original article

Liraglutide, leptin and their combined effects on feeding: additive intake reduction through common intracellular signalling mechanisms

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Aim: To investigate the behavioural and intracellular mechanisms by which the glucagon like peptide-1 (GLP-1) receptor agonist, liraglutide, and leptin in combination enhance the food intake inhibitory and weight loss effects of either treatment alone.

Methods: We examined the effects of liraglutide (a long-acting GLP-1 analogue) and leptin co-treatment, delivered in low or moderate doses subcutaneously (s.c.) or to the third ventricle, respectively, on cumulative intake, meal patterns and hypothalamic expression of intracellular signalling proteins [phosphorylated signal transducer and activator of transcription-3 (pSTAT3) and protein tyrosine phosphatase-1B (PTP1B)] in lean rats.

Results: A low-dose combination of liraglutide (25 μg/kg) and leptin (0.75 μg) additively reduced cumulative food intake and body weight, a result mediated predominantly through a significant reduction in meal frequency that was not present with either drug alone. Liraglutide treatment alone also reduced meal size; an effect not enhanced with leptin co-administration. Moderate doses of liraglutide (75 μg/kg) and leptin (4 μg), examined separately, each reduced meal frequency, cumulative food intake and body weight; only liraglutide reduced meal size. In combination these doses did not further enhance the anorexigenic effects of either treatment alone. *Ex vivo* immunoblot analysis showed elevated pSTAT3 in the hypothalamic tissue after liraglutide-leptin co-treatment, an effect which was greater than that of leptin treatment alone. In addition, s.c. liraglutide reduced the expression of PTP1B (a negative regulator of leptin receptor signalling), revealing a potential mechanism for the enhanced pSTAT3 response after liraglutide-leptin co-administration.

Conclusions: Collectively, these results show novel behavioural and molecular mechanisms underlying the additive reduction in food intake and body weight after liraglutide-leptin combination treatment.

Keywords: exendin-4, GLP-1, obesity, rat, synergy, weight loss

Introduction

The prevalence of obesity in the USA has increased by 75% since the early 1980s [1,2], with obesity-related US healthcare costs currently exceeding $140 bn per year [3]. The sustained weight loss needed to substantially reduce risk for negative health comorbidities in obese individuals is rarely achieved by diet and exercise alone. The incretin hormone glucagon-like peptide-1 (GLP-1) is a promising target biological system for the pharmacological treatment of obesity [4,5]. Liraglutide and exenatide (synthetic exendin-4) are long-acting GLP-1 receptor agonists that improve glycaemic control and are US Food and Drug Administration (FDA)-approved for the treatment of type 2 diabetes mellitus. While not yet FDA-approved for obesity treatment, liraglutide and exenatide have been found to slow gastric emptying and reduce food intake and body weight in human populations and animal models [4,6–8].

These GLP-1 analogues produce some degree of weight loss as a monodrug therapy; however, recent attention has been focused on the potential for combination-based pharmacotherapies to increase the magnitude of weight loss effects [9,10]. Indeed, a peripheral treatment combination of leptin, a hormone produced by adipose tissue with potent anorectic effects, and the GLP-1 receptor agonist exendin-4, produces greater intake reduction and weight loss in rats than does either treatment alone [11,12]. Furthermore, central GLP-1 receptor blockade via forebrain intracerebroventricular (ICV) delivery of exendin-(9-39) attenuates food intake suppression by ICV leptin [13,14]. Hindbrain neurons are a critical site for leptin receptor (LepRb) and GLP-1 receptor interaction as exendin-4 and leptin co-delivered to the hindbrain (fourth ICV exendin-4 and leptin administration) yields additive food intake and body weight reduction, and fourth ICV exendin-(9-39) attenuates the intake reduction by hindbrain leptin delivery [15]. The hypothalamus is also critical for LepRb and GLP-1 receptor interaction as leptin increases GLP-1 peptide [16] and GLP-1 receptor mRNA expression [17] in hypothalamic neurons.

The behavioural mechanisms through which leptin and GLP-1 receptor agonists combine to reduce feeding are

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unknown. Peripheral exendin-4 treatment in rhesus macaques reduces feeding through suppression of meal size, with no effect on meal frequency [18]. In rodents, peripheral exendin-4 reduces both meal size and meal frequency [19], whereas exendin-4 administration to the ventral tegmental area [20] or to the ventral hippocampus [21] reduces intake via a specific reduction in meal size. Similarly, peripheral liraglutide treatment reduces food intake via meal size reduction in minipigs [8].

