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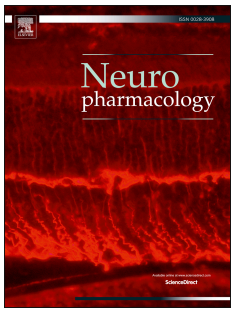

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Brainstem GLP-1 signalling contributes to cancer anorexia-cachexia syndrome in the rat

Tito Borner[1,2,3], Claudia G. Liberini[1,2,3], Thomas A. Lutz[1,2], Thomas Riediger[1,2]

[1] Institute of Veterinary Physiology, University of Zurich, Zurich, Switzerland

[2] Zurich Center of Human Integrative Physiology, University of Zurich, Zurich, Switzerland

[3] Zurich Center for Clinical Studies, University of Zurich, Zurich, Switzerland

Running title: Brainstem-derived GLP-1 mediates cancer anorexia-cachexia

Corresponding author:

Thomas Riediger

Institute of Veterinary Physiology, University of Zurich

Winterthurerstrasse 260

8057 Zurich, Switzerland

Phone: +41-44-635-8815

Fax: +41-44-635-8932

e-mail: triedig@vetphys.uzh.ch

ABSTRACT

The cancer anorexia-cachexia syndrome (CACS) is a frequent and severe condition in cancer patients. Currently, no pharmacological treatment is approved for the therapy of CACS. Centrally, glucagon-like peptide-1 (GLP-1) is expressed in the *nucleus tractus solitarii* (NTS) and is implicated in malaise, nausea and food aversion. The NTS is reciprocally connected to brain sites implicated in the control of energy balance including the *area postrema* (AP), which mediates CACS in certain tumour models. Given the role of GLP-1 as a mediator of anorexia under acute sickness conditions, we hypothesized that brainstem GLP-1 signalling might play a role in the mediation of CACS.

Using hepatoma tumour-bearing (TB) rats, we first tested whether the chronic delivery of the GLP-1R antagonist exendin-9 (Ex-9) into the fourth ventricle attenuates CACS. Second, we investigated whether a genetic knockdown of GLP-1 expression in the NTS ameliorates CACS.

Ex-9 attenuated anorexia, body weight loss, muscle and fat depletion compared to TB controls. Similarly, TB animals with a knockdown of GLP-1 expression in the NTS had higher food intake, reduced body weight loss, and higher lean and fat mass compared to TB controls.

Our study identifies brainstem GLP-1 as crucial mediator of CACS in hepatoma TB rats. The GLP-1R represents a promising target against CACS and possibly other forms of disease-related anorexia/cachexia.

Key terms: cancer, food intake, energy balance, brainstem, muscle degradation.

1. INTRODUCTION

The cancer anorexia-cachexia syndrome (CACS) causes a decline of the clinical status of cancer patients and often becomes a life-threatening condition (Laviano et al., 2003). CACS increases mortality, reduces treatment success and produces severe psychological suffering of patients and their families (Gordon et al., 2005; Kauth and Metz, 1987). We recently demonstrated the importance of the *area postrema* (AP) of the brainstem for the mediation of CACS in hepatoma tumour-bearing (TB) rats (Borner et al., 2016a). In order to identify a possible therapeutic target, our current study aimed to explore the role of the neuropeptide glucagon-like peptide-1 (GLP-1) in the brainstem as a mediator of CACS.

GLP-1 is a posttranslational product of the peptide preproglucagon (PPG), which is expressed in enteroendocrine L-cells of the small intestine, in pancreatic α-cells (Eissele et al., 1992; Kauth and Metz, 1987) and in the *nucleus tractus solitarii* (NTS) (Merchenthaler et al., 1999), which is an integrative and relay center for enteroceptive signals controlling food intake (Grill and Hayes, 2009, 2012). GLP-1-expressing NTS neurons project to various GLP-1R expressing brain sites (Jin et al., 1988; Llewellyn-Smith et al., 2011) implicated in the control of food intake and energy balance (Dunphy et al., 1998; Merchenthaler et al., 1999).

Systemic and central administration of GLP-1 or the GLP-1R agonist exendin-4 reduce food intake and body weight (Hayes, 2012; Hayes et al., 2010; Turton et al., 1996) and brainstem GLP-1 signalling might also play a role in the physiological control of energy balance (Alhadeff et al., 2016; Barrera et al., 2011). Furthermore, GLP-1 signalling induces anorexia, nausea and food aversion in rodents and humans (Calara et al., 2005; Kanoski et al., 2012; Kinzig et al., 2002; Thiele et al., 1998).

The AP/NTS region is activated in TB rodents (Johnen et al., 2007; Ruud and Blomqvist, 2007), by the inflammatory endotoxin LPS**,** by the emetic agent LiCl**,** and by the chemotherapeutic drug cisplatin (Endo et al., 2004; Horn et al., 2007; Rinaman, 1999b)**.** LPS and cisplatin-induced anorexia are attenuated in rats receiving fourth ventricular injection of the GLP-1R antagonist exendin-9 (Ex-9) (De Jonghe et al., 2016; Grill et al., 2004). Furthermore, central administration of Ex-9 attenuated LiCl-induced anorexia and blunts LiCl-induced AP activation (Rinaman, 1999a; Thiele et al., 1998). Therefore, we hypothesized that GLP-1 might also contribute to anorexia and cachectic body weight loss under chronic cancer conditions.

