Original Article

The effect of melanocortin (Mc3 and Mc4) antagonists on serotonin-induced food and water intake of broiler cockerels

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The current study was designed to examine the effects of intracerebroventricular injections of SHU9119 [a nonselective melanocortin receptor (McR) antagonist] and MCL0020 (a selective McR antagonist) on the serotonin-induced eating and drinking responses of broiler cockerels deprived of food for 24 h (FD24). For Experiment 1, the chickens were intracerebroventricularly injected with 2.5, 5, and 10 μg serotonin. In Experiment 2, the chickens received 2 nmol SHU9119 before being injected with 10 μg serotonin. For Experiment 3, the chickens were given 10 μg serotonin after receiving 2 nmol MCL0020, and the level of food and water intake was determined 3 h post-injection. Results of this study showed that serotonin decreased food intake but increased water intake among the FD24 broiler cockerels and that these effects occurred in a dose-dependent manner. The inhibitory effect of serotonin on food intake was significantly attenuated by pretreatment with SHU9119 and MCL0020. However, the stimulatory effect of serotonin on water intake was not altered by this pretreatment. These results suggest that serotonin hypophagia and hyperdipsia were mediated by different mechanisms in the central nervous system, and that serotonin required downstream activation of McRs to promote hypophagia but not hyperdipsia in the FD24 chickens.

Keywords: chicken, food and water intake, MCL0020, serotonin, SHU9119

Introduction

Over the past decades, many studies have investigated brain neurochemical systems that regulate food and water intake in animals. From these, a number of major central neurotransmitter circuits that appear to contribute to these behaviors have been identified. Catecholaminergic and serotonergic systems are amongst the most extensively investigated [15]. However, information for avian species is scarce and controversial. The effect of serotonin central injection has been studied on a limited scale in avian species, but appears to depend on the lineages (breeding lines) examined. In Leghorn chickens and turkeys deprived of food for 24 h (FD24) or satiated, intracerebroventricular (ICV) injections of serotonin induce a significant decrease in food intake [2] while increasing water intake among sated chickens but decreasing water consumption among the fasted birds [4]. These data emphasize the need to evaluate differences associated with bird strains or feeding habits in studies of systems that regulate avian food intake.

Proopiomelanocortin (POMC) is a precursor for three major peptide families: the adrenocorticotropins, melanocortins and the β-endorphins. The α-melanocyte stimulating hormone (α-MSH) derived from a multifunctional POMC inhibits food intake in mammals. ICV administration of α-MSH or its potential agonist (MTII), suppresses food intake [5,19] while the ICV injection of a melanocortin antagonist, SHU9119 or HS014, stimulates feeding behavior in rats [5]. Furthermore, targeted disruption of melanocortin receptors (McRs) results in obesity, hyperphagia, and hyperinsulinemia [9]. ICV injection of α-MSH in neonatal fasting chickens leads to significantly decreased food intake [12]. These reports suggest that α-MSH is essential for regulating feeding behavior and bodyweight by reducing food intake in mammals and birds. However, the effects of α-MSH on food and water intake in avians have not been fully elucidated.

Based on findings in the literature and considering that serotonin and melanocortin have identical effects on feeding behavior in birds, we hypothesized that melanocortin

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mediates serotonin signaling in the avian hypothalamus. Therefore, the current study was designed to determine for the first time whether blocking melanocortin 3 and 4 receptors (Mc3-Rs and Mc4-Rs) affects serotonin-induced feeding and drinking responses in FD24 broiler cockerels. We also assessed whether Mc3-Rs and Mc4-Rs are necessary downstream targets for serotonin-induced food and water intake in fasted chickens.

Materials and Methods

Animals

Seventy two broiler cockerels (Eshragh, Iran) were reared in heated batteries with continuous lighting until 3 weeks of age. The chickens were provided a mash diet (21% protein and 2,869 kcal/kg of metabolizable energy; Pars animal Feed, Iran) and water *ad libitum*. At approximately 2 weeks of age, the chickens were transferred to metabolic cages that each contained one chicken. The room was maintained at a temperature of 22 ± 1°C with 50% humidity, and continuously lighted [20]. Animal handling and experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (USA) and the current laws of the Iranian government.

