LHA expressed p-CREB, while this rate was determined to be 0.96±0.30% in control groups treated with saline. When the number of activated neurons was compared between subjects given antagonists and those given no antagonists, a decrease to 3.20±0.61% in neuronal activation was observed, and the difference was statistically significant ($p<0.001$) (Figure 2). The number of p-CREB-positive neurons, which was approximately 1.63±0.56% in control subjects, increased to 85.78±8.13% with AMPA injection, while the non-NMDA receptor antagonist CNQX reduced this number to approximately 2.82±0.91%, demonstrating a statistically significant change ($p<0.001$) (Figure 3). It was determined that the application of the specific antagonist in the NMDA injection groups resulted in a statistically significant decrease in the activation of MCH neurons in the lateral hypothalamic area. The number of p-CREB-positive MCH neurons was determined to be 1.36±0.65% in control subjects, increased to 84.77±2.55% with NMDA injection, and was reduced to 22.27±4.91% with MK801 application ($p<0.001$) (Figure 4).

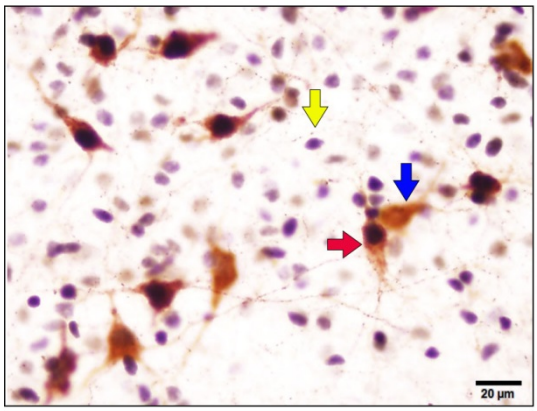


Figure 1.
pCREB expression in MCH neurons located in the lateral hypothalamic area of the experimental group. In the dual indirect immunoperoxidase labeling technique, MCH-positive neurons are distinguished by brown cytoplasmic staining (blue arrow), while p-CREB-positive neurons expressing in the nucleus are distinguished by black nucleus staining (yellow arrow). MCH neurons expressing p-CREB are indicated by a red arrow.

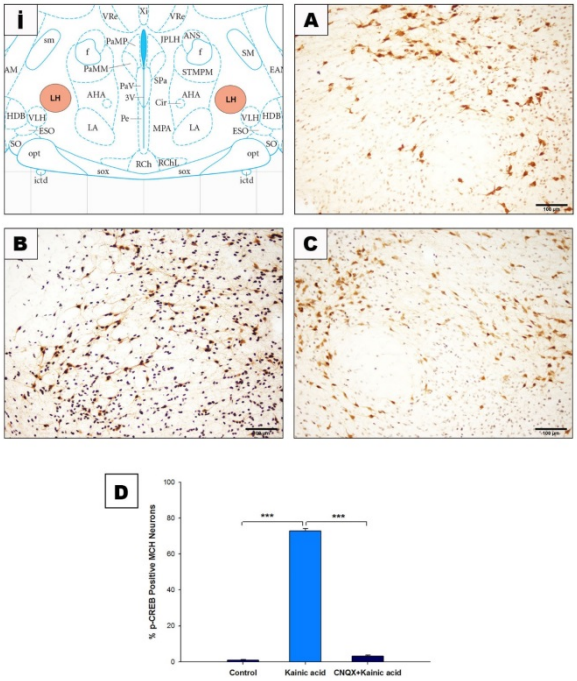


Figure 2.
*The effect of the glutamate agonist kainic acid and the antagonist CNQX on the activation of MCH neurons in the LH in male subjects. (i) Localization of the LH according to the Paxinos and Watson rat atlas [9]. (A) Control, (B) Kainic acid, and (C) CNQX administrations are shown to monitor the activation of MCH neurons localized in the LH. Activated MCH neurons are distinguished by the presence of p-CREB-positive nuclei. (D) Graphical representation of the effect of kainic acid agonists and antagonists on MCH neurons (***$p<0.001$).*

Discussion and Conclusion

In this study, we tested the hypothesis that the glutamatergic system contributes to the activation of melanin-concentrating hormone (MCH) neurons and provided histomorphological evidence supporting this hypothesis. First, robust and specific MCH immunolabeling was achieved, consistent with established descriptions of the MCH system in the rat brain, thereby ensuring reliable identification of the neuronal population of interest[2]. Using double immunohistochemistry, we quantified the colocalization of phosphorylated CREB (p-CREB)—a widely used marker of neuronal activation—with MCH-immunoreactive neurons and statistically compared the proportions of double-labeled cells across experimental groups.