Like GLP-1 receptors, activation of LepRbs reduces food intake, at least in part, by reducing meal size. Chronic peripheral leptin treatment reduces meal size with minimal impact on meal frequency [22,23]. Similarly, leptin-deficient mice [24] and rats with virally mediated, chronic LepRb 'knockdown' in the nucleus tractus solitarius (NTS) neurons [25] consume larger meals without substantially altered meal frequency compared with controls. In contrast to these findings, acute ICV leptin administration reduces meal frequency in rats with only minimal effect on meal size [26]. It is unknown whether the additive anorectic effects of GLP-1 receptor and LepRb co-administration occur by reducing meal size, meal frequency or both. The present study examines the cumulative food intake, body weight and meal pattern effects of low and moderate dose combinations of peripheral [subcutaneous (s.c.)] liraglutide and central (third ICV) leptin co-administration in rats.

The experiments in the present study also examine whether common or complementary intracellular signalling mechanisms underlie the additive food intake reduction achieved with combined GLP-1 receptor and LepRb activation. LepRb binding induces rapid tyrosine-phosphorylation of signal transducer and activator of transcription-3 (pSTAT3), a transcription factor that is commonly used as a marker of LepRb-mediated neuronal activation [27]. Recent data show that exendin-4 also elevates pSTAT3 in hypothalamic and hindbrain neurons after ICV administration [28]; therefore, the intracellular pSTAT3 signalling pathway is a potential molecular target underlying the enhanced food intake and body weight reduction by combined leptin and GLP-1 receptor agonists. Using an *ex vivo* approach, we examined whether combined s.c. liraglutide and third ICV leptin administration increases pSTAT3 beyond the increases observed after leptin treatment alone. The impact of s.c. liraglutide treatment on activation of protein tyrosine phosphatase-1B (PTP1B), a negative regulator of LepRb signalling, was investigated as a potential mechanism for the augmented leptin-mediated pSTAT3 effects after liraglutide-leptin co-treatment. Collectively, the results of the present study show novel behavioural and neurobiological mechanisms through which leptin and GLP-1 receptor agonist combination treatment enhance the feeding and body weight suppressive effects of either treatment alone.

Materials and Methods

Subjects

Adult male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA; 325–450 g during experimental procedures) housed individually under a reverse 12-h light/dark cycle (lights on 10:00 hours) for at least 2.5 weeks before procedures, had *ad libitum* access to chow (LabDiet 5001; LabDiet, St Louis, MO, USA) and water, except where noted. All procedures conformed to and received approval from the University of Pennsylvania Institutional Animal Care and Use Committee.

Surgery

Under ketamine (90 mg/kg), xylazine (2.7 mg/kg) and acepromazine (0.64 mg/kg) anaesthesia and analgesia (Metacam, Fisher Scientific, Waltham, MA, USA; 2 mg/kg), guide cannulae (Plastics One, Roanoke, VA, USA; 26-gauge) cemented to the skull using jewellers screws were implanted with its tip stereotaxically positioned 2.0 mm above the third ventricle at the following co-ordinates: 2.0 mm caudal to bregma; 7.7 mm ventral to skull surface; on midline. The anatomical positions of third ICV injection sites were evaluated 1 week after surgery by measurement of the cytoglucopenia-induced sympatho-adrenal mediated glycaemic effect resulting from 210 μg (2 μl) of 5-thio-D-glucose [29].

Procedures

Food Intake, Meal Patterns and Body Weight Analyses. Food was removed 30 min before the first injection, and rats received third ICV injections (1 μl) of leptin (National Hormone & Peptide Program) or vehicle (NaHCO$_3$). Fifteen minutes later, immediately before lights off, each rat received a s.c. injection (1 ml/kg) of liraglutide (gift from Novo Nordisk, Bagsvaerd, Denmark) or vehicle (sterile saline). Pharmacological studies used a within-subjects design, with treatments separated by 3–4 days. Doses were selected to be in the low to moderate range for food intake reduction based on dose–response curves for peripheral liraglutide [30] and ICV leptin [31]. Cumulative intake was measured with an automated feeding system (DiaLog Instruments). Individually housed rats had access to a food cup on a load cell circuit that communicated with an interface and computer with customized software (LabVIEW, National Instruments software). The weight of the food cup was measured every 10 s, enabling assessment of meal variables. A meal was defined as an episode of feeding in which at least 0.25 g was ingested, with the meal termination defined as the beginning of a pause in ingestion >10 min, as previously described [25,32]. Data were objectively calculated using a custom Microsoft Excel macro.