Drugs

Serotonin, SHU9119 (a nonselective Mc3-R and Mc4-R antagonist) and MCL0020 (a selective Mc4-R antagonist) were purchased from Tocris Bioscience (Tocris, UK). All solutions were prepared in a 0.9% pyrogen-free NaCl solution (saline) that served as the vehicle control.

Surgical preparation

At 3 weeks of age, the broilers were anesthetized with xylazin [1 mg/kg body weight, intramuscular (IM) injection] and ketamine (30 mg/kg bodyweight, IM) [29]. A 23-gauge thin-walled stainless steel guide cannula (Razipakhsh, Iran) was then stereotaxically implanted into the right lateral ventricle using a technique previously described by Denbow *et al.* [3]. The stereotaxic specifications were anterior/posterior: 6.7 mm, lateral: 0.7 mm and horizontal: 3.5~4 mm below the dura mater with the head oriented as described by Van Tienhoven and Juhász [30]. The cannula was secured with three stainless steel screws placed into the calvaria surrounding each guide cannula. Acrylic dental cement (Pars Acryl, Iran) was then applied to the screws and guide cannula. An orthodontic No. 014 wire (American Orthodontics, USA) trimmed to the exact length of the guide cannula was inserted into the guide cannula when the chickens were not being used for the experiments. Lincospectin (Razak, Iran) was applied to the incision to prevent possible infections. The chickens were allowed a minimum of 5 days to recover prior to receiving injections

of solutions.

Experimental procedures

To evaluate the possible involvement of central McRs in serotonin induction of eating responses, the effects of centrally administered SHU9119 and MCL0020 on serotonin-associated eating responses of the chickens were investigated. Injections were delivered with a 29-gauge, thin-walled stainless steel injecting cannula (Razipakhsh, Iran) that extended 1.0 mm beyond the guide cannula. This injecting cannula was connected through 60-cm polyethylene-20 tubing (Parsian tube, Iran) to a 10-μL Hamilton syringe (Hamilton, Switzerland). All drugs were injected over a period of 60 sec. The solution was then allowed to diffuse from the tip of the cannula into the ventricle for an additional 60 sec.

All experimental procedures were performed from 10:00 am to 4:00 pm. The chickens were removed from the cages, restrained by hand, and then returned to the cages after receiving the injection. The birds were handled and mock injected daily during the 5-day recovery period in order for them to adapt to the injection procedure. Twenty-four h before initiating the experiments, the animals were deprived of food but still had access to water *ad libitum*. Immediately after receiving the injections, the chickens were returned to their individual, computerized metabolic cages (Altromin, Germany) where food and water intake was automatically measured. Fresh food and water were given at the time of injection (or after the second injection for Experiments 2 and 3), and cumulative food (g) and water (mL) intake were recorded 30, 60, 90, 120, 150, and 180 min after the injection. Placement of the guide cannula into the ventricle was verified by the presence of cerebrospinal fluid and an ICV injection of 10 μL methylene blue followed by an analysis of frozen brain tissue sections performed at the end of the experiments.

Experiment 1 was designed to examine the effect of ICV injections of serotonin on the food and water intake of FD24 chickens (n = 6 per group). The chickens received 2.5, 5, and 10 μg serotonin in 10 μL of saline. The control group was injected with 10 μL of saline. For Experiment 2, each chicken received two injections. The first injection contained either 0 or 2 nmol SHU9119 in 5 μL of saline. The second injection consisted of either 0 or 10 μg serotonin in 5 μL saline delivered 15 min after the first injection as described in Table 1 (n = 6 per group). All injections for the control group contained only 5 μL of saline. Experiment 3 was conducted similar in a manner similar to Experiment 2 except that the chickens received 0 or 2 nmol MCL0020 instead of SHU9119. All animals in Experiments 1, 2, and 3 were deprived of food for 24 h prior to initiating the study. All doses of serotonin, SHU9119 and MCL0020 were calculated based on previous and pilot studies [4,24].

