Prokineticin 2 Is a Hypothalamic Neuropeptide That Potently Inhibits Food Intake

James V. Gardiner,[1] Attia Bataveljic,[1] Neekhil A. Patel,[1] Gavin A. Bewick,[1] Debabrata Roy,[1] Daniel Campbell,[1] Hannah C. Greenwood,[1] Kevin G. Murphy,[1] Saira Hameed,[1] Preeti H. Jethwa,[2] Francis J.P. Ebling,[2] Steven P. Vickers,[3] Sharon Cheetham,[3] Mohammad A. Ghatei,[1] Stephen R. Bloom,[1] and Waljit S. Dhillo[1]

OBJECTIVE—Prokineticin 2 (PK2) is a hypothalamic neuropeptide expressed in central nervous system areas known to be involved in food intake. We therefore hypothesized that PK2 plays a role in energy homeostasis.

RESEARCH DESIGN AND METHODS—We investigated the effect of nutritional status on hypothalamic PK2 expression and effects of PK2 on the regulation of food intake by intracerebroventricular (ICV) injection of PK2 and anti-PK2 antibody. Subsequently, we investigated the potential mechanism of action by determining sites of neuronal activation after ICV injection of PK2, the hypothalamic site of action of PK2, and interaction between PK2 and other hypothalamic neuropeptides regulating energy homeostasis. To investigate PK2's potential as a therapeutic target, we investigated the effect of chronic administration in lean and obese mice.

RESULTS—Hypothalamic PK2 expression was reduced by fasting. ICV administration of PK2 to rats potently inhibited food intake, whereas anti-PK2 antibody increased food intake, suggesting that PK2 is an anorectic neuropeptide. ICV administration of PK2 increased c-fos expression in proopiomelanocortin neurons of the arcuate nucleus (ARC) of the hypothalamus. In keeping with this, PK2 administration into the ARC reduced food intake and PK2 increased the release of α-melanocyte–stimulating hormone (α-MSH) from ex vivo hypothalamic explants. In addition, ICV coadministration of the α-MSH antagonist agouti-related peptide blocked the anorexigenic effects of PK2. Chronic peripheral administration of PK2 reduced food and body weight in lean and obese mice.

CONCLUSIONS—This is the first report showing that PK2 has a role in appetite regulation and its anorectic effect is mediated partly via the melanocortin system. ***Diabetes*** **59:397–406, 2010**

Prokineticin 2 (PK2) is an 81–amino acid cysteine-rich protein structurally related to prokineticin 1 (PK1), with which it shares 44% sequence homology (1–3). Both bind to two related G-protein–coupled receptors, termed prokineticin receptor 1 (PKR1) and prokineticin receptor 2 (PKR2) (4–6). The prokineticins are so called because the first effect ascribed to them was the stimulation of guinea pig ileum smooth muscle contraction (2). Subsequently, PK2 has been shown to be involved in several developmental and physiological processes (7). It is thought to be critical for the development of the central nervous system (CNS) because mice lacking either PK2 or PKR2 have poorly developed olfactory bulbs (8–10). In addition, these mice have hypogonadotrophic hypogonadism due to abnormal gonadotrophin-releasing hormone neuronal migration (3,11). The same phenotype occurs in humans with mutations of PK2 or PKR2 (11–13).

PK2 is expressed in several regions of the adult brain but is found in highest concentrations in the suprachiasmatic nucleus (SCN), the site of the master circadian oscillator. PK2 expression in the SCN varies with timing of the circadian cycle (14). These data suggest a role for PK2 in the regulation of the circadian clock (15). In accordance with this, mice with targeted deletion of either PK2 or PKR2 exhibit alterations in the circadian control of locomotor activity, thermoregulation, and sleep (16,17). Thus, PK2 may act as an output molecule for the SCN circadian clock (18).

The hypothalamus is important in the regulation of energy homeostasis. Because PK2 receptors are expressed in hypothalamic nuclei known to regulate appetite (19,20), we hypothesized that PK2 may play a role in the control of appetite regulation. Indeed, intracerebroventricular (ICV) administration of an amphibian homologue of PK2 (Bv8) reduces food intake in rats (19). However, there are currently no reports of the effects of PK2 on appetite. We therefore investigated the role of PK2 in the control of energy homeostasis. Our data suggest that PK2 is a novel hypothalamic regulator of food intake.

RESEARCH DESIGN AND METHODS

Materials. PK2 was purchased from Peprotech (London, U.K.), PK2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and control IgG was generated from a random sequence peptide epitope (21).

Wistar rats and C57BL/6 mice. Adult male Wistar rats weighing 200–250 g and adult male C57BL/6 mice weighing 20–25 g (Harlan, Bicester, U.K.) were maintained in individual cages (width 24.5 cm, length 41.5 cm, and depth 18.5 cm) at 21–23°C with a 12-h light/dark cycle with ad libitum access to food (RM1 diet; SDS, Witham, U.K.) and water unless specified in procedure protocol.

Diet-induced obese mice. Studies were performed in C57BL/6 mice when they had developed diet-induced obesity and their body weight was stable (see supplementary Methods 1, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1198/DC1). Animal studies were approved under British Home Office Scientific Procedures Act 1986.

Effect of nutritional status on hypothalamic PK2 mRNA expression rats. Three groups of rats (ad libitum fed, fasted for 12 or 24 h, $n = 24$) were killed at the beginning of the light phase. Whole hypothalami were dissected and total RNA was extracted using an RNAqueous-4PCR kit (Applied Biosystems, Austin, TX) according to the manufacturer's protocol. cDNA was synthesized from 0.5 μg of RNA using a High Capacity cDNA Reverse

From the [1]Department of Investigative Medicine, Imperial College London, London, U.K.; the [2]School of Biomedical Sciences, University of Nottingham, Nottingham, U.K.; and [3]RenaSci Consultancy Ltd, Nottingham, U.K.
Corresponding author: Stephen R. Bloom, s.bloom@imperial.ac.uk.
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J.V.G., A.B., and N.A.P. contributed equally to this study.

Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative real-time PCR was performed in triplicate using the following primer sets: 18S RNA primer assay ID:4310893E, PK2 primer assay ID:Rn00593837_m1.

Effect of ICV administration of PK2 on food intake. ICV cannulation was performed as previously described (22). Male rats (n = 10–12/group) were injected intracerebroventricularly with saline or PK2 at doses of 0.005, 0.015, 0.05, and 0.15 nmol/rat at the beginning of the dark phase. All rats were injected within a single 20-min period with individual times of injection noted. The subsequent measurements of food intake are relative to the recorded injection time. Food intake was measured 1, 2, 4, 8, and 24 h after injection for these studies.

In a second study, rats (n = 10–12/group) were injected intracerebroventricularly with saline or PK2 at doses of 0.15, 0.5, and 1.5 nmol/rat at the beginning of the dark phase. To determine whether PK2 has anorectic effects in animals refeeding after a fast, rats (n = 10–12/group) fasted for 24 h were injected intracerebroventricularly in the early light phase with saline or PK2 at doses of 0.15, 1.5, or 4.5 nmol/rat. Food intake was measured 1, 2, 4, 8, and 24 h after injection for these studies.

Effect of ICV administration of PK2 on locomotor activity. Rats (n = 10–12/group) were injected intracerebroventricularly with saline or PK2 1.5 nmol/rat at the beginning of the dark phase. This dose of PK2 was used because it potently reduced food intake. The ambulatory activity of each animal was measured simultaneously using the optical beam technique (Opto M3; Columbus Instruments, Columbus, OH) (supplementary Methods 2).

Effect of ICV administration of PK2 on behavior. Adult male Wistar rats weighing 200–250 g (n = 10–12/group) were injected intracerebroventricularly with saline or PK2 1.5 nmol/rat and behavioral patterns monitored continuously for 120 min after injection. Behavior was classified into eight categories: feeding, drinking, grooming, burrowing, rearing, locomotion, head down, and sleeping, as previously described (23). Abnormal behavior was defined by a significant increase in locomotor activity, rearing, head down or burrowing, or reduced sleeping or grooming, as previously described (23,24).

Effect of ICV administration of PK2 on energy expenditure. Rats (n = 10–12/group) were injected intracerebroventricularly with saline or PK2 1.5 nmol/rat at the beginning of the dark phase and food was removed after injection but water was available ad libitum. Oxygen consumption was measured by indirect calorimetry using an open-circuit Oxymax system of the Comprehensive Lab Animal Monitoring System (Columbus Instruments) (25).