Ex vivo Immunoblot pSTAT3 and PTP1B Analyses. For pSTAT3 immunoblot analyses, pharmacological treatments were carried out (four groups, n = 6–7 per group), with the same injection timings as described above, using a 6.0-μg dose of leptin (ICV) and a 50-μg/kg dose of liraglutide (s.c.). Consistent with our previous work that combined behavioural and *ex vivo* approaches [32,33], higher doses were used for *ex vivo* signalling analyses compared with the behavioural analyses in order to optimize the ability to detect activated intracellular signalling pathways. Forty-five minutes after the s.c. injections (1 h after ICV injections), the rats were sacrificed by decapitation. The brains were rapidly removed and the hypothalamus was extracted and flash-frozen in isopentane and stored at
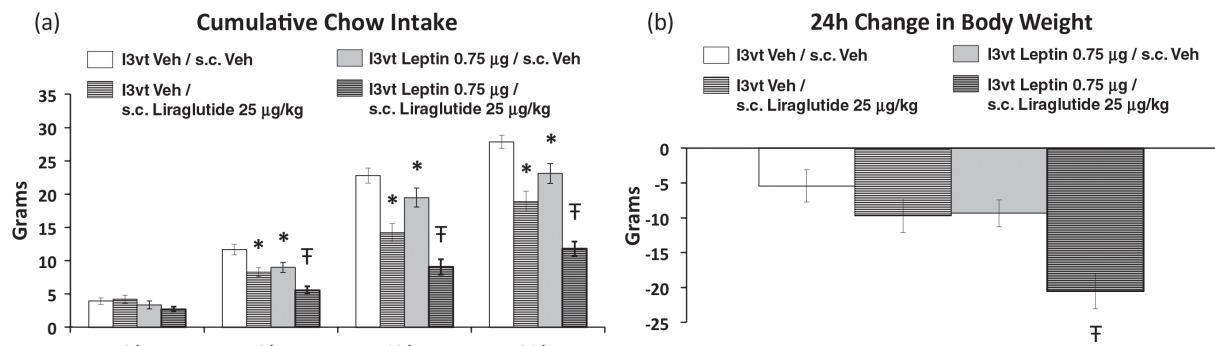
Figure 1. (a) Cumulative chow intake and (b) 24-h change in body weight after third intracerebroventricular (I3vt) delivery of 0.75 μg leptin and subcutaneous (s.c.) administration of 25 μg/kg liraglutide. Liraglutide and leptin each significantly reduced chow intake. Co-administration of liraglutide and leptin further reduced chow intake and body weight such that these effects were significantly greater than that of either drug alone. *p < 0.05 vs I3vt vehicle/s.c. vehicle, Ŧ p < 0.05 vs all other treatments (I3vt vehicle/s.c. vehicle, I3vt vehicle/s.c. liraglutide, I3vt leptin/s.c. vehicle). Veh, vehicle.
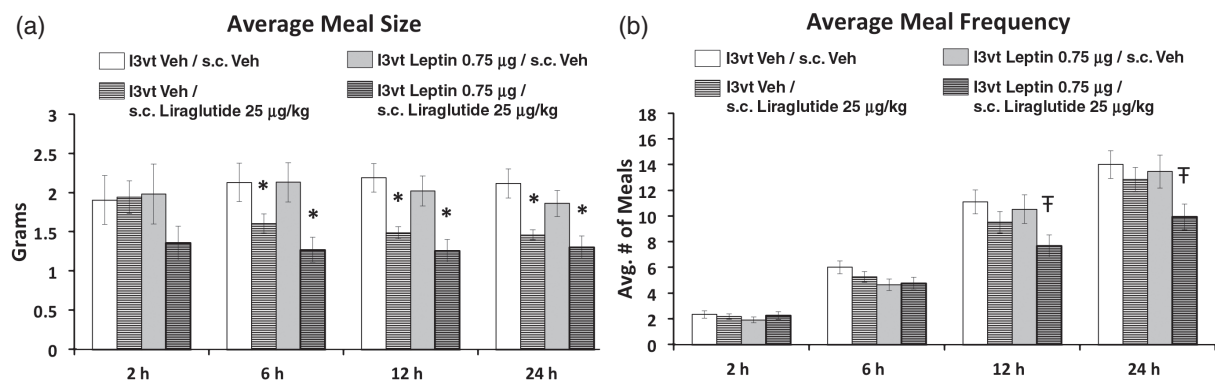

Figure 2. (a) Average meal size and (b) meal frequency after third intracerebroventricular (I3vt) leptin (0.75 μg) and subcutaneous (s.c.) liraglutide (25 μg/kg). Liraglutide treatment alone reduced meal size. Meal frequency was significantly reduced by liraglutide and leptin co-administration, without any effects with either drug alone. *p < 0.05 vs I3vt vehicle/s.c. vehicle, Ŧ p < 0.05 vs all other treatments (I3vt vehicle/s.c. vehicle, I3vt vehicle/s.c. liraglutide, I3vt leptin/s.c. vehicle). Veh, vehicle.

−80 °C. For PTP1B immunoblot analyses (n = 9 per group), the rats were sacrificed 45 min after s.c. injections.