We examined whether a pharmacological blockade of brainstem GLP-1R by chronic fourth ventricular infusion of Ex-9 blunts CACS. To confirm the involvement of local brainstem GLP-1 as a mediator of CACS, we knocked down PPG gene expression in the NTS by a lentiviral short-hairpin RNA (shRNA) approach.

2. MATERIALS AND METHODS

2.1 Animals and housing conditions

Male Buffalo rats (Charles River Laboratory, USA) were housed at controlled temperature (21±1°C) under a 12-hour artificial light cycle with ad libitum access to standard laboratory rat chow (89025W16, Provimi Kliba, Switzerland). All experiments were approved by the Veterinary Office of the Canton Zurich (#95/2013).

2.2 Cell culture and tumour model

The hepatoma tumour model and the Morris hepatoma 7777 cell culture procedures were described previously (Borner et al., 2016a).

2.3 Fourth ventricular cannulation

Rats (250-280 g) were placed in a stereotactic frame under isoflurane anaesthesia. A guide cannula (9mm 326OPG/Spc, Plastics One, USA) was implanted into the fourth ventricle. The coordinates for cannula placement (bregma -11.6 mm, lateral 0.0 mm, dorsoventral -7.2 mm) were chosen according to the rat brain atlas (Paxinos and Watson, 2006). After recovery, the correct placement of the cannula was verified by measuring the hyperglycaemic response to fourth ventricular infusion of 5-thio-D-glucose (Ritter et al., 1981).

2.4 Lentiviral vectors

High-titer (>10^9 IU/ml) lentiviral vectors dissolved in PBS, produced by Sigma-Aldrich, were kindly provided by Dr. Shin Lee, ETHZ, Zurich, Switzerland. These viral vectors expressed a non-target sequence (control) shRNA or a shRNA directed against the rat PPG mRNA under the control of the human U6 promoter; the PPG shRNA oligonucleotide sequence was *tgccaaacgtcatgatgaatt*. In addition, the anti-PPG vector contained an independent enhanced green fluorescent protein (turboGFP)

cassette that was used a reporter gene to confirm successful genomic integration and expression.

2.5 Lentiviral shRNA knockdown of PPG gene expression in the NTS

Rats (260-320 g) were anesthetized with an intraperitoneal injection of 50mg/kg ketamine and 6mg/kg xylazine and placed in a stereotactic frame with the head in a ventroflexed position. A midline incision was made above the occipito-atlantal joint, the muscles were retracted and the joint capsule was opened. A 10µl glass-syringe (Nanofil, World Precision Instrument, USA) equipped with a 35G bevelled needle (World Precision Instrument, USA) was attached to a stereotactic arm. Using a microinfusion pump (World Precision Instrument, USA), 2.0 µl of virus solution were infused bilaterally into the NTS over 20 min (400 µm lateral and 100 µm caudal to the obex, and 600 µm below the surface of the brain). These coordinates were chosen to target the area of the NTS with the highest density of PPG neurons (Barrera et al., 2011; Larsen et al., 1997a).

2.6 Tissue collection, qPCR, and confirmation of PPG knockdown

After sacrifice, micro-punch samples (2 mm diameter) containing the AP/NTS region were obtained under a dissecting microscope on 3 consecutive 250 µm-thick coronal sections previously cut on a cryostat (Leica microsystem, Germany) and pooled together. Sections were collected starting 250 µm caudal to the obex. RNA was extracted and purified according to the manufacturer's instructions (ReliaPrep, Promega, USA). cDNA was generated from the extracted RNA using the high

capacity cDNA reverse transcription kit (Applied Biosystems, USA). Quantitative polymerase chain reaction was performed using pre-designed Taqman probes (Thermoscientific, USA). Rat S18 (Rn01428913) was used as housekeeping reference gene and the following probes were used to amplify PPG (Rn00562293), GLP-1R (Rn00562406), IL-1β (Rn00580432), TNF-α (Rn01525860), and IFN-γ (Rn00594078) transcripts. The relative expression was calculated using the comparative delta-delta Ct method. Sample were run in duplicates.

2.7 Measurement of energy expenditure

The experiments involving Ex-9 treated rats were conducted in an open-circuit indirect calorimetric system (TSE Phenomaster, Germany). The animals were single-housed in metabolic cages and adapted to the housing conditions for 7 days before the start of the experiment as previously described (Borner et al., 2016a). Food intake and body weight was measured daily before dark onset.

2.8 Computed tomography (CT) scanning and muscle weight measurements

Total carcass lean and fat volumes were measured by quantitative microcomputed tomography (La Theta LCT-100A scanner, Hitachi-Aloka Medical, Japan) as previously described (Borner et al., 2016b). After the CT scanning, the left gastrocnemius, tibialis and soleus muscles were dissected at the level of their upper to lower tendons and weighed.

