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RESEARCH PAPER

A role for neuropeptide Y in the gender-specific gastrointestinal, corticosterone and feeding responses to stress

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BACKGROUND AND PURPOSE

Exposure to an acute stress inhibits gastric emptying and stimulates colonic transit via central neuropeptide Y (NPY) pathways; however, peripheral involvement is uncertain. The anxiogenic phenotype of $NPY^{-/-}$ mice is gender-dependent, raising the possibility that stress-induced gastrointestinal (GI) responses are female-dominant through NPY. The aim of this study was to determine GI transit rates, corticosterone levels and food intake after acute restraint (AR) or novel environment (NE) stress in male and female $NPY^{-/-}$ and WT mice.

EXPERIMENTAL APPROACH

Upper gastrointestinal transit (UGIT) (established 30 min after oral gavage) and corticosterone levels were determined under basal or restrained conditions (30 min) and after treatment i.p. with Y_1 antagonist BIBO3304 or Y_2 antagonist BIIE0246. Faecal pellet output (FPO) was established after AR and treatment i.p. with NPY in the NE, as were colonic bead expulsion rates.

KEY RESULTS

UGIT and FPO were similar in unrestrained male and female mice. $NPY^{-/-}$ females displayed significantly slower UGIT than $NPY^{-/-}$ males after AR, but both genders displayed significantly higher FPO and reduced food intake relative to WT counterparts. Peripheral NPY treatment increased bead expulsion time in WT mice. AR male $NPY^{-/-}$ mice had higher levels of corticosterone than male WT mice; whilst in AR WT mice, after peripheral Y_1 and Y_2 receptor antagonism in males, and Y_2 antagonism in females, corticosterone was significantly elevated.

CONCLUSIONS AND IMPLICATIONS

NPY possesses a role in the gender-dependent susceptibility to stress-induced GI responses. Furthermore, NPY inhibits GI motility through Y_2 receptors and corticosterone release via peripheral Y_1 and Y_2 receptors.

Abbreviations

AR, acute restraint; ARC, arcuate nucleus; BIBO3304, (*R*)-*N*-[[4-(aminocarbonylaminomethyl)-phenyl]methyl]-N^2-(diphenylacetyl)-argininamide trifluoroacetate); BIIE0246, (*S*)-N^2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6*H*)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-*N*-[2-[1,2-dihydro-3,5(4*H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl] ethyl]-argininamide; CRH, corticotrophin-releasing hormone; DMSO, dimethyl sulfoxide; DPPIV, dipeptidyl peptidase IV; ENS, enteric nervous system; FPO, faecal pellet output; GI, gastrointestinal; HPA, hypothalamic–pituitary–adrenal; IBS, irritable bowel syndrome; NPY, neuropeptide Y; NPY(3–36), neuropeptide Y(3–36); PVN, paraventricular nucleus; PYY, peptide YY; PYY(3–36), peptide YY(3–36); UGIT, upper gastrointestinal transit; WT, wild type

Introduction

Individuals are continuously exposed to different forms of psychological, social and physical stressors; and this chronic or repeated exposure is widely believed to play a major role in the pathogenesis, or exacerbation of symptoms, of many diseases (Heraclides *et al.*, 2011). Often stress-associated disorders, such as functional gastrointestinal (GI) diseases (Maunder and Levenstein, 2008) or eating disorders (Blehar, 1995) are more prevalent among women than men but the mechanisms underlying this gender differentiation are largely unknown.

The 36-amino-acid neuropeptide Y (NPY) is one of the most abundantly expressed neuropeptides in the central and peripheral nervous systems (Dumont *et al.*, 1998) and a key mediator in the responses to both acute and chronic stress. Many experimental stressors induce NPY release (Thorsell *et al.*, 1999) and up-regulate both NPY mRNA and its receptors' mRNA (Y_1, Y_2 and Y_5; nomenclature follows Alexander *et al.*, 2011), which are responsible for the physiological actions of NPY in the periphery and brain (Michel *et al.*, 1998). Acute stress up-regulates NPY in the hypothalamic arcuate (ARC) (Kas *et al.*, 2005) and paraventricular nuclei (PVN) (Dube *et al.*, 1992), where metabolic and stress-related signals are integrated and appropriate feeding, neuroendocrine and visceral responses are initiated. In the PVN, NPY activates the hypothalamic–pituitary–adrenal (HPA) axis and modulates the visceral stress responses mediated through corticotrophin-releasing hormone (CRH) pathways (Dimitrov *et al.*, 2007). Additionally, NPY is potently anxiolytic (Karl *et al.*, 2008), acting through Y_1 receptors in the amygdala to inhibit CRH signalling and terminate the behavioural stress and anxiety responses (Kask *et al.*, 2001). In humans, haplotype-driven NPY expression is able to predict responses to stress challenges and is inversely correlated with trait anxiety levels (Zhou *et al.*, 2008). Furthermore, NPY concentrations in the cerebrospinal fluid of patients with post-traumatic stress disorder are low (Sah *et al.*, 2009). Ablation of the NPY gene from mice results in a gender-dependent anxiogenic phenotype, whereby males and females display different anxiogenic responses in behavioural tests, indicating NPY has a sexually dimorphic role in behavioural stress responses (Painsipp *et al.*, 2011). Additionally, GI inflammation, which is known to enhance anxiety in a gender-dependent manner, produces different behavioural responses to stress challenges in female and male $NPY^{-/-}$ mice (Painsipp *et al.*, 2011).

