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Y4 receptors and pancreatic polypeptide regulate food intake via hypothalamic orexin and brain-derived neurotropic factor dependent pathways

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ABSTRACT

Gut-derived peptides are known to regulate food intake by activating specific receptors in the brain, but the target nuclei and neurons influenced are largely unknown. Here we show that peripherally administered pancreatic polypeptide (PP) stimulates neurons in key nuclei of the hypothalamus critical for appetite and satiety regulation. In the lateral hypothalamic area (LHA), also known as the feeding center, neurons expressing the orexigenic neuropeptide orexin co-localize with the early neuronal activation marker c-Fos upon i.p. injection of PP into mice. In the ventromedial hypothalamus (VMH), also known as the satiety center, neurons activated by PP, as indicated by induction of c-Fos immunoreactivity, express the anorexigenic brain-derived neurotrophic factor (BDNF). Activation of neurons in the LHA and VMH in response to PP occurs via a Y4 receptor-dependent process as it is not seen in Y4 receptor knockout mice. We further demonstrate that in response to i.p. PP, orexin mRNA expression in the LHA is down-regulated, with Y4 receptors being critical for this effect as it is not seen in Y4 receptor knockout mice, whereas BDNF mRNA expression is up-regulated in the VMH in response to i.p. PP in the fasted, but not in the non-fasted state. Taken together these data suggest that PP can regulate food intake by suppressing orexigenic pathways by down-regulation of orexin and simultaneously increasing anorexigenic pathways by up-regulating BDNF.

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1. Introduction

Pancreatic polypeptide (PP), released from F-cells of the pancreatic islets in response to food intake, plays an important role in the control of food intake and long-term energy balance (Ueno et al., 1999; Batterham et al., 2003; Asakawa et al., 2003). PP dose-dependently reduces food intake in freely fed and fasted mice, and this is mediated through the Y4 receptor since the effect is abolished in Y4 receptor knockout mice (Balasubramaniam et al., 2006; Lin et al., 2009).

Y4 agonism with PP is thought to mediate effects on appetite and energy balance by actions within the brainstem, resulting in modulation of digestive processes such as gastric secretion, motility and emptying by modulating vagal cholinergic pathways (Ueno et al., 1999; Asakawa et al., 2003; McTigue et al., 1997; Adrian et al., 1976). Besides indirect effects via the brain stem on gastrointestinal functions, the hypothalamus also appears to be involved in mediating PP's effects on food intake. Repeated administration of PP to mice over 24 h significantly reduced mRNA expression of the orexigenic neuropeptide Y (NPY) and orexin in the whole hypothalamus (Asakawa et al., 2003). However, despite the powerful anorexigenic effects of PP and its possible role in the etiology and treatment of obesity, it is not known in which hypothalamic nuclei and via which hypothalamic pathways Y4 agonism with PP regulates appetite.

Using electrolytic lesions, two areas of the hypothalamus have been identified as having opposite effects on feeding behavior in rodents. Lesions in the lateral hypothalamic area (LHA) cause hypophagia and weight loss (Bernardis et al., 1992), whereas lesions of the ventromedial hypothalamus (VMH) are associated with hyperphagia and the development of obesity (King, 2006). Prominent neuropeptides that are know to regulate appetite and that are expressed in the LHA or the VMH are the potent orexigenic peptides orexin A and orexin B, derived from the common precursor prepro-orexin, and brain-derived neurotrophic factor (BDNF), respectively.

In the hypothalamus, prepro-orexin is expressed in neurons of the perifornical area, the posterior hypothalamic area and the LHA, with no expression in other hypothalamic areas known to express regulators of energy homeostasis, notably the paraventricular nucleus (PVN), the VMH or the arcuate nucleus of the hypothalamus (ARC) (Sakurai et al., 1998; Campbell et al., 2003; Rodgers

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et al., 2002). Orexins, particularly orexin A, powerfully stimulate food intake after intracerebroventricular administration to sated or fasted rats (Sakurai et al., 1998; Haynes et al., 1999), as well as having effects on wakefulness, pleasure and reward pathways and hormone secretion (Ganjavi HaS, 2007). On the other hand, fasting markedly up-regulates orexin mRNA expression in the LHA, further supporting its important role in appetite regulation (Sakurai et al., 1998).

Orexin-expressing neurons in the LHA may be important mediators of effects of PP on energy homeostasis, because Y4-like immunoreactivity and mRNA for Y4 receptors was found on orexin cell bodies in the LHA (Campbell et al., 2003). Additionally, repeated administration of PP to mice over 24 h significantly reduced orexin mRNA expression in the whole hypothalamus (Asakawa et al., 2003), albeit this change was not localized to a particular hypothalamic nucleus.

While the VMH has long been recognized as a satiety center, new evidence suggests that BDNF, which is highly expressed in the VMH, may contribute to this function. BDNF exerts hypophagic and weight-reducing effects similar to MC4 receptor agonism with α-MSH or synthetic analogues such as MTII (Xu et al., 2003). Melanocortin signaling may mediate effects via the BDNF system, because acute intracerebroventricular injection of MTII led to increased BDNF mRNA expression in the VMH of fasted mice (Xu et al., 2003). Moreover, lack of BDNF as in BDNF mutant mice or lack of BDNF signaling through its tyrosine kinase receptor B (trkB) leads to an obesity syndrome that mimics obesity due to lack of MC4 receptor signaling (Xu et al., 2003). Central or peripheral administration of BDNF has been shown to reduce food intake, increase energy expenditure, and reduce circulating insulin and glucose levels in *db/db* mice (Tsuchida et al., 2001). In keeping with a role of BDNF in the day-to-day regulation of energy homeostasis, food deprivation reduces BDNF mRNA levels in the VMH (Xu et al., 2003), and genetically obese *ob/ob* mice show increased VMH expression of BDNF mRNA (Komori et al., 2005), perhaps as an adaptive measure to oppose increased adiposity. Moreover i.v. administration of leptin at a dose sufficient to activate downstream signaling in the hypothalamus lead to up-regulation of BDNF mRNA and protein levels in the VMH, specifically in the dorsomedial aspect (Komori et al., 2006). Taken together, these data suggest that BDNF may mediate the anorexigenic and weight-reducing effects of melanocortin and leptin signaling, and lead us to hypothesize that BDNF in the VMH may also contribute to the anorexigenic effects of PP.