2.9 Experiment 1: Chronic infusion of Ex-9 in healthy and tumour-bearing rats

First, we investigated the effect of Ex-9 on food intake, body weight, metabolic rate and respiratory exchange ratio (RER) in non-tumour-bearing (NTB) rats. Primed osmotic minipumps (model 2ML2, ALZET, DURECT corporation, USA) were implanted subcutaneously into the left flank and connected to the cannula via a subcutaneous vinyl catheter (C312VT, Plastics One, USA). Minipumps were filled with Ex-9 (0,83 mg/ml) or saline and released a constant amount of Ex-9 (100 µg/day) directly into the fourth ventricle. In tumour-bearing rats, minipumps were implanted on day 9 after tumour inoculation, i.e. at the typical onset of the anorectic response (Borner et al., 2016a; Borner et al., 2016b). The infusion lasted for 11 days. Animals were euthanized with a lethal intraperitoneal dose of pentobarbital (100 mg/kg) injected shortly before dark onset (see above) for tissue collection and carcass analysis.

2.10 Experiment 2: Effects of PPG gene expression knockdown in the NTS prior and during tumour growth

Three weeks after the lentivirus injection, rats were single-housed and adapted to the housing conditions for 14 days before tumour cell injection. PPG knockdown (PPG-KD) and control animals were kept in BIODAQ cages (Research Diets, USA). Daily food intake and body weight were measured daily shortly

before dark-onset. At the end of the experiment, animals were euthanized for tissue collection and carcass analysis (see above).

2.11 Experiment 3: Measurements of brainstem PPG and GLP-1R expression, and circulating GLP-1 levels

PPG and GLP-1R mRNA expression levels were analysed in non-operated TB, NTB, and NTB/pair-fed (PF) rats (220-250 g). Moreover, circulating GLP-1 levels were also measured. Rats were single-housed in BIODAQ cages. Daily food intake and body weight were measured shortly before dark-onset. Starting from nine days after tumour induction of the TB group, PF animals received the same amount of food that was consumed the previous day by the TB rats. Nineteen days after tumour-induction, animals were euthanized shortly before dark onset and brains were processed for gene expression analysis as described above. Blood was collected from the right ventricle of the heart in EDTA-coated tubes (Sarstedt, Germany) containing a DPP-IV inhibitor (1:100, Millipore, USA) and centrifuged at 7'000 x g (4°C, 7 min) to obtain plasma. The levels of GLP-1 were measured in triplicates using a commercial kit (Meso Scale Discovery, USA) according to the manufacturer instructions.

2.12 Data evaluation and statistical analysis

All data were expressed as mean ± SEM. In the Ex-9 studies, body weight changes were calculated by subtracting the weight of the animal at the day of minipump

implantation from the final body weight. In the experiments involving PPG-KD animals, body weight change was calculated by subtracting the body weight before the onset of the anorectic response (day 8 after tumour induction) from the body weight at the end of the experiment. Metabolic rate and RER were calculated as described previously (Borner et al., 2016b). For the experiment involving Ex-9 treatment in healthy rats, the average values obtained from 2 consecutive days prior to minipump implantation were used as baseline and compared to the average values of treatment days 3 and 4. In the experiment involving TB animals treated with Ex-9, the values obtained between days 2-3 after tumour inoculation were used as baseline (i.e., prior to the onset of anorexia) and compared to the average values on days 12-13 after tumour induction (i.e. treatment days 3-4).

Unpaired Student's *t*-test was used for comparison between two groups. Statistical comparisons between multiple groups were performed using one-way ANOVA followed by Tukey's post-hoc test. For all statistical tests, a *p*-value less than 0.05 was considered significant. Data were analysed using Prism GraphPad 5.0.

3. RESULTS

3.1 Chronic fourth ventricular infusion of Ex-9 attenuated CACS in hepatoma tumour-bearing rats but did not affect energy balance in healthy rats

Vehicle treated TB (Vehicle/TB) animals showed a clear anorectic response. In Ex-9 treated TB (Ex-9/TB) rats, anorexia was markedly attenuated compared to controls (Figure 1A). On average, daily food intake was about 17% higher in Ex-9 treated rats compared to controls during the treatment period (Ex-9/TB: 16.3±0.4 g vs. Vehicle/TB: 13.9±0.6 g; $p < 0.01$; Figure 1B). Tumour-induced body weight loss was attenuated in rats receiving Ex-9 (Figure 1C). This appeared to be mainly due to a preservation of fat mass because Ex-9/TB rats had higher fat mass but similar total lean mass compared to Vehicle/TB animals (Figure 1D). However, Ex-9/TB rats had higher gastrocnemius and total hind limb muscle weights (i.e. sum of gastrocnemius, tibialis and soleus muscle mass) than TB controls (Fig. 1E). Relative to baseline conditions, control rats had significantly lower RER values on days 12-13 reflecting an increased lipid metabolism. While the tumour-dependent reduction in RER was not observed in the Ex-9/TB group, the RER of these animals did not significantly differ from the vehicle treated group (Fig. 1F). The metabolic rate was similar between Ex-9/TB and Vehicle/TB animals before and during the treatment period (Fig. 1G). In addition, no differences in metabolic rate were detected when EE data were not corrected for body weight (data not shown). Importantly, tumour weight did not differ significantly between Ex-9/TB and Vehicle/TB rats at the end of the experiment (17.5±1.1 vs. 14.2±1.2 g).