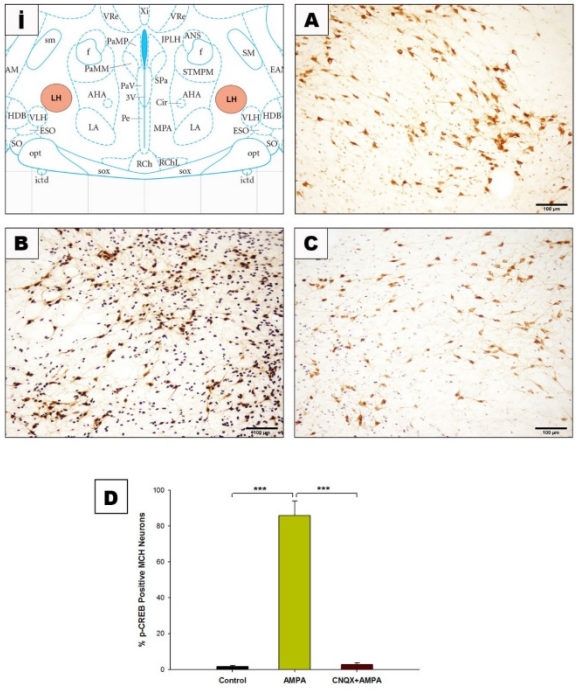


Figure 3.

*The effect of glutamate agonist AMPA and antagonist CNQX on the activation of MCH neurons in the LH in male subjects. (i) Localization of the LH according to the Paxinos and Watson rat atlas [9]. (A) Control, (B) Kainic acid, and (C) CNQX administrations are shown to monitor the activation of MCH neurons localized in the LH. Activated MCH neurons are distinguished by the presence of p-CREB-positive nuclei. (D) Graphical representation of the effect of AMPA agonists and antagonists on MCH neurons (***p<0.001).*

MCH is a key orexigenic neuropeptide, synthesized primarily in the lateral hypothalamic area (LHA)[3,8] and the incerto-hypothalamic area (IHA), and exerts its effects mainly through MCHR1 receptors[18-20]. Central administration of MCH stimulates food intake in different species, while chronic intracerebroventricular infusion promotes weight gain and obesity-like phenotypes in rodents[21-23]. Conversely, pharmacological blockade of MCH1R signaling reduces food intake, supporting a functional role for MCH signaling in energy balance regulation[24]. Consistent with these functional data, the anatomical distribution of MCH projections is broad and includes multiple hypothalamic nuclei involved in feeding control—such as the arcuate, paraventricular, supraoptic, ventromedial, dorsomedial nuclei, and other hypothalamic regions—as well as extra-hypothalamic targets including the nucleus accumbens and hippocampus[19,25]. In the nucleus accumbens, dense MCHR1 expression and the established role of this structure in reward and motivation suggest that MCH may also participate in the hedonic, non-homeostatic components of feeding behavior[26,27]. Moreover, MCH has been reported to modulate downstream neuropeptide systems by stimulating NPY and AgRP release and inhibiting CART and αMSH, indicating that it can shift hypothalamic networks toward an orexigenic state[28].

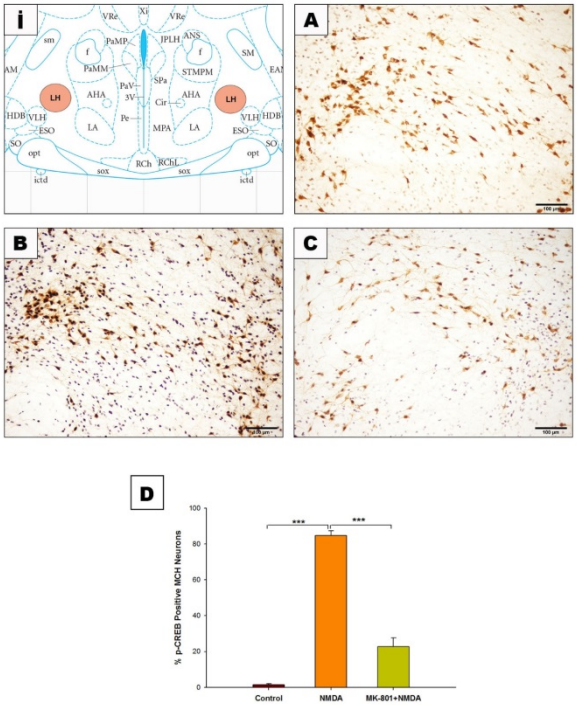


Figure 4.