Hypothalamic tissues were homogenized in radioimmunoprecipitation assay buffer. Hypothalamic lysates were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes for immunoblot analysis, as previously described [32]. Immunoreactivity was visualized using enhanced chemiluminescence (Chemidoc XRS; BioRad, Hercules, CA, USA). Phosphorylated and total STAT3 antibodies (Cell Signaling; 1 : 1000 dilution) were used to evaluate pSTAT3 activity normalized to total STAT3. PTP1B (Santa Cruz Biotechnology, Dallas, TX, USA; 1 : 500 dilution) protein expression was normalized to β actin loading control. Blots were quantified using densitometry analysis in software (Image J software; National Institute of Health).

Data and Statistical Analyses

All statistical analyses for behavioural measures used repeated measures analysis of variance (ANOVA; separate analysis conducted for each time point) using the ICV and s.c. drugs as factors. When significant main effects of either drug were detected, Newman–Keuls *post hoc* tests were used to compare

individual treatments. Immunoblot analyses were analysed using one-way ANOVA. The α level for significance was 0.05. Statistical analyses were conducted using STATSOFT software (Statistica V10).

Results

Low-Dose Combination: Cumulative Food Intake, Change in Body Weight and Meal Patterns

Subcutaneous liraglutide (25 μg/kg) and ICV leptin (0.75 μg) each reduced cumulative food intake relative to vehicle-vehicle treatment at 6, 12 and 24 h, (Figure 1; p values <0.05). Combined treatment yielded significantly greater intake reduction than either treatment alone at 6, 12 and 24 h, (p values <0.05 compared with each treatment). Body weight was significantly reduced 24 h after injections only by the combination treatment, which was significantly different from either drug treatment alone and from vehicle-vehicle treatment (p < 0.05). The s.c. liraglutide treatment and the combined treatment reduced meal size compared with vehicle-vehicle treatment at 6, 12 and 24 h (Figure 2; p values <0.05). The combination treatment effect on meal size, however, was not different from

s.c. liraglutide alone at any time point. Meal frequency was only reduced by the combination treatment at 12 and 24 h, which was significantly different from each of the other three treatments at these time points (Figure 2; p values <0.05).

Moderate Dose Combination: Cumulative Food Intake, Change in Body Weight and Meal Patterns

Subcutaneous liraglutide (75 μg/kg), ICV leptin (4 μg) and the combination treatment each reduced cumulative food intake relative to vehicle-vehicle treatment at 6, 12 and 24 h, and change in body weight at 24 h (Figure 3; p values <0.05). Combined treatment was not significantly different from either drug treatment alone at any time point for cumulative intake, nor for 24-h change in body weight. S.c. liraglutide treatment and combined treatment reduced meal size compared with vehicle-vehicle treatment at 12 and 24 h (Figure 4; p values <0.05), whereas the combination treatment was not different from s.c. liraglutide treatment alone at any time point. Meal frequency was reduced by each drug treatment alone and by the combination treatment at 6, 12 and 24 h compared with

vehicle-vehicle treatment (Figure 4; p values <0.05), whereas the combined treatment did not differ from either drug treatment alone at any time point.

Intracellular Signalling Pathways: pSTAT3 and PTP1B

The ICV leptin treatment alone (6 μg) increased pSTAT3 protein expression relative to vehicle-vehicle treatment, whereas s.c. liraglutide (50 μg/kg) alone had no effect (Figure 5a; p < 0.05). pSTAT3 activation was significantly higher after the combination treatment compared with either drug treatment alone (p values <0.05). PTP1B expression following s.c. liraglutide (50 μg/kg) was significantly lower than observed with vehicle treatment (Figure 5b; p < 0.05).

