British Journal of Pharmacology (1997) 122, 1405–1410

Increased feeding in fatty Zucker rats by the thiazolidinedione BRL 49653 (rosiglitazone) and the possible involvement of leptin and hypothalamic neuropeptide Y

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1 The thiazolidinedione BRL 49653 (rosiglitazone) induces hyperphagia and weight gain in obese, insulin-resistant fatty Zucker rats but not in lean insulin-sensitive rats. We investigated whether these responses might involve neuropeptide Y (NPY), leptin and insulin.

2 BRL 49653 (1 mg kg^{-1} day^{-1}, orally) was given for 7 or 20 days to fatty and lean Zucker and Wistar rats.

3 In lean rats of either strain, BRL 49653 had no effect on food intake, body weight, plasma insulin and corticosterone, NPY or NPY mRNA levels.

4 Fatty rats given BRL 49653 showed a 30% increase in food intake and accelerated body weight gain (both $P<0.01$) after 7 and 20 days, but without significant changes in regional hypothalamic NPY or NPY mRNA levels.

5 Plasma leptin levels were twice as high in untreated fatty Zucker rats as in lean rats ($P<0.01$), but were unaffected by BRL 49653 given for 20 days. However, BRL 49653 reduced insulin levels by 42% and increased corticosterone levels by 124% in fatty rats (both $P<0.01$).

6 Hyperphagia induced in fatty Zucker rats by BRL 49653 does not appear to be mediated by either a fall in circulating leptin levels or increased activity of hypothalamic NPYergic neurones. The fall in plasma insulin and/or rise in corticosterone levels during BRL 49653 treatment may be involved, consistent with the postulated role of these hormones in the control of food intake.

Keywords: BRL 49653; rosiglitazone; thiazolidinedione; insulin; leptin; NPY

Introduction

The thiazolidinediones enhance insulin action and lower blood glucose concentrations in obese, insulin-resistant animals and patients with glucose intolerance or non-insulin-dependent diabetes (NIDDM) (Saltiel & Olefsky, 1996). They are ligands at the peroxisome-proliferator activator receptor-γ (PPAR-γ) in the nuclei of adipocytes and inhibit lipolysis; the resulting reduction in non-esterified fatty acid (NEFA) production is thought to be responsible, via the Randle cycle, for the improvement in glucose tolerance (Oakes *et al*., 1994; Saltiel & Olefsky, 1996).

In the fatty (*fa*/*fa*) Zucker rat, which displays morbid obesity with severe insulin resistance and mild glucose intolerance, troglitazone and the potent novel thiazolidinedione BRL 49653 (rosiglitazone) improves glucose tolerance, lower hyperinsulinaemia and up-regulate insulin receptors in peripheral tissues (Fujiwara *et al*., 1988; Smith *et al*., 1993). Lean Zucker and Wistar rats show no such effects, suggesting that these compounds act preferentially in conditions of insulin resistance. Preliminary studies (Smith *et al*., 1993; Eldershaw *et al*., 1995) indicate that BRL 49653 also stimulates feeding and induces slight but significant weight gain in fatty Zucker, but not in lean rats. Although weight gain has not been observed in clinical trials of troglitazone (Nolan *et al*., 1994; Saltiel & Olefsky, 1996), it is clearly important to characterize this effect and to understand its mechanism in obese rats.

