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Muscarinic receptor antagonism causes a functional alteration in nucleus accumbens μ -opiate-mediated feeding behavior

Michelle L Perry^{1,2}, Brian A. Baldo, PhD², Matthew E Andrzejewski³, and Ann E. Kelley^{1,2,**}

¹Molecular and Cellular Pharmacology, University of Wisconsin-Madison, 6001 Research Park Blvd, Madison, WI 53719, Phone: 608-265-6078, Fax: 608-265-3050

²Department of Psychiatry, University of Wisconsin-Madison, 6001 Research Park Blvd, Madison, WI 53719, Phone: (608) 262-5392, Fax: 608-265-3050

³Waisman Center University of Wisconsin-Madison, 225 Waisman Center, 1500 Highland Ave, Madison, WI 53705, Phone: 608-263-3148

E-mail: Michelle L Perry [mlperry@wisc.edu] Brian A. Baldo [babaldo@wisc.edu] Matthew E Andrzejewski [andrzejewski@waisman.wisc.edu] Ann E. Kelley [aekelley@wisc.edu]

Obesity is currently a major health problem in the world today. While a great deal of research has focused on mechanisms controlling food intake and appetite, there has been little success in developing effective pharmacological treatments. Considerable progress has been made in better understanding metabolically driven feeding, particularly the communication between the periphery (i.e. leptin and ghrelin signals) and the brain (i.e. hypothalamus). However, homeostatic controls are not the only factors controlling ingestive behavior. Consideration of food cravings and the consumption of high calorie foods driven by palatability, events controlled by motivational and hedonic processes, must be taken into account.

The ventral striatum (nucleus accumbens, Acb) has received much attention as a component of higher order control of food motivation [11]. This forebrain region receives afferent projections from affect-related 'limbic' structures while sending efferent projections to the extrapyramidal motor control regions. These anatomical arrangements contributed to the hypothesis that the Acb plays a pivotal role in the translating of motivational signals into behavioral output [14]. The Acb also contains multiple neurotransmitter systems that have been shown to play critical, yet dissociable, roles in the control of appetitively motivated behaviors [11,12]. For example, stimulation of Acb-localized μ -opioid receptors induces voracious eating of highly palatable foods in satiated animals and augments operant responding for food reward [1,15,20,26,29]. In contrast, the blockade of glutamate input or enhancement of GABA within the Acb shell, a subregion of the ventral striatum, increases food consumption [23] without a concomitant increase in food-reinforced instrumental responding [28]. Also, augmentation of Acb dopamine release increases operant responding for food and food-associated stimuli, but

Corresponding Author: Michelle L. Perry, Molecular and Cellular Pharmacology, University of Wisconsin-Madison, 6001 Research Park Blvd, Madison, WI 53719, E-mail: mlperry@wisc.edu, Phone: 608-265-8710, Fax: 608-263-9340.

Ann E. Kelley, University of Wisconsin-Madison, 6001 Research Park Blvd, Madison, WI 53719, Phone: (608) 262-5392, Fax: 608-265-3050

** Unfortunately, Dr. Kelley passed away last August. While this paper was written after her death, the bulk of the work was carried out while she was still alive, under her supervision.

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does not strongly affect intake [2-5,21]. Thus, striatal connections and discrete neurotransmitter systems both play important roles in the forebrain control of feeding and food-seeking behavior.

To expand our understanding of intra-striatal neurochemical interactions in the control of feeding, our laboratory has begun examining the role of the striatal acetylcholine (ACh) system. The cholinergic interneurons in the striatum comprise only about 1-2% of the neurons in the striatum, but their large dendritic and axonal processes allow for possible influence of large striatal areas [30]. The ACh terminals synapse onto Acb neurons containing the opioid peptide enkephalin (ENK), and μ -opioid receptor mRNA has been found in ACh interneurons [8,18, 19]. Thus, ACh and ENK neurons are positioned to undergo reciprocal interactions.