In light of these findings, we hypothesized that Y4 receptor agonism with PP may play a direct and/or indirect role in modulating orexin and BDNF neurons and thereby providing a means by which PP may control food intake. To test this hypothesis we investigated the activation state of orexin and BDNF neurons in the LHA and the VMH, respectively, upon i.p. injection with PP by measuring immunoreactivity for the early neuronal activation marker c-Fos in these cells using double immunostaining for c-Fos and orexin or double immunostaining and radioactive *in situ* hybridization for c-Fos and BNDF mRNA. We also examined the regulation of orexin and BDNF mRNA expression in the LHA and the VMH in response to peripheral PP administration. Additionally, to investigate the role of Y4 receptors in these processes, we compared responses in wild type and in Y4 receptor deficient ($Y4^{-/-}$) mice.

2. Experimental procedures

2.1. Animals

All research and animal care procedures were approved by the Garvan Institute/St. Vincent's Hospital Animal Ethics Committee. Mice were housed in groups of 3–5 in cages (Techniplast, Rydalmere, NSW, Australia) 29.0 cm × 11.5 cm × 12.5 cm high, equipped with environmental enrichment: a metal ring for climbing integrated in the metal cage lid, tubes or domes for hiding, soft particulate bedding and cellulose paper as nesting material. Mice were housed under conditions of controlled temperature (22 °C) and illumination (12-h light cycle, lights on at 07:00 h) with *ad libitum* access to water and normal chow (6% kilojoules from fat, 21% kilojoules from protein, 71% kilojoules from carbohydrate, 2.6 kilocalories/g, Gordon's Speciality Stock Feeds, Yanderra, NSW, Australia) unless otherwise stated. Fifteen to sixteen week-old male wild type and Y4 receptor knockout ($Y4^{-/-}$) mice on a mixed C57/Bl6-129SvJ background, generated as described previously (Sainsbury et al., 2002a), were used. Unless otherwise stated, all mice were fasted prior to administration of PP in order to maximize effects of PP on food intake and other parameters, as fasting has been shown to enhance the ability of gut-derived satiety hormones to elicit significant hypophagic effects (Challis et al., 2003).

2.2. Immunohistochemistry for determination of PP-induced changes in brain c-Fos expression

Twenty wild type and 20 $Y4^{-/-}$ mice that had been fasted for 24 h were injected with PP (i.p. 200 μg/kg body weight) or saline vehicle in a volume of 10 ml/kg between 10:00 and 12:00 h. At 30 or 90 min after i.p. injection, mice were deeply anaesthetized with ketamine/xylazine (100 and 20 mg/kg from Parke Davis-Pfizer, Sydney, Australia and Bayer AG, Leverkusen, Germany, respectively) and perfused via the left ventricle and right carotid artery with 25 ml phosphate buffered saline following by ice-cold 4% paraformaldehyde in phosphate buffered saline. Our previous experience with another molecule that regulates food intake, macrophage inhibitory cytokine-1 (MIC-1) (Johnen et al., 2007), revealed that this 30 min time point is important for determining specific neurons in the brain that are activated after peripheral administration of MIC-1. However, to more comprehensively investigate the activation of c-Fos in response to i.p. PP administration, we also looked at a 90-min time point. Importantly, our 30-min time point used throughout these studies is earlier than time points at which physiological responses to PP have been observed. For instance, PP has been shown to significantly reduce food intake when measured at 1 (Asakawa et al., 1999) or 2 and 4 (Balasubramaniam et al., 2006) h after i.p. administration. Brains from the five mice in each group were immediately removed and placed in 4% paraformaldehyde for 30 min and then in phosphate buffered saline containing 30% sucrose in which they remained overnight. Coronal slices of 30 μm thickness were mounted on glass slides (Menzel-Glaser, Braunschweig, Germany) and washed in 1% H_2O_2 in 50% alcohol for 20 min to abolish endogenous peroxidase activity. Sections were incubated overnight at room temperature with the primary antibody; rabbit-anti-mouse c-Fos (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted at 1:4000 in phosphate buffered saline containing 0.1% Triton X-100. After three 10-min washes in phosphate buffered saline-Triton, sections were incubated for 3 h with the biotinylated secondary antibody (Sigma–Aldrich, St. Louis, MO, USA), diluted 1:250 in phosphate buffered saline. Sections were washed three times for 10 min each in phosphate buffered saline and then incubated with Avidin–Biotin-Peroxidase Vectastain® (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Sections were rinsed in phosphate buffered saline and treated with diaminobenzidine (Dako, Carpinteria, CA, USA) for 5 min. Slides were rinsed in water and dehydrated through to xylene before cover slipping. Sections were visualized for c-Fos-like immunoreactivity using a Zeiss Axiophot microscope equipped with the ProgRes digital camera (Carl Zeiss Imaging Solutions GmbH, Munich, Germany).