Discussion

Recent studies have begun to explore the role of long-acting GLP-1 analogues in combination pharmacotherapy, including its combination with leptin, as a strategy to enhance the feeding and body weight loss effects. Co-administration of leptin
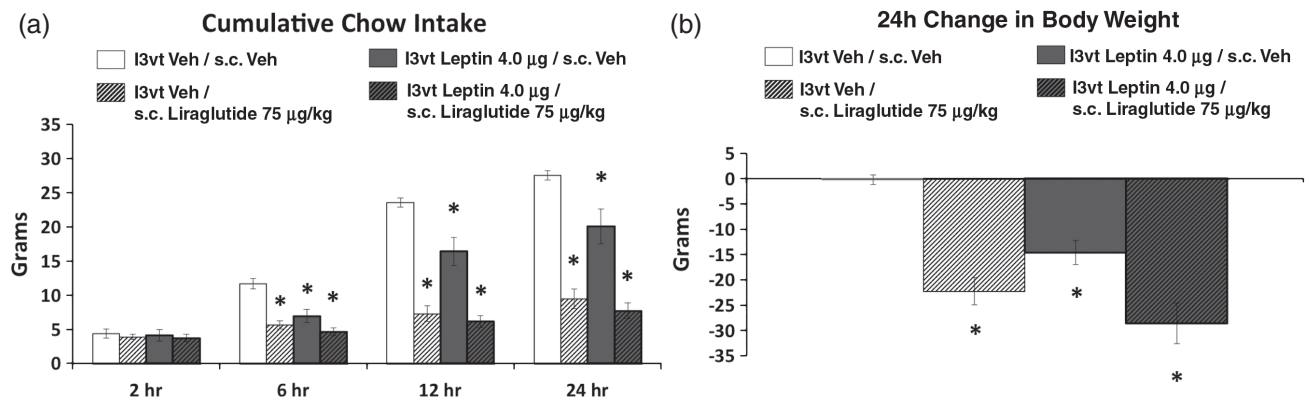
Figure 3. (a) Cumulative chow intake and (b) 24-h change in body weight after third intracerebroventricular (I3vt) leptin (4 μg) and subcutaneous (s.c.) liraglutide (75 μg/kg). Both liraglutide and leptin significantly reduced chow intake and body weight but the combinatorial effects were not greater than either drug alone. * p < 0.05 vs I3vt vehicle/s.c. vehicle. Veh, vehicle.
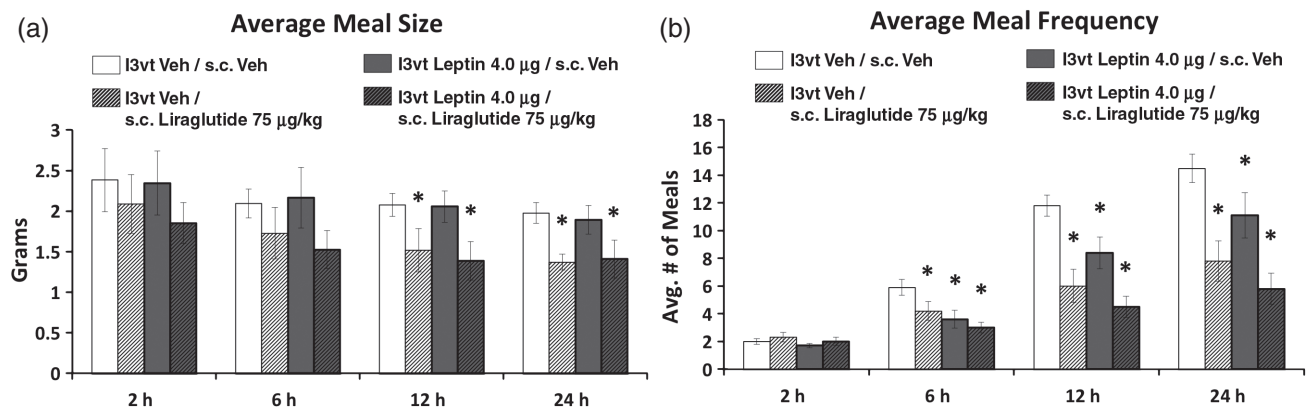
Figure 4. (a) Average meal size and (b) meal frequency after third intracerebroventricular (I3vt) leptin (4 μg) and s.c. liraglutide (75 μg/kg). Subcutaneous (s.c.) liraglutide reduced meal size and meal frequency while I3vt leptin reduced meal frequency only. Co-administration of liraglutide and leptin reduced meal size and meal frequency but the meal size effect was not greater than liraglutide alone and the meal frequency effect was not different from either liraglutide or leptin. *p < 0.05 vs I3vt vehicle/s.c. vehicle. Veh, vehicle.
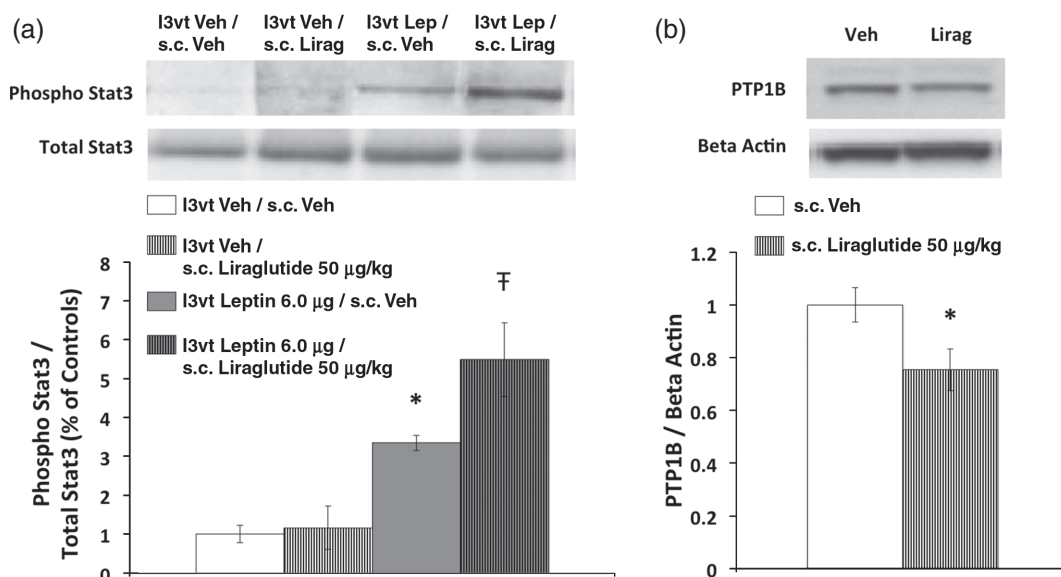
Figure 5. (a) Phosphorylated STAT3 (pSTAT3) protein levels in hypothalamic tissue after third intracerebroventricular (I3vt) leptin (6 μg) and subcutaneous (s.c.) liraglutide (50 μg/kg); and (b) hypothalamic protein tyrosine phosphatase-1B (PTP1B) protein after s.c. liraglutide (50 μg/kg). Leptin alone significantly increased pSTAT3 levels. Hypothalamic pSTAT3 was further increased with co-administration of liraglutide and leptin. Liraglutide alone reduced PTP1B levels in the hypothalamus. Upper panel: representative immunoblots of pSTAT3, STAT3, PTP1B and β actin. Lower panel: quantitative densitometric data of pSTAT3 normalized to total STAT3 (n = 6–7 per group), and PTP1B normalized to β actin (n = 9 per group). *p < 0.05 vs vehicle, Ŧ p < 0.05 vs all other treatments (I3vt vehicle/s.c. vehicle, I3vt vehicle/s.c. Liraglutide, I3vt Leptin/s.c. vehicle). Veh, vehicle.