Recent data from our laboratory and others has suggested that striatal ACh mediates consumption of food, including opioid-mediated fat intake. It has been shown that intra-striatal infusion of scopolamine, a general muscarinic antagonist, can decrease the amount of sucrose an animal will consume and reduce the break point in a progressive ratio schedule **while increasing locomotor activity immediately following drug treatment** [16]. It has also been demonstrated that chow consumption is decreased and that preproenkephalin (PENK) mRNA is reduced for a 24 hour period, suggesting a relatively long lasting effect of scopolamine treatment [17]. Recently we have shown that co-infusion of the μ -opioid specific agonist D-Ala2, NMe-Phe4, Glyol5-enkephalin (DAMGO) and scopolamine within the Acb decreased DAMGO stimulated fat-intake over a one-hour test session [27]. In the present study, we tested the hypothesis that scopolamine may exert a relatively long lasting effect on μ -opioid-mediated feeding behavior. To analyze the duration of the effect of scopolamine on opioid-induced feeding, we designed a time course study in which animals received intra-Acb infusions of scopolamine followed by intra-Acb DAMGO at varying post-scopolamine intervals. Additionally, we aimed to determine whether the effect of intra-Acb scopolamine is specific to food intake, or affects other types of ingestive behaviors, such as drinking.

Forty-two male Sprague-Dawley rats (Harlan, Madison, WI, USA) were housed in pairs in clear plastic cages. Rats received food and water *ad libitum*, unless otherwise specified by the experimental design, and were maintained on a 12-h light/dark cycle (lights on at 07:00 h). Procedures and animal care were performed according to NIH guidelines on the use of animals in research, following the approval of the University of Wisconsin-Madison Medical School Animal Care and Use Committee. All animals underwent standard aseptic surgery for implantation of 10-mm stainless steel guide cannulae (30 gauge) bilaterally above the Acb (1.3 mm anterior and ± 1.7 mm lateral to bregma; 5.3 mm ventral to skull surface). A Ketamine-Xylazine mixture (100-10 mg/kg) was used for anesthesia. Guide cannulae were affixed to the skull with the use of screws and dental acrylic and stylets were placed to prevent cannulae from becoming occluded. Animals received an IM injection of buprenorphine (0.30 mL) for pain and recovered for at least 7 days prior to behavioral testing.

For all experiments, the competitive muscarinic acetylcholine receptor antagonist scopolamine methyl bromide (Sigma) (1 or 10 $\mu\text{g}/0.5\mu\text{l}/\text{side}$), and the μ -opioid specific agonist D-Ala2, NMe-Phe4, Glyol5-enkephalin (DAMGO) (Bachem Biosciences Inc. King of Prussia, PA) (0.25 $\mu\text{g}/0.5\mu\text{l}/\text{side}$), were each dissolved in sterile 0.9% saline. Drugs were administered bilaterally using a microdrive pump (Harvard Apparatus, South Natick, MA) connected via polyethylene tubing (PE-10) while animals were gently handheld. Thirty-three gauge injectors were used, extending 2.5 mm beyond the end of the guide cannulae. The final injection site was 7.8 mm ventral from skull. Total volume infused was 0.5 μl . Injectors then were removed and stylets were replaced before placing the subjects in the test cages. Following behavioral testing, brains were removed and placed in 10% formalin-20% sucrose overnight. Frozen serial sections (60 μm) were collected through the entire extent of the injections site, mounted on gelatinized slides, stained with Cresyl violet and cover slipped. Cannulae placements were then

assessed with light microscopy by an observer blind to the behavioral results of the animals. Photomicrographs of representative acceptable placements are shown in Figure 1.

To assess the duration of scopolamine's effect on DAMGO-induced feeding, animals were placed in a room separate from the animal colony containing automated locomotion/feeding cages (Med Associates, St. Albans, VT). Food intake monitors (Med Associates) were mounted on the sides of the cage and were able to measure food weight with an accuracy of 0.1 g. Two arrays of infrared photobeams were mounted on the front and back of the cage to measure locomotor activity of the animals. Pre-weighed water bottles were attached to the side of the cage and the tub below the food intake monitor was filled with high-fat diet (Teklad Diets, Madison, WI, USA). Animals were habituated to these boxes until a stable baseline food intake was observed (about 5 days). The pretreatment times tested were 30 minutes, 4, 10, and 24 hours. Animals received intra-accumbens scopolamine followed by an infusion of DAMGO at the determined pretreatment time. Immediately following DAMGO infusion, animals were placed in the locomotion/feeding cages for one hour where they *ad libitum* access to food and water. A clear dose-dependent reduction in DAMGO-induced feeding at the 30 minute intra-accumbens infusion of scopolamine (1 or 10 μg / 0.5 μl) was observed (Fig 2). One way analysis of variance demonstrated a significant main effect of scopolamine pretreatments ($F_{(2,18)}=16.82$, $p<0.01$). Post-hoc using Tukey's test analysis showed a significant difference of all pairwise multiple comparisons (saline vs. 10 μg , $p<0.001$; saline vs. 1 μg , $p<0.01$; 1 μg vs. 10 μg , $p<0.05$).