2.3. Double immunostaining for PP-induced c-Fos and orexin immunoreactivity in the LHA

Five wild-type mice that had been fasted for 24 h were injected via the intraperitoneal route with PP (200 μg/kg body weight) in a volume of 10 ml/kg saline between 10:00 and 12:00 h. At 30 min after injection, mice were deeply anaesthetized with ketamine/xylazine and perfused, and brains were isolated, sectioned and treated to abolish endogenous peroxidase activity as described above. Sections were incubated overnight at room temperature with the primary antibodies, goat-anti-mouse c-Fos (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and rabbit-anti-mouse orexin A (Alpha Diagnostics, San Antonio, TX, USA), diluted at 1:4000 and 1:2000, respectively, in phosphate buffered saline containing 0.1% Triton X-100. After three 10-min washes in phosphate buffered saline-Triton, sections were incubated for 2 h with biotinylated anti-goat secondary antibody (Chemicon, Temecula, CA, USA) diluted 1:250 in phosphate buffered saline. Sections were then washed three times in phosphate buffered saline for 10 min each and incubated for 30 min at room temperature with avidin–biotin-peroxidase (Sigma–Aldrich, St. Louis, MO, USA). Sections were washed in phosphate buffered saline and treated with 0.05% diaminobenzidine tetrahydrochloride (Sigma–Aldrich, St. Louis, MO, USA) and 0.007% hydrogen peroxide in the presence for 0.04% nickel ammonium sulphate to stain for c-Fos immunoreactivity. Section were then washed in phosphate buffered saline and incubated for 2 h with the biotinylated anti-rabbit secondary antibody (Sigma–Aldrich, St. Louis, MO, USA) diluted 1:250 in phosphate buffered saline. Sections were then washed three times for 10 min each in phosphate buffered saline and incubated for 30 min at room temperature with Avidin–Biotin-Peroxidase Vectastain® (Vector Laboratories, Burlingame, CA, USA). Sections were rinsed in phosphate buffered saline and treated with diaminobenzidine (Dako, Carpinteria, CA, USA) to detect orexin. Slides were rinsed in water and dehydrated through to xylene before cover slipping.

2.4. Double immunostaining and in situ hybridization for PP-induced c-Fos immunoreactivity and BDNF mRNA in the VMH

Five wild-type male mice that had been fasted for 24 h were injected via the intraperitoneal route with PP (200 μg/kg body weight) in a volume of 10 ml/kg saline between 10:00 and 12:00 h and were killed by cervical dislocation and decapitation 30 min later. Brains were immediately removed and frozen on a metal plate on dry ice. Coronal sections, 20 μm thick, of fresh frozen brains were thaw-mounted on charged slides (Menzel-Glaser, Braunschweig, Germany) and stored at −20 °C until use. For *in situ* hybridization, mouse BDNF DNA oligonucleotides (5′-CCGAACCTTCTGGTCCTCATCCAGCAGCTCTTCGATGACGTGCTCA-3′) were labelled with [35S] thio-dATP, with the ^{35}S-labelled probe being diluted to 10^6 cpm/ml with hybridization solution prepared as previously described (Sainsbury et al., 2002b). Sections were incubated in hybridization solution for 16 h at 42 °C, then washed twice in 2×SSC with 50% formamide and 100 ml of 1 M dithiothreitol at 40 °C for 15 min and twice in 2×SSC with 50% formamide at 40 °C for 15 min. Sections were rinsed in 0.1 M phosphate buffered saline and then incubated in 1% H_2O_2 in 50% alcohol for 20 min to abolish endogenous peroxidase activity. Sections were incubated with a primary antibody for c-Fos as described for c-Fos and orexin double immunostaining, above. After incubation with the secondary antibody as described above and washing with phosphate buffered saline for 30 min, c-Fos immunoreactivity was visualized by incubation with Avidin–Biotin-Peroxidase Vectastain® (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Sections were washed in phosphate buffered saline and treated with diaminobenzidine (Dako, Carpinteria, CA, USA) for 5 min. Slides were rinsed in water then 70% ethanol, air-dried and exposed to autoradiography film for 2 days and then dipped in photo-emulsion for 6 days as previously described (Sainsbury et al., 2002b).

2.5. In situ hybridization for quantification of orexin and BDNF mRNA expression

Wild type and *Y4*$^{-/-}$ mice, half of which were freely fed and half of which had been fasted for 24 h, were injected with PP (i.p. 200 μg/kg body weight) or saline vehicle in a volume of 10 ml/kg between 10:00 and 12:00 h and were killed by cervical dislocation and decapitation 30 min later. Sections from fresh frozen brains from 5 to 6 mice per group were prepared as described above. DNA oligonucleotides complementary to mouse orexin (5′-CTTTCCCAGAGTCAGGATAC-CCGCAGCGTGGTTGCCAGCTCCGTGC-3′) and mouse BDNF DNA (see above) were labelled with [35S] thio-dATP and expression levels of orexin and BNDF mRNA were evaluated by measuring silver grain densities over individual neurons from photo-emulsion-dipped sections as described previously (Sainsbury et al., 2002b).

2.6. Statistical analyses

All data were assessed by factorial ANOVA to assess differences due to genotype (wild type or *Y4*$^{-/-}$), treatment (vehicle or PP injection) or nutritional status (fed or fasted). Where a significant effect or interaction effect was observed, Fisher's or Contrasts post hoc tests were used to determine differences among groups. Statistical analyses were performed using Super-ANOVA (Abacus Concepts Inc., CA, USA). For all statistical analyses, $p < 0.05$ was accepted as being statistically significant.

