Prokineticin 2 potently inhibits food intake

Running title: Prokineticin 2 potently inhibits food intake

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**Objective:** Prokineticin 2 (PK2) is a hypothalamic neuropeptide expressed in CNS 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 following 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 whilst 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 alpha-MSH from *ex-vivo* hypothalamic explants. In addition ICV co-administration of the alpha-MSH antagonist agouti related peptide blocked the anorexigenic effects of PK2. Chronic peripheral administration of PK2 reduced food and bodyweight 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 partly mediated via the melanocortin system.
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) since 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 (GnRH) 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. Since 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 Ltd (London, UK), PK2 antibody from Santa Cruz Biotechnology Inc. (California, USA) and control IgG was generated to a random sequence peptide epitope (21).

**Animals—**

**Wistar Rats and C57BL/6 Mice:** Adult male Wistar rats weighing 200-250g and adult male C57BL/6 mice weighing 20-25g (Harlan, UK) were maintained in individual cages (width 24.5cm, length 41.5cm and depth 18.5cm) (at 21-23°C) with ad libitum access to food (RM1 diet; SDS Ltd, Witham, UK) and water unless specified in procedure protocol.

**Diet induced obese (DIO) mice:** Studies were performed in C57BL/6 mice when they had developed diet induced obesity and their bodyweight was stable (see Supplementary Methods 1 which can be found in an online appendix at http://care.diabetesjournals.org).

Animal studies were approved under the 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 24h, 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, Texas, USA) according to the manufacturer’s protocol. cDNA was synthesized from 0.5μg of RNA using a High
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Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Texas, USA) 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 ICV with saline or PK2 at doses of 0.005, 0.015, 0.05 and 0.15nmol/rat at the beginning of the dark phase. All rats were injected within a single 20 minute 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 24h post-injection for these studies.

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

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

Effect of ICV administration of PK2 on behavior: Adult male Wistar rats weighing 200-250g (n=10-12/group) were injected ICV with saline or PK2 1.5nmol/rat and behavioral patterns monitored continuously for 120 minutes after injection. Behavior was classified into eight categories: feeding, drinking, grooming, burrowing, rearing, locomotion, head down, and sleeping as previously described (23). Abnormal behaviour 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 ICV with saline or PK2 1.5nmol/rat at the beginning of the dark phase and food removed following 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, Ohio, USA) (25).

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

Effect of ICV administration of PK2 on c-fos expression: Rats were cannulated into the lateral ventricle as previously described (26). PK2 (1.5nmol/rat) or saline (n=4/group) was injected into the lateral ventricle of ad libitum fed rats over 1min 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 (200-250g) had permanent indwelling, unilateral, 26 gauge stainless steel guide cannulae (Plastics One, Roanoke, VA) stereotactically implanted into the supra optic nucleus (SON), arcuate nucleus (ARC), paraventricular nucleus (PVN), anterior...
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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 cross-over design. Each animal received both injections (saline or 0.025nmol PK2) in a random order four days apart. Food intake was measured 1,2,4,8 and 24h post-injection. The dose of PK2 was chosen based on previous studies which 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 200-250g
Rats were killed by decapitation and hypothalamic explants prepared as previously described (29). The hypothalami were incubated for 45min in 600µl—artificial cerebrospinal fluid (aCSF) (basal period). The tissues were then exposed to PK2 (10, 100 or 1000nM) in 600 µl— aCSF for 45minutes (n=9-12/treatment). Finally, the viability of the tissue was verified by a 45minute exposure to 56mM KCl. Alpha-MSH, cocaine and amphetamine regulated transcript (CART), thyrotropin releasing hormone (TRH), corticotrophin releasing hormone (CRH), neuropeptide Y (NPY) and agouti related peptide (AgRP) in the aCSF were measured using established radioimmunoassay (22, 30-34).

Effect of melanocortin receptor antagonism on the anorectic effects of PK2: Rats (n=10-12/group) were ICV injected with either (i) saline, (ii) AgRP(1nmol/rat), (iii) alpha-MSH(1nmol/rat), (iv) alpha MSH(1nmol/rat) and AgRP(1nmol/rat), (v) PK2(0.15nmol/rat), (vi) PK2(0.15nmol/rat) and AgRP(1nmol/rat) at the beginning of the dark phase. The doses of alpha-MSH and AgRP were chosen based on previous studies (35). Food intake was measured 1,2,4,8 and 24h post-injection.

