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ORIGINAL ARTICLE

# Peptide $YY_{3-36}$ and Pancreatic Polypeptide Differentially Regulate Hypothalamic Neuronal Activity in Mice *In Vivo* as Measured by Manganese-Enhanced Magnetic Resonance Imaging

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Peptide YY (PYY) and pancreatic polypeptide (PP) are two appetite suppressing hormones, released post-prandially from the ileum and pancreas, respectively. $PYY_{3-36}$, the major circulating form of the peptide, is considered to reduce food intake in humans and rodents via high affinity binding to the auto-inhibitory neuropeptide Y receptor Y2R, whereas PP is considered to act through the Y4R. Current evidence indicates the anorexigenic effects of both peptides occur via signalling in the brainstem and arcuate nucleus (ARC) of the hypothalamus. Manganese-enhanced magnetic resonance imaging (MEMRI) has previously been used to track hypothalamic neuronal activity *in vivo* in response to both nutritional interventions and gut hormone treatment. In the present study, we used MEMRI to demonstrate that s.c. administration of PP results in a significant reduction in signal intensity (SI) in the ARC, ventromedial hypothalamus and paraventricular nucleus of fasted mice. Subcutaneous delivery of $PYY_{3-36}$ resulted in a nonsignificant trend towards decreased SI in the hypothalamus of fasted mice. We found no SI change in the area postrema of the brainstem after s.c. injection of either peptide. These differences in hypothalamic SI profile between PP and $PYY_{3-36}$ occurred despite both peptides producing a comparable reduction in food intake. These results suggest that separate central pathways control the anorexigenic response for PP and $PYY_{3-36}$, possibly via a differential effect of Y4 receptor versus Y2 receptor signalling. In addition, we performed a series of MEMRI scans at 0–2, 2–4 and 4–6 h post-injection of $PYY_{3-36}$ and a potent analogue of the peptide; $PYY_{3-36}$ (LT). We recorded a significant reduction in the ARC SI 2–4 h after $PYY_{3-36}$ (LT) injection compared to both saline and $PYY_{3-36}$ in fasted mice. The physiological differences between $PYY_{3-36}$ and its analogue were also observed in the long-term effects on food intake, with $PYY_{3-36}$ (LT) producing a more sustained anorexigenic effect. These data suggest that MEMRI can be used to investigate the long-term effects of gut peptide delivery on activity within the hypothalamus and brainstem.

**Key words:** pancreatic polypeptide, peptide YY, neuropeptide Y receptor, hypothalamus, neuronal activity, manganese enhanced MRI.

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Obesity is a major growing health concern resulting in serious adverse health consequences; including cardiovascular disease, type 2 diabetes, muscoskeletal disorders and some cancers. The gastrointestinal tract is the largest endocrine organ in the body, producing hormones that play key sensing and signalling roles in the regulation of energy homeostasis (1). A better understanding of the role gut hormones in body weight regulation has enabled the development of potential therapies for obesity based on their pathways of activation. Two such hormones, pancreatic polypeptide (PP) and peptide YY (PYY), are members of the neuropeptide Y (NPY) family and share major structural homologies (2,3). Following a meal, PP is secreted from the F cells of pancreatic islets by a vagal cholinergic

mechanism (4), whereas $PYY_{1-36}$ is released from endocrine L-cells of the small and large bowel (5). $PYY_{1-36}$ is truncated to $PYY_{3-36}$, the major circulating form of the peptide, in the plasma by the enzyme, dipeptidyl peptidase IV (6).

Peripheral administration of both $PYY_{3-36}$ and PP has been shown to reduce food intake in rodents and humans, an effect considered to be mediated by hypothalamic Y-family receptors (7–10). Electrophysiological data and the expression pattern of the immediate early gene *c-fos* indicated that the effects of peripherally injected $PYY_{3-36}$ are mediated by the activation of Y2R located on pro-opiomelanocortin (POMC) neurones in the arcuate nucleus (ARC) (7). Furthermore, the anorexigenic effect of peripheral $PYY_{3-36}$ is absent in Y2R knockout mice and is blocked by a Y2R antagonist (7,11). However, $PYY_{3-36}$ remains effective in POMC knockout animals (12) and electrophysiological studies indicate that $PYY_{3-36}$ inhibits both NPY and POMC neurones (13). Additional evidence indicates that vagal afferents in the brainstem are required to mediate the anorexigenic effects of peripherally injected $PYY_{3-36}$ (11,14). However, this appears to be specific to rats because neither surgical, nor chemical vagotomy in mice prevents the anorexigenic effects of peripheral $PYY_{3-36}$ (15,16).

PP dose-dependently reduces food intake in *ad lib.* fed and fasted animals, which is considered to be mediated via Y4R, since the anorexigenic effect of the peptide is lost in Y4R knockout mice (17,18). The appetite suppressing effect of PP is abolished in vagotomised rodents, suggesting the major site of action of PP is via the brainstem (19). However, peripheral administration of PP has also been shown to decrease the hypothalamic expression of the orexigenic peptides NPY, ghrelin and orexin, suggesting that PP may also work via the hypothalamus (19). Therefore, it is not known whether PP and $PYY_{3-36}$ work via direct action upon the hypothalamus or the brainstem, or indeed both.

Functional neuroimaging techniques are increasingly being used to study aspects of metabolic physiology in both rodents and humans (20). The ability of paramagnetic manganese ($Mn^{2+}$) ions to shorten the proton $T_1$ signal, yielding an increased signal intensity (SI) in specifically weighted magnetic resonance imaging (MRI) scans, combined with their capacity to enter cells via permeation through voltage-gated calcium channels, has propelled their use as an indirect marker for neuronal activation in MRI (21,22). Previous work carried out by our laboratory in a murine model indicates that, even in the presence of an intact blood–brain barrier (BBB), $Mn^{2+}$ ions are capable of entering the hypothalamus, enabling detection of significant differences in SI between fed and fasted states (23). Using this paradigm, we have further applied manganese-enhanced magnetic resonance imaging (MEMRI) to demonstrate distinct patterns of activation in the hypothalamus and brainstem in response to administration of various gut hormones; including ghrelin (24), glucagon-like-peptide-1 (GLP-1) and oxyntomodulin (OXM) (25,26).

In the present study, we used MEMRI to investigate the actions of peripheral injection of $PYY_{3-36}$ and PP on neuronal activity within the hypothalamus and brainstem. In addition, we have used a series of MEMRI scans to investigate the time-course activation of hypothalamic activity after the injection of $PYY_{3-36}$ and $PYY_{3-36}$ (LT), a potent anorexigenic analogue of the peptide.

## Materials and methods

### Materials

Peptides were purchased from Bachem (St Helens, UK). All peptides were dissolved in 100 $\mu$l of saline (0.9%) solution in preparation for s.c. injection.

