Peptides 32 (2011) 776–780

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Nitric oxide is a central component in neuropeptide regulation of appetite

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ARTICLE INFO

Article history:
Received 12 October 2010
Received in revised form
22 December 2010
Accepted 23 December 2010
Available online 22 January 2011

Key words:
Nitric oxide
NPY
CCK
Ghrelin
Appetite
Food intake
NOS KO mice

ABSTRACT

In recent years, there have been a large number of neuropeptides discovered that regulate food intake. Many of these peptides regulate food intake by increasing or decreasing nitric oxide (NO). In the current study, we compared the effect of the food modulators ghrelin, NPY and CCK in NOS KO mice. Satiated homozygous and heterozygous NOS KO mice and their wild type controls were administered ghrelin ICV. Food intake was measured for 2 h post injection. Ghrelin did not increase food intake in the homozygous NOS KO mice compared to vehicle treated NOS KO mice, whereas food intake was increased in the wild type controls compared to vehicle treated wild type controls. NPY was administered ICV and food intake measured for 2 h. Homozygous NOS KO mice showed no increase in food intake after NPY administration, whereas the wild type controls did. In our final study, we administered CCK intraperitoneally to homozygous and heterozygous NOS KO mice and their wild type controls after overnight food deprivation. Food intake was measured for 1 h after injection. CCK inhibited food intake in wild type mice after overnight food deprivation, however, CCK failed to inhibit food intake in the NOS KO mice. The heterozygous mice showed partial food inhibition after the CCK. The current results add further support to the theory that NO is a central mediator in food intake.

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1. Introduction

Studies of peptides that are involved in the pathway of energy intake and expenditure have suggested that nitric oxide (NO) is a common regulator in the function of all these peptides. A number of studies have shown that NO is involved in the regulation of food intake in a variety of species including rats, chicks, broilers, Leghorns, marsupial *Sminthopsis crassicaudata*, mice, and dogs [21,22,24,27,28,31,36,38]. The complex pathway involved in energy intake and expenditure includes agouti-related protein (AgRP), neuropeptide Y (NPY), proopiomelanocortin (PMOC) and cocaine- and amphetamine-related transcript (CART) in the arcuate neucleus and the paraventricular nucleus, melanocortin 4 receptor (MC4R) in the paraventricular and ventromedial hypothalamus (PVH/VMH) and orexin and melanin-concentrating hormone (MCH) in the lateral hypothalamus [14]. In addition, multiple other areas in the brain, e.g., cortico-limbic system and brainstem are involved in regulating food intake. Substances from the periphery that regulate the process in these regions include leptin, insulin, peptide in YY (PYY), glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK) and ghrelin. Previous studies have shown nitric oxide regulates the function of many of these peptides including of leptin, NPY, orexin and ghrelin [12,17,25]. Peptides that enhance feeding increase NO, whereas peptides that decrease food intake decrease NO. Nitric oxide has been shown in mice to increase with advancing age [26].

NO is synthesized within cells by nitric oxide synthase (NOS) from arginine with the aid of molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADPH). In neurons, NO is produced by neuronal nitric oxide synthase (nNOS), representing one of the three NOS isoforms expressed in most tissues [29,35] Studies have linked nNOS to many physiological processes, including cardiac function, immune function, reproductive function, CNS neuronal function, muscular function and feeding [1–3,9,33].

Food intake enhanced by food deprivation or the administration of ghrelin, NPY or orexin-A can each be blocked by co-administration of Nω-nitro-L-arginine methyl ester (L-NAME) a nitric oxide synthase (NOS) inhibitor [12,16,17]. Leptin which decreases food intake has been found to decrease NO levels in the hypothalamus. Both short-term and chronic injections of leptin have been found to decrease nitric oxide synthase and, in nNOS

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0196-9781/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.peptides.2010.12.015

knockout mice, the leptin affect on feeding was greatly reduced [5]. Chronic blockage of NO synthesis reduces adiposity and improves insulin resistance [34].

To further examine whether NO is involved in the regulation of peptides which alter food intake, we examined the ability of NPY and ghrelin to increase feeding and CCK to decrease feeding in a neuronal NOS knockout mouse (NOS KO) compared to the wild type controls.

2. Methods

2.1. Mice

The subjects for the experiments were 12 week male and female mice homozygous, heterozygous NOS KO mice and wild type controls (C57BL/J6) obtained from our breeding colony which was started from breeding pairs obtained from Jackson Laboratories (BarHarbor, ME). Phenotypically they are similar to the mice described by Haung et al. [18]. Sentinels from the colony are tested regularly to ensure the colony is virus and pathogen free. Food (5001 Rodent Diet, PMI Nutrition International, LLC, Brentwood, MO) and water were available on an ad libitum basis and the rooms have a 12 h light–dark cycle with lights on at 06:00 h. Experiments were conducted between 07:30 and 11:00 h.

