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Atropine methyl nitrate increases myenteric but not dorsal vagal complex Fos-like immunoreactivity in the rat

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Abstract

Atropine methyl nitrate (AMN, 0.05, 0.5 and 25 mg/kg) intraperitoneally increased Fos-like immunoreactivity (Fos-LI) in the myenteric plexus, but not the dorsal vagal complex (DVC, the area postrema (AP), nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus (DMV)) in adult, male Sprague–Dawley rats. A 3 mg/kg AMN dose decreased intake of 15% sucrose, but failed to increase Fos-LI in both locations. In conclusion, the myenteric plexus may play a local role in the behavioral response evoked by AMN.
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1. Introduction

The muscarinic cholinergic blocker atropine methyl nitrate (AMN), a synthetic atropine that does not cross the blood brain barrier [1], inhibits sham feeding in rats [2,3]. Lorenz et al. suggested that this reduction is due to activation of a peripheral cholinergic “inhibitory mechanism specific for feeding behavior” [2]. The goal of the current study is to elucidate possible peripheral pathway(s) that has a role in this response by AMN.

We quantified Fos-like immunoreactivity (Fos-LI), a marker for neuronal activation, in three nuclei of the dorsal vagal complex (DVC) in the caudal medulla of the brainstem in response to various doses of AMN given intraperitoneally (i.p.). These nuclei include the area postrema (AP), nucleus tractus solitarii (NTS) and dorsal motor nucleus of the vagus (DVM), which have a role in the central control of food intake. In addition, although AMN is a peripheral antagonist, the central locations can be stimulated by incomplete vagal blockade or increase parasympathetic tone. This effect takes place especially at high doses of AMN [1].

We also quantified Fos-LI in the myenteric plexus of the duodenum and jejunum, as possible peripheral sites of AMN actions. The myenteric plexus, a component of the enteric nervous system (ENS) of the gut, is potentially an important peripheral site for AMN actions due to at least two reasons. First, the ENS contains millions of cholinergic, and others, neurons organized in two main nerve plexuses, myenteric and submucosal [4]. Second, the ENS controls many gut functions, which may be related directly or indirectly to AMN-evoked functions, e.g., motility, secretion or both [4]. For example, inhibition of sham feeding by AMN may involve secretion of satiety hormones, again such as CCK, which may ultimately inhibit sham feeding, gastric emptying or gallbladder contraction. The myenteric plexus contributes directly to such functions because it controls the internal environment of the gut. Therefore, determining if AMN can activate this plexus is a step in elucidating a possible peripheral pathway by which this muscarinic antagonist evokes, at least some of its functions.

2. Materials and methods

2.1. Animals

The Tuskegee University Animal Care and Use Committee approved the experiments. We used 25, 17-week-old, male

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Sprague–Dawley rats, averaging 320 g per rat (Harlan, IN). The animals were housed singly in wire-mesh cages, in a controlled environment (lights were on from 0600 to 1800 and temperature was maintained at 21.5 °C). Rats had ad libitum access to water and pelleted rodent chow (Teklad, WI). To enhance adaptation to the laboratory, we handled each rat for 10 min/day for the first 7 days and each rat was given a daily i.p. injection of 0.5 ml saline.

2.2. Experimental procedure

All rats were deprived of food, but not water, beginning at 6:00 p.m. on the day prior to the experiment to avoid food-induced c-*fos*. At 9:00 a.m., the rats were divided into five treatment groups ($n=5$ rats per group) and received an i.p. injection of AMN (Sigma, 0.05, 0.5, 3 and 25 mg/kg) or 0.5 ml saline.