### Animal preparation

All studies were performed in accordance with the Animals (Scientific Procedures) Act 1986 (UK) (license number 70/6402). C57BL/6 male mice (20–25 g; Harlan, Hillcrest, UK) were singly-housed under a 12 : 12 h light/dark cycle (lights on 07.00 h) at 21–23 °C with *ad lib.* access to RM1 diet (Special Diet Services, Witham, UK). Animals were acclimatised to laboratory conditions for a minimum of 1 week and handled daily, during which time they received two s.c. injections of saline to minimise stress on the study days.

### MRI parameters

Spin-echo multislice $T_1$-weighted sequence imaging was carried out with a 9.4-T horizontal-bore MR scanner (Varian Inc., Palo Alto, CA, USA) using the scanning parameters; repetition time = 1800 ms, echo time = 5.2 ms, matrix = 256 × 256, field of view = 25 × 25 mm. The in-plane resolution of each voxel was approximately 100 $\mu$m. In each acquisition, 46 contiguous transverse slices of 0.4 mm thickness were imaged, giving a scan time of 1 min 57 s. A further 63 acquisitions were subsequently obtained, taking approximately 2 h. The slice offset for each mouse was aligned to identifiable anatomical features with reference to a standard mouse brain atlas to ensure that the region of brain scanned was in register for all mice. The hypothalamus was placed at the foremost point of the transverse slices to enable measurement of $Mn^{2+}$ accumulation in both brainstem and hypothalamic regions, concurrently.

### Experiment 1: Comparison of $Mn^{2+}$ uptake in the hypothalamus and brainstem of fasted and *ad lib.* fed mice

The degree of manganese accumulation within the hypothalamus and brainstem of fasted and *ad lib.* fed mice was carried out to provide control SI profiles for subsequent comparison with those generated after $PYY_{3-36}$ and PP administration. Animals had their chow removed 20 h before scanning and were allowed *ad lib.* access to water. All scans were performed in the early light phase as described previously (23). Mice (n = 5 per group) were anaesthetised with isoflurane via a facemask (1.5% isoflurane–oxygen mixture for induction, reduced to 1% for maintenance). The tail vein was cannulated for i.v. infusion of $MnCl_2$ with a s.c. catheter sited. The head was centrally located inside a quadrature mouse head coil with an internal diameter of 25 mm (Magnetic Resonance Laboratories, Oxford, UK) and a phantom consisting of a glass tube containing 0.9% saline was scanned simultaneously with each animal. After five baseline MRI image acquisitions, each mouse received a bolus s.c. injection of saline (0.9%, 100 $\mu$l) and an infusion of $MnCl_2$ (5 $\mu$l/g of 100 mM $MnCl_2{\cdot}4H_2O$; Sigma-Aldrich Company Ltd, Poole, Dorset, UK) at a rate of 0.2 ml/h via syringe pump (PHD 2000; Harvard Apparatus, Holliston, MA, USA). A further 63 acquisitions were obtained, each acquisition commencing immediately after completion of previous acquisition, lasting approximately 120 min. Respiration was monitored by a respirator (SA Instruments, Inc., Stony Brook, NY, USA) taped to the chest of each individual mouse, whereas temperature was monitored by rectal probe and maintained at 37 ± 0.5 °C via a heating system.

## Experiment 2: The effect of s.c. injection of PP, $PYY_{3-36}$ and $PYY_{3-36}$ (LT) on food intake in fasted mice

Feeding studies were performed to investigate the anorexigenic effects of s.c. injection of PP, $PYY_{3-36}$ and $PYY_{3-36}$ (LT). Male mice were fasted for 20 h, with *ad lib.* access to water and body weights were recorded. Subcutaneous injections of saline control (0.9%), $PYY_{3-36}$ (50, 500 nmol/kg), $PYY_{3-36}$ (LT) (50, 500 nmol/kg) or PP (500 nmol/kg) (n = 9 per group) were administered in the early light phase (08.00–09.00 h). After injection, animals were returned to their home cages containing a pre-weighed amount of chow, which was re-weighed at 2, 4, 8 and 24 h post-injection. These doses were selected on the basis that once daily s.c. administration of 500 nmol/kg $PYY_{3-36}$ (LT) promotes significant weight loss, whereas $PYY_{3-36}$ is without effect (unpublished observations M.K.H. and M.L.A.). A receptor binding assay demonstrating comparable binding at the Y2R for $PYY_{3-36}$ and $PYY_{3-36}$ (LT) is shown in the Supporting information (Fig. S1). The dose of PP was retrospectively chosen because it produced a comparable reduction in food intake compared to the dose of $PYY_{3-36}$.

## Experiment 3: The effect of peripheral administration of PP and $PYY_{3-36}$ on $Mn^{2+}$ uptake in the hypothalamus and brainstem of fasted mice

To measure the effects of $PYY_{3-36}$ and PP upon neuronal activation within the hypothalamus and brainstem, MEMRI scans were performed, as in Experiment 1, on mice fasted overnight (20 h). Following five baseline MRI image acquisitions, mice were injected s.c. with $PYY_{3-36}$ (500 nmol/kg, n = 6), PP (500 nmol/kg, n = 4) or saline (0.9%, 100 $\mu$l) (n = 5).

## Experiment 4: The long-term effects of peripheral administration of $PYY_{3-36}$ and $PYY_{3-36}$ (LT) on $Mn^{2+}$ uptake in the hypothalamus and brainstem of fasted mice

Peripheral injection of $PYY_{3-36}$ has a sustained anorexigenic effect beyond 2 h post-injection (27). Therefore, to assess the long-term effects of s.c. $PYY_{3-36}$ delivery on neuronal activity in the hypothalamus and brainstem, we performed a series of MEMRI scans post-injection. In addition, we compared the effects of $PYY_{3-36}$ with $PYY_{3-36}$ (LT), a potent anorexigenic analogue of the peptide. Male mice were fasted overnight (20 h) and, in the early light phase, injected s.c. with $PYY_{3-36}$ (500 nmol/kg), $PYY_{3-36}$ (LT) (500 nmol/kg) or saline (0.9%) (n = 15 per group). MEMRI scans, taking approximately 2 h, were performed (as in Experiment 1) at three different time points: (i) immediately post-injection (0–2 h); (ii) 2 h post-injection (2–4 h); and (iii) 4–6 h post-injection (4–6 h) with five mice per group [$PYY_{3-36}$, $PYY_{3-36}$ (LT) or saline] at each time point.