This study focused on the possible role of neurones in the hypothalamic arcuate nucleus (ARC) which express neuropeptide Y (NPY). The ARC NPY neurones project to the paraventricular (PVN) and dorsomedial nuclei (DMH) (Chronwall *et al*., 1985), where NPY injection powerfully induces hyperphagia, inhibits sympathetically mediated thermogenesis and induces obesity (Stanley *et al*., 1986; Billington *et al*., 1991; Zarjevski *et al*., 1993). These neurones may function physiologically to detect and counteract energy deficits (Dryden *et al*., 1994). Interestingly, the ARC NPY neurones are overactive in certain genetically obese rodents, namely the *fa*/*fa* (fatty) Zucker rat and *ob*/*ob* and *db*/*db* mice, and this may contribute to their obesity (Dryden *et al*., 1994; Erickson *et al*., 1996b). Overactivity of these NPY neurones in these mutants is apparently due to loss of inhibition by leptin, the product of the *ob* gene, which is secreted by adipose tissue into the circulation and enters the CSF and mediobasal hypothalamus (Zhang *et al*., 1994; Caro *et al*., 1996; Banks *et al*., 1996). The ARC NPY neurones express the OB-Rb leptin receptor (Mercer *et al*., 1996) and are inhibited by leptin (Stephens *et al*., 1995; Schwartz *et al*., 1996; Wang *et al*., 1997); this may partly explain the central hypophagic, thermogenic and anti-obesity effects of leptin (Campfield *et al*., 1995; Pelleymounter *et al*., 1995). The *fa* mutation, in the leptin receptor, induced leptin insensitivity, which may disinhibit the NPY neurones (Takaya *et al*., 1996; Cusin *et al*., 1996).

Insulin, like leptin, enters the ARC (where insulin receptors are expressed) and may inhibit the NPY neurones (Schwartz *et al*., 1992; Dryden *et al*., 1994). Insulin has been postulated to function as a circulating satiety factor, and its ability to suppress feeding, stimulate thermogenesis and cause weight loss could also be mediated by its inhibition of the ARC NPY neurones (Schwartz *et al*., 1992; Dryden *et al*., 1994). By contrast, glucocorticoids appear to stimulate the ARC NPY neurones, which carry type II glucocorticoid receptors (Hisano *et al*., 1988; Tempel & Leibowitz, 1993). However, the relative importance of leptin, insulin and glucocorticoids in regulating

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the NPY neurones and controlling energy homeostasis remains uncertain.

The main aim of this study was to test the hypothesis that further overactivity of the ARC NPY neurones mediates hyperphagia induced by BRL 49653 in the *fa/fa* Zucker rat. We sought evidence of increased NPY levels in the ARC, PVN and DMH and of raised NPY mRNA levels, as these changes occur in other states, including starvation and diabetes, in which hyperphagia is thought to be driven by NPY (Williams *et al*., 1989; Dryden *et al*., 1994). We also investigated the possible roles of leptin, insulin and corticosterone, and studied lean Zucker and Wistar rats, in which thiazolidinediones have much less activity.

Methods

Animals

Male Wistar rats and male lean (Fa/?) and obese (*fa/fa*) Zucker rats aged 10 weeks (Harlan Olac Ltd, Bicester, Oxon, U.K.), were housed in wire-bottomed cages and kept at $22 \pm 2^{\circ}C$ under a 12 h light-dark cycle (09 h 00 min–21 h 00 min). Water and standard laboratory chow (CRM Biosure, Cambridge, U.K.) were freely available. Food intake and general condition were checked daily.

Experimental protocols

Effects of BRL 49653 in lean Wistar rats BRL 49653 was prepared fresh daily by dissolving it in 10% sucrose (1 mg ml^{-1}) and given orally by means of a syringe to 13 rats at a dose of 1 mg kg^{-1} day^{-1} (3 μmol kg^{-1} day^{-1}), at 09 h 00 min each day for 7 days. Control animals ($n = 13$) were given an equivalent volume of 10% sucrose.

At the end of the study, the rats were killed, immediately exsanguinated by cardiac puncture and the brains removed for microdissection. In each group, 8 rats were used for measuring regional hypothalamic NPY levels and 5 for NPY mRNA. Plasma was stored at $-40^{\circ}C$ for subsequent radioimmunoassay of insulin and corticosterone.