Table 1. Treatment procedure for Experiment 2

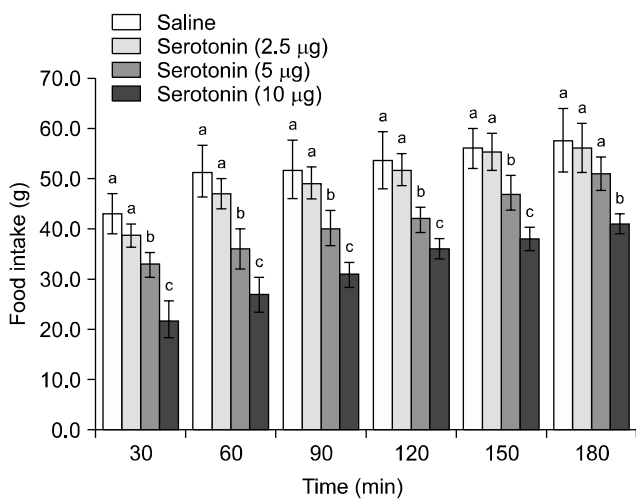| Treatment	First injection (5 μL)	Second injection (5 μL)
S + S (n = 6)	Saline	Saline
S + serotonin (n = 6)	Saline	Serotonin
SHU + S (n = 6)	SHU	Saline
SHU + serotonin(n = 6)	SHU	Serotonin

S: saline, control, SHU: SHU9119.

Fig. 1. Effect of intracerebroventricular (ICV) injection of serotonin at different doses on food intake in chickens deprived of food for 24 h (FD24). Data are presented as the mean ± SE. Lowercase letters (a, b, and c) indicate significant differences between the treatments ($p \leq 0.05$).
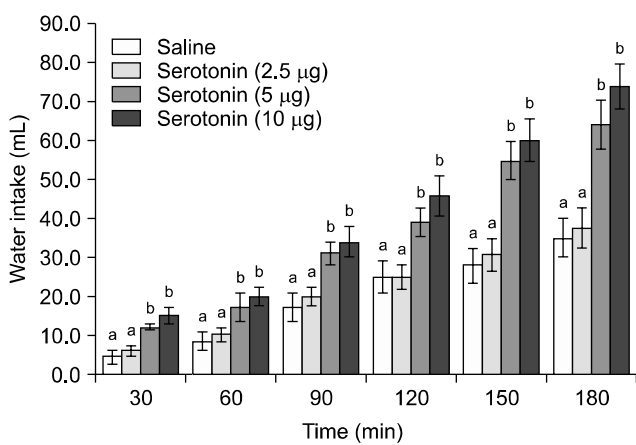

Fig. 2. Effect of ICV injection of serotonin at different doses on water intake in FD24 chickens. Data are presented as the mean ± SE. Lowercase letters (a and b) indicate significant differences between the treatments ($p \leq 0.05$).

Statistical analysis

Cumulative food intake was analyzed by a one-way analysis of variance (ANOVA) and is presented as the mean ± SE. For treatments found to have an effect according to the ANOVA, mean values were compared with post hoc Bonferroni and Dunnett tests. p-values $\leq$ 0.05 were considered to indicate significant differences between the treatments.

Results

The feeding and drinking responses of the broiler chickens to ICV injections of serotonin, SHU9119, and MCL0020 are shown in Figs. 1~6. In Experiment 1, serotonin injected into the lateral ventricle of FD24 chickens caused a dose-dependent decrease in food consumption and increase in water intake compared to the control group [Figs. 1 and 2; $p \leq 0.05$; f (3, 25) = 12.43 and f (3, 25) = 15.68, respectively]. Serotonin (5 and 10 μg doses) had significant anorexic and dipsogenic effects that lasted for at least 180 min. For the subsequent experiments, a 10-μg dose of serotonin was used because it was found to significantly decrease food consumption but increase water intakes in the FD24 birds without affecting other non-ingestive behavioral parameters.