3. Results

3.1. PP activates neurons in the LHA and VMH

We have previously shown that PP dose-dependently reduces food intake in freely fed and fasted mice and this effect was mediated through the Y4 receptor as it was completely abolished in Y4 receptor knockout mice (Balasubramaniam et al., 2006). To determine whether the LHA and/or the VMH may be involved in PP's effects on food intake and to map the neuronal pathways concerned, we investigated the expression of the early neuronal marker c-Fos in response to peripherally-injected PP. Increases in c-Fos immunoreactivity were seen in both the LHA and the dorsomedial aspect of the VMH (VMHDM) of wild-type mice at 30 and at 90 min after i.p. PP injection compared to the levels seen in saline-injected control mice (Fig. 1a–f). In the LHA of wild-type mice, the number of c-Fos-like immunoreactive neurons at 30 min after saline injection was 18 ± 3 compared to 42 ± 4* and 74 ± 12* c-Fos immunoreactive neurons at 30 and 90 min after i.p. PP injection, respectively (data are mean ± SEM of five wild-type mice per group, $F(3,20) = 19.675$, $^*p < 0.0001$ versus wild-type saline-injected mice); in the VMHDM of wild-type mice, the number of c-Fos-like immunoreactive neurons at 30 min after saline injection was 19 ± 2 compared to 70 ± 5* and 66 ± 3* c-Fos immunoreactive neurons at 30 and 90 min after i.p. PP injection, respectively (data are mean ± SEM of five wild-type mice per group, $F(3,20) = 381.548$, $^*p < 0.0001$ versus wild-type saline-injected mice). These increases in c-Fos immunoreactivity in response to PP were not observed in Y4 receptor knockout mice (Fig. 1g–j). Indeed, the number of c-Fos-like immunoreactive neurons in the LHA (16 ± 2) and VMHDM (18 ± 3) of *Y4*$^{-/-}$ mice at 30 min after i.p. PP injection was not sig-

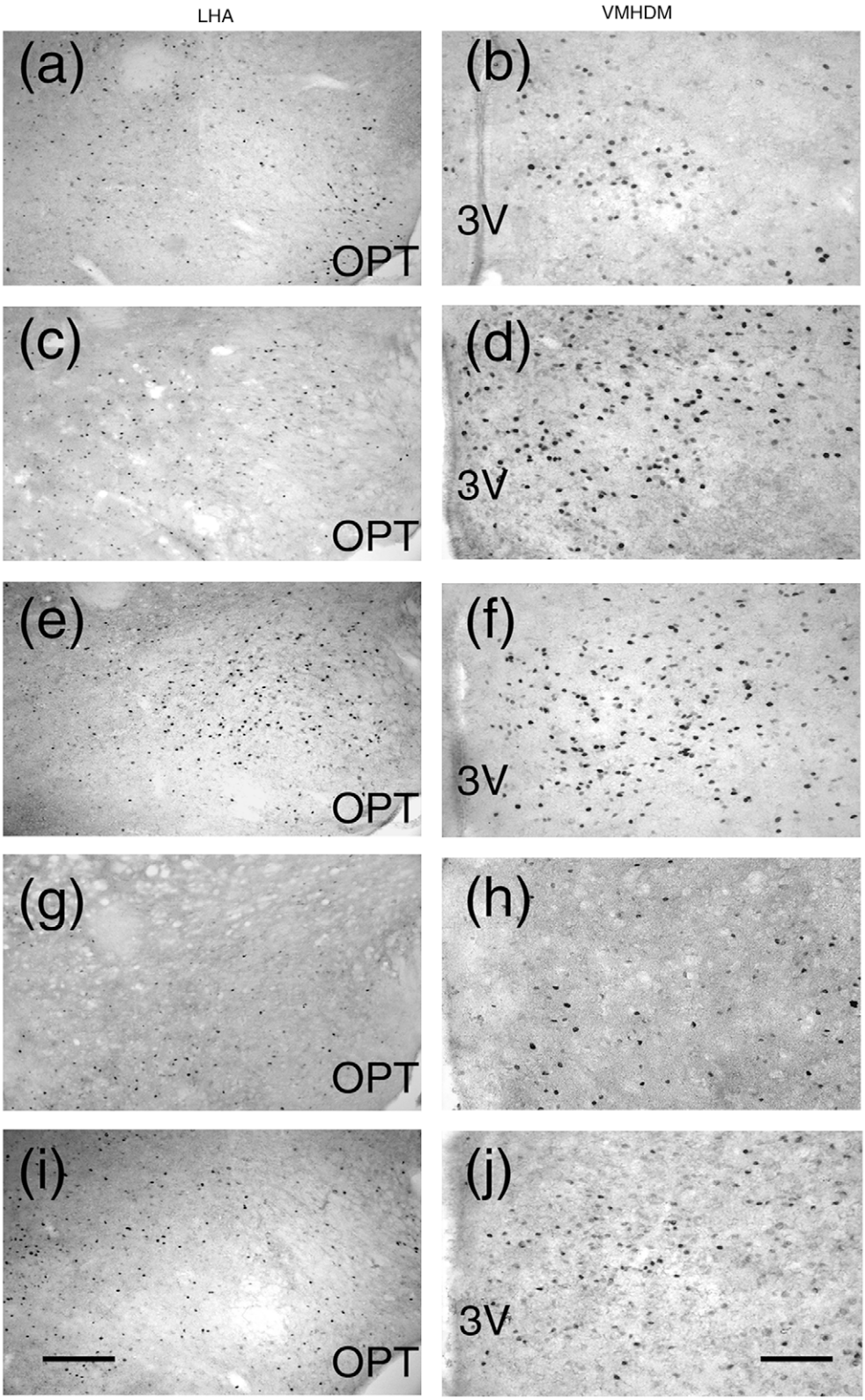

Fig. 1. Pancreatic polypeptide (PP) injection induces a Y4 receptor-dependent increase in c-Fos immunoreactivity in the lateral hypothalamic area (LHA) and the ventromedial hypothalamus (VMH). Photomicrographs of brains from wild-type mice showing c-Fos immunoreactivity at 30 min after i.p. injection of saline (a and b) or PP (c and d), or at 90 min after i.p. PP injection (e and f). Brains from Y4 receptor knockout mice ($Y4^{-/-}$) showing c-Fos immunoreactivity at 30 min after i.p. injection of saline (g and h) or PP (i and j). Photomicrographs are representative of five animals per group. Scale bar for panels a, c, e, g and i = 150 μm; scale bar for panels b, d, f, h and j = 40 μm. OPT, optic tract; 3 V, third cerebral ventricle; VMHDM, dorsomedial aspect of the ventromedial hypothalamus.

nificantly different from that of saline-injected wild-type mice (data are mean ± SEM of five mice per group), demonstrating that PP activates neurons in the LHA and the VMHDM specifically via Y4 receptors.