Under non-pathological conditions, chronic 11-day infusion of Ex-9 into the fourth ventricle did not affect daily food intake (Fig. 1H-I), body weight and body weight gain (Fig. 1J-K) compared to control animals. Similarly, no change in body composition occurred between Ex-9 treated rats compared to controls, and Ex-9 treated and control animals did not differ in body composition (data not shown). Moreover, metabolic rate and RER were similar in both groups and no significant changes occurred during treatment (Fig. 1L-M).

3.2 Knockdown of PPG expression in the NTS attenuated CACS

Fig. 2A shows a representative histological photomicrograph of the right caudal NTS displaying GFP fluorescence 7 weeks after the lentiviral vector injection. The indicated target region (Fig. 2B) corresponds to the caudal NTS where PPG neurons are located (Larsen et al., 1997a). Seven weeks after the lentivirus injection, PPG-KD rats expressed significantly (-47%) less PPG mRNA in the AP/NTS region than controls (Fig. 2C). No change in GLP-1R expression occurred between the experimental groups (Fig. 2D). Furthermore, mRNA levels of TNF-α, IL-1β, and IFN-γ in the AP/NTS did not differ between PPG-KD and controls. The expression values of these markers were similar and not significantly different in non-operated NTB rats (TNF-α; NTB: 1.00±0.13, Ctrl-KD: 0.95±0.41, PPG-KD: 0.85±0.24 ($p>0.9$ for all comparisons), IL-1β; NTB: 1.00±0.12, Ctrl-KD: 1.73±0.94, PPG-KD: 1.21±0.39 ($p>0.7$ for all comparisons) and IFN-γ; NTB: 1.00±0.08, Ctrl-KD: 1.94±1.32, PPG-KD: 1.31±0.86 ($p>0.7$ for all comparisons). Hence, there was no indication of local inflammatory responses caused by the injection of lentivirus.

Both experimental groups showed similar food intake in the week prior to tumour cell inoculation (PPG-KD: 23.0±1.0 vs. controls 23.5±1.0 g/day) and prior to tumour anorexia (Fig 2E). Food intake of control animals decreased 10 days after tumour induction leading to a 30% reduction on the last day of the experiment compared to baseline (i.e. days 1-2 after tumour induction, Fig. 2E). On average, mean daily food intake was 26% higher in PPG-KD than controls from day 8 to the end of the experiment (Fig. 2G). Anorexia in control animals was associated with marked loss of body weight. This effect was significantly attenuated in PPG-KD animals beginning at day 13 (Fig. 2F-H). The body weight change between day 8 after tumour induction and the end of the experiment was -6.4±2.7 vs. -23.9±4.7 g (PPG-KD vs. controls; $p < 0.01$). The differences in body weight were due to both higher lean and fat volumes (Fig. 2I). Despite the higher lean mass of the PPG-KD group, differences in hind limb muscle mass did not reach statistical significance (not shown). Tumour weight did not differ significantly between the two groups (PPG-KD: 11.9±1.1 vs. controls: 12.1±0.9 g).

3.3 Tumour growth decreased PPG expression without affecting circulating GLP-1 and GLP-1R expression in the AP/NTS.

Circulating active GLP-1 levels were not significantly different between NTB, PF and TB rats (Fig. 3A). Tumour-bearing animals had lower PPG expression compared to NTB rats. However, pair-feeding also reduced PPG expression to the level of the TB animals (Fig. 3B). No significant difference in AP/NTS GLP-1R expression levels occurred between the experimental groups (Fig. 3C).

4. DISCUSSION

We identified GLP-1 as a neurochemical mediator of brainstem-dependent CACS providing first evidence that GLP-1 signalling in the caudal brainstem mediates cancer anorexia and subsequent body weight loss. A pharmacological and genetic blockade of GLP-1R signalling in the brainstem attenuated CACS.

Under non-pathological conditions a blockade of brainstem GLP-1R does not affect energy intake or body weight development (Grill et al., 2004; Hayes et al., 2009; Zhao et al., 2012). Our findings are consistent with these studies because neither GLP-1R blockade nor PPG-KD affected food intake or body weight in NTB rats. Consequently, the anti-CACS effects described in our experiments are likely due to a specific blockade of a pathological activation of the GLP-1 system.

Although peripherally administered GLP-1 reduces food intake and body weight (Punjabi et al., 2014; Ruttimann et al., 2009), our findings do not suggest a role of peripheral GLP-1 in the mediation of CACS. There was only a tendency of increased circulating GLP-1 in TB rats but the levels were not significantly different from NTB or pair-fed animals. Further, intestinal GLP-1 seems to act locally on GLP-1R on terminals of intestinal vagal afferents to reduce food intake (Krieger et al., 2016; Ruttimann et al., 2009), but vagal afferents are not required for CACS in this tumour model (Borner et al., 2016a). Our current study rather supports a role of brainstem-derived GLP-1 and central GLP-1R in the mediation of CACS. The brainstem GLP-1 system mediates acute behavioural sickness responses induced by LPS, LiCl and cisplatin (De Jonghe et al., 2016; Grill et al., 2004; Rinaman, 1999a). Food avoidance following malaise is a commonly known phenomenon, which not only occurs under these experimental conditions but also in cancer patients (Bernstein, 1985, 1999).