Effect of ICV administration of PK2 on c-fos expression in arcuate POMC neurons: Animals (n=5 per group) were injected into the lateral ventricle with 1.5nmol PK2. In situ hybridization (ISH) for proopiomelanocortin (POMC) and ICC for c-fos was 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 co-localized cells were expressed as a percentage of the total number of POMC and c-fos neurones.

Effect of acute peripheral administration of PK2 on food intake in lean rats and mice: Rats (n=10-12/group) were injected intraperitoneally (ip) with saline or PK2 at doses of 2.3, 7 or 20nmol/kg at the beginning of the dark phase. Food intake was measured 1,2,4,8 and 24h post-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 540nmol/kg.

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

A similar study was carried out in C57BL/6 DIO mice which were randomized to 1) saline treated with ad libitum access to food, 2) PK2 treated (540nmol/kg per injection) with ad libitum access to food, 3) pair fed group: saline treated but food restricted to the daily median food consumed by the PK2 treated mice over the previous 24h 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’s test except for the intranuclear food intake study which was analyzed using the Holm Bonferroni test. Food intake data expressed as a change compared to saline treated animals was 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 studies the Generalized Estimating Equation and the Mann-Whitney U test were used. In all cases p < 0.05 was considered to be statistically significant.

**RESULTS**

**Expression of PK2 is reduced during fasting:** The expression levels of hypothalamic neuropeptides which reduce food intake e.g. alpha 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 24h (Figure 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 intake which at doses greater than 0.15nmol/rat produced an 85% reduction in food consumed in the first hour following injection (Figure 2A,B, Supplementary Figure 1 and Supplementary Table 2 A,B). When administered ICV in the early light phase to fasted rats, PK2 caused a similarly potent inhibition of food intake (Figure 2C and Supplementary Table 2C). In addition, rats injected with 1.5 and 4.5nmol PK2 had a 30% reduction in 24h food intake compared to saline injected rats (Figure 2D and Supplementary Table 2C).

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 to saline injected animals (Figure 2E,F and Supplementary Table 2). However, consistent with an increase in satiety, ICV PK2 significantly reduced the number of feeding episodes (Supplementary Table 23). Many regulators of food intake also regulate energy expenditure. However, this does not appear to be the case for PK2, since ICV administration of PK2 did not alter oxygen consumption, a surrogate for energy expenditure (Figure 2G). These data suggest that ICV administration of PK2 specifically reduces food intake for up to 24h.

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

PK2 mediates part of its anorectic effects via the melanocortin system: PK2 significantly stimulated the release of alpha-MSH (Figure 5A) but did not alter the release of the other hypothalamic neuropeptides measured (Supplementary Table 3). 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 co-administered AgRP with PK2. In this paradigm AgRP attenuated the effect of PK2 suggesting that in part PK2 may reduce food intake via the melanocortin system (Figure 5B). To confirm that the dose of AgRP used was antagonizing alpha MSH we demonstrated that ICV administration of alpha MSH reduced 0-2h food intake in rats, whilst co-administration of AgRP with the same dose of alpha MSH abolished the anorectic effect of alpha MSH (Figure 5B).

To determine if ICV administration of PK2 resulted in activation of ARC POMC neurones (which produce alpha MSH) we performed co-localization studies of c-fos and POMC following ICV administration of PK2. ICV administration of PK2 significantly increased the number of arcuate POMC-expressing neurones exhibiting c-fos immunoreactivity (Figure 5C 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:** A single ip injection of PK2 (20nmol/kg) reduced food intake by 45% in the first hour following injection but did not affect food intake at the other time points studied (Figure 6A and Supplementary Table 6A).

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

Chronic peripheral administration of PK2 decreases food intake and bodyweight in lean and obese mice:

**Lean mice:** Twice daily ip injection of PK2 for 5 days in lean mice significantly decreased cumulative food intake (Figure 7A) and bodyweight compared to saline injected controls (Figure 7B). The reduction in food intake following each single injection of PK2 was similar in magnitude throughout the study period, suggesting PK2 remained equally potent following recurrent administration (Supplementary Table 4).

**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 if DIO mice were sensitive to PK2 we administered PK2 twice daily by ip
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injection for 5 days to DIO mice. PK2 significantly reduced cumulative food intake (Figure 7C) and bodyweight (Figure 7D) compared to saline injected controls. DIO mice pair fed to the PK2 treated group lost a similar amount of bodyweight as the mice given PK2 (Figure 7D), suggesting the effect of PK2 on bodyweight was predominantly mediated via a reduction in food intake. As in lean mice, injection of PK2 was equally potent at reducing food intake on each of the five days of the study (Supplementary Table 5). These data suggest that repeated administration of PK2 results in reduced food intake and bodyweight without tachyphylaxis in lean and obese mice.