3.2. PP activates orexin-containing neurons in the LHA and BDNF-expressing neurons in the VMH

To characterize the chemical nature of neurons activated by i.p. PP, we performed double immunohistochemistry and double immunohistochemistry/*in situ* hybridization. In the LHA, clear overlap of immunostaining for c-Fos and orexin was detected at 30 min after i.p. PP injection (Fig. 2). In fact, 29 ± 1% of c-Fos positive neurons in the LHA were positive for orexin immunoreactivity, and 75 ± 10% of orexin positive neurons in the LHA were positive for c-Fos immunoreactivity after PP injection (data are mean ± SEM of 3–4 sections from five mice). In the VMH, cells that were positive for c-Fos immunoreactivity after i.p. PP injection also expressed mRNA for the anorexigenic acting BDNF, this effect being particularly obvious in the dorsomedial aspect of the VMH (Fig. 3). Indeed, 33 ± 5% of c-Fos immunoreactive neurons in the VMHDM were positive for BDNF mRNA, and 86 ± 4% of BDNF-expressing neurons in the VMHDM were positive for c-Fos immunoreactivity after PP injection (data are mean ± SEM of 3–4 sections from five mice).

3.3. PP inhibits orexin mRNA expression in the LHA and alters BNDF mRNA in the VMH

In wild-type animals that received i.p. injection of saline, fasting caused a significant increase in orexin mRNA expression in the LHA (Fig 4a, b and g, $F(5,19) = 24.46$, $p < 0.0001$). PP injection significantly reduced the orexin mRNA expression levels in non-fasted and fasted wild-type mice, such that the fasting-induced increase in orexin mRNA expression was no longer seen in PP-injected wild types (Fig. 4c and d). This effect of PP on orexin mRNA expression was mediated via Y4 receptors, because orexin mRNA expression in the LHA of non-fasted or fasted PP-injected $Y4^{-/-}$ mice was no different from that of saline-injected wild-type mice of the same nutritional status (Fig. 4e and f).

Fasting in saline-injected wild-type mice caused a significant drop in BDNF mRNA expression in the VMH (Fig. 5a, b, e, $F(3,16) = 20.11$, $p < 0.0001$). Expression levels of BDNF mRNA in non-fasted wild-type mice were also significantly reduced by i.p. PP injection (Fig. 5c and e). However, when animals were injected with PP in the fasted state, the fasting-induced drop in BDNF mRNA expression was reversed by the activation of Y4 signaling with PP (Fig. 5d and e). Together, these results suggest that in response to i.p. PP injection, Y4 signaling in the brain, either directly or indirectly, causes changes in expression pattern of orexigenic and also anorexigenic neuropeptides, which in turn could influence food intake.

4. Discussion

The data obtained in this study suggest that peripherally administered PP can control food intake by modulating neuronal activity and expression levels of key molecules in hypothalamic centers that control feeding (the LHA) and satiety (the VMH). This is also consistent with recent data using the same technologies showing altered c-Fos activation in other parts of the hypothala-

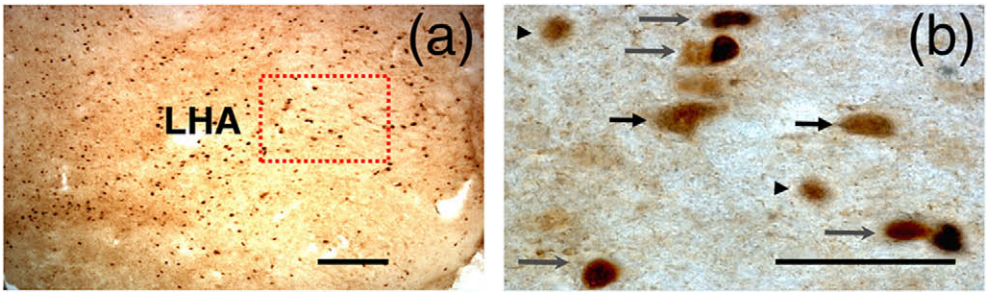

Fig. 2. Pancreatic polypeptide (PP) injection induces c-Fos immunoreactivity in orexin-immunopositive neurons in the lateral hypothalamic area (LHA). Brightfield micrographs showing co-expression of c-Fos and orexin in the LHA at 30 min after i.p. injection of PP. (a) Low-magnification and (b) boxed area from a is shown in high-magnification. Black arrows indicate neurons positive only for orexin. These neurons show feint, irregularly shaped cytoplasmic staining. Black arrowheads indicate neurons positive only for c-Fos. These neurons are smaller or more darkly stained than those stained for orexin, consistent with nuclear staining. Grey spearheads indicate neurons double-labelled for orexin and c-Fos. These neurons show a lighter-stained 'halo' of cytoplasmic orexin staining near a darker c-Fos-stained nucleus. Images are representative of five wild-type animals. Scale bar: a = 200 μm and b = 5 μm. 3 V: third cerebral ventricle.