Anorexia and body weight loss induced by chronic treatment with the GLP-1R agonist exendin-4 is paralleled by nausea (pica behaviour) (Kanoski et al., 2012). Whether tumour-dependent food avoidance and aversive responses contribute to anorexia under our experimental conditions remains to be elucidated.

Our studies do not identify the location of postsynaptic GLP-1R mediating CACS in our model. Fourth but not third ventricular injection of Ex-9 attenuates the anorectic effect of LPS indicating that blockade of hindbrain GLP-1R is sufficient to reverse acute sickness anorexia. Acute fourth ventricular infusions appear to be ineffective in the forebrain due to the rostral-to-caudal flow of the cerebrospinal fluid (Daniels and Marshall, 2012; Grill et al., 2004), but it cannot be excluded that Ex-9 might reach forebrain areas during chronic infusion into the fourth ventricle. A possible relevance of GLP-1-ergic signalling between the NTS and the AP is suggested by our recent finding that a specific lesion of the AP attenuates CACS (Borner et al., 2016a). However, the presence of axon terminals from PPG neurons to the AP has so far only been confirmed in mice (Llewellyn-Smith et al., 2011). Since AP neurons may also function as first order upstream neurons that activate downstream PPG neurons in the NTS, it is currently unknown how the AP interacts with the NTS in the mediation of GLP-1 dependent CACS.

Other possible downstream targets for PPG neurons reside in the lateral parabrachial nucleus (LPBN), the dorsal raphe nuclei, and in the hypothalamus (e.g. paraventricular nucleus, PVN). GLP-1ergic projections to calcitonin gene-related peptide (CGRP) expressing neurons in the LPBN suppress food intake. Interestingly, CGRP neurons mediate cancer anorexia in mice (Campos et al., 2017).

There are also projections from PPG neurons of the NTS to serotonergic neurons located in brainstem raphe nuclei (Llewellyn-Smith et al., 2011). The serotoninergic

system is implicated in CACS (Laviano et al., 2005; van Norren et al., 2017) and LPS-induced anorexia (von Meyenburg et al., 2003). Recently, GLP-1R-dependent reduction in food intake and body weight has been linked to activation of serotonin (5-HT) neurons in the dorsal raphe nucleus (Anderberg et al., 2017). Whether 5-HT is a downstream mediator of GLP-1 in the context of CACS is unknown. Axonal terminals from GLP-1 neurons of the NTS form synaptic contacts with Corticotropin-releasing hormone (CRH) neurons in the PVN (Ghosal et al., 2013). Central infusion of GLP-1 activates CRH neurons in the PVN and leads to increased adrenocorticotropin (ACTH) and corticosterone plasma levels (Kinzig et al., 2003; Larsen et al., 1997b). Therefore, a GLP-1 dependent activation of the PVN might not only contribute to anorexia, but also to a glucocorticoid-induced loss of body weight, as it has been demonstrated under conditions of acute and chronic stress (Ghosal et al., 2017).

It might appear surprising that the positive effect of Ex-9 treatment on hind limb muscle mass was not paralleled by increased whole body lean mass. Vice versa, PPG-KD rats had higher total lean mass but no increased hind limb muscle mass. It should be mentioned, however, that PPG-KD animals were sacrificed 4 days earlier than those in the Ex-9 experiment. Hence, muscle atrophy may not have been advanced at that stage, which could account for the lack of a statistically significant treatment effect on muscle mass.

While GLP-1 blockade improved energy intake in TB rats, the metabolic rate was not affected. The cachectic effect in hepatoma TB rats is partly independent of reduced energy intake because TB rats lose more body weight and muscle mass than pair-fed

NTB animals (Borner et al., 2016a). Therefore, a relative increase in energy expenditure appears to contribute to CACS in this tumour model. While we did not explore the effects of peripheral Ex-9 administration on CACS, additional beneficial effects might occur in the periphery also with respect to energy balance. At least in diet-induced obese mice, peripheral treatment with a GLP-1R agonist increased basal energy expenditure presumably via increased expression of uncoupling proteins in different tissues including muscle (Tomas et al., 2015). Moreover, stimulation of GLP-1R increased oxygen consumption and thermogenic gene expression in muscle cells, which can be blocked by Ex-9 (Choung et al., 2017).

GLP-1R expression levels in the AP/NTS were unaffected by tumour growth or pair-feeding. Tumour growth decreased PPG expression levels in the NTS. However, no difference was found between PF and TB animals, indicating that the decrease in PPG expression during tumour growth is likely due to a change in eating or body weight rather than specific tumour-dependent effects. Despite the lower NTS GLP-1 expression levels following tumour growth, GLP-1 blockade was effective in attenuating CACS. Changes in PPG mRNA expression may not necessarily correlate with GLP-1 abundance or synaptic release. Further studies are required to investigate this aspect.