To further examine the time course of scopolamine's feeding effect, 4, 10, and 24 h pretreatment times were examined (Fig 3). A two way analysis of variance showed a significant main effect of treatment ($F_{(1,18)} = 6.57$, $p<0.02$) with no main effect of time ($F_{(2,18)}=0.97$, $p=0.40$). More interesting, the Tukey post-hoc analysis revealed a significant difference in the time point versus treatment comparison ($F_{(2,18)} = 5.11$, $p<0.02$). At the 4 hour time point the scopolamine treated group's intake was significantly lower than that of the saline group ($p<0.05$) while no significant difference was observed at the 10 and 24 h time points ($p<0.05$). Thus, the 30 min and 4 hour prior treatment with scopolamine was able to potently inhibit DAMGO-induced feeding, an effect which dissipated by 10 hours following scopolamine treatment. In contrast, water intake was also analyzed, and no significant differences were observed between groups (data not shown).

Previous data has demonstrated that scopolamine modulates pharmacologically induced feeding states and feeding in hungry animals, but it is not clear whether or not this effect is specific to food intake or is general to all types of ingestive behavior. To further explore the specificity of scopolamine's modulation of appetitive behavior, we separately deprived subjects of either food or water 18 hours prior to scopolamine infusion (10 μg / 0.5 μl). A paired t-test demonstrated a significant effect of treatment for chow consumption ($t_{(11)}5.74$, $p<0.001$) (Figure 4a), but no difference was observed for water intake ($t_{(11)}=0.08$, $p=0.94$) (Figure 4b). It can be observed from Figure 4 that intra-accumbens scopolamine has a marked effect on animals in negative energy balance (4a), but no effect on water intake of water-deprived rats (4b). Thus, scopolamine effects on appetitive behavior appear to display some specificity for feeding.

The present study describes the time course of scopolamine-opioid interactions and indicates the specificity of intra-accumbens cholinergic modulation of feeding. It was demonstrated that this decrease in DAMGO-induced feeding was an enduring effect, likely outlasting the direct pharmacological effects of scopolamine. Fat intake was observed to be decreased significantly 30 minutes and 4 hours after scopolamine infusion while, 10 hours later, only a negligible decrease in intake was observed. **Anatomical examination of injection sites (shell vs. core, anterior vs. posterior Acb) revealed no systematic differences in scopolamine's effect**

among Acb regions (data not shown). Additionally, present findings suggest that scopolamine's actions are specific to the consumption of food and not generalizable to other behaviors, such as drinking. Scopolamine-treated animals deprived of water drank a similar amount compared to controls; in contrast, food-deprived animals infused with scopolamine ate significantly less chow than those treated with saline. The current studies add to a recent and growing body of literature implicating striatal-cholinergic-opioid interactions in the motivational control of food intake [17,19,27].

Our food and water deprivation studies revealed that blockade of cholinergic muscarinic receptors appears to specifically diminish feeding behavior; this effect does not generalize to a different type of ingestive behavior, drinking. This result is in agreement with previous work that demonstrated separate control mechanisms for feeding and drinking within the Acb [3, 22,24]. The fact that drinking was left unaltered by intra-accumbens infusion of scopolamine would also suggest that scopolamine's effect 1) is not due to malaise, 2) is not due to motor impairment, and 3) is not due to a general motivational impairment. It also should be added that previous studies have shown that intra-accumbens scopolamine does not produce a decrease in locomotor activity [6,10,16].

