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The orexigenic effect of kyotorphin in chicks involves hypothalamus and brainstem activity and opioid receptors

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ABSTRACT

Kyotorphin (KTP), first isolated in the bovine brain and now having been identified in a variety of species, is known most extensively for its analgesic-like properties. KTP indirectly stimulates opioid receptors by releasing methionine enkephalin (met-enkephalin). Stimulation of opioid receptors is linked to hunger perception. In the present study, we sought to elucidate the effect of KTP on food intake in the neonatal chick. Intracerebroventricular injection of 0.6, 3.0 and 12 nmol KTP increased feeding up to 60 min post-injection. KTP treated chicks increased pecking efficiency and decreased time spent in deep rest, 20 and 30 min following injection, respectively. Gastrointestinal transit rate was not affected by KTP. Blocking mu, delta, and kappa opioid receptors suppressed orexigenic effects of KTP, suggesting that all three types are involved in KTP's stimulatory effect. The lateral hypothalamus (LH) and arcuate nucleus (ARC) of the hypothalamus and the nucleus of the solitary tract (NTS), within the brainstem had increased numbers of c-Fos immunoreactive cells following KTP treatment. In conclusion, KTP caused increased feeding in broiler-type chicks, likely through activation of the LH, ARC, and NTS.

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1. Introduction

Kyotorphin (KTP) is a central dipeptide (L-tyrosyl–L-arginine) first isolated in the bovine brain (Takagi et al., 1979) and since found in other vertebrates including mice, rats, guinea pigs, rabbits (Shiomi et al., 1981; Ueda et al., 1980) and humans (Nishimura et al., 1991). Studied most extensively for its analgesic properties, KTP stimulates met-enkephalin release in the spinal cord, which through associations with opioid receptors (Hughes et al., 1975) produces long-lasting analgesia (Takagi et al., 1979; Shiomi et al., 1981; Janicki and Kipkowski, 1983). Roles for KTP in other physiological systems have also been documented (for review see Dzambazova and Bocheva, 2010); KTP was found to affect aversive pecking in chicks (Kastin et al., 1981) and inhibit rat's attention towards various sensory stimuli (Kolaeva et al., 2000). Because stimulation of opioid receptors is linked to food intake regulation (Brown and Holtzman, 1974; Grandison and Guidotti, 1977) and KTP interacts with these receptors, we sought to examine appetite-related roles for KTP in the present study using Cobb-500 chicks.

KTP's effect on food intake was evaluated by intracerebroventricularly (ICV) administering KTP to chicks and recording food intake up to 180 min post injection. Since KTP causes hypothalamic effects (Dzambazova et al., 2008), we quantified c-Fos immunoreactive cells as a measure of neuronal activity in several hypothalamic nuclei including the lateral hypothalamus (LH), ventromedial hypothalamus (VMH), paraventricular nucleus (PVN), dorsomedial nucleus (DMN), and arcuate (ARC), as well as in the nucleus of the solitary tract (NTS), following ICV KTP. Because KTP is associated with opioid receptor stimulation (Hughes et al., 1975), we hypothesized that interactions between KTP and the chick's innate opioid system may contribute to KTP's effect on food intake. Thus, we examined relationships between KTP-induced food intake stimulation and three subtypes of opioid receptors by using the selective antagonists, betafunaltrexamine (β-FNA), ICI-174,864 (ICI), and norbinaltorphimine (BNI), which block mu, delta, and kappa opioid receptor subtypes, respectively. A comprehensive behavior analysis was conducted to determine whether KTP exerts other behavioral effects not related to ingestion. Finally, we examined KTP's effect on total alimentary canal transit time. The results from this study may provide a novel role for KTP in food intake regulation.

2. Materials and methods

2.1. Animals

Unsexed Cobb-500 broiler chicks (*Gallus gallus*) from breeders 30 to 40 weeks of age were obtained from a commercial hatchery

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on the morning of hatch. They were caged individually in a room at 30 ± 2 °C and 50 ± 5% relative humidity with access to a mash diet (20% crude protein and 2685 kcal ME/kg) and tap water. In all experiments, chicks were injected at day 4 post-hatch, and each experiment was conducted using chicks from separate hatches. Experiments were conducted sequentially in the order described below. All experimental procedures were performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals and were approved by the Radford University Institutional Animal Care and Use committee.

2.2. Intracerebroventricular (ICV) injection procedure

Chicks were ICV injected using a method adapted from Davis et al. (1979). The head of the chick was briefly inserted into a restraining device that left the cranium exposed and allowed for free-hand injection. Injection coordinates were 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 2 mm deep targeting the left lateral ventricle. Anatomical landmarks were determined visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the needle. The needle remained *in vivo* in the un-anaesthetized chick for 5 s to reduce backflow. Chicks were assigned to treatments at random. KTP (Tyr–Arg, 337.4 molecular weight, American Peptide, Sunnyvale, CA, USA) was dissolved in avian artificial cerebrospinal fluid (Anderson and Heisey, 1972) as a vehicle for a total injection volume of 5 μL with 0.06% Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and its head sectioned coronally to verify the injection site. Data from chicks without dye present in the lateral ventricle system were eliminated from statistical analysis. Sex was determined visually by dissection.