Effect of ICV administration of anti-PK2 antibody on food intake. Rats (n = 10–12/group) were injected intracerebroventricularly with either control IgG or anti-PK2 antibody (10 or 30 pmol) in the early light phase. Food intake was measured 1, 2, 4, 8, and 24 h after injection.

Effect of ICV administration of PK2 on c-fos expression. Rats were cannulated into the lateral ventricle as previously described (26). PK2 (1.5 nmol/rat) or saline (n = 4/group) was injected into the lateral ventricle of ad libitum–fed rats over 1 min in the early light phase. Immunocytochemistry (ICC) for c-fos was performed on brain sections from animals as previously described (27). Total numbers of c-fos–positive cells were counted bilaterally in matched sections from hypothalamic nuclei.

Effect of intranuclear administration of PK2 on food intake. Rats (200–250 g) had permanent indwelling, unilateral, 26-gauge stainless steel guide cannulae (Plastics One, Roanoke, VA) stereotactically implanted into the supraoptic nucleus (SON), arcuate nucleus (ARC), paraventricular nucleus (PVN), anterior hypothalamic area (AHA), ventromedial hypothalamus (VMH), dorsomedial nucleus (DMN), SCN, and lateral hypothalamic area (LHA) of the hypothalamus (coordinates listed in supplementary Table 1) (n = 16/nucleus), as previously described (28). The study was of a randomized crossover design. Each animal received both injections (saline or 0.025 nmol PK2) in a random order 4 days apart. Food intake was measured 1, 2, 4, 8, and 24 h after injection. The dose of PK2 was chosen based on previous studies that show that 10% of the effective ICV dose results in significant effects when directly administered into responsive hypothalamic nuclei and minimizes nonspecific activation (28). Cannula placement was determined at the end of the study by injection of Indian ink (28).

Effect of PK2 on the release of neuropeptides known to affect appetite. Adult male Wistar rats weighing 200–250 g were killed by decapitation and hypothalamic explants prepared as previously described (29). The hypothalami were incubated for 45 min in 600 μl artificial cerebrospinal fluid (aCSF; basal period). The tissues were then exposed to PK2 (10, 100, or 1,000 nmol/l) in 600 μl aCSF for 45 min (n = 9–12/treatment). Finally, the viability of the tissue was verified by a 45-min exposure to 56 mmol/l KCl. α-Melanocyte–stimulating hormone (α-MSH), cocaine and amphetamine–regulated transcript, thyrotropin-releasing hormone, corticotrophin-releasing hormone, neuropeptide Y, and agouti-related peptide (AgRP) in the aCSF were measured using established radioimmunoassay (22,30–34).

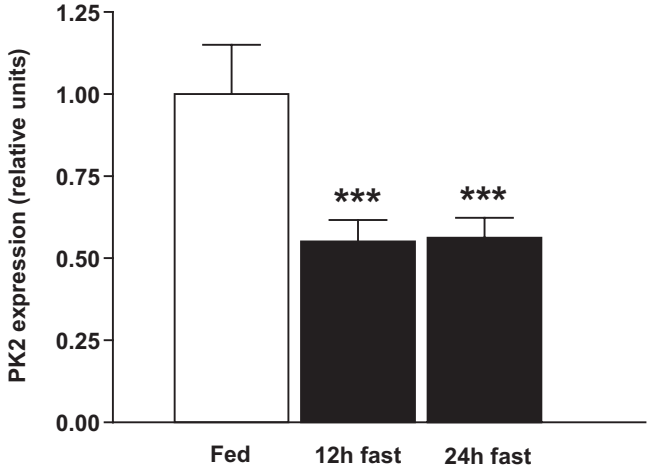

FIG. 1. Fasting reduces hypothalamic expression of PK2. Hypothalamic expression of PK2 mRNA among ad libitum–fed, 12-h fasted, and 24-h fasted rats (n = 24 per group) is shown. Results are expressed as mean ± SEM. ***P < 0.001 versus fed group.

Effect of melanocortin receptor antagonism on the anorectic effects of PK2. Rats (n = 10–12/group) were intracerebroventricularly injected with either *1*) saline, *2*) AgRP (1 nmol/rat), *3*) α-MSH (1 nmol/rat), *4*) α-MSH (1 nmol/rat) and AgRP (1 nmol/rat), *5*) PK2 (0.15 nmol/rat), or *6*) PK2 (0.15 nmol/rat) and AgRP (1 nmol/rat) at the beginning of the dark phase. The doses of α-MSH and AgRP were chosen based on previous studies (35). Food intake was measured 1, 2, 4, 8, and 24 h after injection.

Effect of ICV administration of PK2 on c-fos expression in arcuate proopiomelanocortin neurons. Animals (n = 5 per group) were injected into the lateral ventricle with 1.5 nmol PK2. In situ hybridization (ISH) for proopiomelanocortin (POMC) and ICC for c-fos were performed on sections including the ARC, as previously described (36,37). A riboprobe corresponding to nucleotides 307–795 of the POMC rat sequence (accession no. NM_139326) was used for ISH. Total numbers of positive cells per animal were counted from matched sections of the ARC, and colocalized cells were expressed as a percentage of the total number of POMC and c-fos neurons.

Effect of acute peripheral administration of PK2 on food intake in lean rats and mice. Rats (n = 10–12/group) were injected intraperitoneally with saline or PK2 at doses of 2.3, 7, or 20 nmol/kg at the beginning of the dark phase. Food intake was measured 1, 2, 4, 8, and 24 h after injection. Due to the limited availability of recombinant PK2, the effects of peripheral administration of PK2 on food intake were further characterized in mice. A similar study was conducted in groups of C57BL/6 mice (n = 10–12/group) injected with saline or PK2 at doses of 7, 20, 60, 180, or 540 nmol/kg.

Effect of chronic peripheral administration of PK2 on food intake and body weight in lean and diet-induced obese mice. Adult male C57BL/6 mice weighing 20–25 g (n = 10/group) were given twice daily intraperitoneal injections (early light phase and just prior to the dark phase) of either saline or PK2 180 nmol/kg for 5 days. Food intake was measured 4 h after the injection at the beginning of the light phase and 1 h after the injection just prior to the dark phase. Daily food intake and body weight were also measured.

A similar study was carried out in C57BL/6 diet-induced obese (DIO) mice that were randomized to *1*) saline treatment with ad libitum access to food, *2*) PK2 treatment (540 nmol/kg per injection) with ad libitum access to food, or *3*) pair-fed group: saline treatment but food restricted to the daily median food consumed by the PK2-treated mice over the previous 24-h period (n = 10/group).

Statistical analysis. Data are shown as mean values ± SEM except c-fos and behavioral analysis data, which are presented as median and interquartile range. The studies of food intake and hypothalamic PK2 expression were analyzed using a one-way ANOVA, followed by post hoc Dunnett test except for the intranuclear food intake study, which was analyzed using the Holm Bonferroni test. Food intake data expressed as a change compared with saline-treated animals were analyzed using a one-way ANOVA, followed by post hoc least significant difference test. For the c-fos immunocytochemistry and dual ISH/ICC studies, the Mann-Whitney U test was used. Data from behavioral studies were compared using Kruskal-Wallis one-way ANOVA on ranks. For the CLAMS (Comprehensive Laboratory Animal Monitoring System) studies, the generalized estimating equation and the Mann-Whitney U test were used. In all cases, $P < 0.05$ was considered statistically significant.

