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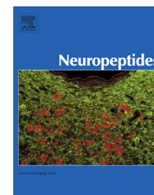

Substance P is associated with hypothalamic paraventricular nucleus activation that coincides with increased urotensin 2 mRNA in chicks

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ABSTRACT

Exogenous administration of substance P (SP) exerts anorexigenic effects in both chicks and rats, but the central mechanism mediating this response is poorly understood. Therefore, this study was designed to elucidate mechanisms of SP-induced anorexia using chicks as models. Chicks that received intracerebroventricular (ICV) injections of SP dose-dependably reduced their food intake with no effect on water intake. Next, the diencephalon was isolated from SP-injected chicks and mRNA expression of neuropeptide Y (NPY), corticotropin releasing factor (CRF), urocortin 3 (UCN 3) and CRF receptors were measured but were not affected. When measured in the hypothalamus, mRNA abundance of these and NPY receptors, urotensin 2 (UTS2) and melanocortin receptor 4 (MCR4) were not affected by SP-injection. Quantification of c-Fos immunoreactivity in appetite-associated hypothalamic nuclei demonstrated that the paraventricular nucleus (PVN) was activated in SP-injected chicks. Finally, in the PVN isolated from SP-injected chicks, there was increased expression of UTS2 mRNA while CRF and UCN3 were not affected. Thus, the anorexigenic effects of SP appear to be mediated by PVN activation and may involve UTS2.
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1. Introduction

Substance P (SP), an eleven residue excitatory neurotransmitter, is a member of the tachykinin family and is distributed throughout the central and peripheral nervous system (Otsuka and Yoshioka, 1993; Gallagher et al., 1992; Quartara and Maggi, 1998), including the hypothalamus (Langevin and Emson, 1982). Some of the first and most well-known research on SP concerns its role in pain transmission (Harrison and Geppetti, 2001; Otsuka and Konishi, 1976). SP is released from a large number of dorsal raphe nuclei, projections from which extend to many parts of the forebrain. In addition, SP is also found in the dorsal root ganglion and primary sensory neurons. This neuropeptide has also been linked to the sensation of pain by the stimulation of neurokinin 1 receptors, which are distributed throughout the central nervous system (Felipe et al., 1998).

Substance P, via three types of G-protein coupled tachykinin receptors (Gerard et al., 1993), is associated with several other aspects of physiology (Harrison and Geppetti, 2001), including appetite regulation. In rats, intracerebroventricular (ICV) injection of SP causes reduced food and water intake (Dib, 1999). The appetite-associated effect of SP has also been observed in male layer chicks (Tachibana et al., 2010), however its central mechanism of action is poorly understood.

Therefore, the purpose of the present study was to evaluate appetite-related responses after central administration of SP and to determine the central mechanism mediating this effect in chicks. We measured appetite-associated mRNA expression in the whole diencephalon, whole hypothalamus and hypothalamic paraventricular nucleus (PVN) in addition to quantifying c-Fos immunoreactivty in the hypothalamus after SP injection.

2. Methods and materials

2.1. Methods

2.1.1. Animals

Cobb-500 chicks were group caged for 1 d, then individually in a room at 30 ± 2 °C and 50 ± 5% relative humidity where they had ad libitum access to a mash diet and tap water. The individual

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cages allowed visual and auditory contact with other chicks. Chicks were handled twice daily to adapt to handling. All trials were conducted between 08:00 and 13:00 h using 4 d post hatch chicks. Experimental procedures were performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Tech Animal Care and Use Committee.

2.2. Intracerebroventricular (ICV) injection procedure

Chicks were injected using a method adapted from Davis et al. (1979) that does not appear to induce physiological stress (Furuse et al., 1999). 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 at injection depth in the un-anesthetized chick for 10 s post injection to reduce backflow. Chicks were assigned to treatments at random. Substance P (American Peptide, Sunnyvale, CA, USA) was dissolved in artificial cerebrospinal fluid (Anderson and Heisey, 1972) as a vehicle for a total injection volume of 5 μL with 0.1% Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and its head sectioned along the frontal plane to determine the site of injection. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Whole blood glucose was measured from trunk blood with hand-held glucometers (Bayer One-Touch Ultra). Sex of chicks was determined visually by dissection.