Treatment with BRL 49653 in fatty and lean Zucker rats BRL 49653 (1 mg kg^{-1}) was given daily for 7 or 20 days. The 7 day experiment employed 27 fatty and 15 lean rats. In the obese group, 14 received BRL 49653 (8 being used for NPY analysis and 6 for NPY mRNA), while 13 controls (8 for NPY and 5 for NPY mRNA) received sucrose alone. Eight lean rats received BRL 49653 and 7 were controls; only regional hypothalamic NPY levels were investigated in this study.

The 20 day experiment also employed 27 fatty and 15 lean rats, allocated to treatment and control groups as in the 7 day study. Plasma leptin, insulin and corticosterone levels were measured in fatty and lean rats.

Hypothalamic microdissection

NPY mRNA was measured in a mediobasal hypothalamic block including the ARC (the only site of significant NPY mRNA content (Morris, 1989)), which was snap-frozen in liquid nitrogen immediately after dissecting and stored at $-70^{\circ}C$ until extraction of RNA.

Regional hypothalamic NPY levels were measured in 8 selected areas microdissected from frontal slices cut with a vibrating microtome (Williams *et al*., 1989). Tissue from each area in each rat was boiled for 10 min in 400 μl of 0.1 M hydrochloric acid, and the samples sonicated for 30 s to disperse the tissue and to extract NPY.

The areas studied were: the medial preoptic area (MPO), lateral preoptic area (LPO), anterior hypothalamic area (AHA), paraventricular nucleus (PVN), ventromedial nucleus (VMH), dorsomedial nucleus (DMH), lateral hypothalamic area (LHA) and a wedge of tissue at the base of the third ventricle which included the arcuate nucleus (ARC). The extracts were frozen at $-40^{\circ}C$ until assayed for NPY and protein concentrations.

Assays

Regional NPY concentrations were measured by RIA by use of an NPY antiserum raised in rabbit against porcine NPY, ^{125}I-labelled porcine NPY (Amersham International, Bucks, U.K.) and synthetic porcine NPY (Bachem Ltd, Essex, U.K.) as standard (McKibbin *et al*., 1991). Cross-reactivity with peptide YY and related peptides was $<1\%$. Assay sensitivity was <0.5 fmol ml^{-1} and the within-assay coefficient of variation (CV) was 5%. All samples from each study were measured in duplicate in a single assay. Protein levels in hypothalamic extracts were measured by a modified Lowry technique, and NPY levels were expressed as fmol μg^{-1} protein.

Corticosterone and insulin concentrations were measured by use of commercial RIA kits (DPC, Caernarfon, U.K. and Pharmacia Diagnostics, St Albans, U.K.); each had a within-assay CV of 4%. Plasma leptin levels were measured by use of a RIA kit (Biogenesis, Poole, U.K.), with a detection limit of 0.2 ng ml^{-1} and an intra-assay CV of 4%.

Neuropeptide Y mRNA measurements

RNA extracted from hypothalamic tissue by use of the guanidinium thiocyanate-phenol-chloroform method was separated by electrophoresis and transferred to a Hybond-N membrane (Amersham) by capillary blotting (Cusin *et al*., 1996). NPY mRNA was measured by hybridization with rat NPY cDNA probe (provided by Dr Steven L Sabol, NIH, U.S.A.), ^{32}P-labelled by the random hexanucleotide method. The signal intensities for NPY mRNA were normalized by use of the signal similarly obtained for tubulin mRNA with a ^{32}P-labelled α-tubulin probe (kindly provided by Prof. Julian Crampton, Liverpool, U.K.). Autoradiographic bands were visualized by overnight exposure to Fuji Medical X-ray film and quantified by scanning densitometry.

Statistical analyses

Data are shown as mean $\pm$ s.e.mean. Food intake, body weight, plasma concentrations of insulin, corticosterone and leptin, and NPY mRNA levels were analysed by use of Student's unpaired t tests. Hypothalamic NPY levels in each study were compared initially by two-way analysis of variance (ANOVA), coupled to a *post-hoc* modified t test.