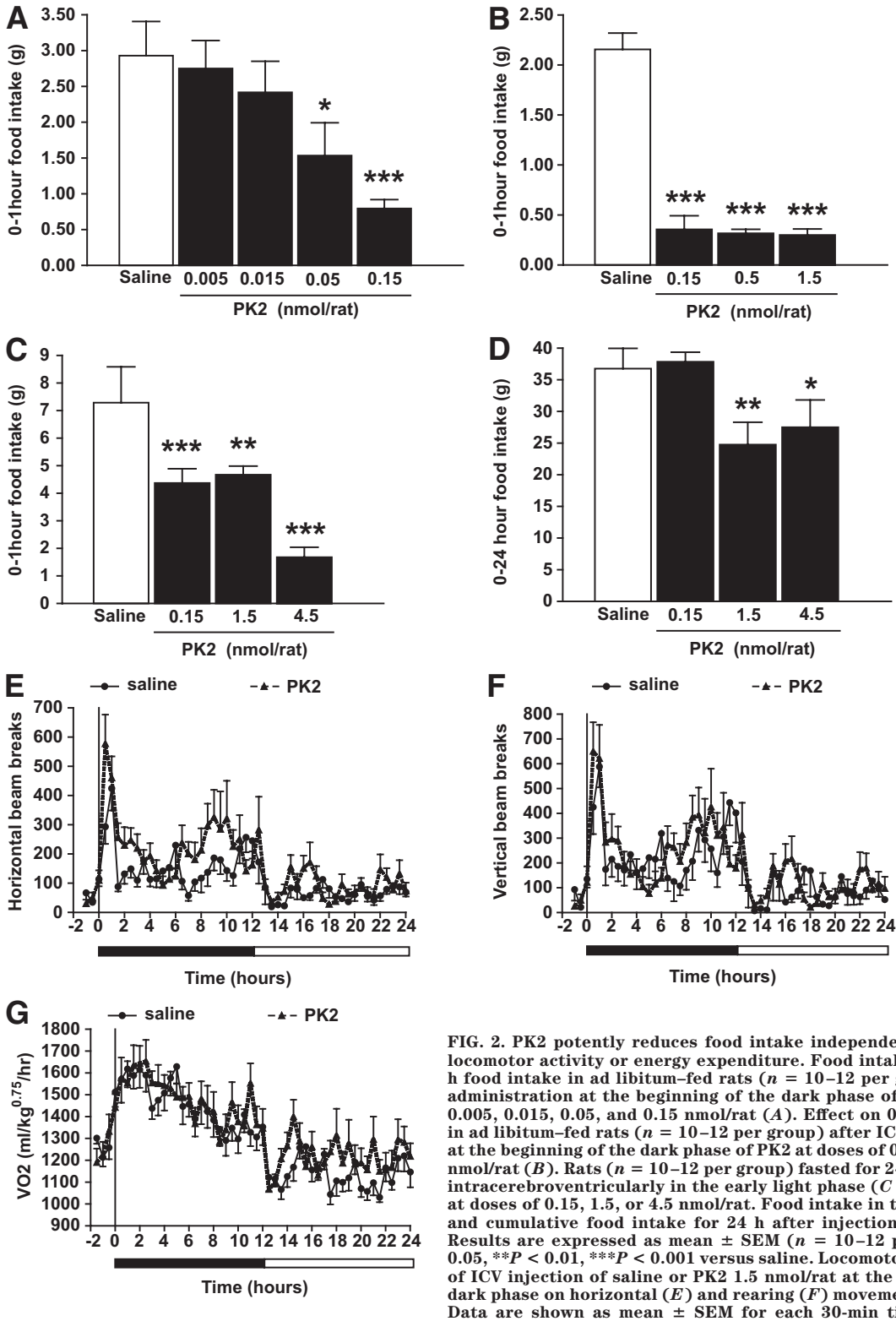

FIG. 2. PK2 potently reduces food intake independent of changes in locomotor activity or energy expenditure. Food intake: Effect on 0–1 h food intake in ad libitum–fed rats (n = 10–12 per group) after ICV administration at the beginning of the dark phase of PK2 at doses of 0.005, 0.015, 0.05, and 0.15 nmol/rat (*A*). Effect on 0–1 h food intake in ad libitum–fed rats (n = 10–12 per group) after ICV administration at the beginning of the dark phase of PK2 at doses of 0.15, 0.50, and 1.5 nmol/rat (*B*). Rats (n = 10–12 per group) fasted for 24 h were injected intracerebroventricularly in the early light phase (*C* and *D*) with PK2 at doses of 0.15, 1.5, or 4.5 nmol/rat. Food intake in the first hour (*C*) and cumulative food intake for 24 h after injection are shown (*D*). Results are expressed as mean ± SEM (n = 10–12 per group). *P < 0.05, **P < 0.01, ***P < 0.001 versus saline. Locomotor activity: Effect of ICV injection of saline or PK2 1.5 nmol/rat at the beginning of the dark phase on horizontal (*E*) and rearing (*F*) movement, respectively. Data are shown as mean ± SEM for each 30-min time period (n = 10–12 per group). Horizontal black bar under the x-axis indicates dark phase and open bar indicates light phase. Energy expenditure: Effect of ICV injection of saline or PK2 1.5 nmol/rat on oxygen consumption (*G*). Horizontal black bar under the x-axis indicates dark phase and open bar indicates light phase.

RESULTS

Expression of PK2 is reduced during fasting. The expression levels of hypothalamic neuropeptides that reduce food intake (e.g., α-MSH) are often elevated in states of positive energy balance and reduced in states of negative energy balance (38). Hypothalamic PK2 expression was significantly reduced by 45% in rats fasted for 12 or 24 h (Fig. 1). This is consistent with the hypothesis that PK2 is an endogenous anorectic hypothalamic neuropeptide.

PK2 reduces food intake without altering locomotor activity or energy expenditure. ICV administration of PK2 caused a dose-dependent significant reduction in food

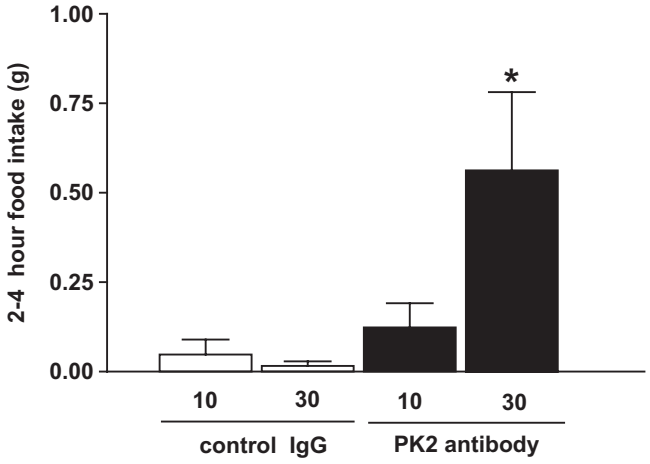

FIG. 3. Immunoblockade of endogenous PK2 increases food intake. The effect on 2–4 h food intake of ICV administration of control IgG or anti-PK2 antibody (10 or 30 pmol) to satiated rats (n = 10–12 per group) at the beginning of the light phase. Results are expressed as mean ± SEM. *P < 0.05 versus 30 pmol control IgG group.

intake that, at doses greater than 0.15 nmol/rat, produced an 85% reduction in food consumed in the first hour after injection (Fig. 2*A* and *B*, supplementary Fig. 1, and supplementary Table 2*A* and *B*). When administered intracerebroventricularly in the early light phase to fasted rats, PK2 caused a similarly potent inhibition of food intake (Fig. 2*C* and supplementary Table 2*C*). In addition, rats injected with 1.5 and 4.5 nmol PK2 had a 30% reduction in 24-h food intake compared with saline-injected rats (Fig. 2*D* and supplementary Table 2*C*).

A reduction in food intake can be due to an indirect effect, for example, changes in locomotor activity or behavior (39). ICV administration of PK2 to ad libitum–fed rats did not significantly alter locomotor activity or result in abnormal behavior compared with saline-injected animals (Fig. 2*E* and *F* and supplementary Table 3). However, consistent with an increase in satiety, ICV PK2 significantly reduced the number of feeding episodes (supplementary Table 3). Many regulators of food intake also regulate energy expenditure. However, this does not appear to be the case for PK2, because ICV administration of PK2 did not alter oxygen consumption, a surrogate for energy expenditure (Fig. 2*G*). These data suggest that ICV administration of PK2 specifically reduces food intake for up to 24 h.

Immunoblockade of endogenous hypothalamic PK2 increases food intake. Rats injected with anti-PK2 antibody ate significantly more than rats injected with control IgG antibody, suggesting that endogenous PK2 may restrain appetite (Fig. 3 and supplementary Table 4).

PK2 reduces food intake via specific hypothalamic nuclei. ICV administration of PK2 resulted in a significant increase in c-fos immunoreactivity in the SON, ARC, PVN, and AHA (Fig. 4*A*–*F*). No significant changes in c-fos expression were observed in the VMH, DMN, SCN, or LHA (Fig. 4*A* and supplementary Fig. 2).

To establish which hypothalamic nuclei mediate PK2's anorectic effects, PK2 was administered into the hypothalamic nuclei showing c-fos activation after ICV administration of PK2. In addition, other nuclei expressing PKR2 were also injected with PK2 as negative controls. PK2 significantly reduced 0–1 h food intake in rats after administration into the SON, ARC, PVN, and AHA (Fig. 4*G*), but there was no significant effect of PK2 after injection into the VMH, DMN, SCN, or LHA (Fig. 4*G*).

PK2 mediates part of its anorectic effects via the melanocortin system. PK2 significantly stimulated the release of α-MSH (Fig. 5*A*) but did not alter the release of the other hypothalamic neuropeptides measured (supplementary Table 5).