## Image analysis

Image processing software (IMAGEJ, version 1.3.1; http://www.rsbweb.nih.gov/ij/) was used to define and calculate the relative SI within specific regions of interest (ROI) from $T_1$-weighted scan data. ROI corresponding to

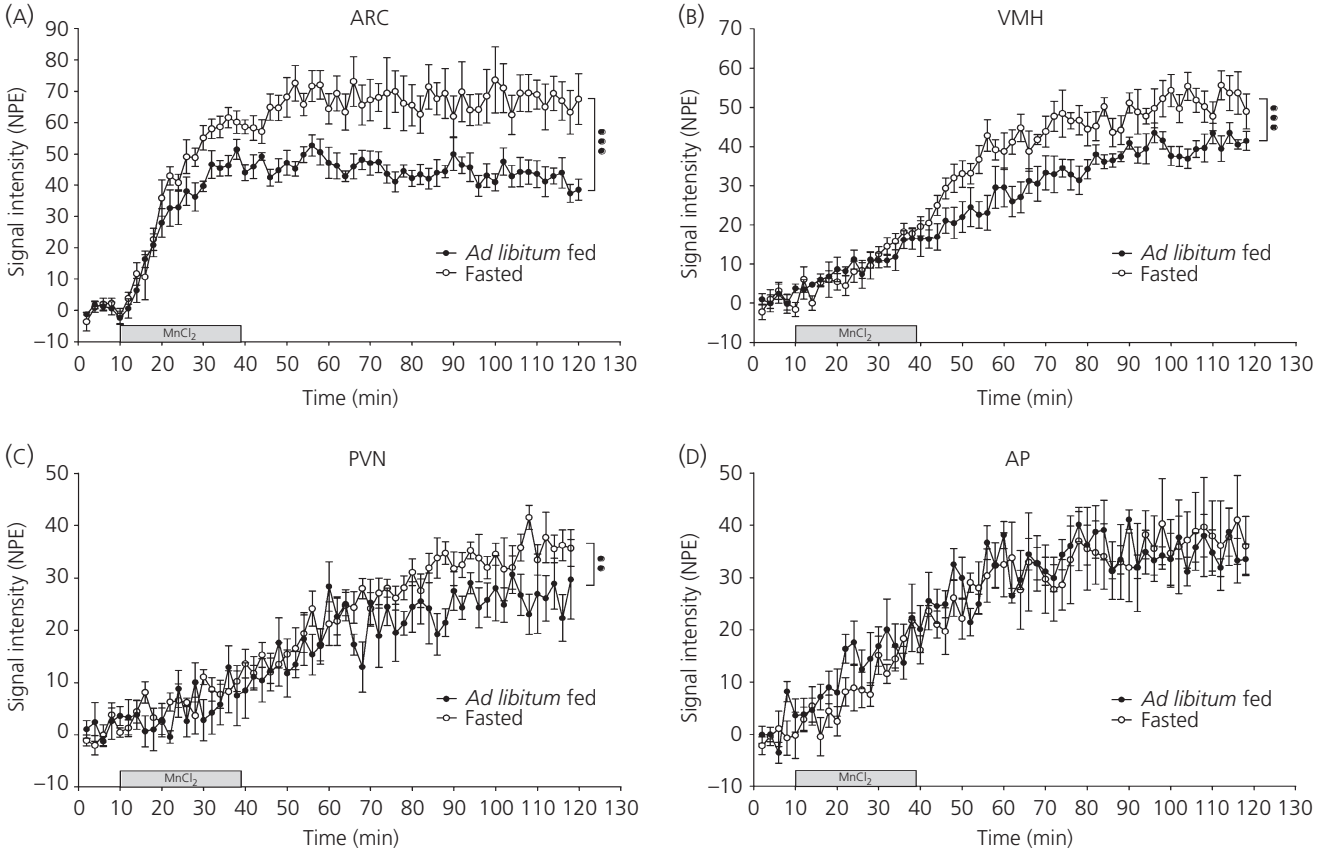


**Fig. 1.** $T_1$-weighted manganese-enhanced magnetic resonance imaging (MEMRI) signal intensity profiles in *ad lib.* fed and fasted mice. Time course of normalised $T_1$-weighted MEMRI signal intensity (SI) change recorded in the (A) arcuate nucleus (ARC), (B) ventromedial nucleus of the hypothalamus (VMH), (C) paraventricular nucleus (PVN) and (D) area postrema (AP), in *ad lib.* and fasted mice. The grey bar indicates the duration of the i.v. infusion; n = 5 per group; ●●●, $P < 0.001$; ●●, $P < 0.01$. Statistical differences were determined by generalised estimating equations. Results are the mean ± SEM.

the ARC, ventromedial nucleus of the hypothalamus (VMH), paraventricular nucleus (PVN), periventricular nucleus (PeN), fourth ventricle (4th V) and the area postrema (AP) were generated with reference to a standard mouse brain atlas (28) (AP: fig. 92, 4th V: fig. 80, ARC VMH, and PeN: fig. 43, PVN: fig. 38) (see Supporting information, Fig. S2). Previous MEMRI analysis using the protocol implemented in the present study has revealed insufficient manganese reaches the nucleus of the solitary tract in the brainstem (25), thereby preventing effective analysis of this region. It is important to mention that this represents a major limitation to our technique when assessing neuronal activity in the brainstem in response to gut hormone treatments. The changes in SI within these regions as a result of nonbiological factors were corrected for by normalising the SI of each target area to that of the saline phantom at the same time point (SI target area/SI of phantom). To correct for slight variations in the time taken for the $MnCl_2$ to enter the circulation, the first enhancing time point of each scan was defined as the acquisition in which the SI in the lateral ventricle was increased > 20% over baseline. The acquisitions were then realigned so that the first enhancing acquisition was in register across all animals. Any animal that exhibited head motion during the scan was excluded from the study. Signal intensity profiles are illustrated as the normalised percentage enhancement, which is the percentage increase in baseline readings recorded 0–10 min before manganese infusion normalised to the SI of the phantom.

### Statistical analysis

All data are presented as the mean ± SEM. Given the cumulative nature of MEMRI signal intensity data differences in SI profile between the ROI in all experimental groups were analysed using generalised estimating equations and the the Mann–Whitney U-test with commercial statistical software (STATA, version 9.1; Statacorp, College Station, TX, USA), which compare profiles for the entirety of the 2-h scan. In addition, we have employed the same statistical test to compare differences in the rate of manganese infusion for the 10–40 min period of manganese infusion. $P < 0.05$ was considered statistically significant. Food intake data was either analysed using one-way ANOVA with Bonferroni post-hoc test (Graphpad Software, Prism, version 5 La Jolla, CA, USA). or Student's two-tailed t-test. In all cases, $P \leq 0.05$ was considered statistically significant.

## Results

### Experiment 1: Comparison of $Mn^{2+}$ uptake in the hypothalamus and brainstem of fasted and *ad lib.* fed mice

We recorded a significant increase in SI in the ARC, VMH and PVN of fasted compared to *ad lib.* fed mice ($P < 0.01$ for all) (Fig. 1A–C). There was no significant difference recorded in the SI profile in the AP of fed compared to fasted animals (Fig. 1D).