Although we identified GLP-1 as neuronal mediator of CACS, it remains unknown how NTS PPG neurons are activated under tumour conditions. Recent studies suggest a role of the macrophage inhibitory cytokine-1 (MIC-1) in CACS, which acts via the AP/NTS region to reduce food intake and body weight (Johnen et al., 2007; Tsai et al., 2014). Our model is characterized by increased levels of MIC-1 in the absence of

other inflammatory circulating cytokines (Borner et al., 2016a; Ruud and Blomqvist, 2007). MIC-1 primarily activates non-catecholaminergic neurons in the NTS (Tsai et al., 2014). GLP-1-expressing neurons are not catecholaminergic (Larsen et al., 1997a), but it is currently unknown whether MIC-1 activates GLP-1 expressing NTS neurons. Interestingly, a specific receptor for MIC-1 (GFRAL, GDNF family receptor alpha like) was recently identified (Emmerson et al., 2017; Mullican et al., 2017; Yang et al., 2017). In the brain, GFRAL is exclusively expressed in the AP/NTS region. Not only the AP but also the NTS is accessible by blood-borne mediators (Broadwell and Sofroniew, 1993). Hence, NTS PPG neurons could be directly or indirectly activated by MIC-1 and possibly other circulating cytokines leading to GLP-1 release in downstream projection sites (Fig. 4).

In summary, we found GLP-1/GLP-1R signalling in the brainstem to be required for the central mediation of CACS in hepatoma TB rats. A blockade of brainstem GLP-1 not only attenuated anorexia, but also body weight loss and muscle wasting. Future studies should address the question whether brainstem GLP-1 might mediate anorexia and cachexia in other cancer or chronic disease models. Moreover, it remains to be established which stimuli activate the brainstem GLP-1 system under these sickness conditions. Based on our findings, GLP-1R antagonists may be therapeutically useful for the treatment of CACS and possibly other form of sickness-anorexia.

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Conflict of interest:

All authors declare that they have no conflict of interest.

Figure legends:

Fig. 1 Chronic central Ex-9 treatment attenuated CACS but did not affect energy balance in non-tumour-bearing rats

A-B) Ex-9 (100 μg/day) administered chronically into the fourth ventricle significantly attenuated the anorectic response induced by tumour growth. C) Ex-9 treated rats showed attenuated tumour-dependent body weight loss following treatment. Ex-9 treated animals had higher fat mass than controls (D) and higher gastrocnemius, and total muscle mass (sum of gastrocnemius, tibialis and soleus muscle mass) compared to tumour-bearing controls (E). F) Following tumour induction, control rats had significantly lower RER values relative to baseline conditions, reflecting a shift towards lipid oxidation. Ex-9 treatment did not prevent this tumour-induced effect. G) Metabolic rate did not significantly differ between groups. In non-tumour-bearing rats, Ex-9 infusion did not affect food intake (H-I), body weight or body weight gain (J-K). Ex-9 treatment did not alter respiratory exchange ratio and metabolic rate (L-M). Baseline data were collected for two consecutive days prior to minipump implantation. Data analysed using the Student's *t*-test, * $p < 0.05$, ** $p < 0.01$ (A-E and H-K) or with one-way ANOVA followed by Tukey's post-hoc test (F-G and L-M). Different letters indicate significant differences ($p<0.05$).

Fig. 2 Lentiviral PPG-specific shRNA injection into the NTS significantly decreased PPG mRNA expression and attenuated anorexia, and loss of body weight by preservation of lean and fat mass

A) Representative image of GFP fluorescence in the NTS seven weeks after lentivirus infusion, indicating successful transfection. Scale bar 50 μm. B) Location of NTS target area for lentiviral PPG knockdown. Bregma level -14.64. Gr, gracile nucleus; SolC, commissural nucleus of the solitary tract; Sol, nucleus of the solitary tract; 10, dorsal motor nucleus of the vagus; CC, central canal. Modified image from The Rat Brain in Stereotaxic Coordinates, Paxinos, G. and Watson, C., Copyright Paxinos, G. and Watson, C. (1998), with permission from Elsevier. C-D) Quantification of PPG and GLP-1R mRNA levels in the AP/NTS of rats injected with lentivirus encoding for

a PPG-specific shRNA or control lentivirus. E-F) PPG knockdown in the NTS strongly attenuated anorexia and body weight loss induced by tumour growth without affecting baseline food intake before tumour induction. G-H) From day 8 onwards, PPG KD animals showed significantly increased food intake and lost significantly less body weight compared to controls. I) The higher body weight of PPG KD animals was due to by higher lean and fat volumes compared to tumour-bearing controls. Data analysed using the Student's *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3 Tumour growth did not affect circulating GLP-1, and PPG and GLP-1R expressions levels in the AP/NTS

A) Circulating GLP-1 did not significantly differ between groups. B) Tumour-bearing rats (TB) and pair-fed animals (PF) had lower PPG expression levels in the AP/NTS compared to non-tumour-bearing controls (NTB). C) GLP-1R expression was similar among all groups. Data analysed with one-way ANOVA followed by Tukey's post-hoc test. Means with different letter are significantly different from each other ($p<0.05$).