We therefore hypothesized that ICV PK2 may mediate part of its anorectic effect via the melanocortin system. To further investigate the relationship between PK2 and the melanocortin system in vivo, we coadministered AgRP with PK2. In this paradigm, AgRP attenuated the effect of PK2, suggesting that PK2 may reduce food intake in part via the melanocortin system (Fig. 5*B*). To confirm that the dose of AgRP used was antagonizing α-MSH, we demonstrated that ICV administration of α-MSH reduced 0–2 h food intake in rats, whereas coadministration of AgRP with the same dose of α-MSH abolished the anorectic effect of α-MSH (Fig. 5*B*).

To determine whether ICV administration of PK2 resulted in activation of ARC POMC neurons (which produce α-MSH), we performed colocalization studies of c-fos and POMC after ICV administration of PK2. ICV administration of PK2 significantly increased the number of arcuate POMC-expressing neurons exhibiting c-fos immunoreactivity (Fig. 5*C* and Table 1). Together these data suggest that PK2 may mediate part of its anorectic effect via the ARC melanocortin system.

Peripheral administration of PK2 acutely reduces food intake in rats and C57BL/6 mice

Rats. A single intraperitoneal injection of PK2 (20 nmol/kg) reduced food intake by 45% in the first hour after injection but did not affect food intake at the other time points studied (Fig. 6*A* and supplementary Table 6*A*).

C57BL/6 mice. Intraperitoneal injection of PK2 in mice reduced 0–1 h food intake at similar doses to those in rats (Fig. 6*B*). Higher doses of PK2 in mice resulted in a further dose-dependent reduction in food intake for up to 24 h after injection (Fig. 6*B* and supplementary Table 6*B*). The highest dose of PK2 (540 nmol/kg) administered produced a 20% reduction in 24-h food intake (Fig. 6*C* and supplementary Table 6*B*).

Chronic peripheral administration of PK2 decreases food intake and body weight in lean and obese mice

Lean mice. Twice daily intraperitoneal injection of PK2 for 5 days in lean mice significantly decreased cumulative food intake (Fig. 7*A*) and body weight compared with saline-injected controls (Fig. 7*B*). The reduction in food intake after each single injection of PK2 was similar in magnitude throughout the study period, suggesting that PK2 remained equally potent after recurrent administration (supplementary Table 7).

DIO mice. DIO mice are commonly used as a rodent model of human obesity (40). Some anorectic factors, for example leptin, are ineffective in obese mice (41). To investigate whether DIO mice were sensitive to PK2, we administered PK2 twice daily by intraperitoneal injection for 5 days to DIO mice. PK2 significantly reduced cumulative food intake (Fig. 7*C*) and body weight (Fig. 7*D*) compared with saline-injected controls. DIO mice pair fed to the PK2-treated group lost a similar amount of body weight as the mice given PK2 (Fig. 7*D*), suggesting the effect of PK2 on body weight was mediated predominantly via a reduction in food intake. As in lean mice, injection of PK2 was equally potent at reducing food intake on each of the 5 days of the study (supplementary Table 8). These

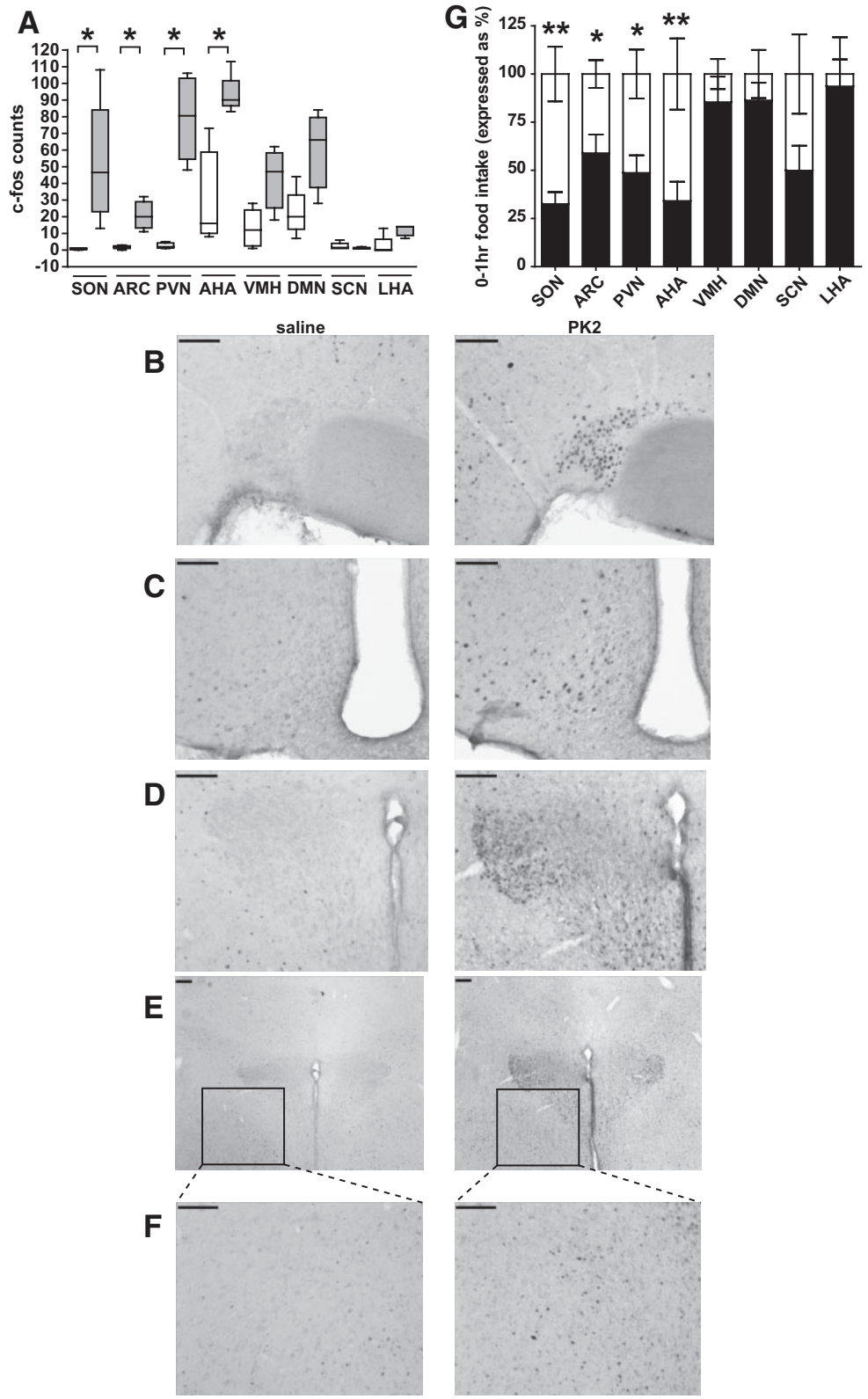

FIG. 4. PK2 mediates its effects via specific hypothalamic nuclei. *A*: Graphical representation of c-fos activation in hypothalamic nuclei of rats after administration of saline or PK2 (1.5 nmol/rat) into the lateral ventricle. Open bars represent saline-injected animals; filled gray bars, PK2-injected animals. Data are shown as median and interquartile range. SON, supraoptic nucleus; ARC, arcuate nucleus; PVN, paraventricular nucleus; AHA, anterior hypothalamic area; SCN, suprachiasmatic nucleus; VMH, ventromedial hypothalamus; LHA, lateral hypothalamic area; DMN, dorsomedial nucleus. *$P < 0.05$ versus saline. *B–F*: Representative brain sections showing c-fos expression in the SON (*B*), ARC (*C*), PVN (*D*), and AHA (*E* and *F*) of rats injected into the lateral ventricle with saline or PK2 (1.5 nmol/rat). Scale bar, 100 μm. Brain sections from rats injected with saline are shown in the panels on the left and those from rats injected with PK2, in the panels on the right. Representative brain sections showing c-fos expression in the VMH, DMN, SCN, and LHA are shown in supplementary Fig. 1. *G*: Effects on food intake of saline or PK2 (0.025 nmol/rat) injection into specific hypothalamic nuclei at the beginning of the dark phase into rats. Food intake consumed in the first hour after PK2 injection (black bar) is shown as mean ± SEM as a percentage of food intake consumed in the first hour after saline injection (white bar) for each nucleus. *$P < 0.05$, **$P < 0.01$ versus saline.