and GLP-1 receptor agonists, delivered by different routes of administration, results in significant reductions in food intake and body weight that are greater than the effects of either drug alone [11,12,15]. In the present study, leptin was administered directly to the third ventricle to achieve anatomically distributed (both forebrain and hindbrain) central effects on energy balance control, whereas liraglutide was delivered s.c. to model its delivery in a clinical setting. Consistent with previous findings, we report that combined acute treatment of low doses of leptin (0.75 μg) and liraglutide (25 μg/kg) reduced cumulative food intake for up to 24 h by a significantly greater magnitude than the effects of either drug alone. The present study extends previous findings by showing that the additive food intake reduction results primarily from a significant reduction in meal frequency that was not observed in response to either treatment alone. This reduction in meal frequency after combined treatment may be mediated by reduced gastric emptying (and thereby increased inter-meal interval), as both peripheral liraglutide and central leptin reduce emptying rates [4,34]. Meal size, conversely, was significantly reduced by 25 μg/kg liraglutide, but not by 0.75 μg leptin. The combined effect of these two treatments on meal size was not significantly different from that of liraglutide treatment alone, although a trend was observed. Interestingly, while both leptin and liraglutide alone at these doses had no significant effect on body weight change 24 h after administration, co-administration of leptin and liraglutide significantly reduced 24-h body weight, supporting a role for the liraglutide-leptin combination in amplifying body weight loss.

It was previously reported that the additive effect of intraperitoneal administration of leptin and GLP-1 on food intake and body weight is obtained primarily with low-dose combinations [12]. Consistent with that finding, we found that the intake inhibitory and weight loss effects of a moderate dose liraglutide-leptin combination treatment (4 μg leptin, 75 μg/kg liraglutide) were not greater compared with either treatment alone. It is possible that the intake and body weight effects of both leptin and liraglutide at these doses had reached a floor effect, thus limiting further reduction in food intake and body weight; however, in our previous work, the food intake reduction produced by a high dose of fourth ICV leptin (20 μg) was further augmented by fourth ICV exendin-4 [15], suggesting that the anorectic effects of high doses of central leptin can be further augmented by GLP-1 receptor activation when both drugs are delivered to the hindbrain. Interestingly, the present results also showed that the intake inhibitory effect of 4 μg ICV leptin by itself is mediated by a reduction in meal frequency with no effect on meal size. These data contrast with those that follow chronic peripheral leptin treatment, which reduces meal size with minimal impact on meal frequency [22,23].

