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ORIGINAL ARTICLE

The importance of acclimatisation and habituation to experimental conditions when investigating the anorectic effects of gastrointestinal hormones in the rat

CR Abbott, CJ Small, A Sajedi, KL Smith, JRC Parkinson, LL Broadhead, MA Ghatei and SR Bloom

Endocrine Unit, Imperial College London, Hammersmith Campus, London, UK

Objective: Peptide YY3-36 ($PYY_{3–36}$), glucagon-like peptide-1 (GLP-1), oxyntomodulin and cholecystokinin (CCK) are gastrointestinal-derived hormones that are released postprandially in proportion to the amount of calories ingested. All significantly reduce food intake following peripheral administration to rodents. We have investigated the effect of handling, exposure to a novel environment or to environmental enrichment on the anorectic effect of these gut hormones.
Results: Results suggest that the transfer of a rat into a novel environment (cage change) inhibits the anorectic response to peripherally administered $PYY_{3–36}$ and oxyntomodulin (1 h food intake reduction (% saline control): PYY/home cage $82.3 \pm 5.9\%$, $P<0.05$; PYY/clean cage $103.4 \pm 9.7\%$; oxyntomodulin/home cage $71.6 \pm 12.1\%$, $P<0.05$; oxyntomodulin/clean cage $103.0 \pm 8.5\%$) and attenuates the anorectic response to GLP-1 and CCK (1 h food intake reduction (% saline control): GLP-1/home cage $68.8 \pm 6.4\%$, $P<0.01$; GLP-1/clean cage $80.0 \pm 9.3\%$; CCK/home cage $49.8 \pm 6.2\%$, $P<0.001$; CCK/clean cage $69.4 \pm 10.6\%$, $P<0.05$). We have also observed that exposure to a novel environment does not alter anorectic effect of peripherally administered melanocortin 3/4 receptor agonist, melanotan II (MTII) (1 h food intake reduction (% saline control): MTII/home cage $32.0 \pm 6.3\%$, $P<0.001$; MTII/clean cage $24.8 \pm 4.2\%$, $P<0.001$). The attenuation in food intake observed following exposure to a novel environment can be attributed, in part, to a significant reduction in the food intake of the saline treated animals. In a further study, the anorectic effect of peripherally administered $PYY_{3–36}$ is attenuated in unhandled rats ($88 \pm 4.2\%$ saline control, $P=$ ns) or rats exposed to environmental enrichment ($103.3 \pm 9.7\%$ saline control, $P=$ ns), but not in animals that were handled extensively prior to the study ($80.1 \pm 7.3\%$ saline control, $P<0.05$).
Conclusion: These studies highlight the importance of handling, acclimatisation and habituation of rodents to experimental conditions prior to investigating the ability of gut hormones to alter food intake.
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Introduction

Peptide YY (PYY) is a gastrointestinal-derived hormone that is released postprandially in proportion to the amount of calories ingested.[1–3] Peripheral administration of $PYY_{3–36}$, significantly reduces food intake and body weight in rodents, and suppresses appetite and food intake when intravenously infused into humans.[4] The preproglucagon products glucagon-like peptide-1 (GLP-1) and oxyntomodulin are released with PYY from the same cells in the gastrointestinal tract in response to nutrient ingestion. Peripheral administration of GLP-1 and oxyntomodulin significantly reduce gastric emptying and food intake in both rodents and humans.[5–7] The secretion of the gastrointestinal hormone cholecystokinin (CCK) is reported to be associated with meal termination and satiety. Peripheral administration of CCK reduces food intake and delays gastric emptying.[8]

The reproducibility of the anorectic effect of $PYY_{3–36}$ on food intake in rodents has recently been questioned.[9] It has been suggested that the anorectic effects of $PYY_{3–36}$ and other gastrointestinal hormones in rodents are influenced by stress.[10] The reduction in food intake associated with the

Correspondence: Professor SR Bloom, Endocrine Unit, Imperial College London, 6th Floor Commonwealth Building, Hammersmith Campus, Du Cane Road, London W12 ONN, UK.
E-mail: s.bloom@imperial.ac.uk
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presence of a stressor may negate the appetite suppressing effects of $PYY_{3–36}$ and other gut hormones. Thus the failure to reproduce previously published anorectic effects of $PYY_{3–36}$ may be attributed to an altered stress state in the rodent models investigated.