Group differences within individual nuclei were then examined by means of Student's unpaired t test, with a significance level of $P < 0.05$.

Results

Lean Wistar and Zucker rats

In normal Wistar rats, 7 days of treatment with BRL 49653 did not affect food intake, body weight, plasma insulin or corticosterone levels (Table 1). In addition, there were no significant changes in NPY concentrations in any of the 8 regions examined (data not shown) or in hypothalamic NPY mRNA levels (BRL 49653, 3.70 ± 0.66 vs control, 3.58 ± 0.41 arbitrary units; $P > 0.05$).

Similarly, lean Zucker rats treated with BRL 49653 for 7 days showed no significant changes in food intake, body weight, plasma insulin or corticosterone levels (Table 1), and regional hypothalamic NPY levels were unchanged (data not shown). In lean Zucker rats given BRL 49653 for 20 days, there were no changes in food intake, body weight, plasma insulin or corticosterone levels (Table 1). NPY concentrations were also unaltered by BRL 49653 treatment (data not shown).

Table 1 Food intake, body weight and plasma hormone levels in Wistar and lean Zucker rats

	Wistar rats (7 days of treatment)		*Lean Zucker rats (7 days of treatment)*		*Lean Zucker rats (20 days of treatment)*	
	Control	*BRL 49653*	*Control*	*BRL 49653*	*Control*	*BRL 49653*
n	13	13	8	7	8	7
Initial food intake (g day^{-1})	31.0±1.9	31.5±0.8	25.3±1.1	24.6±1.2	21.5±0.7	22.1±0.8
Final food intake (g day^{-1})	28.4±1.1	29.0±1.0	22.6±1.9	23.2±1.7	22.9±0.7	23.3±0.6
Initial body weight (g)	259±7	261±7	235±8	232±6	222±5	221±5
Final body weight (g)	331±8	339±7	268±7	267±6	306±7	310±6
Insulin (mu l^{-1})	42.2±4.8	32.3±6.9	32.0±8.4	28.2±4.3	26.7±1.9	23.1±2.5
Corticosterone (ng ml^{-1})	29.2±8.4	26.4±4.9	19.4±4.1	23.3±6.4	11.1±0.5	10.9±1.3
Leptin (ng ml^{-1})	–	–	–	–	3.7±0.3	–

None of the differences between treated and appropriate control groups reached statistical significance ($P<0.05$).

Table 2 Food intake, body weight and plasma hormone levels in fatty Zucker rats

	Fatty Zucker rats (7 days of treatment)		*Fatty Zucker rats (20 days of treatment)*	
	Control	*BRL 49653*	*Control*	*BRL 49653*
n	13	14	13	14
Initial food intake (g day^{-1})	33.9±1.2	34.8±1.4	35.6±0.9	34.9±0.9
Final food intake (g day^{-1})	34.1±0.8	44.7±0.7**	32.2±0.8	44.5±1.0**
Initial body weight (g)	334±6	332±7	329±8	325±8
Final body weight (g)	390±5	414±7**	4567±11	528±9**
Insulin (mu l^{-1})	397.4±26.8	279.1±20.8*	398.9±24.1	217.1±10.7**
Corticosterone (ng ml^{-1})	58.9±22.3	132.1±22.0**	58.70±10.1	131.7±10.1**
Leptin (ng ml^{-1})			8.7±0.5	9.1±0.5

Statistical significance of differences vs corresponding controls; *$P<0.05$; **$P<0.01$.

Fatty Zucker rats

In fatty Zucker rats given BRL 49653 for 7 days, food intake was significantly increased by 31% over control values ($P<0.01$) and the increase in body weight during the 7 day period was 43% greater than in controls ($P<0.05$) (Table 2). Plasma insulin levels were 10 fold higher than in lean Zucker rats and fell by 42% during treatment with BRL 49653 ($P<0.05$) (Table 2). Plasma corticosterone levels were over 3 fold higher than in lean Zucker rats and were further increased by 124% ($P<0.01$) in treated rats (Table 2).