DISCUSSION

The hypothalamus is a key centre 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 since PK2 or PKR2 null mice display abnormal circadian rhythms and locomotor activity (16-18). ICV administration of PK2 potently inhibited food intake in rats up to 24h post-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 as the increase in food intake following 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 to 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 bodyweight compared to 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 to 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, as, for example, has been suggested to occur with NPY and AgRP (42). A post embryonic or conditional knockout model of PK2, or animal studies in which local hypothalamic PK2 expression is reduced may help to further determine the role of endogenous PK2 in appetite regulation.

In order to investigate the hypothalamic sites which 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
Prokineticin 2 potently inhibits food intake and may be mediated directly via these nuclei. Following 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 alpha MSH from hypothalamic explants ex vivo and ICV co-administration 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 which produce alpha MSH. Together this these data suggests that PK2 may mediate part of its anorectic effect via the hypothalamic ARC melanocortin system.

To investigate if peripheral administration of PK2 had anorectic effects we investigated the effects of ip administration of PK2 on food intake and bodyweight 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 bodyweight. Since repeated administration of anorectic agents can cause tachyphylaxis (44), resulting in an attenuated effect following repeated administration. PK2 administration to lean or obese mice caused a similar reduction in food intake following each injection and resulted in a significant reduction in bodyweight suggesting that tachyphylaxis to the anorectic effects of PK2 does not occur using this administration protocol.

Leptin reduces food intake and bodyweight 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 investigated the effect of repeated ip 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 following each injection, and reduced cumulative food intake and bodyweight 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 to lean mice.

In both lean and DIO mice the effect of PK2 on bodyweight is likely to be predominantly mediated via a reduction in food intake since control mice pair-fed to PK2-injected mice lost a similar amount of weight to 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 as the prokineticin receptor agonist Bv8 has actually been shown to slightly increase, rather than reduce, water intake (19). This phenonmenum 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 utilisation of glycogen stores, this being especially true in rodents. This is followed by a period in which fat is lost which coincides 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 in part mediated via the ARC melanocortin system. Repeated peripheral administration of PK2 for 5 days
Prokineticin 2 potently inhibits food intake and bodyweight in obese mice. Further studies investigating the effects of longer term administration of PK2 on food intake and bodyweight will determine the potential of PK2 as a target for the development of anti-obesity agents.

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REFERENCES
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**Table 1: Expression of c-fos in arcuate POMC neurons following ICV administration of PK2:** Data are presented as median (interquartile range) from matched sections throughout the ARC. Co-localized labeling is expressed as a percentage as mean ± S.E.M. * = p < 0.05 vs. saline; ** = p < 0.01 vs. saline; n = 5 per group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total POMC cell counts</th>
<th>Total c-fos cell counts</th>
<th>Colocalised POMC/c-fos cell counts</th>
<th>% POMC cells colocalised with c-fos</th>
<th>% c-fos cells colocalised with POMC</th>
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<tbody>
<tr>
<td>Saline</td>
<td>425 (424 – 435)</td>
<td>12 (7 – 18)</td>
<td>5 (4 – 7)</td>
<td>1.2 ± 0.3</td>
<td>48.1 ± 5.5</td>
</tr>
<tr>
<td>PK2</td>
<td>440 (412 – 446)</td>
<td>51 (26 – 65)*</td>
<td>32 (16 – 33)**</td>
<td>7.0 ± 1.4</td>
<td>64.0 ± 6.6</td>
</tr>
</tbody>
</table>
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FIGURE LEGENDS

Figure 1: Fasting reduces hypothalamic expression of PK2
Hypothalamic expression of PK2 mRNA between ad libitum fed, 12h fasted and 24 hour fasted rats (n=24 per group) is shown. Results are expressed as mean ± SEM. *** = p<0.001 vs. fed group.

