Food intake in lean and obese mice after peripheral administration of glucagon-like peptide 2

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Abstract

We investigated the potential anorectic action of peripherally administered glucagon-like peptide 2 (GLP2) in lean and diet-induced obese (DIO) mice. Mice, fasted for 16 h, were injected i.p. with native GLP2 or [Gly²]GLP2, stable analog of GLP2, before or after GLP2 (3–33), a GLP2 receptor (GLP2R) antagonist, or exendin (9–39), a GLP1R antagonist. Food intake was measured at intervals 1, 2, 4, 8, and 24 h postinjection. In addition, we tested in lean mice the influence of [Gly²]GLP2 on gastric emptying and the effects of GLP1 alone or in combination with [Gly²]GLP2 on food intake. [Gly²]GLP2 dose dependently and significantly inhibited food intake in lean and DIO mice. The reduction of food intake occurred in the first hour postinjection and it was sustained until 4 h postinjection in lean mice while it was

sustained until 2 h postinjection in DIO mice. GLP2 significantly inhibited food intake in both lean and DIO mice but only in the first hour postinjection. The efficiency of [Gly²]GLP2 or GLP2 in suppressing food intake was significantly weaker in DIO mice compared with lean animals. The [Gly²]GLP2 anorectic actions were blocked by the GLP2R antagonist GLP2 (3–33) or by the GLP1R antagonist exendin (9–39). The coadministration of [Gly²]GLP2 and GLP1 did not cause additive effects. [Gly²]GLP2 decreased the gastric emptying rate. Results suggest that GLP2 can reduce food intake in mice in the short term, likely acting at a peripheral level. DIO mice are less sensitive to the anorectic effect of the peptide.

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Introduction

Glucagon-like peptide 2 (GLP2) is a 33-amino acid peptide, produced by the processing of the proglucagon gene within the mucosal L-cells of the intestine and specific neurons located in the brainstem. The actions of GLP2 are transduced by the GLP2 receptor (GLP2R), which is localized within the gastrointestinal tract to enterendocrine cells, subepithelial myofibroblast cells, and in the neurons of the enteric nervous system (Estall & Drucker 2006). In the CNS the GLP2R is expressed in the hypothalamic nucleus, the hippocampus, the nucleus of the solitary tract, the parabrachial nucleus, the supramammillary nucleus, and the substantia nigra (Vrang & Larsen 2010). The main stimulus for the GLP2 release is represented by the presence of nutrients, specifically fats and carbohydrates, in the intestinal lumen (Brubaker 2006). After secretion, GLP2 is degraded by the enzyme dipeptidyl peptidase-IV (DPP-IV), which renders the peptide inactive by N-terminal truncation of the alanine at position 2 (Drucker *et al.* 1997). The degradation-resistant analog of GLP2, [Gly²]GLP2 has increased efficacy compared with native GLP2 and is currently in clinical trials as a therapeutic for a variety of intestinal insufficiencies and diseases (Rowland & Brubaker 2011).

The main site of action for the hormone peptide is the gastrointestinal tract where the peptide exerts trophic properties (Estall & Drucker 2006). In addition to promoting expansion of the gastrointestinal mucosal surface area, GLP2 has been shown to affect gastrointestinal motility in humans and rodents. In fact, it inhibits gastric emptying (Wøjdemann *et al.* 1998, Nagell *et al.* 2004), decreases gastric fundic tone leading to enhancing gastric capacity (Amato *et al.* 2009), reduces intestinal transit *in vivo* (McDonagh *et al.* 2007), and reduces small and large intestinal motility *in vitro* (Amato *et al.* 2010, Cinci *et al.* 2011). The inhibitory effect on gastric tone, at least in mice, seems to depend on the nutritional state of the animal, being largely more evident in mice fed a high fat diet (HFD), which after 14 weeks develop obesity syndrome (Collins *et al.* 2004). In fact, diet-induced obese (DIO) animals have higher GLP2R expression in gastric fundus than mice fed a standard diet (lean animals) (Rotondo *et al.* 2011).

