Central administration of Y5 receptor antisense decreases spontaneous food intake and attenuates feeding in response to exogenous neuropeptide Y

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Abstract

A number of neuropeptide Y (NPY) receptor subtypes, including the recently cloned Y5 receptor, have been implicated in the stimulation of food intake. In the present study, Y5 receptor antisense oligodeoxynucleotides (ODNs) were used to assess the potential involvement of the Y5 receptor in the regulation of spontaneous as well as NPY-induced food intake. Repeated central administration of Y5 antisense ODN significantly decreased spontaneous food intake and subsequently resulted in a significant weight loss. Furthermore, Y5 antisense ODN

pre-treatment significantly inhibited the robust feeding response elicited by central administration of NPY (5.3 ± 0.8 vs 1.08 ± 0.28 g, vehicle $\pm$ s.e.m. vs Y5 ODN $\pm$ s.e.m.). The present results provide evidence that central Y5 receptors are involved in both spontaneous as well as NPY-induced food intake, which may prove to be a new therapeutic route in the treatment of obesity and other disorders of appetite.

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Introduction

Food intake in rodents is regulated by a multitude of neuropeptides, some increasing and others decreasing food intake. On a molar basis, neuropeptide Y (NPY) is the most powerful orexigenic agent discovered to date. NPY is a widely distributed neurotransmitter found in high concentrations in many hypothalamic nuclei (Tatemoto *et al.* 1982). In the hypothalamus, NPY is primarily synthesised in neurones in the ventromedial part of the arcuate nucleus (ARC). These neurones project predominantly to the paraventricular nucleus (PVN) and the dorsomedial nucleus (DMH), contributing to the abundant NPY-immunoreactive terminal field found in both nuclei (Bai *et al.* 1985). NPY is believed to exert its impact on feeding and the control of energy expenditure via interaction with specific NPY receptors in both the PVN and the DMH (Tomaszuk *et al.* 1996).

Studies of the orexigenic properties of NPY have led to a better understanding of the pathophysiology of several rodent obesity syndromes. Thus, chronic treatment with NPY leads to obesity that is virtually identical to genetic obesity and hyperphagia models hallmarked by hyperinsulinaemia, insulin resistance, hyperphagia and reduced brown adipose tissue activity (Beck *et al.* 1992, Tomaszuk

et al. 1996). Furthermore, chronic i.c.v. administration of NPY antisense oligodeoxynucleotides (ODNs) and antisera to NPY decreases starvation-induced feeding and leads to hypophagia and loss of body weight (Akabayashi *et al.* 1994, Burlet *et al.* 1995). Pharmacological characterisation of NPY actions has suggested the presence of at least five NPY receptors and to date four of these have been cloned from the rat central nervous system (Eva *et al.* 1990, Gerald *et al.* 1995, 1996, Hu *et al.* 1996). The protein sequence predicted from the cloned cDNAs of all known members of the NPY receptor gene family shows the typical architecture common to G protein coupled seven transmembrane-spanning receptors, but the NPY receptor subtypes share little sequence homology with each other (Herzog *et al.* 1997). Based on the agonist binding characteristics of the NPY Y5 receptor subtype, it has been suggested that this receptor is responsible for mediating the orexigenic properties of NPY (Gerald *et al.* 1996, Hu *et al.* 1996). However, with the advent of selective Y1 and Y5 antagonists it has become clear that simple receptor phrenology is unable to support the early held notion, one receptor one action, because both Y1 and Y5 antagonists are capable of inhibiting food intake in rodents (Kanatani *et al.* 1996, Dominic *et al.* 1997, Schaffhauser *et al.* 1997).

To evaluate the importance of the Y5 receptor subtype, we used antisense ODN directed against the Y5 receptor mRNA (Y5 ODN), which in theory should cause translation arrest resulting from either interference with ribosomal activity, or ribonuclease H-mediated degradation, or both. This method has been shown to be useful in a number of *in vitro* and *in vivo* receptor modulation experiments (Akabayashi *et al.* 1994, Pilowsky *et al.* 1994, Wahlstedt 1994, Hulse *et al.* 1995, Schaffhauser *et al.* 1997).

Thus, we hypothesise that by lowering endogenous expression of NPY Y5 receptor, spontaneous food intake is reduced and body weight consequently reduced. Furthermore, decreased central Y5 receptor expression would also give rise to an attenuated feeding response to exogenous NPY.

Part of this study has been presented in abstract form (Tang-Christensen *et al.* 1997).

Materials and Methods

Animals

Thirty-six male Wistar rats (Panum Institute, Copenhagen, Denmark) weighing between 230 and 270 g were housed under 12 h light:12 h darkness (lights on at 0600 h) and controlled ambient temperature (21–23 °C) and humidity (70%). All experiments were conducted in accordance with the internationally accepted principles of the care and use of laboratory animals and approved by the Danish Committee for Animal Research.