*The effect of glutamate agonist NMDA and antagonist MK801 on the activation of MCH neurons in the LH in male subjects. (i) Localization of the LH according to the Paxinos and Watson rat atlas [9]. (A) Control, (B) Kainic acid, and (C) CNQX applications are observed to affect the activation of MCH neurons localized in the LH. Activated MCH neurons are distinguished by the presence of p-CREB-positive nuclei. (D) Graphical representation of the effect of NMDA agonists and antagonists on MCH neurons (***p<0.001).*

Accumulating evidence indicates that MCH neuronal activity is regulated by neurotransmitters[10]. Electrophysiological and pharmacological studies have shown that MCH neurons respond to both glutamatergic and GABAergic inputs[5]. However, to our knowledge, histomorphological data directly addressing the intracellular signaling correlates of glutamatergic stimulation in MCH neurons have been lacking. In addition, prior reports emphasize receptor expression on MCH neurons predominantly in the context of inhibitory signaling (e.g., GABAergic receptors)[11,29], whereas evidence regarding ionotropic glutamate receptor involvement at the cellular level has remained limited.

Our findings demonstrate that glutamatergic agonists acting via ionotropic non-NMDA and NMDA receptors activate a substantial fraction of MCH neurons and that this activation is associated with increased p-CREB expression, implicating CREB phosphorylation as part of the intracellular pathway mediating glutamatergic excitation in this neuronal population. Although it is not within the scope of this study to identify the possible mediators of CREB activation, one or more of the intracellular pathways, including the PI3K/Akt pathway[30], the cAMP-PKA signaling pathway[31,32], and the Ca2+/CaMK-IV pathway[33], may be responsible for leading to CREB phosphorylation. Importantly, we further show that this response can be attenuated by antagonists targeting distinct glutamate receptor subtypes, supporting the interpretation that functional ionotropic glutamate receptors contribute to excitatory drive onto MCH neurons. Given that hypothalamic orexigenic and anorexigenic circuits are shaped not only by direct peripheral signals but also by interneuron-mediated neurotransmission, the presence of functional excitatory receptor mechanisms is essential for these neurons to integrate upstream inputs. In this context, the glutamatergic system is the principal excitatory neurotransmitter network, and our results provide histological support for the notion that MCH neurons are responsive to glutamatergic transmission via receptor-specific mechanisms. In broader terms, it can be suggested that glutamate may influence food intake through MCH neurons, which may be involved in CREB-mediated activation. Additional studies using this knowledge may lead to comprehensive preclinical and clinical studies investigating the causes of obesity or eating disorders.

One limitation of this study is the use of immunohistochemistry. The results of this technique can only be shown within the scope of the antibody specificity. Although the antibodies used are highly specific for the respective proteins, some neurons may not be detected during the staining. Another limitation is the lack of comparison between the genders. Due to insufficient financial support, we were able to use only male rats. Performing the same protocol on female rats would have given a chance to make a comparison between genders, since hormonal profiles may potentially affect the outcome.

Taken together, these data extend prior functional observations by adding a cellular and intracellular signaling dimension: ionotropic glutamate receptor agonists (NMDA, AMPA, and kainic acid) increase MCH neuronal activation, and this effect is suppressible with receptor-specific antagonists. This pattern strongly suggests that MCH neurons express functional glutamatergic receptors and that CREB phosphorylation is a candidate signaling event downstream of this activation. Overall, the present work contributes new evidence regarding glutamate-dependent regulation of MCH neurons and identifies p-CREB as a relevant activation-associated intracellular marker within this pathway.

Researcher Contribution Statement:
Idea and design: Ö.E.; Data collection and processing: D.G.Y., M.B., G.T., C.O.; Analysis and interpretation of data: D.G.Y., M.B., G.T., C.O.; Writing of significant parts of the article: D.G.Y., Ö.E.
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The authors of the article have no conflict of interest declarations.
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