One still open question regarding the actions of GLP2 is about its importance in controlling food intake. Although GLP2 has been shown to decrease food intake in rodents when administered centrally (Tang-Christensen *et al.* 2000, Lovshin *et al.* 2001), it does not affect food consumption after peripheral administration in rodents or avian species,

at least in long-term observations (Tsai *et al.* 1997, Shousha *et al.* 2007), probably because of the activity of GLP2 degrading enzyme DPP-IV. Thus, due to the lack of information about the effects of GLP2 on food consumption in the short term, the purpose of the present study was to clarify the potential role of GLP2 on feeding behavior within 24 h. The efficiency of the long-acting GLP2 analog, [Gly²]GLP2, and of the native peptide was examined also in DIO mice to reveal any possible difference in comparison with lean animals.

Materials and Methods

Animals

Adult male C57BL/6J mice (Harlan Laboratories, San Pietro al Natisone, Udine, Italy) weighing about 18 g were housed individually in cages under standard conditions of light (12 h light:12 h darkness cycle) and temperature (22–24 °C). We used adult male animals to avoid the effects of female reproductive hormones on food intake. Food and water were available *ad libitum* except otherwise indicated. All experimental procedures were approved by Ministero della Sanità (Rome, Italy) and were in compliance with the guidelines of the European Communities Council Directive of 24 November 1986.

Diets

After 1 week of free access to a pelletized rodent diet (GLP4RF21; Mucedola, S.r.L. Italy), mice were divided into two groups. One group were kept on the standard diet, which consisted of 18.5% proteins, 60% carbohydrates, and 3% fats; the other group were fed a HFD (PF4051/D; Mucedola), which consisted of 23% proteins, 38% carbohydrates, and 34% fats (60% caloric fat content) for at least 14 weeks. At the end of this period, the body weight reached 38.22 ± 1.1 g ($n=30$) in HFD-fed mice and it was significantly different from the first group of animals (lean) (27.4 ± 0.9 g; $n=30$).

Peptides

The following peptides were used: GLP2, GLP11 (7–36), exendin (9–39) (Tocris Bioscience, Bristol, UK), [Gly²]GLP2 and GLP2 (3–33) (Caslo Laboratory, Lyngby, Denmark).

Experimental protocol

Fasted (16 h) mice were injected i.p. with 100 µl of either vehicle (PBS), [Gly²]GLP2 (0.15, 0.30, 0.60, 0.90 µg/g b.w.), GLP1 (0.30 µg/g b.w.), GLP2 (3–33) (0.90 µg/g b.w.), exendin (9–39) (0.20 µg/g b.w.), or GLP2 (0.90 µg/g b.w.) in the early light phase (0800–0900 h). Prior to the initial study, mice received a daily i.p. injection of 100 µl PBS for 7 days to

habituate them to the procedure. To verify the mechanism of action some animals were injected i.p. with GLP2 (3–33) or exendin (9–39) immediately followed by PBS or [Gly²]GLP2 or GLP1. The combined effects of [Gly²]GLP2 (0.60 µg/g b.w.) and GLP1 (0.30 µg/g b.w.) were also analyzed. These concentrations for the joint study were chosen because they separately reduced food intake by 50% in the first hour.

Peptides were dissolved in PBS to concentrations that allowed delivery of dose in 100 µl. Doses were selected on the basis of previously published studies (Hartmann *et al.* 2000, Neary *et al.* 2005, Talsania *et al.* 2005, McDonagh *et al.* 2007) and pilot studies in our laboratory. A minimum of 72 h was allowed between each trial in the same mouse. Following injection, each mouse was returned to its home cage with a preweighed amount of chow. The food intake was determined at 1, 2, 4, 8, and 24 h following peptide or vehicle administration, by measuring the difference between the preweighed chow and the weight of chow at the end of each time interval. Any spillage was collected and weighed.

Gastric emptying

Lean mice were food deprived for 18 h with free access to water. At $t=0$ animals were given free access to preweighed standard chow for 1 h, and then were injected i.p. with 100 µl of either PBS or [Gly²]GLP2 (0.90 µg/g b.w.). Mice were deprived of food for 3 h after i.p. administration and then sacrificed. To determine the amount of food remaining in the stomach after the 3-h postinjection period, the stomach was excised and the gastric contents were collected, frozen, lyophilized overnight, and weighed. Total food intake during the 1-h feeding period was determined by measuring the difference between the preweighed standard chow and the weight of chow and spill. Gastric emptying (%) was calculated as: $(1 - (\text{dry weight of food recovered from the stomach} / \text{total food intake}) \times 100)$ (Talsania *et al.* 2005).