An inhibition of gastric emptying and a stimulation of colonic motility are hallmark GI responses to an acute stress, demonstrated by a number of experimental stressors including exposure to painful stimuli and anger in humans (Rao *et al.*, 1998) or restraint stress in experimental animals (Martinez *et al.*, 2004). PVN Y_1 receptors and $CRHR_2$ receptors have been implicated in the central component of stress-induced upper GI motor alterations (Tache *et al.*, 1987; Chen *et al.*, 1997; Martinez *et al.*, 2004), in addition to peripheral sympathetic pathways involving α-adrenoceptors (Nakade *et al.*, 2005), whilst PVN Y_1 and downstream $CRHR_1$ receptors are implicated in stress-stimulated colonic motility (Monnikes *et al.*, 2000; Tebbe *et al.*, 2005). In support of these findings, centrally administered NPY or CRH inhibits gastric emptying and stimulates colonic transit in conscious rodents (Matsuda *et al.*, 1993; Monnikes *et al.*, 2000; Martinez *et al.*, 2004). However, NPY is also involved in the peripheral modulation of GI function, where it may counterbalance its central stress-induced stimulating effects on colonic motility (Tough *et al.*, 2011). NPY is found within the enteric nervous system (ENS) in inhibitory secretomotor submucosal nerves co-localized with vasoactive intestinal polypeptide (VIP; Fantaguzzi *et al.*, 2009) and in inhibitory myenteric motor neurons (Sang and Young, 1996). Endogenous NPY inhibits electrolyte secretion (a combination of Y_1- and Y_2-mediated effects; Tough *et al.*, 2011) and contracts longitudinal smooth muscle *in vitro* (Hyland *et al.*, 2003), in addition to inhibiting colonic motility *in vivo* by activating neuronal Y_2 receptors (Wang *et al.*, 2010; Tough *et al.*, 2011).

Feeding behaviour is also affected by stress, and although the feeding response often depends on the type of stress delivered, an acute stress such as restraint consistently suppresses food intake experimentally (Kas *et al.*, 2005). Signalling within the feeding circuitry of the ARC includes the orexigenic NPY neurons and affects eating behaviour under adverse conditions. Here, NPY potently stimulates food intake and induces weight gain through the PVN Y_1 and Y_5 receptors (Chaudhri *et al.*, 2006).

The sexually dimorphic role of NPY in behavioural stress responses revealed in $NPY^{-/-}$ mice (Painsipp *et al.*, 2011) raises the possibility that physiological stress responses mediated through NPY pathways may also differ between males and females. Given the critical role of NPY in both GI motility (Tough *et al.*, 2011) and food intake in addition to stress reactivity, it is conceivable that a gender specific role for NPY could contribute to the marked female predominance of stress-associated diseases. The present investigation therefore set out to establish if there were differences in stress-induced upper and lower GI motor function and food intake in male and female $NPY^{-/-}$ mice. In addition, the role of peripheral NPY and the Y_1 and Y_2 receptors in the modulation of stress-induced changes in GI transit was established, with previous evidence indicating Y_2 receptors play a role in inhibiting transit (Wang *et al.*, 2010). Another objective was to further elucidate the role of peripheral NPY in stress-induced corticosterone release, which Y receptors were involved, and to determine whether there were any gender differences at this level.

Methods

Targeted deletion of NPY

$NPY^{-/-}$ mice without the entire coding sequence of NPY, including the initiation start codon, were generated by homologous recombination in embryonic stem cells as described previously (Karl *et al.*, 2008). These mice exhibited no obvious abnormalities and appeared healthy (Edelsbrunner *et al.*, 2009). WT and $NPY^{-/-}$ were on a mixed 129/SvJ/C57BL/6 background and housed under controlled conditions (12:12 h light/dark cycle, lights on 07:00 h, 22 ± 2°C) and provided with standard chow and water *ad libitum* except during experimentation. All mice were aged 10–16 weeks and weight-matched within genders where possible (mean weight

of WT female mice: 24.0 ± 0.4 g, WT male: 27.3 ± 0.5 g, NPY$^{-/-}$ female: 21.2 ± 0.3 g and NPY$^{-/-}$ male mice: 26.4 ± 0.6 g, n = 26–30). The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All experiments were performed in accordance with the Animals (Scientific procedures) Act 1986. Upper GI transit (UGIT), bead propulsion experiments and blood sampling for corticosterone levels were conducted between 10:00 and 13:00 h, whilst faecal pellet output (FPO) experiments were conducted between 10:00 and 14:00 h, to minimize any influence of circadian rhythm.

Upper GI under non-restrained and restrained conditions

Mice were deprived of food for 16 h prior to experimentation, although water was provided *ad libitum*. UGIT was determined by identifying the leading front of an intragastrically administered charcoal meal marker (10% plant charcoal in 5% gum acacia) in the small intestine as described by Pol *et al.* (2005). Mice were then either restrained (see below) or placed back in the home cage. After 30 min, animals were killed by dislocation of the neck, and the small intestine was isolated by cutting at the pyloric and ileocaecal junctions. The length of the small intestine was measured, and the distance travelled by the charcoal meal was determined. For each mouse, UGIT was calculated as the % of the distance travelled by the charcoal, relative to the total length of the small intestine.