The aim of these studies was to investigate the effect of handling, exposure to novel environment or to environmental enrichment on the anorectic effect of the gastrointestinal hormones $PYY_{3–36}$, oxyntomodulin, GLP-1 and CCK. It has previously been shown that transfer of a rat from a dirty to clean environment induces an increase in blood pressure and heart rate and elicits a behavioural pattern normally consistent with a mild stress response.[11,12] To investigate the effect of this mild novel environmental stress, rats were injected intraperitoneally (i.p.) with either saline, $PYY_{3–36}$, oxyntomodulin, GLP-1 or CCK and returned to their original dirty cage or to a clean cage post injection and food intake measured. It has previously been reported that the melanocortin 3/4 receptor agonist, melanotan II (MTII) significantly reduced food intake in rats that were unresponsive to $PYY_{3–36}$.[9] We have examined the feeding response to peripherally administered MTII following exposure to a novel environment. To investigate the effect of handling and environmental enrichment on the anorectic effect of gastrointestinal hormones, rats were either handled extensively, unhandled or exposed to an environmental enrichment paradigm for a period of 14 days prior to i.p. injection with either saline or $PYY_{3–36,}$ and food intake measured.

Methods

Materials

$PYY_{3–36}$, oxyntomodulin, GLP-1, CCK-8 and MTII were all purchased from Bachem UK Ltd (Merseyside, UK). All other reagents, unless stated, were purchased from BDH (Poole, Dorset, UK).

Animals

Male Wistar rats (250–350 g), unless stated, were maintained in individual cages under controlled temperature (21−23°C) and light (12 h light, 12 h dark, lights on at 0700) conditions with *ad libitum* access to chow (RM1 diet, SDS UK Ltd) and water. All animal procedures undertaken were approved by the British Home Office Animals Scientific Procedures Act 1986 (Project license No. 70/5516). Unless stated otherwise, rats were handled daily for a period of 1 min/rat by the same individual, and accustomed to the i.p. injection procedure by injection of 0.5 ml saline followed by the measurement of food intake for two days prior to the initial study.

Study 1: Investigation of the effect of exposure to a novel environment on the effect of peripherally administered hormones on food intake and plasma corticosterone in the rat

Rats were maintained in their home cages for a period of 7 days without cleaning prior to each study. Rats fasted for 24 h ($n=12$/group) were injected i.p., during the early light phase, with either saline or $PYY_{3–36}$ (12.5 nmol/kg) (0.5 ml). Following injection, rats were immediately returned to home or clean cages containing a known amount of chow. Food intake was measured at 1, 2, 4, 6 and 24 h postinjection using an ATP Instrumentation GW 600 balance (ATP Instrumentations Ltd., Ashby-De-La-Zouche, Leicestershire, UK) recording to the nearest 0.1 g. This protocol was followed in subsequent studies in which the effects of GLP-1 (40 nmol/kg), oxyntomodulin (300 nmol/kg), CCK (10 nmol/kg) and MTII (3000 nmol/kg) on food intake were examined. The doses of gastrointestinal hormones have previously been shown to significantly reduce 1 h food intake (but not have maximal inhibition) following i.p. injection in the rat.[4,5,9] The doses were not expected to alter 24 h food intake but rather to examine the effect of a mild stressor on sub maximal food intake. The dose of MTII was selected to be comparable to that used by Tschop *et al*.[9] and previously reported to decrease food intake.

In order to investigate whether exposure to this novel environment altered the hypothalamo-pituitary–adrenal axis, a separate group of rats ($n=12$/group) were maintained in their home cages for a period of 7 days without cleaning prior to being injected i.p., during the early light phase, with 0.5 ml saline and immediately returned to home or clean cages. The rats were then decapitated at 15 min postinjection and blood collected into plastic lithium heparin tubes containing 0.6 mg of aprotinin. Plasma was immediately separated by centrifugation and stored at −20°C. Plasma corticosterone levels were measured by the radioimmunoassay method, using a commercially available kit (MP Biomedicals, Costa Mesa, CA).