Behavioral analysis

Because it is possible that GLP2 inhibits food intake via nonspecific taste aversion, mice were observed for 1 h postinjection using a behavioral score sheet as described previously (Dakin *et al.* 2001). Four different behaviors (still, head down, burrowing, and locomotion) were scored in animals treated with PBS or GLP2 (0.90 µg/g b.w.) by observers blinded to the experimental conditions.

Statistical analysis

All data are expressed as means $\pm$ S.E.M. Statistical significance was determined by ANOVA followed by Bonferroni's post-hoc test using Prism Version 4.0 software (GraphPad Software, Inc., San Diego, CA, USA). A P value < 0.05 was considered to be statistically significant.

Figure 1 Effects of [Gly²]GLP2 on food intake in (A) lean and (B) DIO mice. Vehicle or [Gly²]GLP2 (0.15, 0.30, 0.60, and 0.90 µg/g b.w.) were injected i.p. in mice and food intake was measured at the intervals 0–1, 1–2, 2–4, 4–8, and 8–24 h postinjection. $n=8-10/\text{treatment}$. Data are means $\pm$ s.e.m. * $P<0.05$ vs vehicle.

Results

Effects of [Gly²]GLP2 administration on food intake in lean and DIO mice

I.p. administration of [Gly²]GLP2 (0.15–0.90 µg/g b.w.) caused a dose-dependent and significant reduction in food intake when compared with vehicle-treated mice (Fig. 1A). The minimal dose of [Gly²]GLP2 that had an effect was 0.30 µg/g b.w. The decrease in food intake occurred from the first hour and was sustained until 4 h postinjection. The anorectic effect of [Gly²]GLP2 (0.30–0.90 µg/g b.w.) was abolished by previous administration of the GLP2R antagonist, GLP2 (3–33) (0.90 µg/g b.w.) (Fig. 2A, B and C), which *per se* did not alter the food intake when compared with vehicle-treated mice (Fig. 3).

In DIO mice [Gly²]GLP2 (0.15–0.90 µg/g b.w.) induced a dose-dependent and significant reduction of food intake compared with PBS-treated mice. The lowest effective dose of [Gly²]GLP2 was 0.30 µg/g b.w. The decrease in feeding was sustained with all doses for up to 2 h after injection. There were no significant differences in interval food intake at any other time point (Fig. 1B). The [Gly²]GLP2 (0.30–0.90 µg/g b.w.) inhibition in feeding was abolished by previous injection of 0.90 µg/g b.w. of GLP2 (3–33),

the GLP2R antagonist (Fig. 2D, E and F). Once more, GLP2 (3–33) *per se* did not alter the food intake when compared with vehicle-treated DIO mice.

The efficiency of i.p. [Gly²]GLP2 (0.30–0.90 µg/g b.w.) in suppressing food intake was significantly weaker in DIO mice than in lean animals (Fig. 4).

Effect of GLP2 administration on food intake in lean and DIO mice

To confirm the anorectic potential of the peptide we used the native GLP2 and measured food intake within 4 h. In both lean and DIO mice administration of GLP2 (0.90 µg/g b.w.) significantly inhibited food intake in the first hour postinjection when compared with vehicle-treated mice and there were no significant changes in the food intake at any further interval. There was no change in still, head down, burrowing, and locomotion episodes in lean or DIO-treated mice compared with their respective controls. Once more,

Figure 2 Effects of GLP2 (3–33), antagonist of GLP2R, on inhibitory effects induced by different doses of [Gly²]GLP2 on food intake in (A, B and C) lean and (D, E and F) DIO mice. Vehicle, [Gly²]GLP2, or GLP2 (3–33) (0.90 µg/g b.w.), followed by [Gly²]GLP2, were injected i.p. in mice and food intake was measured at the intervals 0–1, 1–2, and 2–4 h postinjection in lean animals ($n=6-8/\text{treatment}$) or at the intervals 0–1 and 1–2 h postinjection in DIO mice ($n=6-8/\text{treatment}$). Data are means $\pm$ s.e.m. * $P<0.05$ vs vehicle.