Acute restraint model

Mice were restrained for 30 min in a capped and ventilated plastic centrifuge tube, which allowed for very limited movement. To establish UGIT or corticosterone levels from trunk blood, the animals were removed from the restraining tubes and killed immediately by dislocation of the neck. In WT mice, BIIE0246 (2 mg·kg^{-1}), BIBO3304 (100 µL of 0.4 mM) or vehicle (100 µL of 10% DMSO), as delivered previously by Tough *et al.* (2011), were administered i.p. 15 min prior to restraint and therefore 45 min prior to death, to determine UGIT or corticosterone levels. In another experimental group, the number of pellets produced during 30 min restraint was counted, and the mice were removed and placed in a cage to monitor food intake for the subsequent 4 h. Normal food intake after acclimatization in the cages for 3 days was also recorded.

Novel environment stress model

Naive mice, or WT and NPY$^{-/-}$ mice administered i.p. with vehicle (100 µL saline) or NPY (8 nmol·kg^{-1}) 10 min previously were taken out of home cages (group housed) and placed individually in rat cages with a grid bottom. FPO was measured after 15 min and 4 h in this environment. Weighed food pellets were provided throughout and then re-weighed after 4 h to establish food intake during this time.

Corticosterone analysis

Blood was collected and allowed to clot for 30 min at room temperature, the blood serum was extracted after 20 min centrifuging at 562× *g* and samples were stored at –20° until required. A commercial RIA kit with sensitivity of 7.5 ng·mL^{-1} was used with intra- and the inter-assay variations of 10.4% and 14.2% respectively.

Bead propulsion

Mice were deprived of food for 16 h prior to experimentation, although water was provided *ad libitum*. Distal colonic propulsion was measured according to the methods described by Koslo *et al.* (1986). Ten minutes after administration of vehicle (100 µL saline) or NPY (8 nmol·kg^{-1}), mice were placed under isoflurane anaesthesia; and a 2 mm bead was inserted 2 cm into the distal colon of each mouse using blunt tubing (Portex, 1.7 × 0.4 mm). The mouse was subsequently placed into a grid bottom cage to which it had been acclimatized and the time to expulsion measured.

Statistical analyses

For UGIT, FPO and bead propulsion measurements, single comparisons between data groups were performed using Student's unpaired *t*-test, whereas multiple comparisons used one-way ANOVA with Bonferoni's *post* test, to compare data within a genotype. Two-way ANOVA with Bonferoni's *post* test was used to compare each gender and genotype or WT response to vehicle and agonist/antagonist treatments, or control/stress treatments. *P*-values ≤ 0.05 were statistically significant.

Materials

BIBO3304 and BIIE0246 were gifts from Boehringer-Ingelheim Pharma KG (Biberach an der Riss, Germany), and stock solutions were dissolved in 10% dimethyl sulfoxide (DMSO) and stored at –20°C. NPY was purchased from Bachem (St. Helens, UK). A commercial corticosterone RIA kit was purchased from MP Biomedicals.

Results

UGIT under basal and restrained conditions

The role of endogenous NPY in UGIT was determined 30 min after the oral gavage of a non-nutritive charcoal meal in male and female WT and NPY$^{-/-}$ mice, under basal and acute restraint conditions. Restraint for 30 min did not affect the rate of UGIT in female WT mice compared with male WT mice, although there was a tendency for slower transit after restraint in both WT genders (Figure 1). In contrast, restraint stress significantly slowed UGIT in female NPY$^{-/-}$ mice compared with unrestrained females, whilst males showed a non-significant slowing of transit. Furthermore, restraint significantly slowed the rate of UGIT in female NPY$^{-/-}$ mice compared with male NPY$^{-/-}$ mice UGIT (Figure 1).

Effect of restraint on FPO and food intake

During restraint stress, female and male NPY$^{-/-}$ mice produced more faecal pellets than WT mice of the same gender, although this difference was only significant in females (Figure 2A). Furthermore, female NPY$^{-/-}$ mice ate significantly less than female WT mice in the 4 h after restraint stress

(Figure 2B); however, male WT mice ate a similar amount as male NPY$^{-/-}$ mice (Figure 2B). Female and male NPY$^{-/-}$ mice ate significantly less in the 4 h after restraint compared with respective non-restrained food intake (Figure 2B).

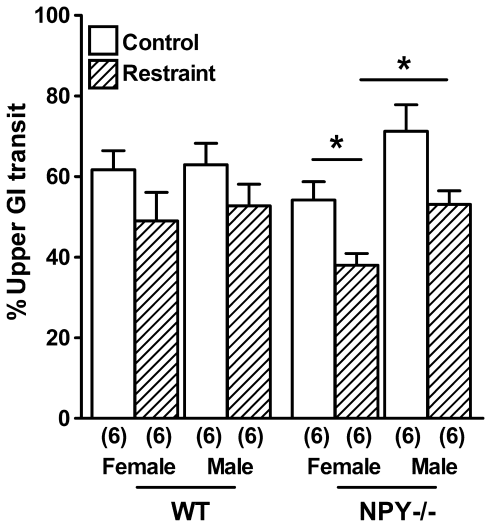

Figure 1

The rates of UGIT in female and male WT mice and NPY$^{-/-}$ mice under normal conditions or after 30 min restraint stress. UGIT was significantly slower in female NPY$^{-/-}$ mice after restraint stress and compared with restrained male NPY$^{-/-}$ mice (*$P < 0.05$). Each column is the mean + 1 SEM from the number of observations shown in parenthesis.