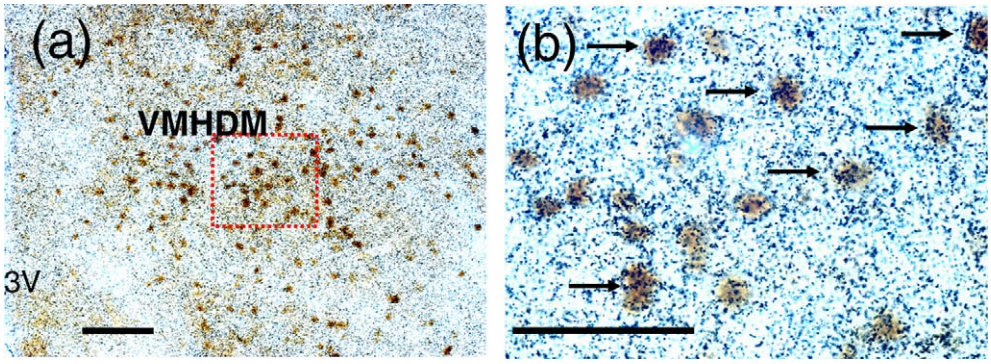

Fig. 3. Pancreatic polypeptide (PP) injection induces c-Fos immunoreactivity in cells expressing bone derived neurotrophic factor (BDNF) mRNA in the ventromedial hypothalamus (VMH). Brightfield micrographs showing c-Fos immunofluorescence and BDNF mRNA by *in situ* hybridization at 30 min after i.p. injection of PP. (a) Low-magnification and (b) boxed area is shown in high-magnification. Arrows indicate neurons double-labelled with c-Fos immunoreactivity and BDNF mRNA. Images are representative of five wild-type animals. VMHDM, dorsomedial aspect of the ventromedial hypothalamus. Scale bar: a = 40 μm and b = 5 μm.