Study 2: Investigation of the effect of handling and environmental enrichment on the effect of peripherally administered $PYY_{3–36}$ on food intake in the rat

Rats were divided into three groups ($n=12$–14/group) and either handled, unhandled or exposed to an enriched environment (plastic house in cage, unhandled) for a period of 14 days. At the end of this period, rats were fasted prior to being injected i.p., during the early light phase, with either saline or 12.5 nmol/kg $PYY_{3–36}$. Following injection, rats were returned to their home cages containing a known amount of food and food intake measured as detailed above.

Statistical analysis

Food intake (g) and plasma corticosterone levels (pmol/ml) are expressed as mean±s.e.m. Food intake data were analysed by ANOVA followed by LSD *post hoc* analysis, and corticosterone levels were compared by Student's *t*-test. Values of $P<0.05$ were considered significant.

Results

Study 1: The effect of exposure to a novel environment on the effect of peripherally administered hormones on food intake and plasma corticosterone in the rat

Peripheral administration of PYY_{3-36} and oxyntomodulin significantly reduced food intake in animals that were returned to their home cages (1 h food intake: PYY_{3-36} study: home cage/saline: 6.4 ± 0.4 g; vs home cage/PYY_{3-36}: 5.2 ± 0.4 g, $P<0.05$; oxyntomodulin study: home cage/saline: 6.4 ± 0.5 g; vs home cage/oxyntomodulin: 4.6 ± 0.7 g, $P<0.05$). However, the anorectic effects of these gastrointestinal hormones were absent in animals that were placed in a clean cage postinjection (1 h food intake: PYY_{3-36} study: clean cage/saline: 4.6 ± 0.3 g vs clean cage/PYY_{3-36}: 4.8 ± 0.5 g, $P=$ns; oxyntomodulin study: clean cage/saline: 4.8 ± 0.5 g; vs clean cage/oxyntomodulin: 4.9 ± 0.4 g, $P=$ns) (Figure 1a and b).

Peripheral administration of GLP-1 also significantly reduced food intake in animals that were returned to their home cages (1 h food intake: home cage/saline: 6.3 ± 0.5 g; vs. home cage/GLP-1: 4.5 ± 0.4 g, $P<0.001$). However, this effect was attenuated in animals that were place in a clean cage postinjection (1 h food intake: clean cage/saline: 4.9 ± 0.5 g; vs clean cage/GLP-1: 3.9 ± 0.5 g, $P=$ns) (Figure 1c).

Peripheral administration of CCK significantly reduced food intake in all animals injected. However, the magnitude of the anorectic effect of the peptide was reduced in those animals that had been returned to clean cages compared to