Diet

The animals were kept on a free-feeding paradigm with fresh chow (Altromin no. 1314 food mixture, C Petersen, Ringsted, Denmark) every morning and tap water.

Surgery

Under tribromethanol (500 mg/kg body weight, Merck, Newark, NJ, USA) anaesthesia, all rats were stereotactically implanted with stainless steel guide cannulas (Bilaney Consultants, Hamburg, Germany) extending 3.75 mm below the external surface of the skull. The cannula was placed into the anterior horn of the lateral ventricle through a trepanation located 1 mm lateral to the bregma. After cannulation, the rats were individually housed in metabolic cages, allowing precise measurements of food and water intake. The animals were allowed a 7 day recovery period during which they were handled daily to habituate them to the injection procedure.

ODNs

The unmodified ODN was purchased from DNA Technology (Aarhus, Denmark). The NPY Y5 receptor

antisense ODN spanned 20 bp downstream from the start codon with the following sequence 5-GTG GAA GAA GAG GAC GTC CAT-3.

Based on this sequence the scrambled ODN was constructed using the same numbers of CTGA nucleotides in a random order.

Both sequences were checked for crossmatches using the NIH Genbank. A sense ODN complementary to the antisense sequence was not employed because of a high homology to antisense sequences of coding regions of a number of constitutively expressed neural tissue proteins.

Injection paradigm

Before experiments were started rats were randomised into three groups. One group received the scrambled ODN, one received the Y5 receptor antisense ODN, and one received vehicle. The rats were injected twice daily for 2 days with 50 µg ODN in 10 µl PBS. Food and water intake was monitored every 12 h (0700 and 1800 h) and body weights were measured every 24 h during the following 2 days.

On the third day (after a total of four injections) rats were deprived of food for approximately 1 h (0700–0800 h) before a central injection of 5 µg NPY (Peninsula, London, UK) in 5 µl PBS was given. Food and water intake was assessed after 90 min.

Three hours post NPY injection rats were decapitated and trunk blood was collected.

Insulin

Plasma insulin was measured at Hagedorn Research Laboratory by Drs M Deckert and T Mandrup-Poulsen.

Statistics

Values for food (g), body weight (% of baseline weight) and plasma insulin (ng/ml) are expressed as means $\pm$ s.e.m. The effects of NPY Y5 antisense ODN, scrambled ODN and vehicle were compared by a one-way ANOVA with post hoc analysis using Scheffé's test. Values of $P < 0.05$ were considered significant.

Results

Central administration of Y5 receptor antisense ODN significantly decreased cumulated food intake throughout the observation period (19.72 ± 3.69 vs 34.33 ± 2.60 g, Y5 antisense ODN vs vehicle), while central administration of scrambled ODN had no effect on cumulated 48 h food intake (34.97 ± 2.08 vs 34.33 ± 2.60 g, scrambled ODN vs vehicle) (Fig. 1). The effect of Y5 antisense ODN treatment on food intake was most prominent within the first three 12 h observation periods

Figure 1 Effect on cumulated food intake in rats with free access to food and water of i.c.v. injections of either 50 µg Y5 antisense ODN, 50 µg scrambled ODN or vehicle twice daily for 2 days. Injections were given every morning and evening (0700 and 1900 h) and food intake was monitored in 12 h periods. Values are means ± S.E.M. with $n=8-11$ for all groups. * $P<0.05$ as determined by ANOVA followed by Scheffe's post hoc analysis.

(50–70% reduction). Apparently, the Y5 ODN treatment lost its anorectic effect in the last 12 h observation period (9.9 ± 1.54 vs 12.43 ± 0.79 g, Y5 antisense ODN vs vehicle, Table 1).

Furthermore, the initial 24 h of Y5 antisense ODN treatment significantly decreased body weight when compared with both vehicle and treatment with scrambled ODN (93.8 ± 1.5 vs $100.2 \pm 0.48\%$ of baseline weight, Y5 antisense ODN vs vehicle) (Fig. 2). The effect of Y5 antisense ODN on body weight lasted for 48 h but no further reduction was observed throughout the second half of the 48 h observation period.