In Experiment 2, an ICV injection of 10 μg serotonin alone decreased food consumption but increased water intake ($p \leq 0.05$) in FD24 chickens. On the other hand, 2 nmol SHU9119 alone had no effect on food or water intake (Fig. 3; $p > 0.05$). Furthermore, the effect of serotonin on food intake was significantly attenuated by pretreatment with 2 nmol SHU9119 [Fig. 3; f (3, 25) = 14.08; $p \leq 0.05$]. However, SHU9119 did not alter the
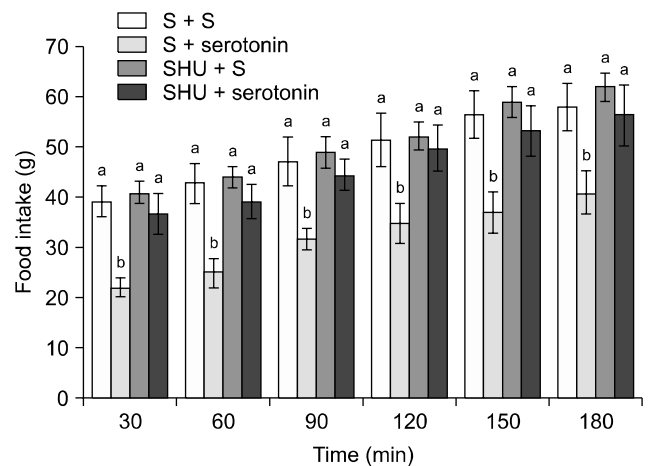

Fig. 3. Effect of ICV injection of SHU9119 (2 nmol) followed by serotonin (10 μg) on food intake in FD24 chickens. Data are presented as the mean ± SE. Lowercase letters (a and b) indicate significant differences between the treatments ($p \leq 0.05$). S: saline, SHU: SHU9119.
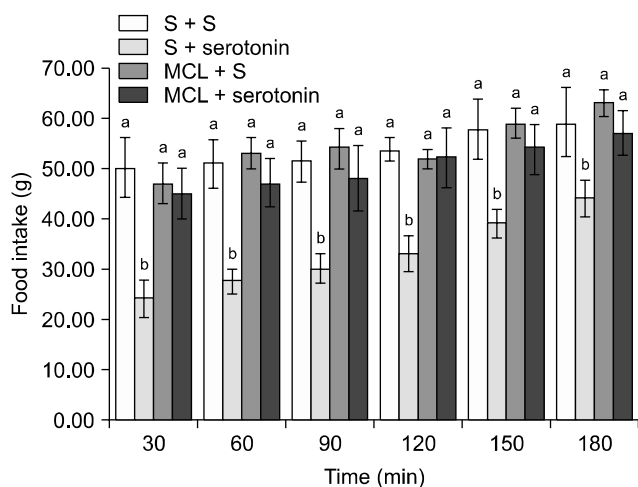
Fig. 4. Effects of ICV injection of MCL0020 (2 nmol) followed by serotonin (10 μg) on food intake in FD24 chickens. Data are presented as the mean ± SE. Lowercase letters (a and b) indicate significant differences between the treatments ($p \leq 0.05$). MCL: MCL0020.
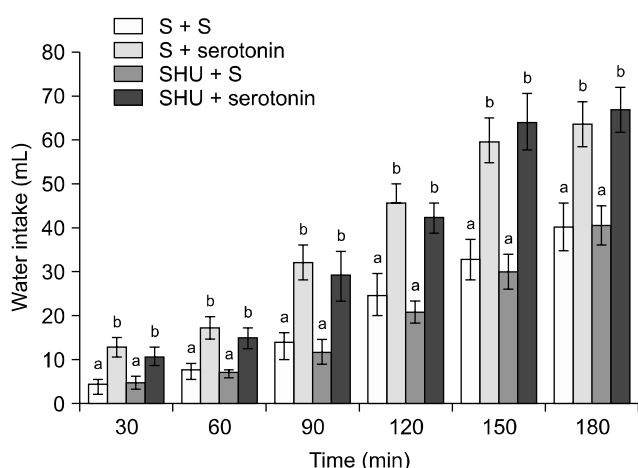

Fig. 5. Effects of ICV injection of SHU9119 (2 nmol) followed by serotonin (10 μg) on water intake in FD24 chickens. Data are presented as the mean ± SE. Lowercase letters (a and b) indicate significant differences between the treatments ($p \leq 0.05$).

dipsogenic effect of serotonin (Fig. 5; $p > 0.05$).