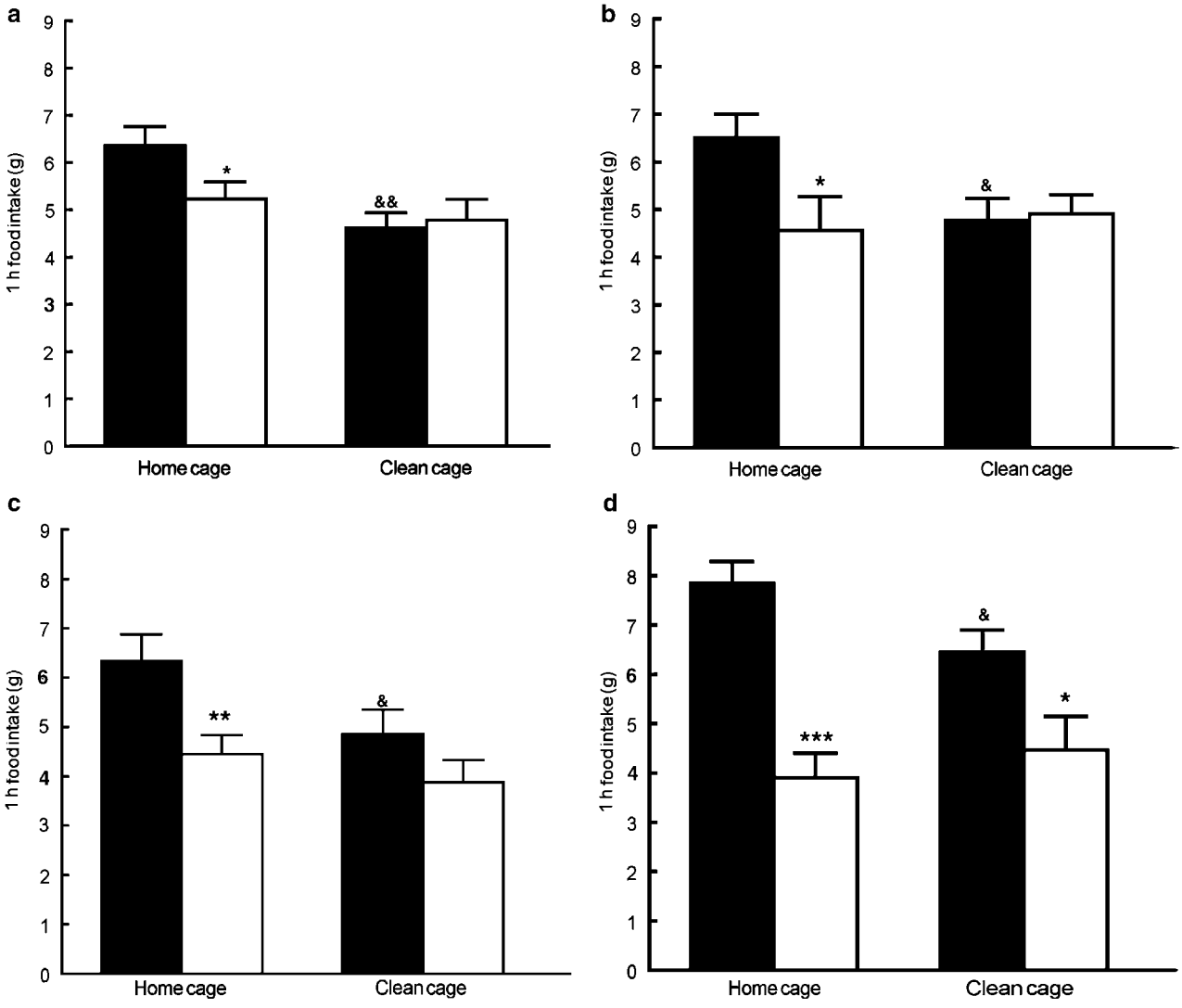

Figure 1 The effect of exposure to a novel environment (cage change) on 1 h food intake following a single i.p. injection of (**a**) PYY_{3-36} (12.5 nmol/kg), (**b**) oxyntomodulin (300 nmol/kg), (**c**) GLP-1 (40 nmol/kg) or (**d**) CCK-8 (10 nmol/kg) when administered in the early light phase to 24 h fasted rats. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ test group (white bars) vs relative saline (black bars), $^{\&}P<0.05$, $^{\&\&}P<0.01$ home cage/saline vs clean cage/saline.