Effect of restraint and either a Y_1 or Y_2 antagonist i.p. on UGIT

Under normal and restrained conditions, the effect of vehicle, a Y_1 (BIBO3304) or a Y_2 (BIIE0246) antagonist was determined on UGIT in female and male WT mice. In both female and male WT mice, BIBO3304 and BIIE0246 non-significantly increased UGIT; however, after acute restraint, BIBO3304 significantly reduced UGIT compared with unrestrained UGIT in both genders (Figure 3). In contrast, after an acute restraint stress, BIIE0246 administration increased UGIT compared with vehicle-treated mice; although this difference was only significant in females.

Effect of restraint and either Y_1 or Y_2 antagonist i.p. on plasma corticosterone levels

Plasma corticosterone levels were also determined in all groups under unrestrained conditions and immediately after acute restraint. WT mice had higher plasma corticosterone after restraint than under normal conditions, and this was significant in females (Figure 4A), whilst both genders of restrained NPY$^{-/-}$ mice displayed significantly higher plasma corticosterone than unrestrained NPY$^{-/-}$ mice of the same gender (Figure 4A). After restraint, NPY$^{-/-}$ males had significantly higher plasma corticosterone than WT males, while female NPY$^{-/-}$ and WT mice displayed similar steroid levels after restraint (Figure 4A).

After administration of a Y_1 or Y_2 receptor antagonist, there was no change in plasma corticosterone under normal conditions in either gender of WT mice. However, administration of BIIE0246 significantly increased plasma corticosterone in restrained female WT mice and non-significantly in male WT mice compared with vehicle controls (Figure 4B), whilst BIBO3304 also increased corticosterone compared with vehicle in both genders of restrained WT mice; this was

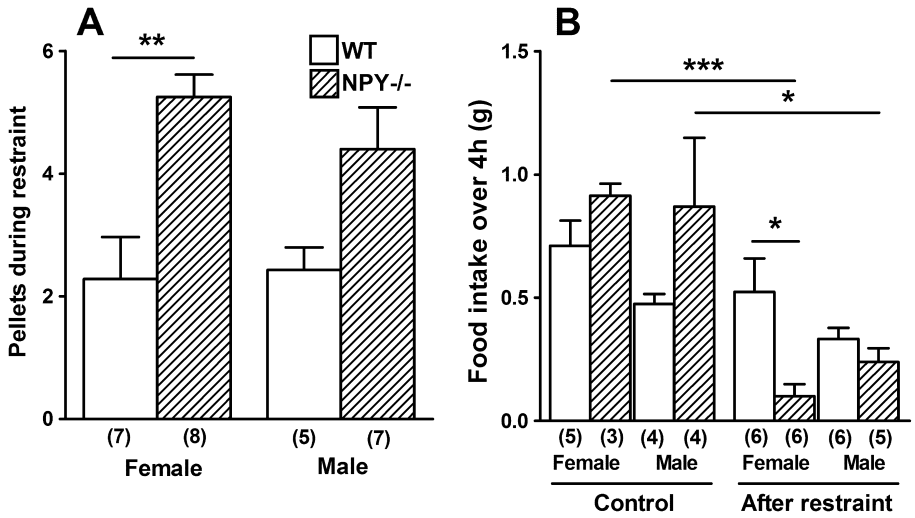

Figure 2

The effect of 30 min restraint stress on (A) the number of faecal pellets produced during restraint and (B) food intake in 4 h under non-stressed conditions (controls) and in the 4 h immediately following restraint in both male and female WT and NPY$^{-/-}$ mice. In (A), NPY$^{-/-}$ females produced significantly more pellets during restraint stress than WT females (**$P < 0.01$), and in (B), NPY$^{-/-}$ females ate significantly less than WT females (*$P < 0.05$), NPY$^{-/-}$ females also ate significantly less than when not restrained and acclimatized to the cages (***$P < 0.001$), as did NPY$^{-/-}$ males (*$P < 0.05$). Each column is the mean + 1 SEM and the number of observations in parenthesis.

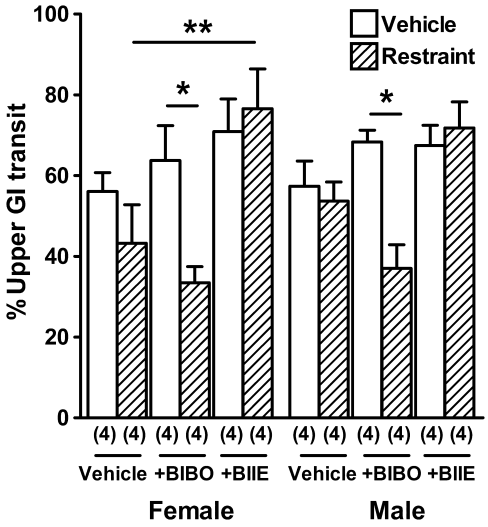

Figure 3

The effect of 30 min restraint on UGIT in the absence and presence of BIBO3304 and BIIE0246. Female and male WT mice displayed significantly slower UGIT after BIBO3304 administration before 30 min restraint compared with normal UGIT after BIBO3304 (*P < 0.05), and female WT mice had significantly faster UGIT after BIIE0246 administration before 30 min restraint compared to vehicle-administered WT mice exposed to 30 min restraint (**P < 0.01). Each column is the mean + 1 SEM from the number of observations shown in parenthesis.

only significant in males (Figure 4B). These data indicate that acute restraint stress is indeed an effective stressor. Interestingly, vehicle treatment in non-stressed female WT mice significantly increased plasma corticosterone compared with basal corticosterone levels (Figure 4A and B).