The results of Experiment 3 showed that the inhibitory effect of serotonin on cumulative food intake was significantly decreased by pretreatment with 2 nmol MCL0020 [Fig. 4; f (3, 25) = 18.56; $p \leq 0.05$]. Additionally, MCL0020 had a modest effect on the dipsogenic response to serotonin [Fig. 6; f (3, 25) = 13.22; $p \leq 0.05$]. The effect of MCL0020 alone on food and water intake was similar to that of SHU9119 (Fig. 6).
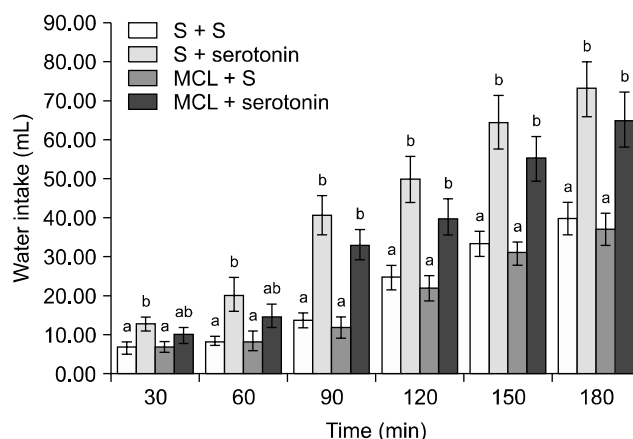

Fig. 6. Effect of ICV delivery of MCL0020 (2 nmol) followed by serotonin (10 μg) on water intake in FD24 chickens. Data are presented as the mean ± SE. Lowercase letters (a and b) indicate significant differences between the treatments ($p \leq 0.05$).

Discussion

In chickens, serotonergic systems are involved in the regulation of numerous physiological functions. Many reports have indicated that serotonin circuitry affects feeding as well as drinking behaviors in pigeons, and that these changes are possibly mediated by independent mechanisms [2,4,15,23]. In the present study, serotonin decreased food intake but increases water consumption in FD24 broiler cockerels. Data from Experiment 1 indicated that serotonin circuitry is involved in the regulation of food as well as water intake in chickens, and suggested that these serotonergic effects are mediated by independent mechanisms. Therefore, species-associated differences in the avian serotonergic mechanism response may not only be restricted to feeding behavior, but also independently affect the mechanisms involved in drinking behavior.

The melanocortin system, particularly Mc4-R, modulates feeding behavior in mammals and birds. Heisler *et al.* [8] reported that the central melanocortin system in chickens appears to be important for regulating feeding activity because POMC mRNA expression is reduced with feed restriction. Mc3-R and Mc4-R both affect energy homeostasis through different mechanisms [9]. On one hand, Mc4-R knockout mice are hyperphagic and obese. In contrast, Mc3-R knockout mice develop a metabolic dysfunction characterized by elevated fat storage and decreased energy expenditure, but food intake and body weight remain unchanged [1]. These findings suggest that Mc3-R is important for body weight gain but the underlying mechanisms remain unclear [21]. In fact, ICV injection of the high-affinity Mc3-R agonist γ-MSH fails to inhibit food intake in rats, suggesting that the Mc3-R does not mediate changes in melanocortin-induced feeding behavior [10]. In addition, Takeuchi and Takahashi reported that Mc4-R is

expressed in chicken brain but Mc3-R is not [26-28]. Concurrently, a selective Mc4-R antagonist, HS014, significantly increases food consumption in doves, indicating that Mc4-Rs mediate the orexigenic effects of endogenous agouti related peptide (AgRP).