### Experiment 2: The effect of s.c. injection of PP, $PYY_{3-36}$ and $PYY_{3-36}$ (LT) on food intake in fasted mice

Subcutaneous injection of PP resulted in a significantly reduced food intake up to 8 h post-injection [0–2 h food intake (FI): PP: 0.56 ± 0.1 g, saline: 1.14 ± 0.15 g; $P < 0.001$ versus saline; 2–4 h: PP: 0.13 ± 0.1 g, saline: 0.52 ± 0.3 g; $P < 0.001$ versus saline; 4–8 h: PP: 0.18 ± 0.1 g, saline: 0.48 ± 0.2 g; $P < 0.01$]. Food intake of PP injected was significantly greater during the 8–24 h period compared to saline controls (8–24 h FI: PP: 4.42 ± 0.4 g, saline: 3.97 ± 0.3 g; $P < 0.01$ versus saline) but remained significantly reduced 24 h post-injection (0–24 h FI: PP: 6.1 ± 0.3 g, saline: 5.3 ± 0.5 g; $P < 0.001$ versus saline) (Fig. 2).

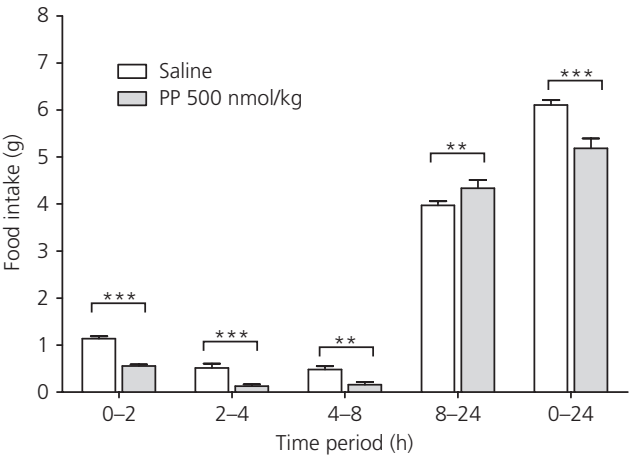


**Fig. 2.** The effects of s.c. injection of pancreatic polypeptide (PP) on food intake in fasted mice. Fasted mice were injected in the early light phases with PP (500 nmol/kg) or saline; n = 9 per group; ***$P < 0.001$ and **$P < 0.01$; data analysed by Student's two-tailed *t*-test.

Fifty nanomol per kilogram doses of both $PYY_{3-36}$ and $PYY_{3-36}$ (LT) significantly reduced food intake for 2 h post-injection compared to saline controls [0–2 h FI: $PYY_{3-36}$ 50 nmol/kg: 0.6 ± 0.2 g, $PYY_{3-36}$ (LT) 50 nmol/kg: 0.59 ± 0.3 g; saline: 1.4 ± 0.3 g; $P < 0.001$ versus saline] (Fig. 3A). Only the 50 nmol/kg of $PYY_{3-36}$ (LT) produced a significantly reduced intake at the 2–4 and 4–8 h time-points (Fig. 3A). Neither the 50 nmol/kg dose of $PYY_{3-36}$, nor $PYY_{3-36}$ (LT) resulted in a significantly reduced food intake over 24 h (Fig. 3A). Five hundred nanomol per kilogram doses of $PYY_{3-36}$ and $PYY_{3-36}$ (LT) caused a significant reduction in food intake 4 h post-injection [0–2 h FI: $PYY_{3-36}$ 500 nmol/kg: 0.44 ± 0.2 g, $PYY_{3-36}$ (LT) 500 nmol/kg: 0.41 ± 0.1 g; saline: 0.95 ± 0.4 g; $P < 0.001$ versus saline; 2–4 h FI: $PYY_{3-36}$ 500 nmol/kg: 0.02 ± 0.04 g, $PYY_{3-36}$ (LT) 500 nmol/kg: 0.001 ± 0.001 g; saline: 0.17 ± 0.18 g; $P < 0.001$ versus saline] (Fig. 3B). However, the 500 nmol/kg dose of $PYY_{3-36}$ (LT) produced a significant reduction in a food intake compared to both $PYY_{3-36}$ and saline at the 4–8, 8–24 and 0–24 h time-points [4–8 h FI: $PYY_{3-36}$ (LT) 500 nmol/kg: 0.005 ± 0.01 g, $PYY_{3-36}$ 500 nmol/kg: 0.77 ± 0.2 g, saline: 0.92 ± 0.2 g; $P < 0.001$ $PYY_{3-36}$ (LT) versus saline and $PYY_{3-36}$; 8–24 h FI: $PYY_{3-36}$ (LT) 500 nmol/kg: 2.6 ± 0.8 g; $PYY_{3-36}$ 500 nmol/kg: 4.13 ± 0.3 g, saline: 3.7 ± 0.3 g; $P < 0.001$ $PYY_{3-36}$ (LT) versus saline and $PYY_{3-36}$; $PYY_{3-36}$ (LT) 500 nmol/kg: 0–24 h FI: $PYY_{3-36}$ (LT) 500 nmol/kg: 3.0 ± 0.8 g, $PYY_{3-36}$ 500 nmol/kg: 5.4 ± 0.4 g, saline: 5.7 ± 0.5 g; $P < 0.001$ $PYY_{3-36}$ (LT) versus saline and $PYY_{3-36}$] (Fig. 3B).

### Experiment 3: The effect of peripheral administration of PP and $PYY_{3-36}$ on $Mn^{2+}$ uptake in the hypothalamus and brainstem of fasted mice

Subcutaneous administration of PP resulted in a significant reduction in SI in the ARC ($P < 0.01$), PVN ($P < 0.05$) and VMH