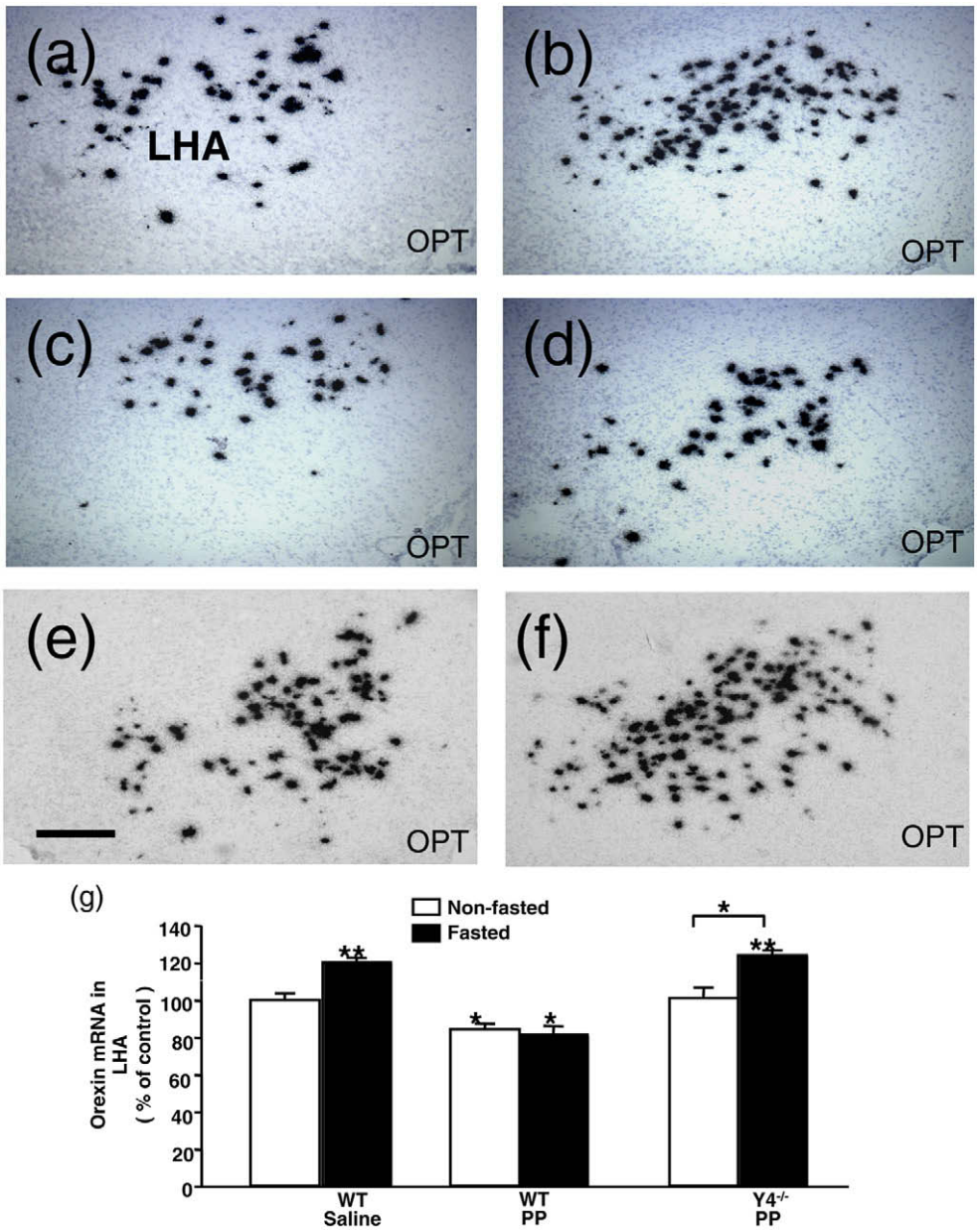

Fig. 4. Pancreatic polypeptide (PP) injection reduces orexin mRNA expression in the lateral hypothalamic area (LHA) of non-fasted and fasted mice via a Y4 receptor-dependent process. Emulsion-dipped autoradiographs (a–f) showing orexin mRNA expression in the LHA at 30 min after (a) i.p. injection of saline into wild-type mice, (b) i.p. injection of saline into wild-type mice after having been fasted for 24 h, (c) i.p. injection of PP into wild-type mice, (d) i.p. injection of PP into wild-type mice after having been fasted for 24 h, (e) i.p. injection of PP into $Y4^{-/-}$ mice, and (f) i.p. injection of PP into $Y4^{-/-}$ mice after having been fasted for 24 h. Scale bar = 25 μm; OPT, optical tract. (g) Quantification of orexin mRNA expression in the LHA, expressed as a percent of non-fasted wild-type saline-injected control mice, in non-fasted and 24-h fasted wild type and $Y4^{-/-}$ mice at 30 min after i.p. injection of saline or PP. Data are mean ± SEM of 5–6 mice per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus non-fasted wild-type saline-injected control mice or the comparison shown by horizontal bars.

mus coinciding with the reduction of food intake (Lin et al., 2009). Here we show that PP injection induced c-Fos activation in orexin immunoreactive neurons in the LHA and down-regulated orexin mRNA expression in this region via Y4 receptor-mediated processes. This change in LHA orexin expression could thereby conceivably reduce the orexigenic drive mediated by this neuropeptide in response to PP. In addition to these changes in the LHA, peripherally-injected PP activated neurons in the VMH via a Y4 receptor-mediated process, as seen by increased c-Fos immunoreactivity in the VMH after PP injection in wild type but not in $Y4^{-/-}$ mice. However, in this region PP-induced c-Fos activation occurred in neurons that express the anorexigenic acting neuropeptide BDNF and resulted in alterations in BDNF expression, thereby likely influencing BDNF's feeding inhibitory action.

PP-induced responses in c-Fos immunoreactivity and changes in neuropeptide mRNA expression could be a direct or also indirect consequence of Y4 receptor signaling on orexin or BDNF neurons. Interestingly, Y4 receptor-like immunoreactivity and Y4 receptor mRNA have been shown to be co-localized with orexin-expressing nerve bodies in the LHA (Campbell et al., 2003). However, it is not clear whether and how circulating PP could access the LHA through the blood brain barrier. Moreover, direct administration of PP into the LHA has effects on food intake and orexin expression that are *opposite* (Campbell et al., 2003) to the anorexia and reduced LHA orexin expression seen in response to i.p.-injected PP in this study.