Figure 3 Effects of GLP2 (3–33) on food intake in lean mice. Mice were injected i.p. with vehicle or GLP2 (3–33) (0.90 µg/g b.w.) and food intake was measured 0–1, 1–2, 2–4, 4–8, and 8–24 h postinjection. ($n=6$ /treatment). Data are means $\pm$ S.E.M.

GLP2 was significantly less effective in DIO animals (Fig. 5A, B and C).

Cross talk between GLP1R and GLP2R

In lean mice the anorectic effects of [Gly²]GLP2 (0.90 µg/g b.w.) were tested after injection of the GLP1R antagonist exendin (9–39) (0.20 µg/g b.w.). Administration of exendin (9–39) blocked the inhibitory effect of [Gly²]GLP2, returning food intake to the same level as animals receiving injections of vehicle (Fig. 6). Exendin (9–39) (0.20 µg/g b.w.) alone did not alter food intake when compared with vehicle-treated mice.

Also GLP1 (0.30 µg/g b.w.) decreased food intake. At the first hour, the reduction was of $-51.5 \pm 6.0\%$ ($n=12$) relative to treatment with PBS and it was similar to that induced by a higher dose of [Gly²]GLP2 (0.60 µg/g b.w.) ($-53.7 \pm 8.0\%$; $n=8$) ($P>0.05$; Fig. 7). This effect was antagonized by exendin (9–39) (0.20 µg/g b.w.) but not by the GLP2R antagonist, GLP2 (3–33) (0.90 µg/g b.w.) (data not shown). Furthermore, coadministration of [Gly²]GLP2 (0.60 µg/g b.w.) and GLP1 (0.30 µg/g b.w.) caused a significant inhibition of feeding compared with PBS-treated animals, but additive inhibition was not observed because the percentage reduction in food intake ($-42.8 \pm 6.5\%$; $n=8$) was not significantly different from the values observed after the peptides were individually administered (Fig. 7).

Gastric emptying

To examine potential mechanisms underlying the peptide anorectic action, the rate of gastric emptying was determined in mice administered PBS or [Gly²]GLP2 (0.90 µg/g b.w.). Mice injected with [Gly²]GLP2 displayed a significant decrease in the rate of gastric emptying by $13.1 \pm 0.7\%$ ($P<0.01$; $n=8$) compared with PBS-treated mice.

Discussion

The results of the present study suggest that GLP2 can enhance satiety in the short-term, although at pharmacological levels. Obese mice fed a HFD are less sensitive to the anorectic action of the peptide.

It is known that some products of the proglucagon, such as GLP1 or oxyntomodulin, are potent inhibitors of food intake when injected intracerebroventricularly in the animal model (Turton *et al.* 1996, Dakin *et al.* 2001) as well as when peripherally administered in both rodents and humans (Flint *et al.* 1998, Verdich *et al.* 2001, Cohen *et al.* 2003, Dakin *et al.* 2004, Liu *et al.* 2010). On the contrary, the potential importance and the mechanism of action responsible for the GLP2-dependent regulation of feeding behavior remain uncertain (Tang-Christensen *et al.* 2000, Lovshin *et al.* 2001, Schmidt *et al.* 2003, Sorensen *et al.* 2003). In fact, in healthy humans GLP2 does not affect appetite or postprandial feeling of satiety, although at concentrations that are not high enough to induce a response (Schmidt *et al.* 2003, Sorensen *et al.* 2003) or in patients with short bowel syndrome (Jeppensen *et al.* 2001). By contrast, GLP2 has been shown to be an inhibitor of food intake in rodents, when centrally injected (Tang-Christensen *et al.* 2000, Lovshin *et al.* 2001), but it is ineffective in long-term experiments when administered peripherally (Tsai *et al.* 1997, Scott *et al.* 1998). In addition, in the Japanese quail neither peripheral nor central administration of GLP2 affected food intake (Shousha *et al.* 2007), even if it is necessary to consider that the amino acid sequence of mammalian GLP2, used in the study shows low homology with the avian GLP2 (Burrin *et al.* 2003), explaining a possible inefficacy of mammalian GLP2 in the quail. In the present study, we examined the anorectic

Figure 4 Comparison of the anorectic effect induced by [Gly²]GLP2 in lean and DIO mice within the (A) first or the (B) second hour. Mice were injected with vehicle or [Gly²]GLP2 (0.30, 0.60, 0.90 µg/g b.w.). Data are means $\pm$ S.E.M. ($n=8-10$ /treatment) and are expressed as a percentage of the mean food intake of the respective vehicle-treated animals. * $P<0.05$ when compared with the respective [Gly²]GLP2 doses of the lean group.