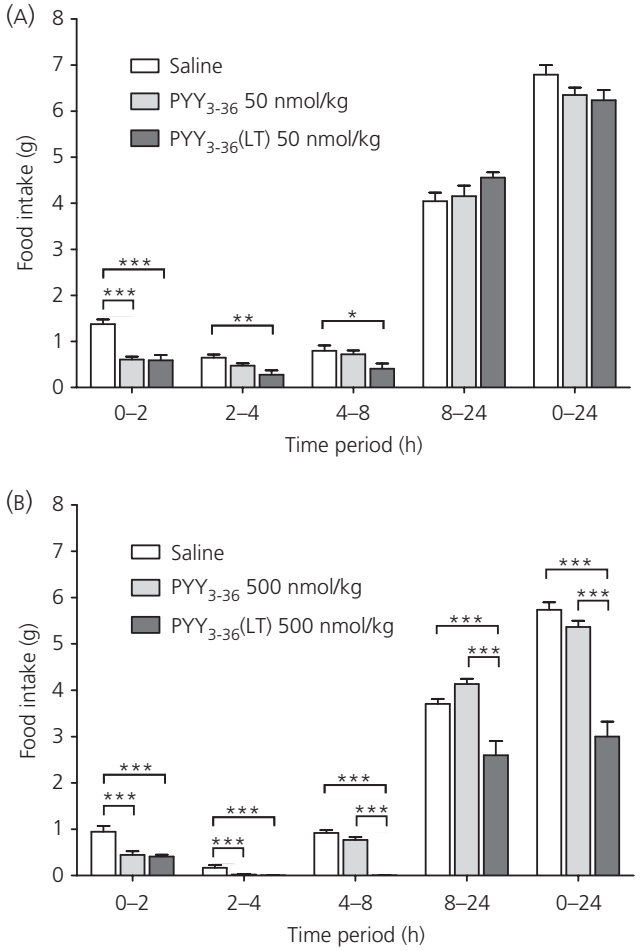


**Fig. 3.** The effects of s.c. injection of peptide YY $(PYY)_{3-36}$, and $PYY_{3-36}$ (LT) on food intake in fasted mice. Fasted mice were injected in the early light phases with (A) 50 nmol/kg of $PYY_{3-36}$, $PYY_{3-36}$ (LT) or saline; or (B) 500 nmol/kg of $PYY_{3-36}$, $PYY_{3-36}$ (LT) or saline; n = 9 per group; ***P < 0.001, **P < 0.01 and *P < 0.05; data analysed by one-way ANOVA with Bonferroni correction.

(P < 0.001) compared to fasted controls (Fig. 4, Table 1). The difference in SI between PP and controls became statistically significant at approximately 10 min post- peptide and $MnCl_2$ infusion (ARC SI at t = 10 min: control: 22.7 ± 8.0; PP: 14.6 ± 10.6, P = 0.03). No significant differences were recorded in hypothalamic or brainstem SI profile after s.c. $PYY_{3-36}$ injection. A comparison of peptide groups revealed a significant reduction in SI in the ARC (P < 0.01), VMH (P < 0.01) and PeN (P < 0.05) of PP compared to $PYY_{3-36}$ injected animals (Fig. 4, Table 1). No significant difference was detected between the SI profiles of treatment groups in the fourth ventricle (Fig. 4, Table 1).

### Experiment 4: The long-term effects of peripheral administration of $PYY_{3-36}$ and $PYY_{3-36}$ (LT) on $Mn^{2+}$ uptake in the hypothalamus and brainstem of fasted mice

There were no significant differences between the SI profiles of mice injected s.c. with $PYY_{3-36}$, $PYY_{3-36}$ (LT) or saline in any ROI recorded during the 0–2 or 4–6 h scans (Table 2). A significant reduction in ARC SI was recorded between $PYY_{3-36}$ (LT) and both saline and $PYY_{3-36}$ groups during the second 2–4 h scan (2–4 h scan ARC SI: $PYY_{3-36}$ (LT) versus saline, P = 0.035; $PYY_{3-36}$ (LT) versus $PYY_{3-36}$, P = 0.045; Table 2, Fig. 5B). There was a trend towards a reduced SI in the PVN of $PYY_{3-36}$ (LT) injected animals during the 2–4 h scan, although this did not reach statistical significance (2–4 h scan PVN SI: $PYY_{3-36}$ (LT) versus saline, P = 0.053; Table 2, see also Supporting information, Fig. S3). No significant differences in SI profile were observed in the VMH, PeN, AP or fourth ventricle (Table 2). There was a trend towards a reduced SI in the ARC of $PYY_{3-36}$ injected animals during the 10–40 min manganese infusion compared to saline-injected animals at the 2–4 and 4–6 h scan. However, this did not reach statistical significance (P = 0.14 and P = 0.54, respectively).

## Discussion

Endogenously-released gut peptides represent a promising therapeutic approach for the treatment of obesity and its associated co-morbidities. PP and $PYY_{3-36}$ are secreted post-prandially in proportion to caloric intake and suppress appetite when administered exogenously in both humans and rodents (7–9,29). Neuronal populations in the hypothalamus, vagus and brainstem have all been implicated in transmitting their effects. However, identifying the specific regions of the central nervous system (CNS) activated by these peptides is confounded by species differences and inconsistent data from knockout and electrophysiological studies (7,12,13,19). In the present study, we have used MEMRI in conjunction with the peripheral administration of PP, $PYY_{3-36}$ and $PYY_{3-36}$ (LT) to determine the pattern of CNS activation induced by these peptides in mice *in vivo*.

In agreement with previous MEMRI studies (23–25), we show a significant increase in SI recorded in the hypothalamic nuclei of fasted compared to *ad lib.* fed animals. It should be noted that the increased hypothalamic SI we repeatedly observed in the fasted state contrasts with analyses of hypothalamic activity carried out using more established immunohistochemical techniques, such as Fos-like-immunoreactivity. Although measurement of Fos-like-immunoreactivity is an effective means of determining neuronal activation, studies indicating no effect of fasting on hypothalamic c-Fos expression (30,31) contrast with those where differences have been detected (32,33). Ultimately, the increased fasted state SI provides a paradigm onto which we are able to measure the effects of peripheral delivery of anorexigenic gut peptides. We have previously shown that the appetite suppressing hormones $GLP\text{-}1_{7\text{-}36amide}$ and OXM both significantly reduce hypothalamic SI in fasted animals; the related peptides producing specific patterns of activation (25,26). We have also shown that $PYY_{3-36}$ significantly reduces SI in the PeN and fourth ventricle in fasted animals (24). In the present study, we demonstrate that s.c. administration of PP in the fasted state results in a similar reduction in SI in the ARC, VMH and PVN compared to both $PYY_{3-36}$ and saline-injected controls.

Recent data suggest that the anorexigenic effects of acute peripheral PP injection are a result of the resultant reduction in or-