BRL 49653 did not significantly alter regional NPY concentrations in any hypothalamic region (Figure 1), or hypothalamic NPY mRNA levels (BRL 49653: 2.17±0.12 vs control: 1.98±0.14 arbitrary units; $P>0.05$).

Similarly, fatty rats treated with BRL 49653 for 20 days showed a 38% increase in food intake ($P<0.01$; Figure 2), and a 62% greater gain in body weight during this period ($P<0.01$; Figure 3), compared with untreated controls. Plasma insulin levels were again significantly lowered, by 45% ($P<0.01$), while corticosterone levels were significantly increased by 124% ($P<0.01$), while corticosterone levels were significantly increased by 124% ($P<0.01$; Table 2). Again, there were no significant changes in NPY levels in any region (Figure 1) or in hypothalamic NPY mRNA levels (BRL 49653: 1.62±0.17 vs control: 1.71±0.15 arbitrary units; $P>0.05$). As previously shown (McKibbin *et al*, 1991), NPY levels in the ARC and DMH were significantly higher (by 65% and 27%, respectively) in the obese Zucker rats than in the lean animals (both $P<0.05$).

Untreated obese rats had leptin levels over twice those of lean rats ($P<0.01$), but 20 days of treatment with BRL 49653 had no effect on plasma leptin levels in obese rats.

Discussion

In lean Zucker or Wistar rats, BRL 49653 given for 7 days did not alter food intake, body weight or plasma corticosterone or insulin, which agrees with previous observations that the thiazolidinediones have little impact unless insulin resistance is present (Saltiel & Olefsky, 1996). There were no changes in regional NPY or in hypothalamic NPY mRNA.

By contrast, fatty Zucker rats treated with BRL 49653 for 7 or 20 days showed a significant fall in their plasma insulin levels, consistent with improved insulin sensitivity. They also displayed enhanced hyperphagia, accelerated weight gain and a further rise in corticosterone. The increased food intake in the already hyperphagic fatty Zucker rats was striking, but does not apparently involve increased activity of the ARC NPY neurones, as there were no significant changes in hypothalamic NPY or NPY mRNA.

The effects of BRL 49653 on food intake and weight gain in fatty rats are probably due to its peripheral actions rather than any direct effect on the CNS, as distribution studies (unpublished) indicate that BRL 49653 does not enter the CNS to an appreciable extent, and PPAR-γ is expressed at only low levels in certain CNS regions (e.g. the hippocampus) and not at all in the hypothalamus (Braissant *et al*., 1996).

We also investigated the possible involvement of leptin, insulin and corticosterone, all of which are implicated in energy homeostasis. Leptin levels were raised in untreated fatty Zucker rats, as has been previously demonstrated (Maffei *et al*., 1995). Certain thiazolidinediones have been shown to decrease leptin expression and levels, although other studies have not found such an effect, possibly because of differences in drug dosage or species (Nolan *et al*., 1996; Zhang *et al*., 1996; De Vos *et al*., 1996). Higher dosages of BRL 49653 (3 mg kg^{-1} day^{-1}) decreased leptin expression in lean Sprague-Dawley rats (De Vos *et al*., 1996), but the dose we used (1 mg kg^{-1} day^{-1}) had no effect on plasma leptin levels in fatty Zucker rats. BRL 49653 could, theoretically, stimulate feeding in the fatty Zucker rat by exacerbating leptin insensitivity in this mutant. However, overall, we suggest that leptin is unlikely to mediate hyperphagia in this context.