Work done by B. Hoebel and colleagues has suggested that ACh transmission in the Acb acts as a satiety signal; specifically it was shown with microdialysis that levels of ACh in the Acb increase during feeding bouts with peak levels at the point of maximal food intake [7,13]. At first glance, these results would seem to contradict the decrease in feeding with muscarinic blockade seen in the present study. Nevertheless, it has been shown that antagonizing subtypes of the muscarinic receptors, M2 and M4, increase ACh levels [9]. The muscarinic M2 and M4 receptors are autoreceptors located presynaptically, and their activation inhibits the release of ACh from the terminal. Thus, the blockade of the M2 and M4 receptors via infusion of scopolamine may cause an increase in synaptic ACh levels. For this reason, the results presented here are not necessarily inconsistent with the results of Hoebel et al. specifically if it is assumed that the postsynaptic receptors mediating the putative satiety-like effects of ACh are not blocked by scopolamine. We also propose that a disruption in the downstream μ -opioid receptor signaling contributes to the anorexic effect of intra-Acb infusion of scopolamine. Previous work in our laboratory has shown that the mRNA of preproenkephalin, the precursor to the opioid modulated peptide enkephalin (an important peptide in reward and positive affective states), is decreased 24 hours after scopolamine infusion [17], and it has been shown to be downregulated 3 hours following intra-striatal scopolamine [25]. If scopolamine does modulate opioid receptor signaling, then this decrease in peptide synthesis would be expected, and suggests the hypothesis that antagonism of the muscarinic receptor leads to a decrease in feeding as a result of alterations in downstream signaling of the opioid and muscarinic receptors.

Our laboratory has previously suggested that a hypothalamic-thalamic-striatal axis controls Acb function in the context of ingestive behaviors [11]. This model proposes that striatal ACh interneurons may play an important role in the integration of information related to energy balance that originates in the hypothalamus. It was suggested that enkephalinergic gene activity may be tonically controlled by ACh interneurons which may indirectly receive information from circulating signals such as leptin and ghrelin. Work presented here agrees with this model insofar as that opioid-induced feeding was modulated by blocking muscarinic receptors. Kelley and colleagues have also suggested that enkephalin plays a primary role in the consummatory phase of feeding by enhancing the hedonic properties of palatable foods [11]. If this is the case, better insight regarding ACh-opioid interactions could allow for new therapeutic developments in the treatment of obesity or other weight related problems. This novel pathway introduces a variety of new drug targets for treatment, particularly the muscarinic receptors themselves, as G-protein coupled receptors have been a major focus for drug development. Hence, further

research into the precise mechanisms underlying functional Ac-opioid interactions in the Acb is warranted.

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Figure 1. Photomicrographs showing representative cannulae placements (right). Schematic diagrams displaying injection sites of 3 animals from each experiment (left) (▲ = Experiment 1 sites; ● = Experiment 2 sites; ■ = Experiment 3 sites). The stereotaxic coordinates shown are in mm anterior of bregma.

Figure 2. DAMGO-induced (0.25 μ g/ 0.5 ul) fat intake is decreased by a 30 minute pretreatment of intra-accumbens scopolamine (1 μ g or 10 μ g/ 0.5 ul) in a dose dependent manner ($n = 6$ / group). Values represent group means ($\pm$ SEM). (* significantly different from saline, $p \leq 0.01$; ♦ significantly different from 1 μ g/ 0.5 ul group, $p \leq 0.05$)

Figure 3. Feeding response to intra-accumbens administration of DAMGO (0.25 μ g/ 0.5 μ l) following 4, 10, and 24 hour pretreatments of scopolamine (10 μ g/ 0.5 μ l) (n = 6 / group). Values represent group means (+ SEM). (* $p \leq 0.05$)

Figure 4.

A) Food deprived animals decreased chow intake with a 30 minute scopolamine pretreatment (10 µg/0.5 ul) (n = 12 / group). Values represent group means (± SEM). (***) $p \leq 0.001$. B) Subjects water deprived for 18 hours were unaffected by a 30 minute pretreatment of scopolamine (10 µg/0.5 ul). Values represent group means (± SEM).