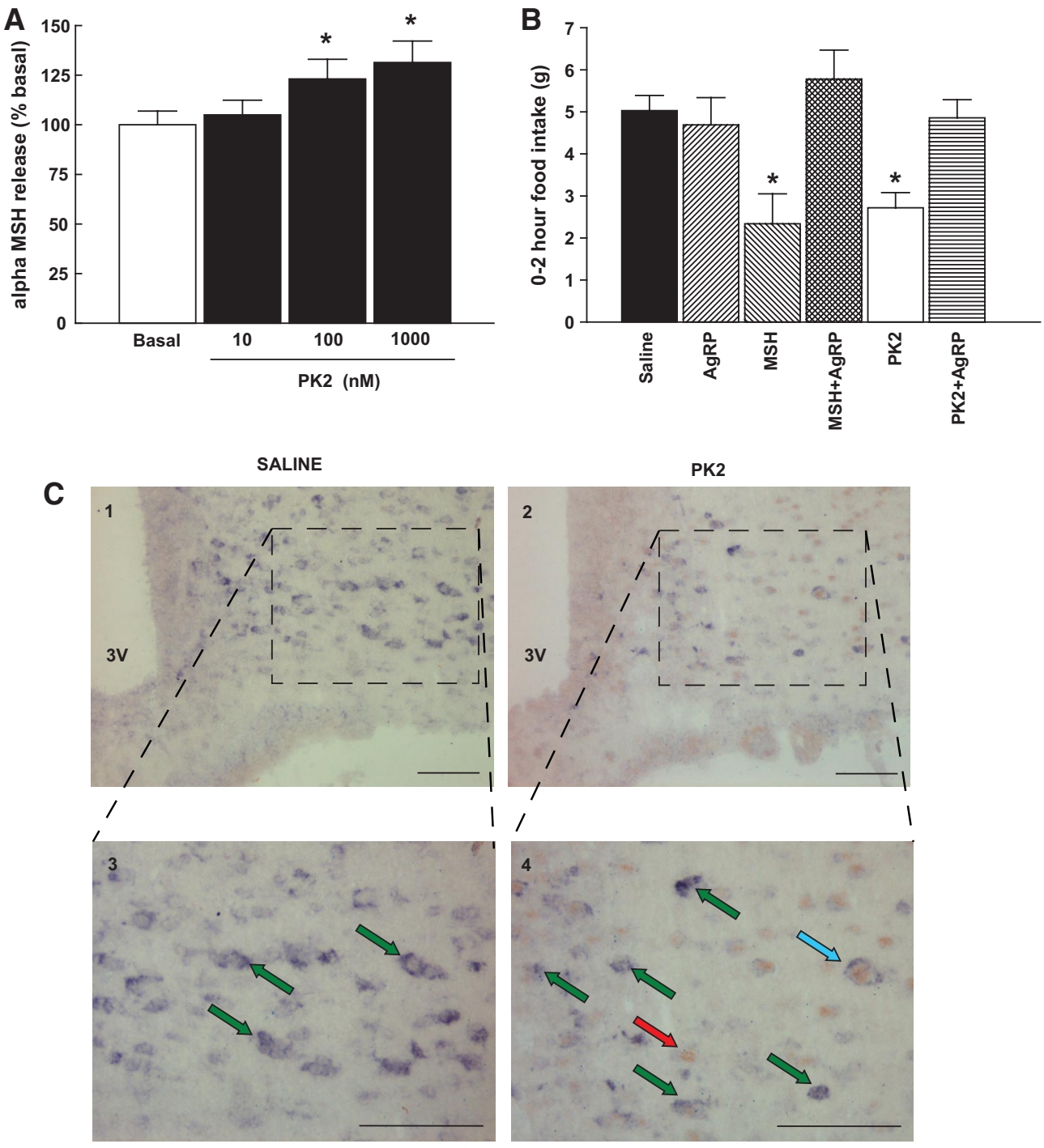

FIG. 5. PK2 mediates part of its anorectic effects via the melanocortin system. *A*: Effect of PK2 on α-MSH release from hypothalamic explants. Peptide release is expressed as percentage of basal (n = 9–12 per treatment). *P < 0.05 versus basal. *B*: Effects of melanocortin receptor antagonism on anorectic effects of PK2. Food intake in the 0–2 h after injection is shown. Results are expressed as mean ± SEM. *P < 0.05 versus saline. *C*: Effect of ICV administration of PK2 on c-fos expression in arcuate nucleus POMC neurons. Arcuate nucleus sections from animals injected with saline (1 and 3) or PK2 (2 and 4) are shown. The green arrows indicate cells expressing only POMC mRNA; the red arrows indicate cells expressing only c-fos; dual-labeled cells are indicated by a blue arrow. 3V, third cerebral ventricle; scale bars, 100 μm (1 and 2 are shown at ×10 magnification; 3 and 4 are shown at ×20 magnification). (A high-quality digital representation of this figure is available in the online issue.)

data suggest that repeated administration of PK2 results in reduced food intake and body weight without tachyphylaxis in lean and obese mice.

DISCUSSION

The hypothalamus is a key center of the brain involved in appetite regulation. PK2 and its receptors are expressed in hypothalamic nuclei known to affect food intake, but the effect of PK2 on food intake has not previously been reported. Our data suggest that PK2 is a novel hypothalamic anorectic neuropeptide. It was important to determine whether the anorectic effects of PK2 were due to behavioral changes because PK2- or PKR2-null mice display abnormal circadian rhythms and locomotor activity

TABLE 1
Expression of c-fos in arcuate POMC neurons after ICV administration of PK2

Treatment	Total POMC cell counts	Total c-fos cell counts	Colocalized POMC/c-fos cell counts	% POMC cells colocalized with c-fos	% c-fos cells colocalized with POMC
Saline	425 (424–435)	12 (7–18)	5 (4–7)	1.2 ± 0.3	48.1 ± 5.5
PK2	440 (412–446)	51 (26–65)*	32 (16–33)†	7.0 ± 1.4	64.0 ± 6.6

Data are median (interquartile range) from matched sections throughout the ARC. Colocalized labeling is expressed as a percentage as mean ± SE. *$P < 0.05$ versus saline; †$P < 0.01$ versus saline; $n = 5$ per group.

(16–18). ICV administration of PK2 potently inhibited food intake in rats up to 24 h after injection, but did not alter locomotor activity or cause abnormal behaviors. These data suggest the anorectic effects of PK2 are not secondary to effects on locomotor activity or other behavioral abnormalities.

To investigate the possibility that endogenous PK2 may affect appetite, we determined the effect of immunoblockade of endogenous hypothalamic PK2 in rats. ICV administration of PK2 antibody in the early light phase (when endogenous CNS PK2 levels are highest [14]) increased food intake in rats, suggesting that elevated CNS PK2 signaling may inhibit food intake in the light phase. However, this finding needs to be interpreted with caution because the increase in food intake after ICV administration of PK2 antibody was small. Conversely, CNS PK2 expression is at its lowest in the dark phase, and this reduction in PK2 inhibitory tone may therefore contribute to the nocturnal increase in food intake. Our results also show that hypothalamic PK2 mRNA expression was lower in fasted rats compared with ad libitum–fed rats, supporting the hypothesis that PK2 may act as an endogenous anorectic signal.

If PK2 is an endogenous inhibitor of food intake, one might expect PK2-null mice to be obese. Mice lacking PK2 have not been reported to have increased body weight compared with their wild-type littermates (8,16). However, PK2 is critical in CNS development and these mice have reduced voluntary and spontaneous locomotor activities, show a reduction in the time spent sleeping compared with wild-type mice (16), and have reduced fertility (11). These factors are likely to confound the effects of the lack of PK2 signaling on energy homeostasis in this mouse model. In addition, it is known that developmental compensation in embryonic knockout models of appetite-regulating factors can mask roles in energy homeostasis, for example, as has been suggested to occur with neuropeptide Y and AgRP (42). A postembryonic or conditional knockout model of PK2, or animal studies in which local hypothalamic PK2 expression is reduced, may help