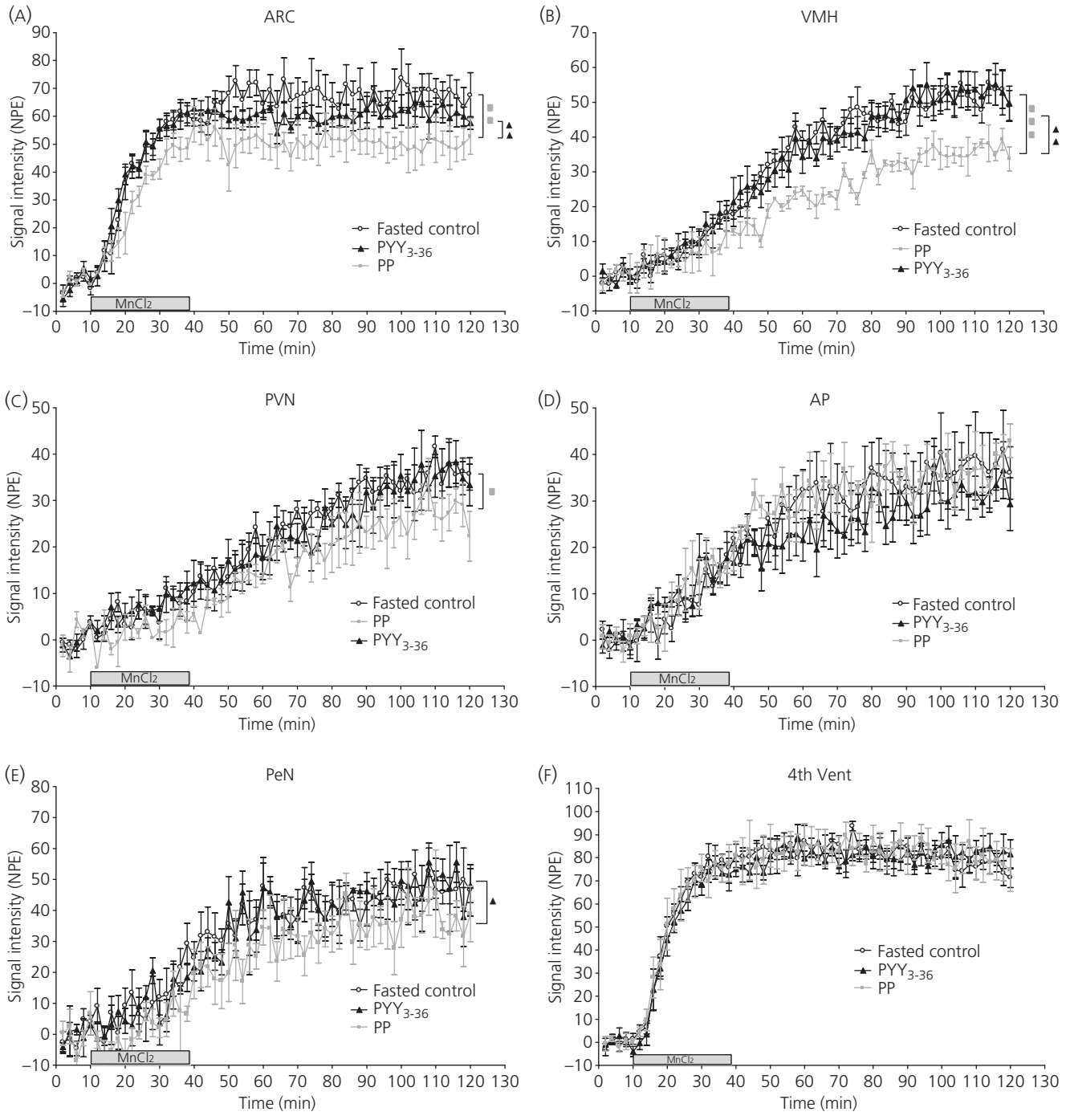


**Fig. 4.** $T_1$-weighted manganese-enhanced magnetic resonance imaging (MEMRI) signal intensity profiles in fasted mice injected s.c. with peptide YY $(PYY)_{3-36}$, pancreatic polypeptide (PP) or saline. Time course of normalised $T_1$-weighted MEMRI signal intensity (SI) change recorded in the (A) arcuate nucleus (ARC), (B) ventromedial nucleus of the hypothalamus (VMH), (C) paraventricular nucleus (PVN), (D) area postrema (AP), (E) periventricular nucleus (PeN) and (F) fourth ventricle (4th Vent). The grey bar indicates the duration of the i.v. infusion; s.c. PP dose: 500 nmol/kg (n = 4); s.c. $PYY_{3-36}$ dose: 500 nmol/kg, (n = 6); saline (0.9%, n = 5). ■, $P < 0.001$ PP versus saline; ▲▲, $P < 0.01$ PP versus $PYY_{3-36}$ and ▲, $P < 0.05$ PP versus $PYY_{3-36}$. Statistical differences were determined by generalised estimating equations. Results are the mean ± SEM.

exin expression in the lateral hypothalamic area (LHA) and increased expression of the anorexigenic factor, brain-derived neurotrophic factor in the VMH (34). Conversely, direct administration of PP into the LHA has the opposite effect on orexin expression and food intake (35). These data suggest an indirect route of hypothalamic activation for circulating PP and are consistent with the findings of Sainsbury *et al.* (34), showing that the level of c-Fos immunoreactivity in the LHA was greater at 90 min than 30 min,

**Table 1.** Comparison of Signal Intensity (SI) Profiles Following s.c Administration of Pancreatic Polypeptide (PP), Peptide YY $(PYY)_{3-36}$, or Vehicle in Fasted Mice.

| | ARC | PVN | VMH | AP | PeN | 4th V |
|---|---|---|---|---|---|---|
| PP versus fasted controls | **< 0.002** | **< 0.022** | **< 0.0001** | 0.755 | 0.102 | 0.910 |
| $PYY_{3-36}$ versus fasted controls | 0.325 | 0.946 | 0.796 | 0.957 | 0.682 | 0.832 |
| PP versus $PYY_{3-36}$ | **< 0.009** | **< 0.252** | 0.0039 | 0.260 | **< 0.045** | 0.799 |

The values represent P values generated from a statistical comparison of the SI profiles in specific regions of interest of fasted mice s.c. administered PP (500 nmol/kg), $PYY_{3-36}$ (500 nmol/kg) or saline control (0.9%). Statistical comparison was performed using generalised estimating equations. ARC, arcuate nucleus; PVN, paraventricular nucleus; VMH, ventromedial nucleus of the hypothalamus; AP, area postrema; PeN, periventricular nucleus; 4th Vent, fourth ventricle.

**Table 2.** Time-course Comparison of Signal Intensity (SI) Profiles at 0–2, 2–4 and 4–6 h Post s.c. Administration of Peptide YY $(PYY)_{3-36}$, $PYY_{3-36}$ (LT) or Vehicle in Fasted Mice.

| | ARC | PVN | VMH | AP | PeN | 4th V |
|---|---|---|---|---|---|---|
| $PYY_{3-36}$ versus fasted controls | | | | | | |
| Scan 1: 0–2 h | 0.325 | 0.796 | 0.946 | 0.457 | 0.682 | 0.832 |
| Scan 2: 2–4 h | 0.677 | 0.405 | 0.09 | 0.547 | 0.282 | 0.604 |
| Scan 3: 4–6 h | 0.253 | 0.527 | 0.372 | 0.933 | 0.851 | 0.508 |
| $PYY_{3-36}$ (LT) versus fasted controls | | | | | | |
| Scan 1: 0–2 h | 0.213 | 0.334 | 0.528 | 0.175 | 0.303 | 0.106 |
| Scan 2: 2–4 h | **0.035** | 0.053 | 0.176 | 0.818 | 0.563 | 0.051 |
| Scan 3: 4–6 h | 0.405 | 0.923 | 0.870 | 0.507 | 0.413 | 0.897 |
| $PYY_{3-36}$ versus $PYY_{3-36}$ (LT) | | | | | | |
| Scan 1: 0–2 h | 0.516 | 0.824 | 0.625 | 0.599 | 0.291 | 0.085 |
| Scan 2: 2–4 h | **0.045** | 0.207 | 0.862 | 0.284 | 0.679 | 0.052 |
| Scan 3: 4–6 h | 0.795 | 0.172 | 0.365 | 0.771 | 0.794 | 0.219 |