Effect of novel environment stress on FPO and food intake

The effect of individual housing in a novel environment on FPO and food intake was determined in NPY$^{-/-}$ and WT mice of either gender after 15 min and up to 4 h. After 15 min, female and male NPY$^{-/-}$ mice displayed significantly higher FPO than WT counterparts (Figure 5A). FPO at 15 min and 4 h in a novel environment resulted in similar numbers of faecal pellets produced by female and male mice of the same genotype (Figure 5A and B). In contrast, after 4 h in the novel environment female NPY$^{-/-}$ mice exhibited significantly greater FPO than female WT mice (Figure 5B). There was no significant difference in food intake between the genders of each genotype over 4 h (Figure 5C). However, female and male NPY$^{-/-}$ mice displayed a significant reduction in food intake during this stress compared with their respective WT counterparts (Figure 5C). Furthermore, both female and male NPY$^{-/-}$ mice ate significantly less in a novel environment than under normal non-stressed conditions (non-stressed food intake shown in Figure 2B).

Effect of exogenous NPY on FPO and colonic bead expulsion

The effect of novel environment stress on FPO was determined in NPY$^{-/-}$ and WT mice of both genders administered vehicle or NPY i.p. After administration of NPY, WT and

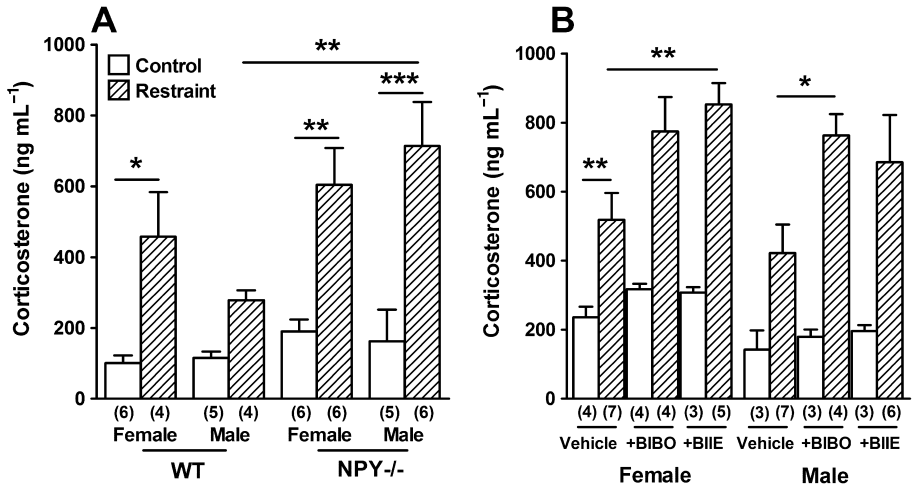

Figure 4

The effect of 30 min restraint on (A) plasma corticosterone in female and male WT and NPY$^{-/-}$ mice compared with normal conditions (B) plasma corticosterone levels in female and male WT mice administered i.p. with vehicle, BIBO3304 or BIIE0246. In (A), male NPY$^{-/-}$ mice had significantly higher plasma corticosterone after 30 min restraint (***P < 0.001) and compared with male WT mice after 30 min restraint (**P < 0.01). Female WT and NPY$^{-/-}$ mice had significantly higher corticosterone after restraint (*P < 0.05 and **P < 0.01 respectively). In (B), BIBO3304-treated WT male mice and BIIE0246-treated female WT mice had significantly higher corticosterone levels than controls after restraint (*P < 0.05 and **P < 0.01 respectively), whilst BIBO3304 and BIIE0246 had no effect on corticosterone in unrestrained mice of either gender (**P < 0.01, *P < 0.05, respectively). On comparing non-stressed vehicle controls (in B) with control WT mice (in A), it was found only female mice had significantly higher corticosterone levels after vehicle treatment (**P < 0.01, asterisk not shown). Each column is the mean + 1 SEM from the number of observations shown in parenthesis.