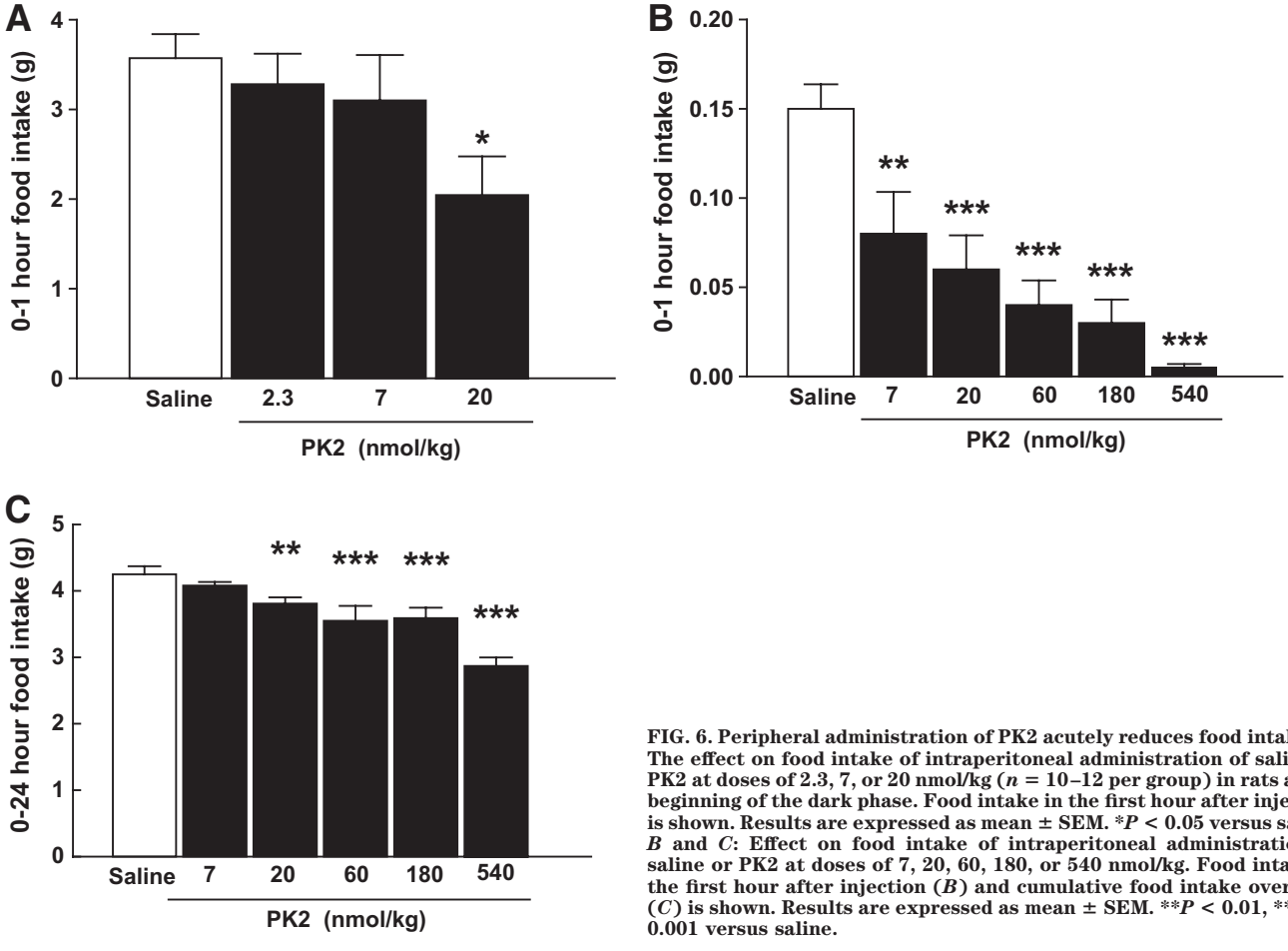

FIG. 6. Peripheral administration of PK2 acutely reduces food intake. *A*: The effect on food intake of intraperitoneal administration of saline or PK2 at doses of 2.3, 7, or 20 nmol/kg ($n = 10–12$ per group) in rats at the beginning of the dark phase. Food intake in the first hour after injection is shown. Results are expressed as mean ± SEM. *$P < 0.05$ versus saline. *B* and *C*: Effect on food intake of intraperitoneal administration of saline or PK2 at doses of 7, 20, 60, 180, or 540 nmol/kg. Food intake in the first hour after injection (*B*) and cumulative food intake over 24 h (*C*) is shown. Results are expressed as mean ± SEM. **$P < 0.01$, ***$P < 0.001$ versus saline.

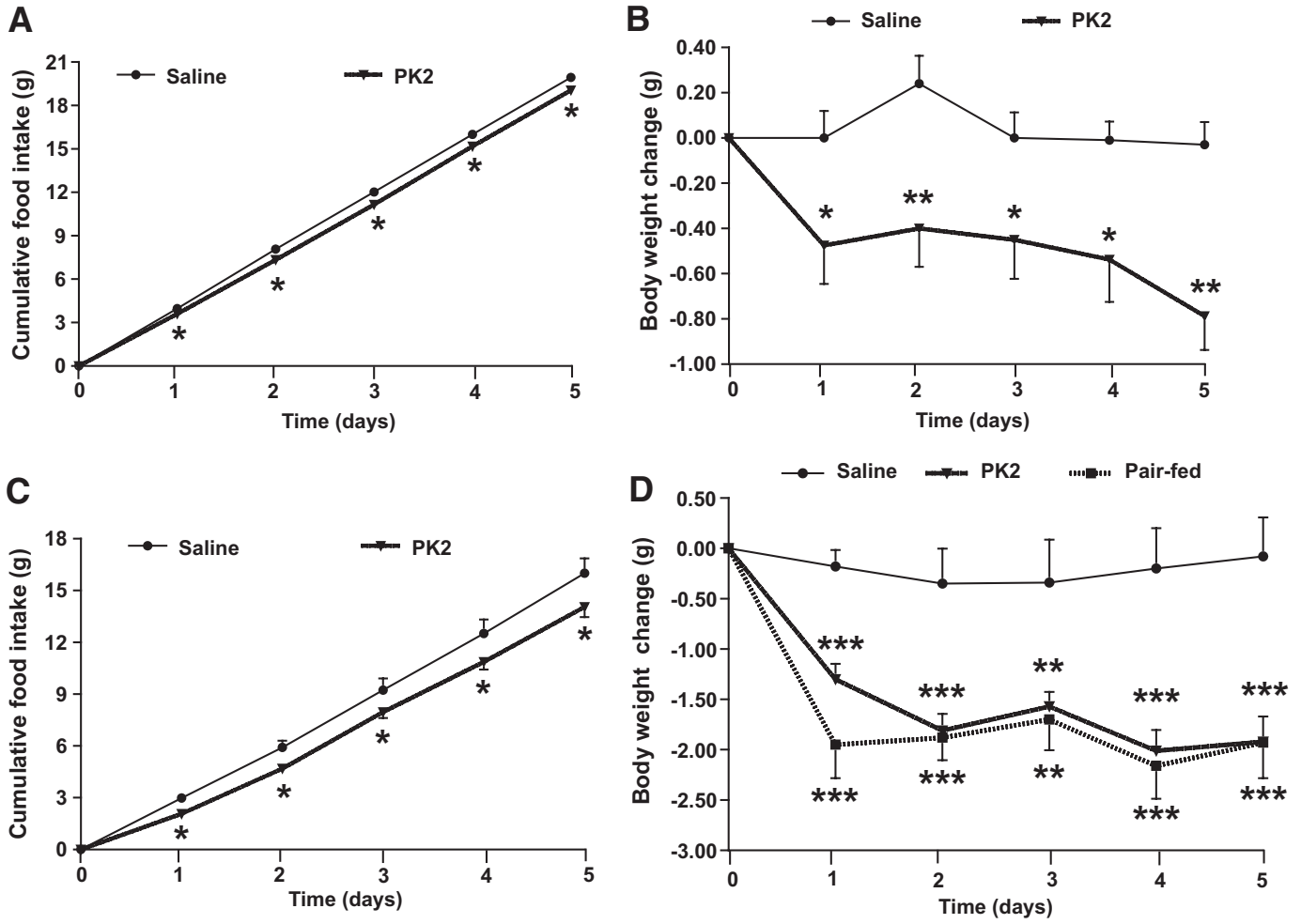

FIG. 7. Chronic peripheral administration of PK2 decreases body weight in lean and obese mice. *A* and *B*: Cumulative food intake (*A*) and change in body weight (*B*) of C57BL/6 mice (n = 10 per group) intraperitoneally injected twice daily for 5 days with either saline or PK2 (180 nmol/kg). *C* and *D*: Effects of intraperitoneal injection of saline or PK2 (540 nmol/kg per injection) twice daily for 5 days to C57BL/6 DIO mice. Cumulative food intake of the mice injected with saline or PK2 throughout the study is shown in *C*. The food intake of the pair-fed group was restricted to the median food intake consumed by the PK2-treated mice over the previous 24-h period. Change in body weight of the saline-treated, pair-fed, and PK2-treated mice throughout the study is shown in *D*. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus saline.

to further determine the role of endogenous PK2 in appetite regulation.