2.3. Experiment 1: food and water intake

Chicks, fasted for 180 min, were randomly assigned to receive either 0 (vehicle only), 2.5, 5.0 or 10.0 nmol (based on Tachibana et al., 2010) SP by ICV injection. After injection, chicks were returned to their individual home cages and given ad libitum access to both food and water. Food and water intake were monitored (0.01 g) every 30 min for 180 min post injection. Water weight (g) was converted to volume (ml; 1 g = 1 ml). Data were analyzed using analysis of variance (ANOVA) at each time point. The initial ANOVA included the main effects of sex and GnIH dose and their interaction. Neither sex nor the interaction was significant, so a second ANOVA was conducted with the main effect of dose. When dose effects were significant, Tukey's method of multiple comparisons was used to separate the means. Statistical significance was set at $P < 0.05$ for all experiments.

2.4. Experiment 2: diencephalon and expression of appetite-associated mRNA

Chicks, fasted for 180 min, were randomly assigned to receive vehicle or 10 nmol SP via ICV injection. Following injection food was withheld to prevent effects associated with food consumption. Sixty min following injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, decapitated, and brains removed. The whole upside-down brain was lowered into liquid nitrogen to the point where the most ventral aspect of the optic lobe was level with the surface of the liquid nitrogen. The brain was left in this position for 11 s. This procedure resulted in brain regions around the hypothalamus freezing and providing firmness necessary to make precise cuts for hypothalamus extraction. Perpendicular to the midline suture a cut was made at the septopallio-mesencephalic tract and at the third cranial nerves. 2.0 mm parallel to the midline two cuts were made. This block was lifted away from the telencephalon and comprised the diencephalon (including the hypothalamus, pretectum, prethalamus and thalamus). It was collected in RNAlater (Qiagen) and homogenized using 5 mm stainless steel beads and 1 mL Isol Lysis reagent (5-Prime, USA) for 2 × 2 min at 20 Hz. After incubation and centrifugation for 10 min for 12,000×*g* at 4 °C, the supernatant was removed and total RNA separated, following the manufacturer's instructions (5-Prime). Following the step of addition to 70% ethanol, mixtures were transferred to spin columns and total RNA purified using the RNeasy Mini Kit (Qiagen, USA), including the optional on-column RNase-free DNase I step (Qiagen, USA). The eluted total RNA samples were evaluated for integrity by agarose-formaldehyde gel electrophoresis and concentration and purity assessed by spectrophotometry at 260/280/230 nm.

Single-strand cDNA was synthesized from 200 ng total RNA in 20 μL reactions with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), following the manufacturer's instructions. Reactions were performed under the following conditions: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. Primers for real time PCR are listed in Table 1 and amplification efficiency was validated for all primer pairs before use (95–100% efficiency). Real-time PCR reactions were performed in duplicate with Fast SYBR Green Master Mix (Applied Biosystems, USA) and 10-fold diluted cDNA. PCR was performed under the following conditions: 95 °C for 20 s and 40 cycles of 90 °C for 3 s plus 60 °C for 30 s. A dissociation step consisting of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s was performed at the end of each PCR reaction to ensure amplicon specificity.

Data were analyzed using the ΔΔCT method, where ΔCT = CT target gene – CT Actin, and ΔΔCT = ΔCT target sample – ΔCT calibrator (Schmittgen and Livak, 2008). Actin served as the endogenous control and the mean of the vehicle-treated group served as the calibrator. Relative quantities, calculated as $2^{-\Delta\Delta CT}$, were used for performing ANOVA. The statistical model included the main effect of treatment. Sex was determined to be non-significant and therefore removed from the model.

2.5. Experiment 3: whole hypothalamus and expression of appetite-associated mRNA

Procedures were identical to those described in Section 2.5 except the hypothalamus was separated from the thalamus by making a cut from the anterior commissure to 1.0 mm ventral to the posterior commissure.

2.6. Experiment 4: c-Fos immunohistochemistry on hypothalamus

Chicks, fasted for 180 min, were randomly assigned to receive either vehicle or 10.0 nmol SP by ICV injection. Food was withheld following injection to prevent c-Fos immunoreactivity associated with food consumption. Sixty min 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 60 min 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 60 μm coronal sections that contained appetite-related nuclei based on anatomies described by Puelles et al. (2007) were collected in 0.02 M phosphate buffered saline (PBS) containing 0.1% sodium azide using a cryostat at −15 °C. The ventromedial hypothalamus (VMH); two parts of the paraventricular nucleus, the magnocellular (PaMC) and

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