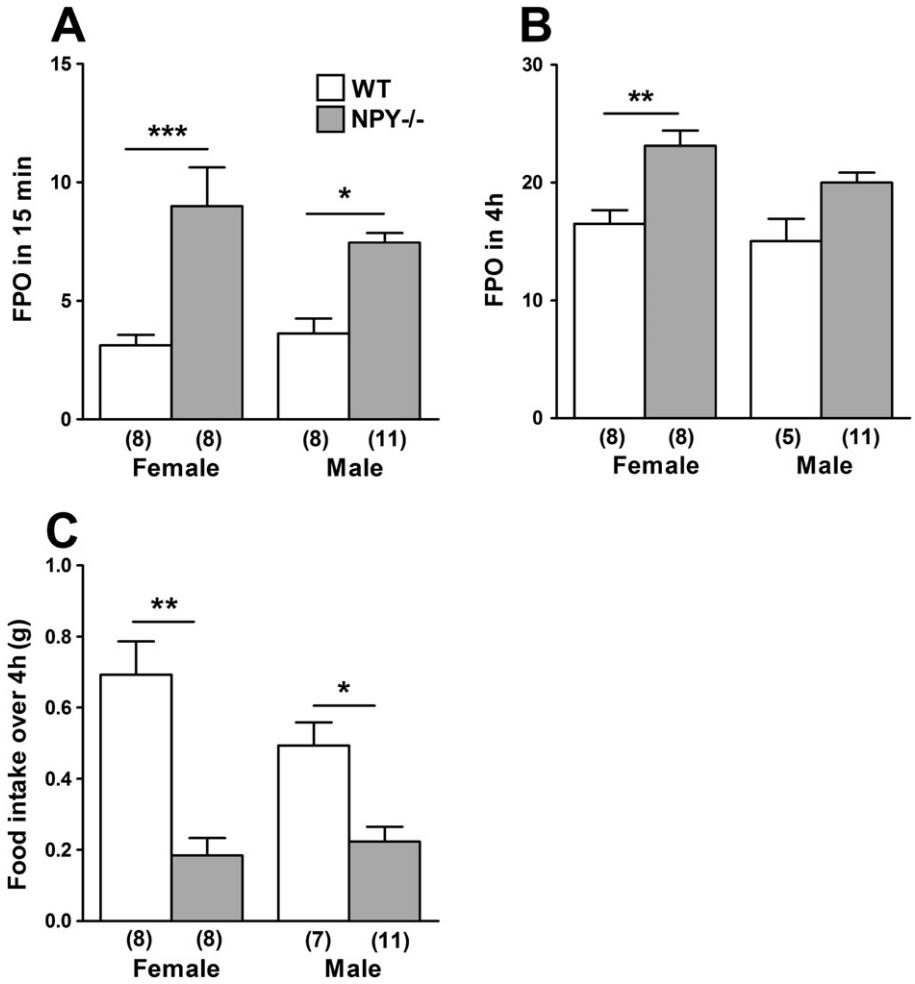

Figure 5

Effect of a novel environment stress in male and female WT and NPY$^{-/-}$ mice on faecal pellet output over (A) 15 min and (B) 4 h, and (C) on food intake in 4 h in the novel environment. In (A), female NPY$^{-/-}$ mice produced significantly more pellets after 15 min in a novel environment than female WT mice (***$P < 0.001$), and male NPY$^{-/-}$ mice produced more pellets than male WT mice (*$P < 0.05$). In (B), female NPY$^{-/-}$ mice produced more pellets than female WT over 4 h (**$P < 0.01$). In (C), female NPY$^{-/-}$ mice ate significantly less than female WT mice (**$P < 0.01$), whilst male NPY$^{-/-}$ mice ate significantly less than male WT mice over 4 h (*$P < 0.05$). Also, on comparing data in (C) with the respective control data in Figure 2B, NPY$^{-/-}$ females were found to eat significantly less in 4 h in the novel environment than when acclimatised to the cages (***$P < 0.001$, asterisk not shown), as did NPY$^{-/-}$ males (*$P < 0.05$, asterisk not shown). Each column is the mean + 1 SEM from the number of observations shown in parenthesis.

NPY$^{-/-}$ mice produced fewer stress-stimulated pellets within 15 min than their vehicle-treated counterparts, although this difference was not statistically significant (Figure 6A).

In WT males, exogenous NPY administration significantly increased the time to expulsion of a bead inserted 2 cm into the distal colon compared with vehicle (Figure 6B). Removal of NPY had no significant effect on bead expulsion time after vehicle administration, although there was increased expulsion time after exogenous NPY treatment, but this was not statistically significant (Figure 6B).

Discussion

The present results show NPY$^{-/-}$ mice display the greatest inhibition of UGIT and elevated plasma corticosterone levels after acute restraint, and the largest FPO and inhibition of food intake when placed in a novel environment, all corroborating the well-established anxiogenic phenotype of NPY null mice (Karl *et al.*, 2008; Painsipp *et al.*, 2011). Importantly, this study also shows for the first time that male and female NPY$^{-/-}$ mice display a different susceptibility to stress-induced GI and feeding responses, whereby females are consistently more vulnerable.

After an acute restraint stress, WT mice (both genders) showed only a non-significant slowing of UGIT compared to normal UGIT rates, whilst restrained female NPY$^{-/-}$ mice had significantly slower UGIT. Previous evidence has suggested a role for NPY in the stress-induced inhibition of gastric emptying (Chen *et al.*, 1997); however, our findings point to a redundancy in the central signalling pathway in the PVN, whereby NPY is not required for an inhibition of gastric