As described in our previous work [5–9], 90 min after the injection, rats were anesthetized with sodium pentobarbital (10 mg/kg, i.p.) and perfused transcardially in two stages. First, the rats were perfused with 500 ml of Krebs solution (Krebs saline formula in mM: 119 NaCl, 4.7 KCl, 1.2 $NaH_2PO_4 \cdot H_2O$, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11.1 glucose and 1 M $CaCl_2$) to collect the small intestine. Second, the rats were perfused with 500 ml of 4% formaldehyde (made in 0.1 M phosphate-buffered saline [PBS]) to collect the brains. The small intestine was exposed through a midline abdominal incision and the duodenum and jejunum were collected. On the basis of our previous experiments [5–9], the duodenal sample was 5–10 cm aborad from the pylorus and the jejunal sample was 20–25 cm aborad from the pylorus. The lumens of the removed segments were rinsed with Krebs solution, and the segments were placed in Sylgard-coated Petri dishes (World Precision Instruments, FL), opened along the mesenteric attachment, stretched and pinned with the mucosal side up, and stored overnight in Zamboni's fixative at 4 °C. On the next day, the tissues were unpinned and cleared three times in 100% dimethyl sulfoxide (DMSO), 10 min each time, followed by three 10-min rinses with 0.1 M PBS, pH 7.4. Whole mounts (approximately 1 cm^2) of longitudinal smooth muscle with adhering myenteric plexus from the duodenum and jejunum were prepared using a dissecting microscope and processed for immunohistochemical detection of Fos-LI.

After collection, the hindbrains were postfixed with 4% formaldehyde for 2 h and placed in 25% sucrose overnight at room temperature. The hindbrains were sectioned at 40 μm on a cryostat at −20 °C. Consistent with our previous work, the areas cut included the following levels of the DVC according to the Paxinos and Watson rat brain atlas [10]: the area postrema (AP, −4.5 mm caudal to interaural plane), nucleus of the solitary tract (NTS, −4.5 and −4.8 mm caudal to interaural plane) and the dorsal motor nucleus of the vagus (DMV, −4.5 and −4.8 mm caudal to interaural plane). Sections were taken at multiple levels of the NTS to insure sampling from sites receiving both gastric and intestinal vagal afferent innervation.

2.3. Immunohistochemistry

Based on our previous methods [4,5], the whole mount preparations and hindbrain sections were incubated for 24 h at room temperature in primary antiserum raised in rabbit against a peptide representing amino acids 4–17 of human Fos (Oncogene, Ab-5, San Diego, CA [1:12,500 dilution]). After a subsequent overnight incubation in biotintylated donkey anti-rabbit serum (Jackson ImmunoResearch Laboratories, PA [1:500 dilution]), the tissues were incubated for 3 h in avidin conjugated to horseradish peroxidase (HRP), then washed with 0.01 M tris PBS and processed to reveal HRP activity using diaminobenzidine (DAB, Sigma, MO) intensified with nickel.

2.4. Counting procedure

We utilized automated computer software (ImagePro Plus, Media Cybernetics) to count Fos-LI in the DVC sections. Fos-positive cells within the AP, NTS and DMV were counted at the −4.5 mm caudal to interaural plane, and within the NTS and DMV were counted at the −4.8 mm caudal to interaural plane according to the rat brain atlas [10], and described in details in our previous work [5–9]. Only one section per level of the DVC was counted and the intensity of Fos-positive nuclei was set to an acceptable level that corresponds with our previous work.

Two observers, blinded to the treatments, counted Fos-positive cells at each intestinal level (duodenum and jejunum) of every animal. The final count for each intestinal level represents the average from 10 non-overlapping, 40× microscopic fields. The process was also described in our previous work [5–9].

2.5. Statistical analysis

To compare the counts of Fos-positive neurons in the different hindbrain levels and myenteric neurons in response to AMN, we performed separate one-way ANOVAs for each brain and intestinal level. All post hoc analyses were done with Bonferroni *t*-test. Differences were considered significant at $p<0.05$. All summary data are presented as mean±S.E.M.

2.6. Food intake

To confirm previous findings by Lorenz et al. in 1978 and Weingarten et al. in 1982, we measured food intake of 15% sucrose solution for 60 min in response to atropine methyl nitrate (3 mg/kg) or saline i.p.

3. Results

3.1. Dorsal vagal complex

3.1.1. Area postrema

One-way ANOVA revealed no significant difference in the counts of Fos-positive neurons between each of the treatment groups and saline [$F(1,8)=9.879$, $p=0.014$; $F(1,8)=0.960$,

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