Figure 5 Effects of native GLP2 on food intake in (A) lean and (B) DIO mice. Vehicle or GLP2 (0.90 µg/g b.w.) were injected i.p. in mice and food intake was measured at the intervals 0–1, 1–2, and 2–4 h postinjection. (C) Comparison of the anorectic action induced by GLP2 at the first hour postinjection. Results are expressed as a percentage of the mean food intake of the respective vehicle-treated animals. Data are means $\pm$ s.e.m. ($n=8$ /treatment). * $P<0.05$ vs vehicle. # $P<0.05$ when compared with lean group.

potential of the peptide within 24 h and evaluated its efficacy also in DIO mice.

We found that [Gly²]GLP2 caused a significant decrease in food intake; the effect was sustained until 4 h postinjection, while it failed to affect food intake in the long term, suggesting that the anorectic effect is transient. Interestingly, the dose of [Gly²]GLP2 that inhibited the food intake by 50% was higher than that of GLP1, which resulted in 50% of inhibition (0.60 vs 0.30 µg/g b.w. respectively). Also the native GLP2 inhibited food intake, although its action was more transient than [Gly²]GLP2 and not detectable after 2 h. Excessive degradation of GLP2 catalyzed by the ubiquitous enzyme DDP-IV (Hansen *et al.* 2007), which apparently renders the peptide inactive by N-terminal truncation, could explain the difference in the effect duration. Moreover, we have ruled out that the reduction of food intake induced by GLP2 is due to nonspecific food aversion because behavioral analysis did not show any malaise signals.

Our results appear to be in contrast with previous reports in humans or rats (Scott *et al.* 1998, Jeppensen *et al.* 2001, Schmidt *et al.* 2003, Sorensen *et al.* 2003). However, Scott *et al.* (1998) did not measure food intake in the short term, but daily food intake. Moreover, in humans a variety of factors, such as psychological or environmental conditions, may influence meals. Another possible explanation may be that subjects were treated with GLP2 (Jeppensen *et al.* 2001, Schmidt *et al.* 2003, Sorensen *et al.* 2003), which is rapidly degraded.

To define the specificity of the anorectic GLP2 response, we tested the effect of [Gly²]GLP2 after the injection with the GLP2R antagonist GLP2 (3–33) (Shin *et al.* 2005, Baldassano *et al.* 2009). GLP2 (3–33) completely blocked the anorectic effect of GLP2 in equivalent doses, without affecting the food intake reduction induced by GLP1, confirming the specificity of the response. The employed dose of GLP2 (3–33) had no effect on food intake *per se*, suggesting that GLP2R is not tonically activated.

Because in rodents a role for GLP1R signaling in the regulation of the central anorectic effects induced by i.c.v. injection of GLP2 has been proposed (Tang-Christensen *et al.*

2000, Lovshin *et al.* 2001), we used exendin (9–39) a GLP1R antagonist (Göke *et al.* 1993) to evaluate a possible cross talk between GLP1R and GLP2R signaling networks regulating food intake. In our experiments, similar to results obtained following i.c.v. infusion of GLP2 in rats (Tang-Christensen *et al.* 2000), the anorectic effects of [Gly²]GLP2 were blocked by pretreatment with the GLP1R antagonist exendin (9–39), suggesting that the effects of GLP2 on feeding require the functional activity of GLP1R signaling. It is unlikely that [Gly²]GLP2 directly interacts with GLP1R because previously it was reported that the peptide has no effect in cells transfected with GLP1R (Lovshin *et al.* 2001) and increases cAMP accumulation in neuronal cultures derived from GLP1R null mice (Lovshin *et al.* 2004). Indeed, the finding that exendin (9–39) eliminates the GLP2-mediated inhibitory effects on food intake might imply that the GLP1R antagonist acts also as a GLP2R antagonist, as in the past it was proposed (Wheeler *et al.* 1995, Munroe *et al.* 1999, Tang-Christensen *et al.* 2000). However, more recent studies clearly demonstrate that exendin (9–39) does not act as a functional GLP2R antagonist (Lovshin *et al.* 2001, 2004). In mice intracerebroventricular GLP2 inhibits dark-phase feeding more potently after GLP1R blockade or complete disruption of GLP1R in GLP1R knockout mice (Lovshin *et al.* 2001). The discrepancy with our results might be due to the route of administration (intracerebroventricular vs i.p.) and consequently a different site of action of the peptide. Results obtained in our experiments following coadministration suggest that GLP1 and GLP2 act through a common pathway because the effects of the peptides on the food intake were not additive.