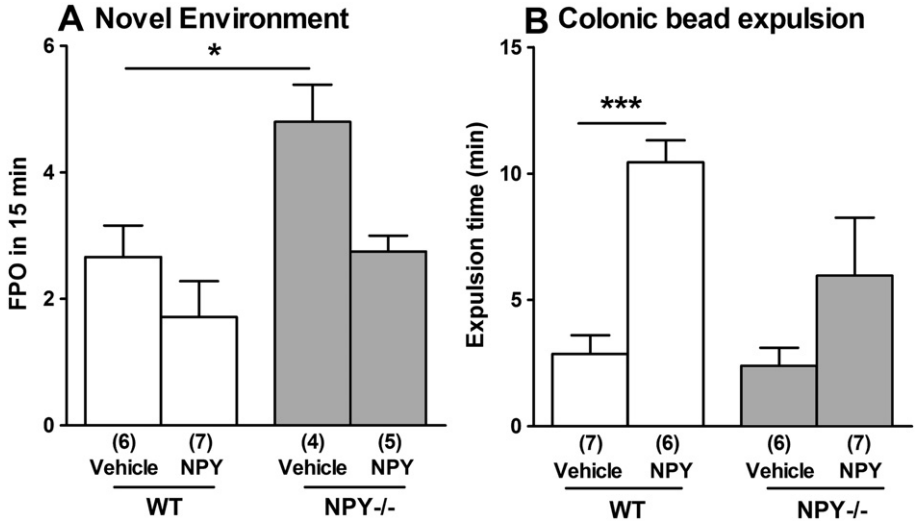

Figure 6

The effect of i.p. administration of NPY (8 nmol·kg^{-1}) on (A) novel environment stress-induced faecal pellet output and (B) on the time taken for expulsion of a 2 mm bead, in WT and NPY$^{-/-}$ mice and compared with vehicle treatment (100 μL saline). In (A), NPY$^{-/-}$ mice produced significantly more pellets than WT mice after vehicle treatment (*P < 0.05). In (B), WT mice treated with NPY had a significantly increased time until bead expulsion compared with vehicle controls (***P < 0.001). Each column is the mean + 1 SEM from the numbers of observations shown in parenthesis.

emptying during stress. Under normal conditions, NPY$^{-/-}$ males displayed faster UGIT, whilst NPY$^{-/-}$ females exhibited slower UGIT than gender-matched WT mice. Taken together, these results suggest endogenous NPY does not contribute significantly to the inhibition of gastric emptying or activation of the ileal brake, a feedback mechanism in the GI tract stimulated by the presence of nutrients in the ileum (Van Citters and Lin, 1999), that is more likely to be mediated by PYY (Lin *et al.*, 1996). These observations are supported by recent findings from our group showing that UGIT was unaffected by NPY removal but significantly faster in PYY null mice (Tough *et al.*, 2011). It follows that NPY is not involved in inhibiting UGIT under normal conditions as NPY is a potent stimulator of feeding in the ARC and induces motor activity in the duodenum which mirrors that of the fasted state (Fujimiya *et al.*, 2000). Nevertheless, female NPY$^{-/-}$ mice displayed slower UGIT than male NPY$^{-/-}$ mice after restraint, indicating stress-induced pathways leading to slower UGIT, known to be at least partially mediated through central CRF receptors (Martinez *et al.*, 2004), are increased in females. It is possible these gender-differences are due to the influence of sex hormones and the oestrus cycle on PVN CRF_2 expression (Iwasaki-Sekino *et al.*, 2009). Additionally, an acute stress did not inhibit UGIT significantly in WT mice of either gender as expected; therefore, a more severe stress may be required to reveal this effect, whilst in anxiogenic NPY$^{-/-}$ mice, this stress was sufficient to reveal the inhibited UGIT, in addition to gender differences.

The present study has highlighted opposing roles of peripheral Y_1 and Y_2 receptors in modulating UGIT under acute stress. In unrestrained male and female WT mice, both peripherally acting, competitive antagonists BIBO3304 and BIIE0246 slightly, but non-significantly, increased UGIT. However, during restraint, BIBO3304 administration significantly reduced UGIT in both genders, whilst BIIE0246 led to a significant increase in UGIT in females and to a lesser degree in males. This is similar to the differential roles we revealed for the Y_1 and Y_2 receptors in colonic motility *in vitro* (Tough *et al.*, 2011). As Y_1 receptors are present on the cell bodies of nitrergic myenteric neurons (Peaire *et al.*, 1997), most of which are motor nerves innervating the circular smooth muscle (Sang and Young, 1996), it is possible that the increased NPY released during stress will promote the activation of Y_1 receptors to hyperpolarize these tonically active inhibitory neurons and reduce their activity. The presence of a Y_1 antagonist would therefore prevent this tonic inhibition, inducing relaxation and slowing transit as we observed for post-restraint UGIT in the presence of the Y_1 antagonist BIBO3304. In contrast, Y_2-mediated stress mechanisms have the opposite effect upon UGIT. Y_2 antagonism with BIIE0246 increased UGIT during stress, particularly in female mice, indicating that Y_2 receptors are involved in a different tonically active intramural mechanism that inhibits UGIT under stressed conditions.

Exposure to a novel environment is an established rodent stressor, which induces colonic motor responses (Wang *et al.*, 2010). In the novel environment, NPY$^{-/-}$ females produced significantly more pellets over 15 min compared with WT females and this difference lasted for up to 4 h. NPY$^{-/-}$ males had higher FPO than WT males within 15 min in a novel environment; however, this difference did not last 4 h. In addition, female NPY$^{-/-}$ mice produced more faecal pellets during 30 min restraint than female WT mice; and the same, although non-significant, trend was observed in males. These data point to a moderate gender-dependent colonic stress response involving NPY. In addition, the greater vulnerability to stress-induced FPO and faster colonic bead expulsion rates in NPY$^{-/-}$ mice *in vivo*, and the ability of i.p. administered NPY

to prevent these defecation responses after NPY removal indicates peripheral NPY has a predominantly inhibitory influence on colonic motility during stress that may be more pronounced in females. We have previously reported that the pellet propulsion in the isolated colon tends to be faster in those from NPY$^{-/-}$ mice (Tough *et al.*, 2011). This all corroborates findings by Wang *et al.* (2010), whereby i.p. administered NPY inhibited FPO induced by a novel environment, in addition to bethanechol-induced diarrhoea, but opposed the central stimulatant effect NPY exerted on colonic motor function through Y_1 and CRH pathways (Monnikes *et al.*, 2000; Tebbe *et al.*, 2005). Nevertheless, peripheral NPY is a neuromodulator via neurogenic Y_2 receptors within both intramural plexi of the colon (Wang *et al.*, 2010), as well as inhibiting upper GI inhibitory motor mechanisms *in vivo* and colonic motility *in vitro* via Y_1 receptors (see above).