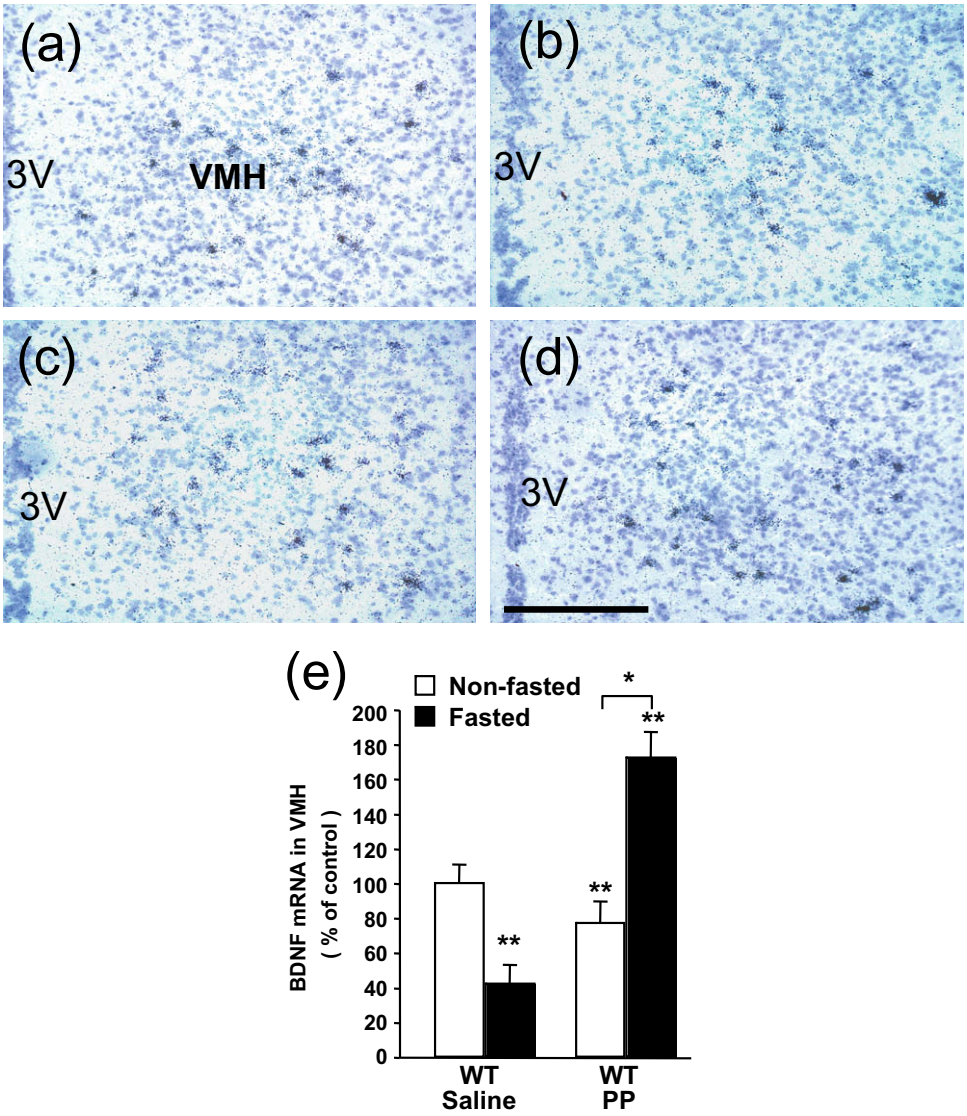

Fig. 5. Pancreatic polypeptide (PP) injection reverses the fasting-induced reduction in bone derived neurotrophic factor (BDNF) mRNA expression in the ventromedial hypothalamus (VMH). Emulsion-dipped autoradiographs (a–d) of BDNF mRNA expression in the VMH of wild-type mice at 30 min after (a) i.p. injection of saline, (b) i.p. injection of saline after having been fasted for 24 h, (c) i.p. injection of PP, and (d) i.p. injection of PP after having been fasted for 24 h. 3 V: third cerebral ventricle. Scale bar = 40 μm. (e) Quantification of BDNF mRNA expression in the VMH, expressed as a percent of non-fasted wild-type saline-injected control mice, in non-fasted and 24-h fasted wild-type mice at 30 min after i.p. injection of saline or PP. Data are mean ± SEM of 5–6 mice per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus non-fasted wild-type saline-injected control mice or the comparison shown by horizontal bars.

Therefore, it is likely that peripherally-derived PP activates orexin-containing neurons and down-regulates orexin mRNA expression in the LHA via indirect neuronal relays, consistent with our observation that the level of c-Fos immunoreactivity in the LHA was stronger at 90 than at 30 min after i.p. PP injection.

In addition to orexin, these studies reveal a possible role for VMH-expressed BDNF in mediating the anorexigenic and weight-reducing effects of PP, at least under fasted conditions or at early time points after meal ingestion. PP was shown to induce c-Fos immunoreactivity in neurons in the dorsomedial part of the VMH that co-express the anorexigenic BDNF, and PP reversed the fasting-induced reduction in BDNF expression in the dorsomedial part of the VMH. It is noteworthy that, contrary to the PP-induced increase in BDNF expression in the VMH observed under fasted conditions, PP significantly reduced BDNF expression in the VMH in non-fasted mice, which would suggest concomitant reduction in anorexigenic effects of BDNF under these conditions. In order to determine whether an increase in BDNF expression may functionally contribute to PP-induced hypophagia, it would be necessary to study effects of PP on food intake in fed and fasted mice with mutation in either BDNF or its trkB receptor.

As the VMH is not readily accessible to blood-borne factors such as PP, it is unlikely that PP regulates BDNF expression via a direct effect. One possible mechanism by which i.p.-injected PP may influence BDNF expression in the VMH and food intake is through effects on the ARC to influence POMC expression and α-MSH transmission to the VMH via neurons expressing γ-aminobutyric acid (GABA), a highly abundant inhibitory amino acid neurotransmitter found in half of all synaptic terminals in the ARC (Decavel and Van den Pol, 1990) and which mediates effects of other gut-derived satiety factors (Cowley et al., 2001). Y4 receptors are known to be expressed in the ARC (Parker and Herzog, 2000), but it is not known on which neurons (e.g., POMC or GABA-ergic neurons) they are expressed. As α-MSH produced in POMC-expressing neurons in the ARC has been shown to stimulate the expression of BDNF in neurons in the VMH via melanocortin 4 (MC4) receptors localized

on these neurons (Xu et al., 2003), it is possible that PP stimulates BDNF expression in the VMH via stimulation of α-MSH signaling from the ARC, and that this increase in BDNF expression could in turn contribute to PP-induced hypophagia. PP-induced activation of α-MSH signaling may be initiated by GABA-ergic pathways, as PP decreases activity of GABA-expressing neurons by presynaptic attenuation of glutamate release (Acuna-Goycolea et al., 2005), GABA-ergic nerve terminals synapse with POMC-containing neurons in the ARC (Cowley et al., 2001; Horvath et al., 1992), and GABA reduces mRNA expression of POMC and inhibits the release of α-MSH in the ARC (Vergoni and Bertolini, 2000; Jegou et al., 1993). In sum, a possible pathway for PP-induced up-regulation of anorexigenic BDNF expression in the VMH could be direct inhibitory action of PP on GABA-ergic neurons in the ARC, release of GABA-ergic inhibition of ARC POMC neurons resulting in up-regulation of POMC expression and enhanced α-MSH-ergic transmission to BDNF-expressing neurons in the VMH, with subsequent MC4 receptor-mediated up-regulation of BDNF expression in the VMH with subsequent anorexigenic effects.

In addition to possible effects on orexin and BDNF pathways, it is conceivable that modulation of NPY-ergic pathways may be involved in reducing food intake in response to i.p. PP. In keeping with this, it was previously shown that four i.p. injections of PP over 24 h (a total dose more than double the single acute dose used presently) significantly reduced NPY mRNA expression in the whole hypothalamus as determined by real-time PCR (Asakawa et al., 2003). However, we have not observed any change in NPY mRNA expression at 30 min after i.p. PP injection (data not shown), suggesting that any effect of PP on hypothalamic NPY expression is not a primary effect of PP action.

In conclusion, we have demonstrated that the endogenously released gut hormone PP acts via Y4 receptors to induce significant changes in hypothalamic feeding and satiety centers, which could subsequently contribute to its anorexic action. Independent of whether these effects on orexin neurons in the LHA and on BDNF neurons in the VMH occur via direct or indirect effects of PP, enhancing the endogenous production of PP or employing a PP-like agent to increase Y4 receptor signaling could provide a new avenue for the potential treatment of obesity.

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