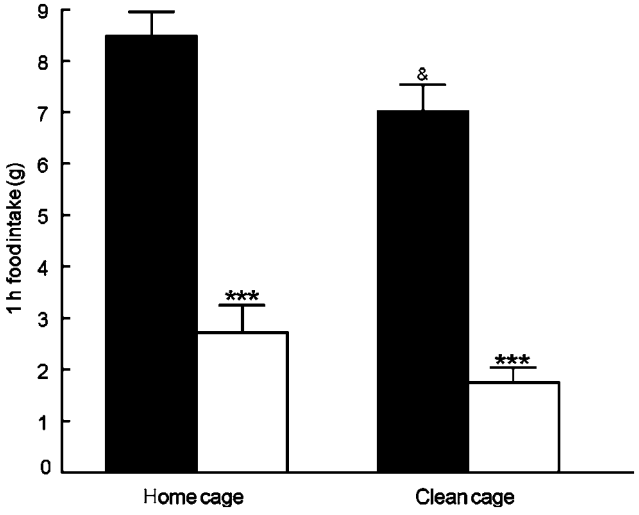

Figure 2 The effect of exposure to a novel environment (cage change) on 1 h food intake following a single i.p. injection of MTII (3000 nmol/kg) when administered in the early light phase to 24 h fasted rats. ***$P<0.001$ MTII (white bars) vs relative saline (black bars), &$P<0.05$, home cage/saline vs clean cage/saline.

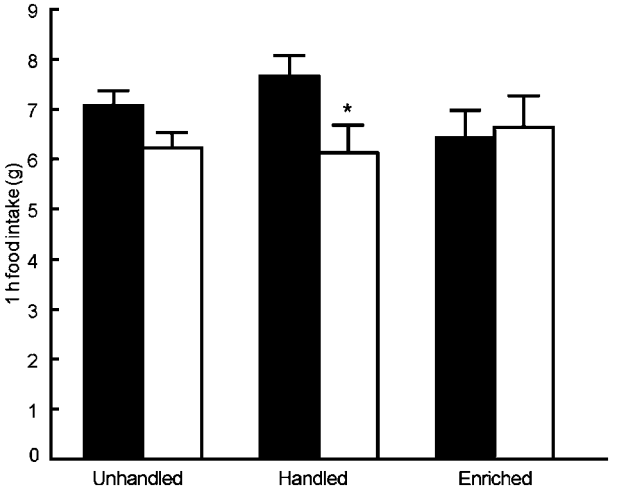

Figure 3 The effect of handling and environmental enrichment on 1 h food intake following a single i.p. injection of PYY_{3-36} (12.5 nmol/kg) when administered in the early light phase to 24 h fasted rats. *$P<0.05$, PYY_{3-36} (white bars) vs relative saline (black bars).

home cages postinjection (1 h food intake: home cage/saline: 7.8 ± 0.4 g; vs home cage/CCK: 3.9 ± 0.5 g; $P<0.001$; clean cage/saline: 6.4 ± 0.5 g; vs clean cage/CCK: 4.5 ± 0.7 g; $P<0.05$) (Figure 1d). Peripheral administration of MTII significantly reduced food intake in all animals and was unaffected by the cage change (1 h food intake: home cage/saline: 8.5 ± 0.5 g; vs home cage/MTII: 2.7 ± 0.5 g; $P<0.001$; clean cage/saline: 7.0 ± 0.5 g; vs clean cage/MTII: 1.8 ± 0.3 g; $P<0.001$) (Figure 2).

Rats that had been injected with saline and were transferred into a clean cage consumed a significantly lower amount of chow compared to those animals that had been injected with saline and returned to their home cages (PYY_{3-36} study: $P<0.01$; all other studies: $P<0.05$; between saline injected groups) (Figures 1 and 2). There was no significant alteration in food intake at later time points in all studies.

There was no significant difference in the plasma corticosterone levels of those animals that were placed in a clean cage compared to those which had been returned to their home cages 15 min postinjection of saline (clean cage: 219.0 ± 28.3 ng/ml; vs home cage: 203.8 ± 31.0 ng/ml, $P=$ns). However, it cannot be excluded that the animals were experiencing a chronic low stress which was not detected using these methods at this time point.

Study 2: Investigation of the effect of handling and environmental enrichment on the effect of peripherally administered PYY_{3-36} on food intake in the rat

Rats that had been handled for 14 days prior to injection with PYY_{3-36} exhibited a significantly reduced food intake compared to those animals that had been handled but received saline (1 h food intake: PYY_{3-36}: 6.1 ± 0.6 g; vs saline: 7.7 ± 0.4 g; $P<0.05$). The anorectic effect of PYY_{3-36} was absent in animals that had not been previously handled (1 h food intake: PYY_{3-36}: 6.2 ± 0.3 g; vs saline: 7.1 ± 0.3 g; $P=$ns) or those animals that were exposed to an enriched environment (1 h food intake: PYY_{3-36}: 6.6 ± 0.6 g; vs saline: 6.4 ± 0.5 g; $P=$ns) (Figure 3).