In rats, HS014 is a potent inducer of feeding, and long-term administration of this compound results in obesity [10]. ICV injection of Mc3/4-R agonists in avian species (chickens and doves) has been shown to significantly decrease food intake [12]. Previous studies showed that the melanocortin agonist MTII suppresses the compensatory feeding responses of food-deprived doves but HS014 stimulates feeding activity when centrally administered [12,24,25]. However, Experiments 2 and 3 of the current study showed that treatment with melanocortin antagonists (SHU9119 and MCL0020) alone had no effect on feeding or drinking responses.

In 2000, Kask et al. [11] reported that α-MSH and β-MSH inhibit fasting-induced food intake by nearly 50% but neither of these two peptides inhibits fluid consumption in water-deprived (24 h) rats. Due to its possible role in controlling food intake, the central melanocortin system is an important downstream target for some hormones and signal transmitters such as insulin, leptin, ghrelin, and neuropeptide Y (NPY) that regulate feeding behavior in avian species [22]. Despite the established clinical effectiveness of serotonin-derived drugs for promoting weight loss, the mechanisms responsible for this effect in birds have remained obscure. Evidence from behavioral studies has shown that the central melanocortin system is the key site of serotonin action associated with food intake in chickens. serotonin has been shown to inhibit AgRP neuronal activity and reduce inhibitory postsynaptic currents onto POMC neurons via the serotonin$_{1B}$ receptor [7].

To determine whether melanocortin circuitry regulation is critical for serotonin-induced feeding and drinking responses, the impact of melanocortin (Mc3 and Mc4) antagonists on food and water consumption promoted by serotonin in FD24 broiler cockerels was examined. Results obtained from Experiments 2 and 3 showed that the anorexic effect of the serotonin ICV injection was attenuated by MCL0020 and SHU9119 pretreatment. Conversely, pretreatment with these two compounds had no effect on water intake in the chickens. These results showed that the effects of serotonin on food intake are modulated by the pathway(s) linked to the Mc4-Rs. Data from the current study indicated that both serotonin and Mc4-R affect the centers of that regulate feeding and energy balance.

Heisler et al. [8] previously showed that the melanocortin pathways are downstream targets for d-fenfluramine (an appetite suppressant that acts on serotonin$_{2C}$ receptor) and CP-94,253 (a high-affinity serotonin$_{2C/1B}$ receptor agonist)

to promote satiety. A mechanism was subsequently proposed in which serotonin regulates feeding behavior via the melanocortin pathways. It has been suggested that some NPY/AgRP terminals are gamma aminobutyric acid and are in contact with nearby POMC neurons, thereby affecting POMC neuronal activity [22]. Clear support for a hypothetical brainstem locus for serotonin hypophagic action has been provided by researchers such as Lee et al. [17] and Grill et al. [6]. For example, injection of CP-93,129 into the lateral parabrachial nucleus (LPBN) reduces food intake in a dose-dependent manner. LPBN is a key site for autonomic regulation and Mc4-Rs are expressed in this region [13,18]. It is possible that serotonin may establish axo-axonal contacts with POMC and AgRP terminals arising from the arcuate nucleus that forms synaptic connections with cells in sites such as the LPBN that express Mc4-Rs [8]. Specifically, if serotonin affects POMC and AgRP neuronal activity, then the release of co-expressed neuropeptides such as cocaine and amphetamine-regulated transcript (CART) and NPY should also be affected. Both CART and NPY alter food intake and the release of these neuropeptides may enhance, interfere with, or have no effect on the actions of serotonin agonists [14,16].

In summary, the present study showed that pretreatment with MCL0020 and SHU9119 blocked serotonin hypophagia but did not alter serotonin induced hyperdipsia in FD24 chickens. These findings suggest that serotonin hypophagia and hyperdipsia were mediated by different mechanisms in the central nervous system, and that serotonin required downstream activation of McRs to promote hypophagia but not hyperdipsia in the FD24 chickens.

Acknowledgments

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