Leptin's acute effect on meal pattern variables after forebrain ICV delivery, to our knowledge, has only been reported in two other studies [26,35]. Consistent with present results, Zorrilla et al. [26] reported little effect of ICV leptin on meal size, whereas meal frequency reduction effects were observed with leptin doses ranging between 1 and 6.25 μg, but not with 0.3 μg leptin. That study included a breakpoint interval between intra-meal pauses and inter-meal intervals [inter-response interval (IRI)] of 5 min to define the end of a meal, whereas in the present study we used a 10-min IRI, both of which are widely accepted in the literature based on extensive behavioural observations and log survivorship analyses [26,36–39]. By contrast, another study, using a ~30-min IRI, reported a reduction

in meal size with no meal frequency reduction after ICV leptin. Thus, the contradictory findings of Flynn et al. [35] may be based on the use of a much longer IRI (30 min) compared with our study (10 min) and the study by Zorrilla et al. [26] (5 min). Future work is needed to determine whether, unlike acute treatment, chronic ICV leptin has a more potent effect on meal size compared with meal frequency, as has previously been observed after chronic peripheral leptin administration.

Subcutaneous liraglutide robustly reduces food intake and body weight in both lean and diet-induced obese rats [30,6,40–42], and reduces food intake in obese minipigs via meal size reduction. The effect of s.c. liraglutide on meal patterns in rats, to our knowledge, has not been previously reported. In the present study, we show that a moderate dose of acute s.c. liraglutide (75 μg/kg) reduced both meal frequency and meal size. In previous studies, the effect of GLP-1 receptor activation on meal patterns appears to be dependent on the route and site of administration [18–21,43,44], with more potent effects generally observed for meal size than for meal frequency reduction. Given that acute liraglutide reduces food intake via activation of both peripheral and central GLP-1 receptors after peripheral administration [40,45], it is possible that the meal pattern effects observed in the present study are mediated by an action in the periphery, centrally, or both. It is also possible that non-specific effects (e.g. nausea) contributed to the meal pattern effects of liraglutide (alone and in combination with leptin), given that under some conditions GLP-1 analogues produce conditioned flavour avoidance learning [30,46].

We investigated the intracellular signalling pathways involved in the combined LepRb and GLP-1 receptor activation in the hypothalamus, where receptors for both leptin and GLP-1 are abundantly expressed [47,48]. The intake inhibitory effect of LepRb signalling is mediated, in part, via the JAK-STAT pathway, where leptin binds to LepRb and results in the phosphorylation of Janus kinase-2(pJAK2) and STAT3 (pSTAT3) [49]. pSTAT3 is also increased after activation of inflammatory cytokine [e.g. interleukin (IL)-1, IL-6] receptors, and central IL-1 and IL-6 signalling is thought to contribute to central GLP-1-induced anorexia [28]. The present results showed that ICV leptin increased hypothalamic pSTAT3 signalling, and this effect was significantly amplified with s.c. liraglutide co-administration; however, we did not observe an increase in pSTAT3 after s.c. liraglutide administration alone, an outcome that differs from a recent study reporting an increase in hypothalamic pSTAT3 after ICV exendin-4 [28]. It is possible that the different route of administration (s.c. vs ICV), GLP-1 analogue (liraglutide vs. exendin-4), or relative dose strength used may have contributed to this difference.

Leptin resistance associated with obesity is often related to increased expression of negative regulators of leptin receptor signalling, such as PTP1B [50]. PTP1B inhibits LepRb signalling by dephosphorylating JAK2 [51,52]. Furthermore, PTP1B genetic deficiency and pharmacological inhibition of PTP1B is known to increase leptin sensitivity [53,54], highlighting the significance of PTP1B in energy balance control. We pursued a mediating role for PTP1B protein expression in the augmented pSTAT3 effect of leptin and
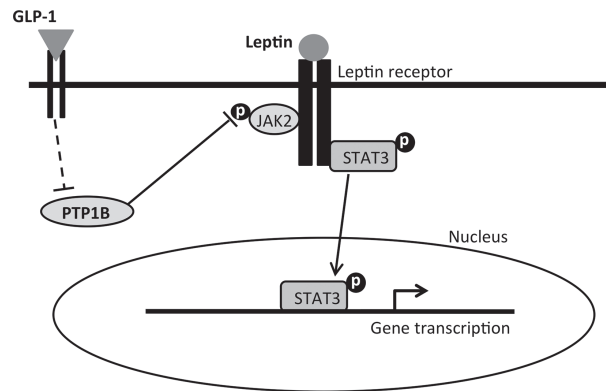