I.c.v. injection of 5 µg NPY caused a robust and identical increase in 60 min food intake in both the scrambled ODN as well as the vehicle group (5.3 ± 0.8 vs 4.9 ± 0.63 g) (Fig. 3). In contrast, the feeding response to exogenous NPY in the Y5 antisense ODN group was reduced by approximately 80% when compared with scrambled and vehicle (1.08 ± 0.28) (Fig. 3). Measurement of circulating insulin levels 3 h post NPY stimulation revealed a trend towards lowered levels in the Y5 antisense

Figure 2 Effect on body weight of i.c.v. injections of either 50 µg Y5 antisense ODN, 50 µg scrambled ODN or vehicle twice daily for 2 days. Body weight was measured daily and expressed as a percentage of initial weight at day 1 (100%). Values are means ± S.E.M. with $n=8-11$ for all groups. * $P<0.05$ as determined by ANOVA followed by Scheffe's post hoc analysis.

ODN group when compared with scrambled and vehicle (100 ± 25 vs 362 ± 100 ng/ml, Y5 antisense ODN vs vehicle, Table 2).

Discussion

This study, together with a recently published study by Schaffhauser *et al.* (1997), provide evidence that central Y5 receptors are important mediators of the orexigenic properties of NPY. A single dose of Y5 antisense ODN significantly reduced spontaneous food intake in *ad libitum* fed rats. The effect on food intake was present in the following three 12 h periods, but there was no significant reduction in the last 12 h period. The loss of sensitivity to Y5 antisense ODN treatment was also mirrored by an initial weight loss after two injections of Y5 ODN and a subsequent stabilisation of body weight after the two following injections. This could imply that other neuronal systems are rapidly recruited during the NPY Y5

Table 1 Effect of i.c.v. injection of either 50 µg Y5 antisense ODN, 50 µg scrambled ODN or vehicle twice daily for 2 days on food intake. Food intake was measured every 12 h 0700 and 1900 h prior to the new i.c.v. injection. Values are expressed as means ± S.E.M. and $n=8-11$ for all groups

Treatment	0–12 h	12–24 h	24–36 h	36–48 h
NPY Y5 antisense	$1.15 \pm 0.38^*$	$6.14 \pm 2.09^*$	$2.35 \pm 0.48^*$	9.90 ± 1.54
Scrambled	4.62 ± 1.06	11.01 ± 0.88	5.51 ± 0.86	13.84 ± 0.79
Vehicle	4.48 ± 0.60	13.38 ± 1.49	4.34 ± 0.52	12.43 ± 0.79

* $P<0.05$ as determined by ANOVA followed by Scheffe's post hoc analysis.

Figure 3 Effect of i.c.v. administration of 5 µg NPY upon food intake in 2 h food deprived rats previously treated with either two daily injections of 50 µg Y5 antisense ODN, 50 µg scrambled ODN or vehicle. Values are means ± S.E.M. with *n*=6 for all groups. **P*<0.05 as determined by ANOVA followed by Scheffe's post hoc analysis.

inhibition, to compensate for the loss of NPY activity, and thus Y5 receptor blockade can only cause a small decrease in body weight. However, to test this more accurately would require continuous infusion of the Y5 ODN over a prolonged period of time. In this antisense study, as well as in others (Akabayashi *et al.* 1994, Hulsey *et al.* 1995), changes in feeding behaviour occurred rapidly, and a possible explanation for this phenomenon by non-specific general behavioural suppression should be considered. Numerous substances cause decreased food intake due to non-specific malaise. However, animals receiving scrambled ODN displayed a normal feeding behaviour and they maintained their body weight throughout the study, emphasising that the mere presence of ODNs in the cerebrospinal fluid has little effect on feeding behaviour.

In theory the reduction of food intake in the antisense-treated group is caused by a reduction in Y5 receptor density. Thus, the response to exogenous NPY should also be blunted. To test this hypothesis we administered 5 µg NPY and monitored the food intake for 1 h. The Y5 antisense ODN-treated animals displayed a severely blunted response to NPY-induced food intake when compared with both the scrambled and the vehicle group.

Table 2 Effect of i.c.v. administration of 5 µg NPY upon circulating insulin levels (3 h post NPY injection) in rats prior-treated with two daily injections of either 50 µg Y5 antisense ODN, 50 µg scrambled ODN or vehicle. Values are expressed as means ± S.E.M. and *n*=8–11 for all groups

Treatment	Insulin concentration (ng/ml)
NPY Y5 antisense	100.3 ± 25.2
Scrambled	303.4 ± 69.0
Vehicle	362.0 ± 100.0

**P*<0.05 as determined by ANOVA followed by Scheffe's post hoc analysis.

Furthermore, we found significantly lower levels of circulating insulin in the Y5 antisense ODN group (Table 2). Central administration of NPY results in a sustained and dose-dependent increase in circulating insulin level both in a fed and fasted state (Moltz & McDonald 1985, van Dijk *et al.* 1994, Marks & Waite 1996, 1997). The observed trend to a (*P*<0.07) difference in circulating insulin levels, between the Y5 antisense ODN on the one hand and vehicle and scrambled ODN on the other, could thus be a result of changes in the number of functional Y5 receptors. But since the rats were not food restricted and ingestion of food by itself stimulated insulin secretion our results are somewhat ambiguous and it needs further experimental work to clarify whether NPY-induced insulin secretion is mediated via the Y5 receptors.