To investigate the hypothalamic sites that may mediate the anorectic effects of PK2, we used the induction of c-fos as a marker of neuronal activation (43). ICV administration of PK2 resulted in c-fos induction in the SON, ARC, PVN, and AHA, and direct injection of PK2 into each of these nuclei reduced food intake, suggesting that the anorectic effects of PK2 may be mediated directly via these nuclei. After ICV administration of PK2 and direct injection of PK2 into the VMH, DMN, SCN, or LHA, which all express prokineticin receptors, there was no induction of c-fos and PK2 did not significantly affect food intake. These data suggest that the anorectic effects of PK2 may be mediated via specific hypothalamic pathways.

ICV administration of PK2 induced c-fos expression in the ARC and direct injection of PK2 into the ARC reduced food intake, suggesting that ICV PK2 may mediate part of its anorectic effect via the ARC. PK2 may therefore mediate its anorectic effects via alteration in hypothalamic ARC neuropeptides. Our results show that PK2 increased the release of α-MSH from hypothalamic explants ex vivo and ICV coadministration of AgRP with PK2 attenuated the anorectic effects of PK2. Consistent with this, ICV administration of PK2 resulted in c-fos activation in ARC POMC neurons that produce α-MSH. Together these data suggest that PK2 may mediate part of its anorectic effect via the hypothalamic ARC melanocortin system.

To investigate whether peripheral administration of PK2 had anorectic effects, we investigated the effects of intraperitoneal administration of PK2 on food intake and body weight in rodents. Peripheral administration of PK2 acutely reduced food intake with similar efficacy in rats and mice. This led us to investigate whether repeated PK2 administration would result in a sustained reduction in food intake and body weight, since repeated administration of anorectic agents can cause tachyphylaxis (44), resulting in an attenuated effect after repeated administration. PK2 administration to lean or obese mice caused a similar reduction in food intake after each injection and resulted in a significant reduction in body weight, suggesting that tachyphylaxis to the anorectic effects of PK2 does not occur using this administration protocol.

Leptin reduces food intake and body weight in lean animals but is ineffective in obese animals (41). This may be due to differences between the appetite circuits of lean and obese animals (45) and/or the development of resistance to leptin in obese animals (46). We therefore inves-

tigated the effect of repeated intraperitoneal PK2 administration in DIO mice, which are commonly used as a model of human obesity (40). PK2 resulted in a similar reduction in food intake after each injection, and reduced cumulative food intake and body weight in DIO mice. Higher doses of PK2 were required to reduce food intake in DIO mice than in lean mice, but this resulted in greater weight loss in DIO mice compared with lean mice.

In both lean and DIO mice, the effect of PK2 on body weight is likely to be mediated predominantly via a reduction in food intake because control mice pair fed to PK2-injected mice lost a similar amount of weight as the PK2-injected group. This is consistent with our studies in rats in which ICV administration of PK2 reduced food intake without affecting energy expenditure. In addition, it is unlikely that this effect is due to an acute reduction in water intake because the prokineticin receptor agonist Bv8 has actually been shown to slightly increase, rather than reduce, water intake (19). This phenomenon of a rapid weight loss on the first day is widely observed in investigations of anorectic agents. The rapid decrease is thought to occur as a result of the rapid utilization of glycogen stores, and is especially true in rodents. The decrease is followed by a period in which fat is lost, coinciding with the less rapid weight loss (47,48).

In summary, our data identify a novel role for PK2 in appetite regulation. The anorectic effects of PK2 appear to be mediated in part via the ARC melanocortin system. Repeated peripheral administration of PK2 for 5 days reduced food intake and body weight in obese mice. Further studies investigating the effects of longer term administration of PK2 on food intake and body weight will determine the potential of PK2 as a target for the development of antiobesity agents.

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REFERENCES

1. Wechselberger C, Puglisi R, Engel E, Lepperdinger G, Boitani C, Kreil G. The mammalian homologues of frog Bv8 are mainly expressed in spermatocytes. FEBS Lett 1999;462:177–181
2. Li M, Bullock CM, Knauer DJ, Ehlert FJ, Zhou QY. Identification of two prokineticin cDNAs: recombinant proteins potently contract gastrointestinal smooth muscle. Mol Pharmacol 2001;59:692–698
3. LeCouter J, Kowalski J, Foster J, Hass P, Zhang Z, Dillard-Telm L, Frantz G, Rangell L, DeGuzman L, Keller GA, Peale F, Gurney A, Hillan KJ, Ferrara N. Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature 2001;412:877–884
4. Lin DC, Bullock CM, Ehlert FJ, Chen JL, Tian H, Zhou QY. Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticins/endocrine gland vascular endothelial growth factor. J Biol Chem 2002;277:19276–19280
5. Masuda Y, Takatsu Y, Terao Y, Kumano S, Ishibashi Y, Suenaga M, Abe M, Fukusumi S, Watanabe T, Shintani Y, Yamada T, Hinuma S, Inatomi N, Ohtaki T, Onda H, Fujino M. Isolation and identification of EG-VEGF/prokineticins as cognate ligands for two orphan G-protein-coupled receptors. Biochem Biophys Res Commun 2002;293:396–402
6. Soga T, Matsumoto S, Oda T, Saito T, Hiyama H, Takasaki J, Kamohara M, Ohishi T, Matsushime H, Furuichi K. Molecular cloning and characterization of prokineticin receptors. Biochim Biophys Acta 2002;1579:173–179
7. Ngan ES, Tam PK. Prokineticin-signaling pathway. Int J Biochem Cell Biol 2008;40:1679–1684
8. Ng KL, Li JD, Cheng MY, Leslie FM, Lee AG, Zhou QY. Dependence of olfactory bulb neurogenesis on prokineticin 2 signaling. Science 2005;308:1923–1927
9. Matsumoto S, Yamazaki C, Masumoto KH, Nagano M, Naito M, Soga T, Hiyama H, Matsumoto M, Takasaki J, Kamohara M, Matsuo A, Ishii H, Kobori M, Katoh M, Matsushime H, Furuichi K, Shigeyoshi Y. Abnormal development of the olfactory bulb and reproductive system in mice lacking prokineticin receptor PKR2. Proc Natl Acad Sci U S A 2006;103:4140–4145
10. Prosser HM, Bradley A, Caldwell MA. Olfactory bulb hypoplasia in Prokr2 null mice stems from defective neuronal progenitor migration and differentiation. Eur J Neurosci 2007;26:3339–3344
11. Pitteloud N, Zhang C, Pignatelli D, Li JD, Raivio T, Cole LW, Plummer L, Jacobson-Dickman EE, Mellon PL, Zhou QY, Crowley WF Jr. Loss-of-function mutation in the prokineticin 2 gene causes Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism. Proc Natl Acad Sci U S A 2007;104:17447–17452
12. Dode C, Teixeira L, Levilliers J, Fouveaut C, Bouchard P, Kottler ML, Lespinasse J, Lienhardt-Roussie A, Mathieu M, Moerman A, Morgan G, Murat A, Toublanc JE, Wolczynski S, Delpech M, Petit C, Young J, Hardelin JP. Kallmann syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. PLoS Genet 2006;2:e175
13. Cole LW, Sidis Y, Zhang C, Quinton R, Plummer L, Pignatelli D, Hughes VA, Dwyer AA, Raivio T, Hayes FJ, Seminara SB, Huot C, Alos N, Speiser P, Takeshita A, Van Vliet G, Pearce S, Crowley WF Jr, Zhou QY, Pitteloud N. Mutations in prokineticin 2 and prokineticin receptor 2 genes in human gonadotrophin-releasing hormone deficiency: molecular genetics and clinical spectrum. J Clin Endocrinol Metab 2008;93:3551–3559
14. Cheng MY, Bittman EL, Hattar S, Zhou QY. Regulation of prokineticin 2 expression by light and the circadian clock. BMC Neurosci 2005;11:17
15. Hastings MH. Circadian rhythms: a gut feeling for time. Nature 2002;417:391–392
16. Li JD, Hu WP, Boehmer L, Cheng MY, Lee AG, Jilek A, Siegel JM, Zhou QY. Attenuated circadian rhythms in mice lacking the prokineticin 2 gene. J Neurosci 2006;26:11615–11623
17. Prosser HM, Bradley A, Chesham JE, Ebling FJ, Hastings MH, Maywood ES. Prokineticin receptor 2 (Prokr2) is essential for the regulation of circadian behavior by the suprachiasmatic nuclei. Proc Natl Acad Sci U S A 2007;104:648–653
18. Cheng MY, Bullock CM, Li C, Lee AG, Bermak JC, Belluzzi J, Weaver DR, Leslie FM, Zhou QY. Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. Nature 2002;417:405–410
19. Negri L, Lattanzi R, Giannini E, De Felice M, Colucci A, Melchiorri P. Bv8, the amphibian homologue of the mammalian prokineticins, modulates ingestive behaviour in rats. Br J Pharmacol 2004;142:181–191
20. Cheng MY, Leslie FM, Zhou QY. Expression of prokineticins and their receptors in the adult mouse brain. J Comp Neurol 2006;498:796–809
21. Jethwa PH, Smith KL, Small CJ, Abbott CR, Darch SJ, Murphy KG, Seth A, Semjonous NM, Patel SR, Todd JF, Ghatei MA, Bloom SR. Neuromedin U partially mediates leptin-induced hypothalamo-pituitary adrenal (HPA) stimulation and has a physiological role in the regulation of the HPA axis in the rat. Endocrinology 2006;147:2886–2892