Rats that had been injected with saline but had been exposed to environmental enrichment or were unhandled prior to the injection exhibited reduced, albeit non-significant, food intake when compared to saline injected animals that were handled prior to the study.

Discussion

We have shown that the anorectic effects of the gastrointestinal hormones PYY_{3-36} and oxyntomodulin are absent and the effects of GLP-1 and CCK attenuated, in rats exposed to a novel environment (clean cage) when compared to saline-injected controls. The attenuation in food intake observed following this cage change can be attributed, in part, to a significant reduction in the food intake of the saline treated animals. It appears that exposure to a novel environment causes a reduction in food intake and this reduction in food intake therefore masks any anorectic effects of gastrointestinal hormone when administered peripherally. Exposure to a novel environment is normally associated with a mild stress response,[11,12] however, in our paradigm, plasma corticosterone levels are not increased. This suggests that although exposure to this mild environmental stress alters feeding patterns in rodents, it is not of a sufficient magnitude to activate the hypothalamo-pituitary–

adrenal axis. In addition, the mild stress may have altered the endogenous hormone environment and this may contribute to the results observed. These results suggest that exposure to a mild environmental stress influences the anorectic effect of peripherally administered gastrointestinal hormones on food intake.

We have also shown that the anorectic effect of $PYY_{3–36}$ is attenuated in rats that have not been handled prior to the study, when compared to their handled counterparts. In support of our findings, the inability of $PYY_{3–36}$ to reduce food intake following peripheral administration to naïve unhabituated mice has recently been shown.[13] Interestingly, the authors suggest that the loss of efficacy of $PYY_{3–36}$ may be due to the effects of stress, as evident by the 32% decrease in food intake in saline-injected unacclimatised compared with saline-injected acclimatised wild-type mice.[13] In addition, we have shown that exposure to an enriched environment also attenuates the anorectic effect of $PYY_{3–36}$. In accordance with our first study, investigating the effect of a novel environment on the anorectic effects of the gastrointestinal hormones, it appears that this attenuation in the anorectic effect of $PYY_{3–36}$ following exposure to cage enrichment is in part due to a reduction in the food intake of the saline treated animals. Thus it appears that habituation and acclimatisation to experimental conditions, and the absence of cage enrichment, is paramount when investigating the effects of gastrointestinal hormones such as $PYY_{3–36,}$ GLP-1 and oxyntomodulin on food intake.

Tschop *et al*.[9] reported that a subset of animals which were unresponsive to $PYY_{3–36,}$ exhibited a marked anorectic response to both CCK and MTII. We have examined the effects of both CCK and MTII in our novel environment paradigm. Both compounds significantly reduced food intake compared to saline controls in rats that had been returned to either their home or clean cage post injection. One explanation for the maintenance of the anorectic effect of these peptides could be the high doses administered. It is interesting to note, in accordance with the previous study, the food intake of the saline treated animals exposed to a novel environment is significantly lower than those animals that were returned to their home cages. These data suggest that peripheral administration of either CCK or MTII are maintained despite a mild stress when administered at these doses.

The exact mechanism by which this mild environmental stress alters the anorectic actions of gastrointestinal hormone is currently unknown and likely to be complex. One possible explanation could be that the hypothalamic mechanisms by which gastrointestinal hormones inhibit food intake are also involved in the control of the hypothalamo-pituitary–adrenal axis, for example neuropeptide Y. However, further extensive investigations are required to elucidate these mechanisms.

In summary, we have shown that the anorectic effects of the peripherally administered gastrointestinal hormones $PYY_{3–36,}$ oxyntomodulin, GLP-1 and CCK are attenuated in rats which have not been handled or have been exposed to a mild environmental stress or an enriched cage environment. These studies highlight the importance of habituation and acclimatisation to experimental conditions, and the absence of cage enrichment, when investigating the effects of gastrointestinal hormones on food intake.

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