Fasted mice were injected s.c. with $PYY_{3-36}$ (500 nmol/kg), $PYY_{3-36}$ (LT) (500 nmol/kg) or saline (0.9%) (n = 15/group). MEMRI scans were performed at three consecutive time-points; 0–2, 2–4 and 4–6 h post injection (n = 5/group per time-point). The values represent P-values generated following a statistical comparison of the SI profiles in specific regions of interest by generalised estimating equations. ARC, arcuate nucleus; PVN, paraventricular nucleus; VMH, ventromedial nucleus of the hypothalamus; AP, area postrema; PeN, periventricular nucleus; 4th Vent, fourth ventricle.

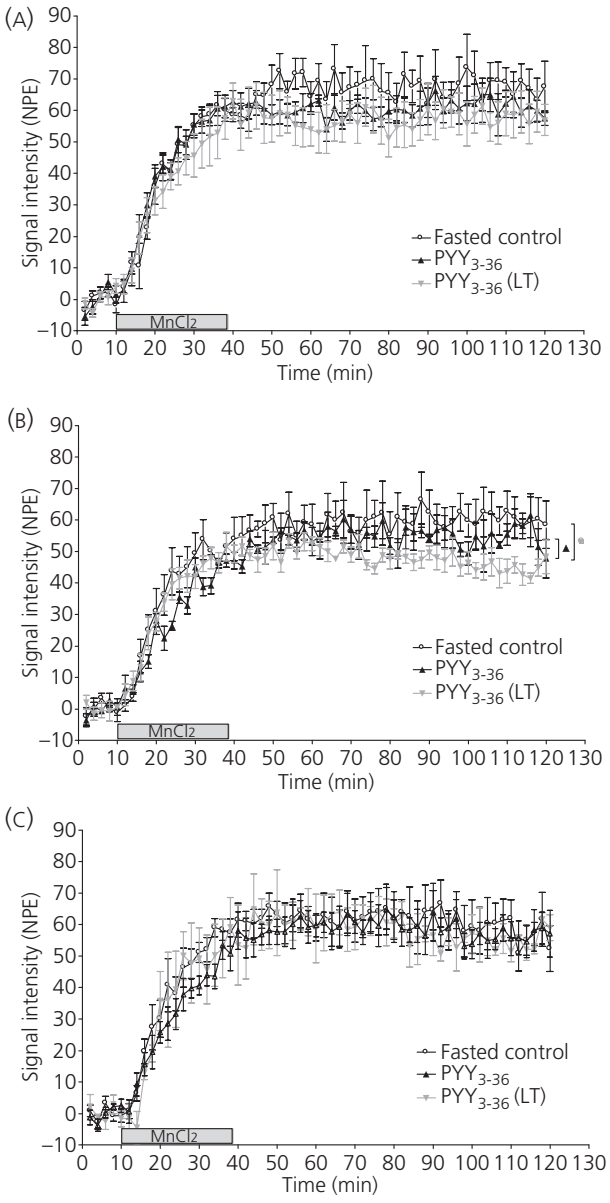


**Fig. 5.** Time-course $T_1$-weighted manganese-enhanced magnetic resonance imaging (MEMRI) signal intensity profiles in the arcuate nucleus of fasted mice injected s.c. with peptide YY $(PYY)_{3-36}$, $PYY_{3-36}$ (LT) or saline. Fasted mice were injected s.c. with $PYY_{3-36}$ (500 nmol/kg), $PYY_{3-36}$ (LT) (500 nmol/kg) or saline (0.9%) (n = 15 per group). MEMRI scans were performed at three successive time-points; (A) 0–2 h, (B) 2–4 h and (C) 4–6 h post injection (n = 5 per group per time-point) and arcuate nucleus (ARC) signal intensity (SI) recorded. The grey bar indicates the duration of the i.v. infusion; ■, P < 0.05 PP versus saline; ▲, P < 0.05 PP versus $PYY_{3-36}$. Statistical differences were determined by generalised estimating equations.

post PP injection in fasted mice. Although the effects of peripheral PP injection on hypothalamic mRNA expression and c-Fos reactivity may be long-term, our MEMRI analysis indicates a rapid hypothalamic response to peripheral PP injection; with a significant reduction in ARC SI after approximately 10 min post-peptide injection. The reduction in ARC SI we have observed after PP administration is consistent with electrophysiology experiments conducted on mouse brain slices (36). A study by Acuna-Goycolea *et al.* (36) revealed that PP decreased spike frequency in GABAergic neurones in the ARC via a presynaptic mechanism and it was recently shown that GABAergic ARC neurones provide a strong orexigenic drive (37). Given that we are measuring the effects of peptides in the fasted state, it is likely that we are weighting our imaging analysis to detect the suppression of orexigenic circuits that will be up-regulated in fasted animals. It may be that the reduction in SI within the VMH, PVN and PeN observed after PP delivery arises as a result

of the decreased activity in first-order orexigenic ARC neurones projecting to second-order neurones within these regions.

It should be noted that changes in SI recorded using MEMRI without BBB disruption are likely to arise from the proximity of a given hypothalamic ROI to the median eminence, the site of manganese entry into the brain (33). The proximity of the ARC to the median eminence means that the SI in the ARC rises most rapidly and to a greater extent compared to more anatomically distant hypothalamic regions, such as the VMH and PVN. Therefore, the earliest detectable difference between MEMRI SI profiles is limited by the necessity for sufficient manganese to reach a given ROI before group differences in SI can be recorded.