Figure 2: PK2 potently reduces food intake independent of changes in locomotor activity or energy expenditure.
Food intake: Effect on 0-1 hour food intake in ad libitum fed rats (n=10-12 per group) following 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 hour food intake in ad libitum fed rats (n=10-12 per group) following ICV administration at the beginning of the dark phase of PK2 at doses of 0.15, 0.50, 1.5 nmol/rat (B). Rats (n=10-12 per group) fasted for 24 hours were injected ICV in the early light phase (C, 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 hours following injection is shown (D). Results are expressed as mean ± SEM. n=10-12 per group. * = p<0.05, ** =p<0.01, *** = p<0.001 vs. saline.
Locomotor activity: Effect of ICV injection of saline or PK2 1.5 nmol/rat at the beginning of the dark phase on horizontal (shown in E) and rearing (shown in F) movement respectively. Data is shown as mean ± SEM for each 30 minute 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.

Figure 3: Immunoblockade of endogenous PK2 increases food intake
The effect on 2-4 hour 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 vs. 30pmol control IgG group.

Figure 4: PK2 mediates its effects via specific hypothalamic nuclei.
(A) Graphical representation of c-fos activation in hypothalamic nuclei of rats following administration of saline or PK2 (1.5nmol/rat) into the lateral ventricle. Open bars = saline injected animals, filled grey bars = PK2 injected animals. Data is shown as median and interquartile range. SON = supra optic 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 vs. saline.
(B-F) Representative brain sections showing c-fos expression in the SON (B), ARC (C), PVN (D) and AHA (E, F) of rats injected into the lateral ventricle with saline or PK2 (1.5nmol/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 Figure 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 following PK2 injection (black bar) is shown as mean ± SEM as a percentage of food intake.
consumed in the first hour following saline injection (white bar) for each nucleus. * = p<0.05, ** = p<0.01 vs. saline.

**Figure 5: PK2 mediates part of its anorectic effects via the melanocortin system**
(A) Effect of PK2 on alpha MSH release from hypothalamic explants. Peptide release is expressed as percentage of basal. n = 9-12 per treatment. * = p<0.05 vs. basal.
(B) Effects of melanocortin receptor antagonism on anorectic effects of PK2. Food intake in the 0-2 hours following injection is shown. Results are expressed as mean ± SEM. * = p<0.05 vs. 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-labelled cells are indicated by a blue arrow. 3V = third cerebral ventricle; scale bars = 100µm. (1 and 2 are shown at 10x magnification; 3 and 4 are shown at 20x magnification).

**Figure 6: Peripheral administration of PK2 acutely reduces food intake**
(A) The effect on food intake of ip 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 following injection is shown. Results are expressed as mean ± SEM. * = p<0.05 vs. saline.
(B, C). Effect on food intake of ip administration of saline or PK2 at doses of 7, 20, 60, 180 or 540 nmol/kg. Food intake in the first hour following injection (B) and cumulative food intake over 24 hours (C) is shown. Results are expressed as mean ± SEM. * = p<0.05, ** = p<0.01, *** = p<0.001 vs. saline.

**Figure 7: Chronic peripheral administration of PK2 decreases bodyweight in lean and obese mice**
(A, B) Cumulative food intake (A) and change in bodyweight (B) of C57BL/6 mice (n=10 per group) ip injected twice daily for 5 days with either saline or PK2 (180 nmol/kg).
(C, D) Effects of ip injection of saline or PK2 (540nmol/kg per injection) twice daily for 5 days to C57BL/6 diet induced obese (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 hour period. Change in bodyweight of the saline, 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 vs. saline.
Prokineticin 2 potently inhibits food intake

Figure 1

![Figure 1](image1)

Figure 2

A

![Figure 2A](image2)

B

![Figure 2B](image3)

C

![Figure 2C](image4)

D

![Figure 2D](image5)
Figure 2 Cont.

![Graph showing horizontal beam breaks over time for saline and PK2 treatments.]

![Graph showing vertical beam breaks over time for saline and PK2 treatments.]

![Graph showing VO2 (mL/kg/hr) over time for saline and PK2 treatments.]

Figure 3

![Bar graph showing 24-hour food intake for control IgG and PK2 antibody treatments.]

Prokineticin 2 potently inhibits food intake
Figure 4

A

Prokineticin 2 potently inhibits food intake

B

saline

PK2

C
Figure 4 Cont.

Prokineticin 2 potently inhibits food intake
Prokineticin 2 potently inhibits food intake

Figure 4 Cont.

G

Hypothalamic nucleus injected with saline or PK2
Prokineticin 2 potently inhibits food intake.

Figure 5

A

B

C

SALINE

PK2
Prokineticin 2 potently inhibits food intake
Prokineticin 2 potently inhibits food intake

Figure 7

A

B

C

D

Cumulative food intake (g)

Time (days)

Bodyweight change (g)

Time (days)