The 30–40% falls in plasma insulin levels during treatment with BRL 49653 could contribute to hyperphagia and weight gain, given the CNS-mediated hypophagic actions of insulin

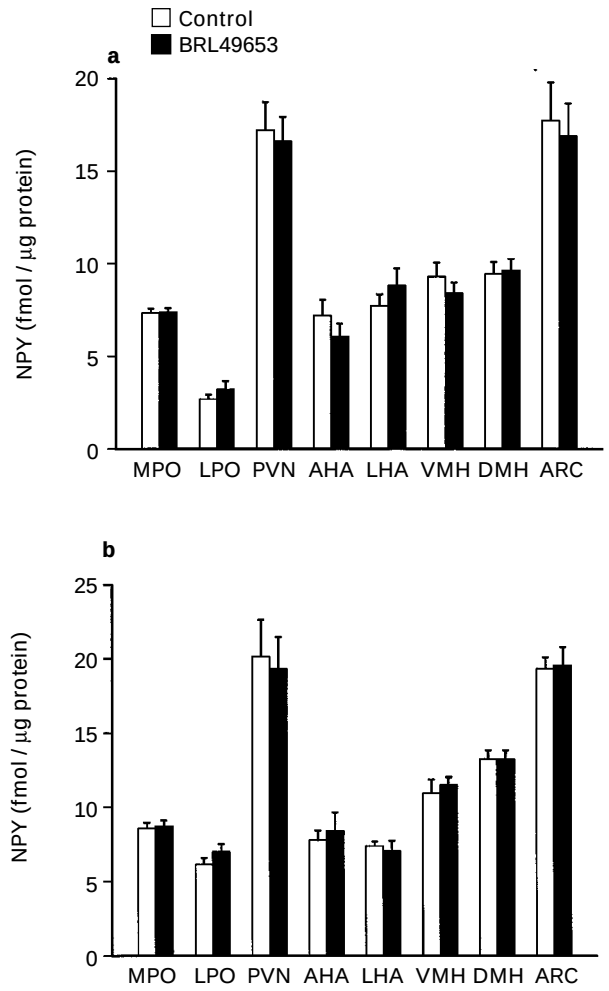

Figure 1 NPY levels in 8 hypothalamic regions of fatty Zucker rats treated with BRL 49653 (rosiglitazone) for 7 days (a) or 20 days (b). Data are mean and vertical lines show s.e.mean ($n=8$). Key to regions: MPO, LPO - medial, lateral preoptic areas; AHA, LHA - anterior, lateral hypothalamic areas; PVN, VMH, DMH, ARC - paraventricular, ventromedial, dorsomedial and arcuate nuclei.

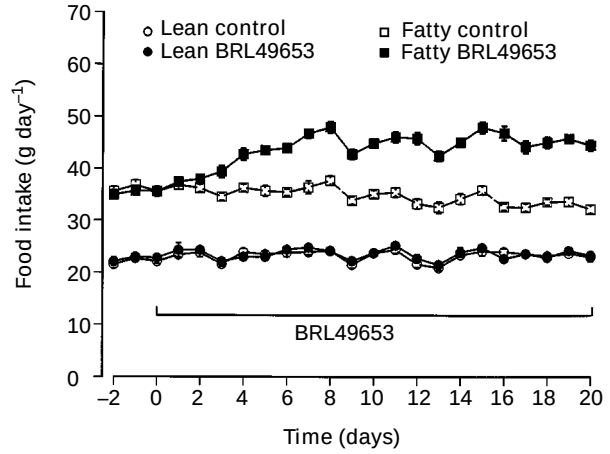

Figure 2 Food intake in fatty and lean Zucker rats during 20 days of treatment with BRL 49653 (3 μmol kg^{-1}) or placebo. Shown are: fatty control ($n=14$), fatty treated with BRL 49653 ($n=15$), lean control ($n=7$) and lean treated with BRL 49653 ($n=8$). Two-way ANOVA showed a significant effect of treatment ($P<0.01$) in the fatty rats between 0–20 days, with no significant effect ($P>0.5$) in lean rats.