Applying the traditionally accepted mechanism of translation–arrest–pool depletion–behavioural effect as an explanation for the currently observed anorexia, the turnover rate of Y5 receptors in neurones mediating the orexigenic effect of NPY must be considerable. However, experimental data from studies of the correlation between numbers of site-specifically expressed Y5 receptors and the behavioural consequences are not available. As first suggested by Hulsey *et al.* (1995) there may be another mechanism to explain the rapid onset of the behavioural effects. The observations that neuronal mRNA and ribosomes are also located in the terminal field (Steward & Banker 1992, Van Minnen 1994) would make it possible for the antisense treatment to induce changes in a distinct package pool of Y5 receptor protein in the terminal field rather than inhibiting translation, transport and packaging of a releasable pool through the endoplasmic reticulum and Golgi apparatus, and thus rapid changes in feeding observed might be explained. Ideally, the effect of antisense ODN treatment upon protein synthesis should be verified by demonstration of lowered numbers of specific Y5 binding sites in hypothalamic nuclei of interest (e.g. PVN and ARC). However, specific Y5 ligands are not available for receptor binding experiments, and the density of hypothalamic NPY binding sites is low, which makes receptor autoradiographic attempts to visualise Y5 binding sites using ¹²⁵I-PYY3–36 in combination with specific cold Y2 ligands less feasible (Widdowson 1997, Widdowson *et al.* 1997). We have unsuccessfully tried to visualise Y5 binding sites using ¹²⁵I-human pancreatic polypeptide (PP) in the presence of unlabelled rat PP, but apparently all human PP binding sites present in the rat hypothalamus are Y4 receptors, and we have not been able to address the question of receptor density in a quantitative manner.

During the last few years, a number of reports have described anorexic effects of both selective Y1 and Y5 compounds (Kanatani *et al.* 1996, Criscione *et al.* 1997, Dominic *et al.* 1997). However, different routes of administration and use of different species make direct comparisons of most of these studies difficult. Thus, central

administration of the peptide Y1 antagonist BW1229 U91 profoundly inhibits NPY- and starvation-induced feeding in rats, and direct injections of the non-peptide Y1 antagonist BIBP3226 and BIBO3304 into the PVN inhibits food intake in both mice and rats (Dominic *et al.* 1997, Doods *et al.* 1997). Knock-out mice which lack expression of either Y5 or Y1 receptor all develop normally and have normal body weights compared with wild type or heterozygous littermates (Marsh *et al.* 1997, Pedrazzini *et al.* 1997). However, the feeding response of Y5 receptor knock-outs to exogenous (NPY) is less vivid, and female Y1 receptor knock-out mice do not mount an adequate hyperphagic response to starvation (Marsh *et al.* 1997, Pedrazzini *et al.* 1997) suggesting that both Y1 and Y5 receptors are involved in regulation of normal feeding behaviour. O'Shea *et al.* (1997) have obtained behavioural feeding data in agreement with involvement of both Y1- and Y5-like sites, which led to the suggestion that an alternative, not yet identified, receptor mediates the orexigenic effect of NPY (Dominic *et al.* 1997, O'Shea *et al.* 1997). However, it is also possible that both Y1 and Y5 receptors mediate NPY-induced feeding. In support of this are a number of recent anatomical reports demonstrating expression of both Y1 and Y5 mRNA in the PVN where NPY elicits its orexigenic effect (Larsen *et al.* 1993, Gerald *et al.* 1996).

The currently observed abolition of NPY-induced feeding by Y5 antisense ODN treatment is unlikely to directly involve Y1 sites because the ODN bears no homology to Y1 encoding Y1 mRNA (Herzog *et al.* 1997). Judging from both our data and the data of Schaffhauser *et al.* (1997), an intact population of Y5 receptors is essential for NPY-induced feeding.

In conclusion, this present study further substantiates the claim made by Schaffhauser *et al.* (1997) that the Y5 subtype is a key receptor in the regulation of feeding behaviour. Y5 antisense ODN-treated rats showed a marked decrease in spontaneous meal size that tended to wear off after four injections. Interestingly the response to exogenous NPY administration was lowered by 80%. By use of selective Y5 agonists and antagonist it may be possible to further evaluate the involvement of the Y5 receptor in feeding behaviour, and thus clarify whether this may turn out to be a new therapeutic route in the treatment of obesity and other disorders of appetite.

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