2.3. Experiment 1: effect of KTP on food intake

In Experiment 1, chicks were randomly assigned to receive 0 (vehicle only), 0.6, 3.0, or 12.0 nmol KTP based on Bungo et al. (1999). Immediately following ICV injection, chicks were returned to their individual cages and given *ad libitum* access to food and water. Food intake was measured (measurement accuracy of 0.01 g) every 30 min for 180 min post injection. Data were analyzed using analysis of variance (ANOVA) at each time point using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). The model included KTP dose, sex and the interaction of sex with KTP dose. Sex and the interaction of sex and KTP dose were not significant and were eliminated from the model (and the effect of sex was not tested in subsequent experiments). If significant treatment effects were found, Tukey's method of multiple comparisons was used to separate the means at each time period. Additionally, treatment effects were partitioned into linear and quadratic contrasts to determine the dose–response relationships at each time period. The number of chicks used in each experiment is given in figure legends. For this and all proceeding experiments, statistical significance was set at $P < 0.05$.

2.4. Experiment 2: c-Fos as an indicator of neuronal activation

Chicks were randomly assigned to receive either vehicle or 6.0 nmol KTP by ICV injection (protocol detailed above). Chicks were allowed *ad libitum* access to food and water until injection, at which time food was withheld to prevent c-Fos immunoreactivity associated with food consumption. One hour post injection (as this is the time expected for the most robust c-Fos expression (Muller and Curran, 1986), chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, then perfused via the carotid artery with ice-cold 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) containing 0.2% picric acid at pH 7.4. Brains were removed from skulls and post-fixed for 1 h in the same solution, after which they were blocked and placed through a series of graded sucrose incubations, consisting of 20%, 30%, and 40% in 0.1 M PB, until they sank. Several 40 μm coronal sections that contained appetite-related nuclei based on anatomies described by Puelles et al. (2007) were collected in 0.02 M PB saline (PBS) containing 0.1% sodium azide using a cryostat at −15 °C. The VMH, PVN, LH, and DMN were collected at interaural 2.08 mm, the ARC at interaural 1.12 mm, and NTS at interaural −7.04 mm. Sections were processed immediately after collection. Procedures for c-Fos immunohistochemistry assay were based on those of Zhao and Li (2010). Free-floating sections were pre-blocked for 1 h with 10% normal goat serum (NGS) and 0.3% Triton X-100 in 0.02 M PBS. To inhibit endogenous peroxidase activity, sections were incubated in 1.5% hydrogen peroxide and 50% methanol in deionized water for 30 min. Following a 3 × 10 min wash in wash buffer (0.05% NGS and 0.3% Triton X-100 in 0.02 M PBS), sections were incubated with rabbit polyclonal anti-c-Fos at a dilution of 1:20,000 (K-25, Santa Cruz, Santa Cruz, CA, USA) in PBS containing 0.3% Triton X-100, 1% NGS, and 1% blocking reagent (11096176001, Roche Diagnostics, MA, DE) for 48 h under slow oscillation at 4 °C. For assay controls, the primary antibody was substituted with normal rabbit serum. Sections were then rinsed 3 × 10 min in wash buffer and incubated with biotinylated goat anti-rabbit secondary antibody at a dilution of 1:200 (Vector Laboratories, CA, USA) in PBS containing 1% NGS for 2 h at room temperature. Following a rinse with PBS, sections were processed with avidin–biotin horseradish peroxidase complex at a dilution of 1:200 (Vectastain Elite ABC Kit, Vector Laboratories). Reactions were visualized with the DAB Substrate Kit for Peroxidase (Vector Laboratories) for 45 s, mounted on gelatin-coated slides and cover-slipped with Vecta-Mount (Vector Laboratories). Anatomy were confirmed and a digital micrograph taken of each section. Overlays containing the respective nuclei boundaries were digitally merged with micrographs and the number of c-Fos immunoreactive cells within each respective nucleus counted by a technician blind to treatment. Data were analyzed by ANOVA using the GLM procedure of SAS.

2.5. Experiments 3, 4, and 5: opioid receptor antagonism on KTP-induced food intake stimulation

Chicks were randomly assigned to either 0, 0.6 nmol KTP, 6 nmol ICI, or 0.6 nmol KTP + 6 nmol ICI. Following ICV injection, chicks were returned to their individual cages with *ad libitum* access to food and water. Food intake was measured 30 min post-injection. Experiment 5 was identical to Experiment 4, however, chicks were ICV injected with 0 (vehicle only), 0.6 nmol KTP, 2.7 nmol BNI, or 0.6 nmol KTP + 2.7 nmol BNI. The experimental procedures in Experiment 6 were identical to those in Experiments 4 and 5, however, chicks were ICV injected with 0 (vehicle only), 0.6 nmol KTP, 41 nmol β-FNA, or 0.6 nmol KTP + 41 nmol β-FNA. Data were analyzed as described for Experiment 1.

2.6. Experiment 6: behavioral effects of KTP

Chicks were kept in individual cages with auditory but not visual contact with each other. They were assigned to receive either 0 or 6.0 nmol KTP. Following ICV injection, chicks were kept in individual 290 × 290 mm acrylic recording arenas with access to food and water. Chicks were recorded from 4 different camera angles on DVD for 30 min post-injection. Data was analyzed in 10-min intervals using ANY-maze behavioral analysis software (Stoelting, Wood Dale, IL). Food and water intake was recorded at 30 min post-injection. Distance moved (m traveled), the amount of time spent standing, sitting, preening, perching, or in deep rest,