On the basis of our results, we cannot conclude about the mechanism of action, i.e. whether the GLP2 inhibitory

Figure 6 Effects of exendin (9–39), antagonist of GLP1R, on inhibitory effects induced by [Gly²]GLP2 on food intake in lean mice. Vehicle or exendin (9–39) (0.20 µg/g b.w.) followed by [Gly²]GLP2 (0.90 µg/g b.w.) were injected i.p. in mice and food intake was measured at the intervals 0–1, 1–2, and 2–4 h postinjection. Data are means $\pm$ s.e.m. ($n=6$ /treatment).

Figure 7 Effects of [Gly²]GLP2 and GLP1, alone or in combination, on food intake in the first hour. Mice were injected i.p. with PBS, [Gly²]GLP2 (0-90 µg/g b.w.), GLP1 (0-30 µg/g b.w.), or [Gly²]GLP2 (0-90 µg/g b.w.) plus GLP1 (0-30 µg/g b.w.). Data are means $\pm$ S.E.M. ($n=8$ /treatment) and are expressed as a percentage of the food intake in PBS-treated animals.

effect directly involves some of the brain centers regulating food intake or if it is indirect through peripheral sites. Indeed, participation of both central and peripheral sites in the GLP2-induced anorectic effect might be considered because a number of studies have shown that peripheral administration of neuropeptides labels the blood-brain barrier-free area postrema and diffuses into the adjacent regions (Whitcomb *et al.* 1990), and GLP2Rs have been described in the nucleus of the solitary tract (Lovshin *et al.* 2004). In addition, functional GLP2R has been localized to the cell bodies of vagal afferents in the nodose ganglion, at least in the rat (Nelson *et al.* 2007), then i.p. GLP2 might activate vagal afferent pathways. However, decreased gastric motility may also be part of the premature inhibition of further ingestion as it constitutes a prandial satiety signal. Inhibition of gastric emptying (Wojdemann *et al.* 1998, Nagell *et al.* 2004) and increased mouse gastric capacity induced by GLP2 have been described (Amato *et al.* 2009). Indeed, the observation that mice injected with [Gly²]GLP2 displayed a significant decrease in the rate of gastric emptying compared

with PBS-treated mice strengthens the hypothesis that reduction in gastric emptying contributes to the short-term reduction in food intake.

In contrast, it is unlikely that the anorectic action of the peptide could be related to its well-known actions in the gastrointestinal tract, where it mediates adaptive increases in mucosal mass and increased transit time associated with increased segmental absorption of water, electrolytes, and nutrients, because the onset time of the anorectic effect is very short.

Another objective of the present study was to examine the effect of GLP2 on food intake in DIO mice, because the release of gut hormones is influenced by the content of the diet, in particular the fat content (Dakin *et al.* 2004), and a different sensitivity to intestinal hormones has been reported between lean and DIO models (Lin *et al.* 2000, Perreault *et al.* 2004). We found that DIO mice were less sensitive to the anorectic action of the peptide than lean animals. In fact, the magnitude and duration of the effects induced by similar doses of [Gly²]GLP2 were less pronounced in obese than lean animals and the native GLP2 was less effective in DIO mice. These observations appear particularly interesting because it has already been described that maintenance of rats on HFD reduces sensitivity to some satiety peptide signals (Covasa & Ritter 1998, Covasa *et al.* 2001), principally the sensitivity to cholecystokinin (Covasa & Ritter 1998, Covasa *et al.* 2001, Nefti *et al.* 2009).

In conclusion, our results show for the first time that the exogenous GLP2, administered peripherally, is able to reduce food intake in the short term, in both lean and DIO mice, although with a different efficacy, likely acting at the peripheral level.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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