Similar to the ARC, the AP in the brainstem represents a receptive site for humoral factors regulating feeding behaviour. Previous MEMRI analysis revealed a significant increase in AP SI in *ad lib.* fed compared to fasted animals, in contrast to the differences observed in the hypothalamus. Furthermore, peripheral injection of GLP-$1_{7\text{-}36amide}$ and OXM in fasted mice resulted in a significantly increased SI, resulting in a SI profile comparable to that of *ad lib.* fed mice (25). The AP and nucleus of the solitary tract in the brainstem are ROIs implicated in transmitting the aversive response to the CNS. Indeed, previous MEMRI analysis has shown a sharp increase in SI in the AP after peripheral injection of the aversive agent lithium chloride (25). Previous studies have suggested that $PYY_{3\text{-}36}$ causes conditioned taste aversion and Fos expression in the AP (15). However, we have recorded no effect of $PYY_{3\text{-}36}$, $PYY_{3\text{-}36}$ (LT) or PP in this region. Furthermore, our behavioural study reveals that the more potent analogue $PYY_{3\text{-}36}$ (LT) did not cause aversive-like symptoms that were observed after LiCl (see Supporting information, Fig. S4). Although these data would indicate that neither PP, nor $PYY_{3\text{-}36}$ alters AP neuronal activity, it should be noted that the peak SI we recorded of approximately 40% was lower than the 120% increase observed in the aforementioned study, despite an identical dosage and infusion rate of manganese. The reduced transverse slice thickness that we have implemented compared to previous studies (0.4 versus 1 mm) may have contributed towards the reduction in signal within the brainstem and our failure to detect a difference between physiological states and peptide administration. Since the peak SI in the NTS was even lower still (data not shown) this prevented effective measurement of neuronal activity in this region. Therefore, our MEMRI protocol is not optimized to assess neuronal activity in the brainstem in response to nutritional states or gut hormone treatments. This is unfortunate as the brainstem is considered to be an important site of interaction in suppressing feeding for various circulating factors such as leptin [44].

The differences that we observe between PP and $PYY_{3\text{-}36}$ groups occur despite both peptides inducing a comparable reduction in food intake, suggesting that the anorexigenic effects of PP and $PYY_{3\text{-}36}$ are mediated by separate CNS pathways. This is further supported by the phenotype of the melanocortin 4 receptor (MC4R) KO mouse in which the anorexigenic effect of $PYY_{3\text{-}36}$ is retained but that of PP is lost (18,45). Since the MC4R is highly expressed in the brainstem and hypothalamus this implicates a brainstem and/or hypothalamic mechanism of action for PP upstream of MC4R signalling but not for $PYY_{3\text{-}36}$. Indeed, a functional MRI study in human volunteers recently implicated corticolimbic and higher cortical centres involved in the hedonic processing of food in the physiological response to $PYY_{3\text{-}36}$ (38). Furthermore, in a rat relapse model of high fat food seeking, peripheral, but not intra-ARC administration of $PYY_{3\text{-}36}$ prevented pellet priming and cue-induced reinstatement of food seeking, suggesting that nonhypothalamic centres are involved in the response to peripheral delivery of the peptide (39). Previous MEMRI studies examining activity within these structures have been carried out in conjunction with hyperosmolar breakdown of the BBB (40). Compromise of the BBB in this fashion facilitates manganese entry into more distal regions of the brain, allowing the activity in reward centres to be studied. However, differential access to hypothalamic nuclei is key to the effects of peripherally circulating peptides such as $PYY_{3\text{-}36}$ (41) and would therefore not be an appropriate method for examining their behaviour *in vivo*. Localised stereotactic injection of $MnCl_2$ into specific brain regions may provide an effective means of monitoring the effects of appetite regulating peptides in ROI beyond the range of manganese diffusion across the BBB (21,42).

To measure the long-term effects of $PYY_{3\text{-}36}$ delivery on SI, we performed a series of scans at 0–2, 2–4 and 4–6 h post-injection. The lack of difference in ARC, PVN or VMH SI observed between $PYY_{3\text{-}36}$ and saline controls at 0–2 h is in line with previous MEMRI data that utilised a lower dose of the peptide (24). It should be noted that we have not replicated the significant effects observed in the PeN and fourth ventricle following $PYY_{3\text{-}36}$ administration in this previous study. It is possible that the higher dose of $PYY_{3\text{-}36}$ that we have implemented in the present study is responsible for these differences. Similar to other functional MRI techniques, MEMRI data represent the net change in SI within a given ROI (21). A decrease in activity in one subset of neurones counterbalanced by an increased activity in another population would result in no net difference in SI. Therefore, the summative nature of MEMRI imaging data may explain why we have failed to detect an effect of $PYY_{3\text{-}36}$ on SI in hypothalamic ROI identified by electrophysiological and immunohistochemical studies as centres for processing the effects of the peptide (7,13,14,43).

Interestingly, s.c. injection of the analogue $PYY_{3\text{-}36}$ (LT) resulted in a significant reduction in ARC SI 2–4 h compared to both control and $PYY_{3\text{-}36}$ groups. $PYY_{3\text{-}36}$ (LT) is more resistant to degradation in the circulation via endogenous peptidases than $PYY_{3\text{-}36}$ (unpublished data M. L. A.) yet binds to the Y2R with a similar affinity to $PYY_{3\text{-}36}$ (see Supporting information, Fig. S1). We surmise that the reduction in ARC SI at 2–4 h and the sustained anorexigenic effect of $PYY_{3\text{-}36}$ (LT) at 4–8 h post-injection reflect the increased circulating levels of the analogue. The longer plasma half-life (unpublished observations J. M.) of $PYY_{3\text{-}36}$ (LT) may therefore generate a sufficiently persistent stimulus to be detectable by MEMRI. The 2-h delay before the reduction in ARC SI implies that $PYY_{3\text{-}36}$ (LT) does not work directly on the hypothalamus. However, we have not recorded a significant effect on AP activity to implicate the brainstem as a possible alternative route of activation. It should also be noted that neither chemical, nor surgical vagotomy in mice diminishes the anorexigenic effect of $PYY_{3\text{-}36}$, suggesting that the brainstem is not an integral part of the neuronal circuitry transmitting the signals generated by this hormone to suppress feeding. (15,16). Future studies using time-course MEMRI scans combined with a

more rigorous measurement of food intake data using metabolic cages should help clarify these effects.

In summary, in the present study, we demonstrate differential patterns of activation after injection of PP and $PYY_{3-36}$, indicating that these peptides manifest their effects via different neuronal pathways. Furthermore, by implementing a series of MEMRI scans, we were able to measure significant differences in the long-term effects of $PYY_{3-36}$ and $PYY_{3-36}$ (LT) on neuronal activity in specific hypothalamic nuclei.

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## Supporting Information

The following supplementary material is available:

**Fig. S1.** Y2R binding assay for peptide YY $(PYY)_{3-36}$ and $PYY_{3-36}$ (LT).

**Fig. S2.** Regions of interest (ROIs).

**Fig. S3.** Time-course $T_1$-weighted manganese-enhanced magnetic resonance imaging (MEMRI) signal intensity profiles in the paraventricular nucleus of fasted mice injected s.c. with peptide YY $(PYY)_{3-36}$, $PYY_{3-36}$ (LT) or saline.

**Fig. S4.** Behavioural effects following peripheral administration of peptide YY $(PYY)_{3-36}$ (LT) and lithium chloride (LiCl) in fasted mice.

This supplementary material can be found in the online article.

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