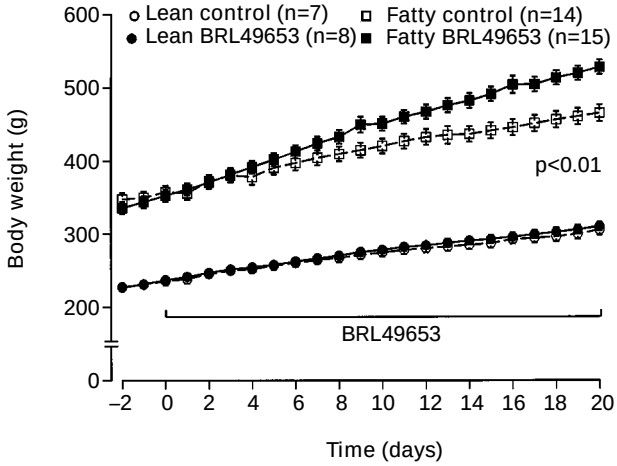

Figure 3 Body weight in Zucker rats during 20 days of treatment with BRL 49653 (3 μmol kg^{-1}) or placebo, as in Figure 2. Two-way ANOVA showed a significant effect of treatment ($P<0.01$) in the fatty rats between 0–20 days.

(Schwartz *et al*., 1992) and evidence that it down-regulates body weight (Strack *et al*., 1995). Interestingly, fatty Zucker rats are apparently resistant to the CNS actions of insulin, in that insulin injected intracerebroventricularly fails to decrease feeding (Ikeda *et al*., 1986), but their hypothalami could still respond to a relative fall in insulin; this would be consistent with the finding that these animals overeat above their usual intake during starvation, when insulin levels fall. The rise in plasma corticosterone with BRL 49653 is unexplained but could also contribute to hyperphagia; together, the fall in insulin and the rise in corticosterone can explain most of the variance in food intake and body weight in hypoadrenal and/or insulin-deficient diabetic rats (Strack *et al*., 1995).

The apparent lack of change in the NPY ARC-PVN neurones is interesting, and challenges the hypothesis that this projection mediates the reciprocal effects of insulin and corticosterone on food intake and body weight (Schwartz *et al*., 1992; Dryden *et al*., 1994; Strack *et al*., 1995). Hyperphagia could be driven by increased NPY release in discrete NPY-sensitive sites such as the PVN, but it seems unlikely that this process could continue for many days without causing changes in NPY or NPY mRNA levels, as are seen in other hyperphagic states such as diabetes and starvation (Dryden *et al*., 1994). The PVN also receives peptidergic terminals from extra hypothalamic neurones (Chronwall *et al*., 1985); selective overeactivity of this input could theoretically lead to increased NPY release in the PVN without an effect on NPY levels in the ARC, but the role of these neurones in appetite regulation is uncertain.

It therefore seems likely that hyperphagia is accentuated by an NPY-independent pathway, as is also the case in certain other conditions such as hypoglycaemia (Corrin *et al*., 1991). The fact that food intake and body weight are normal in the NPY-knockout mouse (Erickson *et al*., 1996a) confirms that other neuronal pathways and neurotransmitters can regulate energy balance, although their identity is uncertain at present.

In conclusion, hyperphagia induced in fatty Zucker rats by BRL 49653 does not appear to involve changes in plasma leptin, and may instead be mediated by a fall in insulin and/or an increase in corticosterone, consistent with the postulated roles of these hormones in regulating food intake. Hyperphagia is apparently mediated by neuronal pathways other than the NPY neurones of the ARC.

Q.W. was supported by a PhD Studentship from SmithKline Beecham Pharmaceuticals and by an Overseas Research Scholarship

awarded by the Committee of Vice-Chancellors and Principals of the United Kingdom; S.D. and C.B. by the Wellcome Trust; H.M.F. by the Tobacco Products Research Trust; and L.P. by the Biotechnology and Biological Sciences Research Council. We are indebted to Lynda Mitchell for technical assistance and to Peter Hynes and his staff for their conscientious care of the animals.

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(Received June 25, 1997
Revised September 10, 1997
Accepted September 12, 1997)