Figure 6. A simplified proposed summary of intracellular signalling mechanisms mediating the intake inhibitory and weight loss effects of leptin-liraglutide combination treatment. Leptin binds to the long form of leptin receptor (LepRb), which activates Janus kinase 2 protein (JAK2) and results in the phosphorylation of various tyrosine residues and the signal transducer and activator of transcription-3 (pSTAT3). pSTAT3 translocates to the nucleus to mediate the transcription of genes related to food intake and body weight control. Glucagon-like peptide-1 (GLP-1), however, binds to GLP-1 receptor and inhibits protein tyrosine phosphatase-1B (PTP1B) via a direct or indirect pathway (broken lines). PTP1B normally dephosphorylates JAK2 and deactivates it. Inhibition of PTP1B therefore disinhibits pJAK2. The disinhibition of pJAK2 by GLP-1 receptor activation, together with the activation of LepRb signalling, results in a combinatorial increase in pSTAT3, which is proposed to mediate the enhanced intake suppression and body weight loss effects of liraglutide and leptin co-administration.

liraglutide co-administration and found that peripheral liraglutide reduced hypothalamic PTP1B relative to vehicle treatment, which suggests a possible intracellular signalling interaction between leptin and liraglutide (Figure 6). The reduction in PTP1B protein was observed 45 min after s.c. liraglutide treatment. Protein reduction within this short time frame is unlikely to be attributable to alterations in transcriptional events, which typically take hours to weeks [55]. Other non-transcriptional mechanisms to account for PTP1B reduction by s.c. liraglutide include rapid protein degradation or re-localization of PTP1B protein to insoluble cellular compartments; however, because the lysis buffer used in the present study (radioimmunoprecipitation assay) breaks down most cellular compartments, it is more likely that the PTP1B protein reduction after s.c. liraglutide administration was a result of rapid PTP1B degradation.

It is unclear whether leptin and GLP-1 bind to receptors located on the same hypothalamic neurons or whether GLP-1 may be reducing PTP1B and augmenting pSTAT3 signalling via a secondary, indirect pathway. The difficulty in obtaining reliable GLP-1 receptor and LepRb antibodies has made it technically difficult to provide anatomical data on the co-localization of both receptors in the hypothalamus. Nevertheless, earlier studies show that there is considerable overlap of leptin-induced and GLP-1-induced cFos-positive cells in the paraventricular nucleus of the hypothalamus [56]. Further, both leptin [57] and peripherally-injected liraglutide [58] bind to proopiomelanocortin- and cocaine- and amphetamine-regulated transcript-expressing neurons in the

arcuate nucleus of the hypothalamus; thus, the paraventricular nucleus of the hypothalamus and the arcuate nucleus of the hypothalamus are potential hypothalamic targets for GLP-1 receptor and LepRb interaction. It is important to note that the hypothalamus is not the only site of action for the anorectic effects of leptin and GLP-1 receptor agonists. Other target sites include the NTS, ventral tegmental area and the hippocampus, when leptin or GLP-1 receptor agonists are administered directly at those sites [15,21,59–61]. Future studies determining the behavioural and intracellular signalling effects after combinatorial leptin and GLP-1 receptor agonists directed to other extrahypothalamic sites are warranted.

The present results identify PTP1B as one mechanism by which liraglutide and leptin co-administration increased pSTAT3. We also acknowledge the possibility of other alternative pathways mediating the enhanced intake inhibitory and weight loss effects of liraglutide-leptin combination treatment. For example, mRNA expression of fibroblast growth factor 21 (FGF21), a growth factor important for the regulation of glucose and lipid homeostasis, increases in the liver after peripheral liraglutide administration [62,63]. FGF21 increases leptin sensitivity in diet-induced obese mice [64] and the metabolic effects of FGF21 appear to be, in part, centrally mediated [65]; therefore, it is possible that liraglutide-induced increases in FGF21 also contributed to the augmented hypothalamic LepRb signalling (increased pSTAT3) after acute liraglutide-leptin co-administration. Alternatively, this combinatorial elevation in pSTAT3 may also be mediated by an increase in LepRb expression as a result of liraglutide treatment. This putative mechanism is less likely, however, given that sub-chronic peripheral liraglutide treatment does not alter LepRb expression in the arcuate nucleus of the hypothalamus of diet-induced obese rats [58]. Nevertheless, these hypotheses remain to be extensively explored.

Results from the present study show that ICV leptin and s.c. liraglutide, when combined at lower but not moderate doses, increased the food intake and body weight-reducing effects of either drug alone. This additive food intake effect after combined administration was based on a significant reduction in meal frequency that was not observed in response to either treatment alone. In conjunction with these behavioural effects we showed for the first time that s.c. liraglutide increased leptin-driven pSTAT3 activation in hypothalamic neurons, an effect that may be based, in part, on liraglutide-mediated reduction in hypothalamic PTP1B expression. Future studies are required to examine whether these intake and body weight-reducing effects of leptin and liraglutide combination treatment are present after other routes of administration and long-term chronic administration, and whether they persist in diet-induced obese rodent models.

Acknowledgements

Conflict of Interest

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