2.2. Drugs

Cholecystokinin octapeptide (CCK), ghrelin (rat) and neuropeptide Y (NPY) were purchased from Bachem Bioscience (King of Prussia, PA). CCK (2 mg/kg) was diluted in saline and then injected i.p. Ghrelin (100 ng) and NPY (1.0 μg) were diluted in saline and then injected intracerebroventricularily in a volume of 2.0 μL per injection. Drug doses were based on previous experiments in our laboratory suggesting that these doses produced maximum effects in mice. Drug concentrations were coded to prevent experimenter bias.

2.3. Genotyping mice

Genotyping of NOS-deficient mice was performed by RT-PCR using 5 μg of tail sample DNA in a 50 μL PCR reaction (MasterTaq kit, Eppendorf Scientific, Inc., Westbury, NY). For identification of the wild-type allele, primer sequences used are 5′-tcagatctgatccgaggagg-3′ and 5′-ttccagagcgctgtcatagc-3′, which amplifies a 117 bp product. For identification of the NOS-deficient primers used are 5′-cttgggtggagaggctattc-3′ and 5′-aggtgagatgacaggagatc-3′, which amplifies a 280 bp fragment of the bacterial neomycin resistant gene. The amplification protocol used is similar to that suggested by The Jackson Laboratory (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol&protocol_id=124). PCR reaction product was then loaded onto a 1.5% polyacrylamide gel containing ethidium bromide and visualized under ultraviolet light.

2.4. Surgery and drug administration

Forty-eight hours prior to testing, the mice were anesthetized with 2,2,2-tribromoethanol, placed in a stereotaxic instrument, and the scalp was deflected. A unilateral hole was drilled 0.5 mm posterior to and 1.0 mm to the right of the bregma. The injection depth was 2.0 mm into the lateral ventricle. Fifteen minutes prior to the introduction of food (Rodent Diet 5001), the mice were again placed under light isoflurane 3% inhaled anesthesia, and injected ICV with 2.0 μL of saline with or without drug. The injection was delivered over 30 s through a 30 gauge needle, which was attached to a 10 μL syringe. After ICV injection, the scalp was closed and the mice were returned to their cages.

Table 1
The average body weights at time of experiments, mean daily food intake and amount eaten during the first 60 min after overnight food deprivation. There was no difference in body weight, however when the mice were food deprived overnight and refed the next morning the NOS KO mice ate significantly less than the wild type controls and the NOS hetrozygote mice.

Strain	Mean body weight (g)	Mean daily food intake (g)	1 h intake (g)
Wild type control	28.61 ± 1.05	3.39 ± 0.15	0.94 ± 0.05
NOS heterozygote	28.43 ± 1.36	3.78 ± 0.16	0.98 ± 0.09
NOS knockout (Homozygote)	26.49 ± 1.24	3.26 ± 0.15	0.55 ± 0.05[a]

[a] indicates $P < 0.01$.

2.5. Experimental protocol

Mice were divided into genotype homozyous, heterozygous or wild type. Within each genotype mice were further divided into two groups of 12. The groups within each genotype were given either drug or vehicle. For CCK, mice were placed in separate cages and food deprived overnight. For ghrelin and NPY the night preceding the experiments, the mice were placed in individual housing and given fresh 5001 Rodent Diet to ensure that they were completely satiated at the start of the trial. Mice were given either CCK, ghrelin or NPY. Mice were given the pellet of a known mass of 5001 Rodent Diet, immediately after receiving CCK or 15 min after injection for ghrelin and NPY, which was weighed 60 or 120 min later.

2.6. Statistics

Results were expressed as means with their standard errors. The food intake amounts were analyzed by one-way analysis of variance (ANOVA) for each group followed by Tukey's or Bonferroni's post hoc analysis. The NOS levels were analyzed by *T*-test.

3. Results

3.1. The effect of overnight food deprivation on food intake in NOS KO mice

We examined the effect of overnight food deprivation on food intake during the first hour after refeeding. The two-way ANOVA for food intake produced a significant effect for strain $F(2,27)$ 25.21, $P < 0.0008$ and time $F(1,27) = 32.31$, $P < 0.0001$, but not for interaction $F(2,27) = 0.89$, P NS. Bonferroni post test indicated that the NOS KO mice ate significantly less than the wild type controls at both 30 and 60 min post food induction. There was no difference between wild type controls and the heterozygous KOs.

The ANOVA for average daily food intake by strain was not significant $F(2,18) = 3.13$, PNS. The ANOVA for average body weight by strain was not significant $F(2,21) = 0.97$, P NS. See Table 1 for summary of results.

3.2. The effect of peripheral administration of CCK on food intake

The two-way ANOVA for food intake at 30 min produced a significant effect for dose $F(1,36) = 37.54$, $P < 0.0001$, but not for strain $F(2, 36) = 1.19$, P NS or dose × strain $F(2, 36) = 2.76$, P NS. Post hoc analysis indicated that the wild type mice which received CCK ate significantly less than the wild type mice which received saline. In addition, the heterozygous mice which received CCK ate significantly less than the heterozygous mice which received saline,