22. Seal LJ, Small CJ, Dhillo WS, Stanley SA, Abbott CR, Ghatei MA, Bloom SR. PRL-releasing peptide inhibits food intake in male rats via the dorsomedial hypothalamic nucleus and not the paraventricular hypothalamic nucleus. Endocrinology 2001;142:4236–4243
23. Smith KL, Patterson M, Dhillo WS, Patel SR, Semjonous NM, Gardiner JV, Ghatei MA, Bloom SR. Neuropeptide S stimulates the hypothalamo-pituitary-adrenal axis and inhibits food intake. Endocrinology 2006;147: 3510–3518
24. Abbott CR, Rossi M, Wren AM, Murphy KG, Kennedy AR, Stanley SA, Zollner AN, Morgan DG, Morgan I, Ghatei MA, Small CJ, Bloom SR. Evidence of an orexigenic role for cocaine- and amphetamine-regulated transcript after administration into discrete hypothalamic nuclei. Endocrinology 2001;142:3457–3463
25. McGowan BM, Stanley SA, Smith KL, Minnion JS, Donovan J, Thompson EL, Patterson M, Connolly MM, Abbott CR, Small CJ, Gardiner JV, Ghatei MA, Bloom SR. Effects of acute and chronic relaxin-3 on food intake and energy expenditure in rats. Regul Pept 2006;136:72–77
26. Dhillo WS, Bewick GA, White NE, Gardiner JV, Thompson EL, Bataveljic A, Murphy KG, Roy D, Patel NA, Scutt JN, Armstrong A, Ghatei MA, Bloom SR. The thyroid hormone derivative 3-iodothyronamine increases food intake in rodents. Diabetes Obes Metab 2009;11:251–260
27. Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, Cone RD, Bloom SR. Gut hormone PYY(3–36) physiologically inhibits food intake. Nature 2002;418: 650–654
28. Kim MS, Rossi M, Abusnana S, Sunter D, Morgan DG, Small CJ, Edwards CM, Heath MM, Stanley SA, Seal LJ, Bhatti JR, Smith DM, Ghatei MA, Bloom SR. Hypothalamic localization of the feeding effect of agouti-related peptide and α-melanocyte–stimulating hormone. Diabetes 2000;49:177–182
29. Dhillo WS, Small CJ, Jethwa PH, Russell SH, Gardiner JV, Bewick GA, Seth A, Murphy KG, Ghatei MA, Bloom SR. Paraventricular nucleus administration of calcitonin gene-related peptide inhibits food intake and stimulates the hypothalamo-pituitary-adrenal axis. Endocrinology 2003;144:1420–1425
30. Kim MS, Small CJ, Stanley SA, Morgan DG, Seal LJ, Kong WM, Edwards CM, Abusnana S, Sunter D, Ghatei MA, Bloom SR. The central melanocortin system affects the hypothalamo-pituitary thyroid axis and may mediate the effect of leptin. J Clin Invest 2000;105:1005–1011
31. Murphy KG, Abbott CR, Mahmoudi M, Hunter R, Gardiner JV, Rossi M, Stanley SA, Ghatei MA, Kuhar MJ, Bloom SR. Quantification and synthesis of cocaine- and amphetamine-regulated transcript peptide (79–102)-like immunoreactivity and mRNA in rat tissues. J Endocrinol 2000;166:659–668
32. Kim MS, Small CJ, Russell SH, Morgan DG, Abbott CR, alAhmed SH, Hay DL, Ghatei MA, Smith DM, Bloom SR. Effects of melanocortin receptor ligands on thyrotropin-releasing hormone release: evidence for the differential roles of melanocortin 3 and 4 receptors. J Neuroendocrinol 2002;14: 276–282
33. Stanley SA, Small CJ, Murphy KG, Rayes E, Abbott CR, Seal LJ, Morgan DG, Sunter D, Dakin CL, Kim MS, Hunter R, Kuhar M, Ghatei MA, Bloom SR. Actions of cocaine- and amphetamine-regulated transcript (CART) peptide on regulation of appetite and hypothalamo-pituitary axes in vitro and in vivo in male rats. Brain Res 2001;893:186–194
34. Kulkarni RN, Wang ZL, Wang RM, Smith DM, Ghatei MA, Bloom SR. Glibenclamide but not other sulphonylureas stimulates release of neuropeptide Y from perifused rat islets and hamster insulinoma cells. J Endocrinol 2000;165:509–518
35. Rossi M, Kim MS, Morgan DG, Small CJ, Edwards CM, Sunter D, Abusnana S, Goldstone AP, Russell SH, Stanley SA, Smith DM, Yagaloff K, Ghatei MA, Bloom SR. A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo. Endocrinology 1998;139:4428–4431
36. Sundquist SJ, Nisenbaum LK. Fast Fos: rapid protocols for single- and double-labeling c-Fos immunohistochemistry in fresh frozen brain sections. J Neurosci Methods 2005;141:9–20
37. Kelley KA, Ho L, Winger D, Freire-Moar J, Borelli CB, Aisen PS, Pasinetti GM. Potentiation of excitotoxicity in transgenic mice overexpressing neuronal cyclooxygenase-2. Am J Pathol 1999;155:995–1004
38. Mizuno TM, Kleopoulos SP, Bergen HT, Roberts JL, Priest CA, Mobbs CV. Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting in *ob/ob* and *db/db* mice, but is stimulated by leptin. Diabetes 1998;47:294–297
39. Aja S, Sahandy S, Ladenheim EE, Schwartz GJ, Moran TH. Intracerebroventricular CART peptide reduces food intake and alters motor behavior at a hindbrain site. Am J Physiol Regul Integr Comp Physiol 2001;281:R1862–R1867
40. Burcelin R, Crivelli V, Dacosta A, Roy-Tirelli A, Thorens B. Heterogeneous metabolic adaptation of C57BL/6J mice to high-fat diet. Am J Physiol Endocrinol Metab 2002;282:E834–E842
41. Niimi M, Sato M, Yokote R, Tada S, Takahara J. Effects of central and peripheral injection of leptin on food intake and on brain Fos expression in the Otsuka Long-Evans Tokushima Fatty rat with hyperleptinaemia. J Neuroendocrinol 1999;11:605–611
42. Flier JS. AgRP in energy balance: will the real AgRP please stand up? Cell Metab 2006;3:83–85
43. Hoffman GE, Smith MS, Verbalis JG. c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. Front Neuroendocrinol 1993;14:173–213
44. Blüher S, Ziotopoulou M, Bullen JW Jr, Moschos SJ, Ungsunan L, Kokkotou E, Maratos-Flier E, Mantzoros CS. Responsiveness to peripherally administered melanocortins in lean and obese mice. Diabetes 2004;53:82–90
45. Enriori PJ, Evans AE, Sinnayah P, Jobst EE, Tonelli-Lemos L, Billes SK, Glavas MM, Grayson BE, Perello M, Nillni EA, Grove KL, Cowley MA. Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons. Cell Metab 2007;5:181–194
46. Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, Friedman JM. Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. Proc Natl Acad Sci U S A 1997;94:8878–8883
47. Yang MU, Van Itallie TB. Composition of weight lost during short-term weight reduction: metabolic responses of obese subjects to starvation and low-calorie ketogenic and nonketogenic diets. J Clin Invest 1976;58:722–730
48. Rothacker DL, Kanerva RL, Wyder WE, Alden CL, Maurer JK. Effects of variation of necropsy time and fasting on liver weights and liver components in rats. Toxicol Pathol 1988;16:22–26