Not completely congruent with the orexigenic role for NPY, previous studies have shown that under basal conditions NPY$^{-/-}$ mice exhibit no changes in food intake or body weight, although they do exhibit blunted hyperphagia and weight gain after fasting (Patel *et al.*, 2006). In this study, both genders of NPY$^{-/-}$ mice ate significantly less than WT mice in a novel environment and after an acute restraint stress, and compared with their respective food intake after acclimatization; thus, it is possible the metabolic consequences of losing NPY becomes more evident physiologically under stressed conditions. Additionally, NPY$^{-/-}$ females showed a greater, although non-significant reduction in feeding compared with NPY$^{-/-}$ males during stress, indicating females may be more susceptible to the stress-induced suppression of feeding. This is supported by the larger reduction in food intake and greater weight loss reported during chronic single housing of female NPY$^{-/-}$ mice compared with WT females and NPY$^{-/-}$ males (Edelsbrunner *et al.*, 2009). Interestingly, male WT mice ate less than female WT mice throughout this study, despite having a higher body weight, although this was not statistically significant.

Another important finding of the present study is that after 30 min restraint, male NPY$^{-/-}$ mice had significantly higher levels of plasma corticosterone than restrained WT counterparts, indicating that endogenous NPY inhibits corticosterone secretion and thus the stress responses. An abundance of data has shown that centrally administered NPY dose-dependently activates the HPA-axis and thus increases corticosterone release (Dimitrov *et al.*, 2007); however, reports concerning the effect of peripheral NPY on regulating corticosterone/cortisol secretion have been contradictory and inconclusive (Renshaw *et al.*, 2000). Despite a lack of significant difference in basal corticosterone levels between any groups in this study, higher basal levels have been reported in female NPY$^{-/-}$ mice (Painsipp *et al.*, 2011). However, as high corticosterone levels have been observed in both genders of NPY$^{-/-}$ mice after the forced swim test, which were similar to the levels observed after restraint stress here, it is possible that stress-induced NPY release may induce the inhibition of corticosterone release (Painsipp *et al.*, 2011). Furthermore, these data point to an inhibitory role for NPY acting via both peripheral Y_1 and Y_2 receptors on corticosterone release during acute stress, as peripherally administered antagonists BIBO3304 and BIIE0246 in restrained WT males, and BIIE0246 in restrained WT females, significantly increased corticosterone levels compared with vehicle. However, this increase in plasma corticosterone was not observed after administration of the antagonists under normal conditions in either gender. Although elevations in corticosterone may be dependent on the type of stressor, support is provided by deletion of the Y_1 or Y_2 receptor from mice leading to high plasma corticosterone concentrations (Cavadas *et al.*, 2006; Painsipp *et al.*, 2008) and high levels previously reported in response to peripheral BIIE0246 administration in WT mice (Kuo *et al.*, 2008). Furthermore, NPY inhibits corticosterone release *in vitro* both from isolated rat adrenocortical cells (Malendowicz *et al.*, 1990) and from human adrenal cortical H295R cells through the Y_1 receptor (Kempna *et al.*, 2010). In humans, NPY administered i.v. inhibits cortisol release (Antonijevic *et al.*, 2000). NPY and the Y_1 and Y_2 receptors are expressed in the human adrenal gland, with binding studies demonstrating that Y_1 receptors are highly expressed in the adrenal cortex (Korner *et al.*, 2004). In the mouse, NPY immunoreactive nerve fibres have been detected around blood vessels and in the cortical cells of the zona glomerulosa (Fernandez-Vivero *et al.*, 1993). Taken together, this indicates a direct Y_1- and probably a presynaptic Y_2-mediated inhibitory action of peripheral NPY, potentially released from intracortical nerve fibres (Li *et al.*, 1999), upon corticosterone release from adrenal cortical cells. Interestingly, non-stressed WT females had a significant increase in corticosterone after vehicle treatment. These cohorts were treated differently, the vehicle cohort being handled previously and having vehicle administered i.p. 45 min before blood collection rather than immediate blood collection under control conditions, possibly explaining the difference.

In conclusion, the present work has shown gender-dependent responses in UGIT, FPO and food intake induced by both restraint and novel environment stresses in NPY$^{-/-}$ mice. Female NPY$^{-/-}$ mice were more susceptible than male NPY$^{-/-}$ mice to the physiological effects of acute stress, suggesting that NPY plays a moderate sexually dimorphic role in some physiological stress responses. Additionally, this work has provided further support that endogenous NPY in both genders plays an inhibitory role in colonic motility, but not in UGIT under normal conditions; however, it has highlighted opposing roles for the Y_1 and Y_2 receptors on UGIT during restraint stress. Removal of NPY from mice and pharmacological interventions in WT mice revealed that NPY inhibits the release of corticosterone through both peripheral Y_1 and Y_2 receptors during stress events. Thus, it is possible that peripheral NPY may activate pathways that contribute to the restoration of GI and colonic homeostasis and inhibit HPA axis activity after a stress event in both genders. These results are significant given the critical role of NPY and its cognate Y receptors in stress/anxiety and energy homeostasis and the increasing prevalence of stress and anxiety-associated diseases, especially in women.

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Conflicts of interest

None.

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