Fig. 4 Possible mechanisms contributing to GLP-1-depedent mediation of CACS.

Humoral mediators such as the macrophage inhibitory cytokine 1 (MIC-1) and other cytokines may directly or indirectly (e.g. via the *area postrema* (AP)) activate GLP-1 expressing neurons located in the *nucleus tractus solitarii* (NTS). In the brain, the MIC-1 receptor GFRAL is exclusively expressed in the AP/NTS region. Local release of GLP-1 in downstream projection sites might lead to activation of neuropeptide/neurotransmitter systems known to be involved in pathological anorexia and body weight loss (see text for further explanations).

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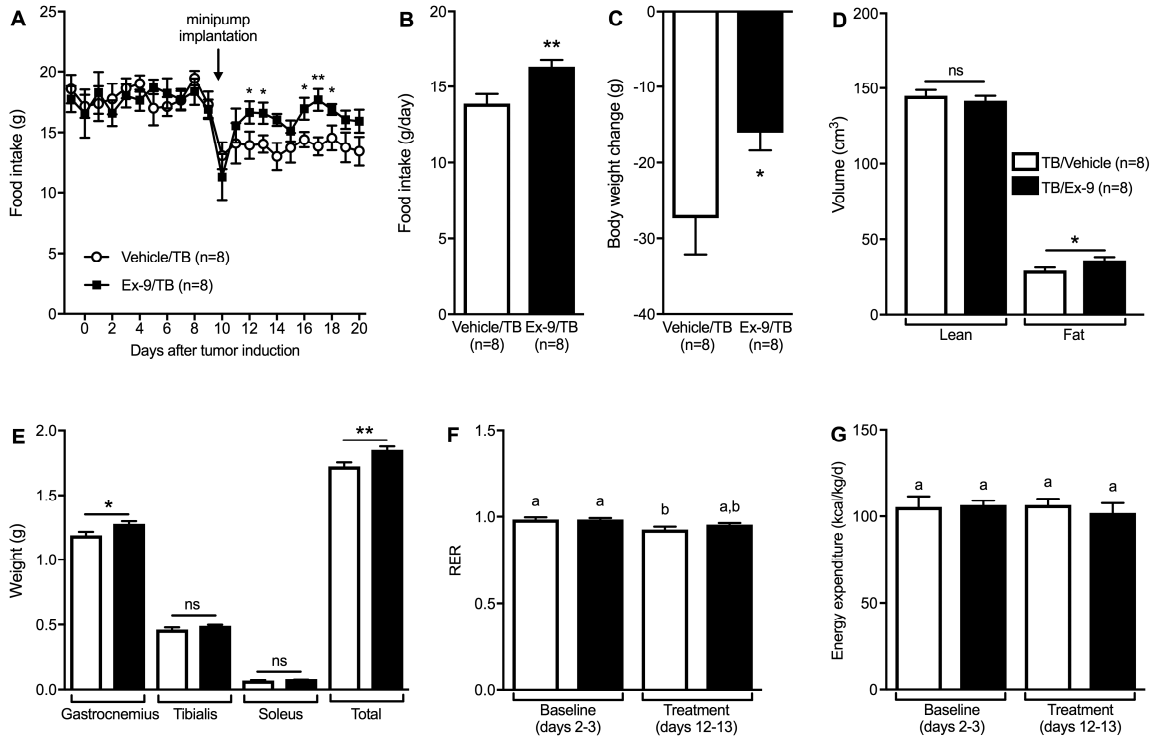
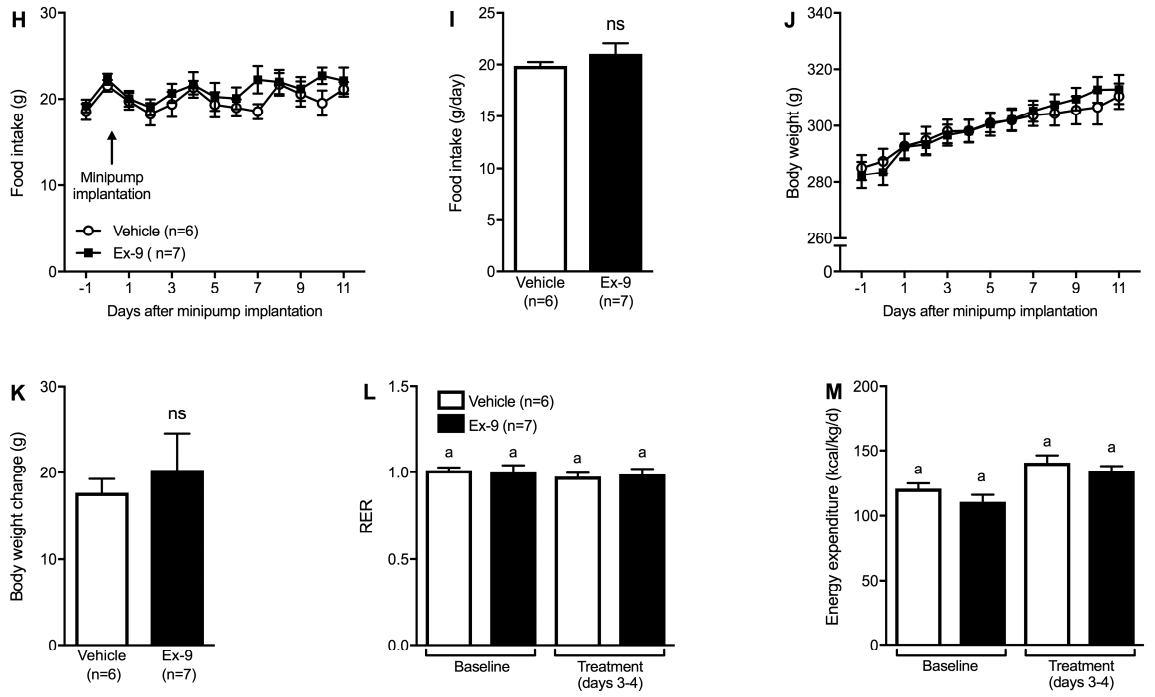
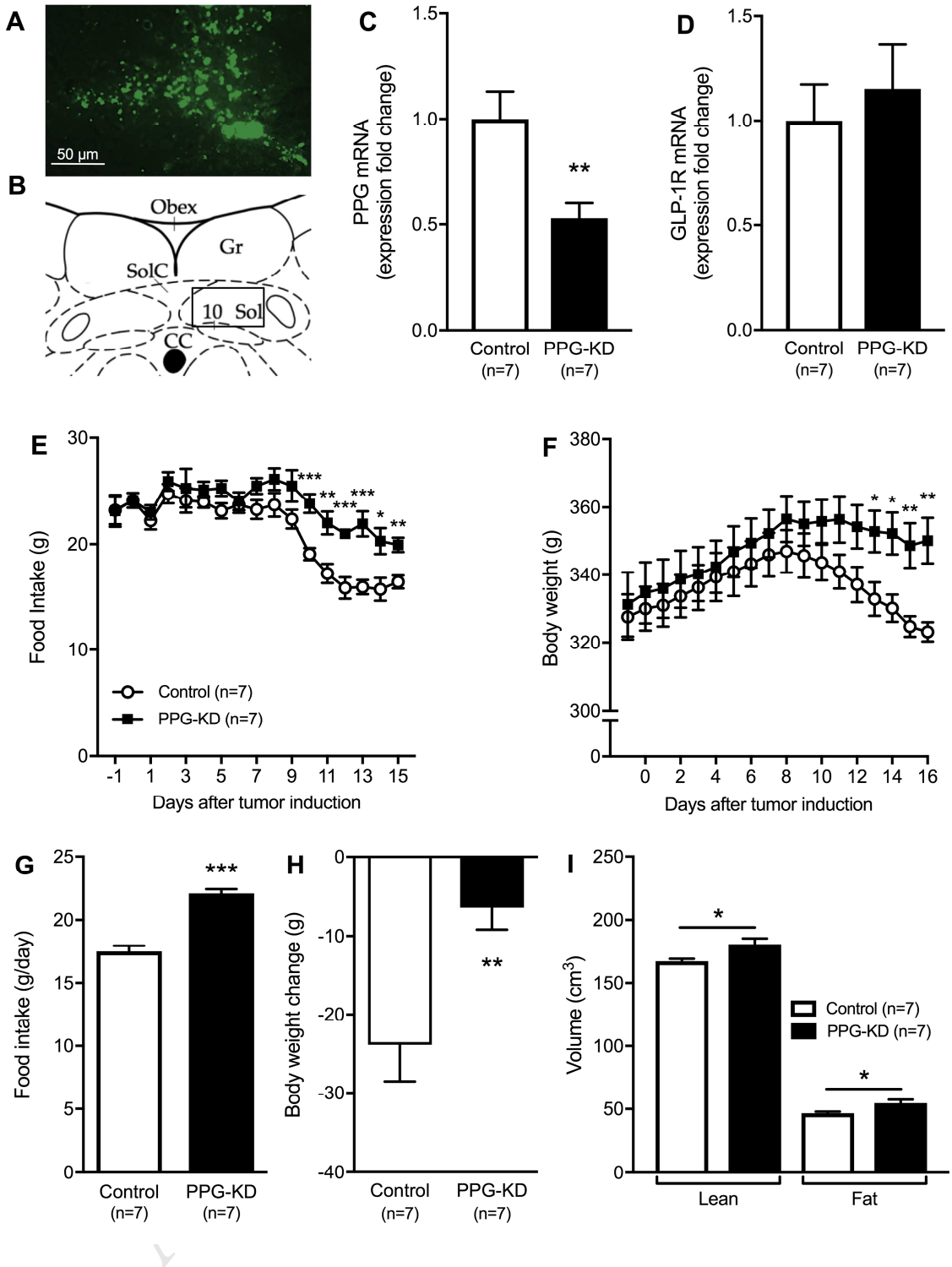
Tumour-bearing rats



Non-tumour-bearing rats

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Control (n=7)
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GLP-1R mRNA (expression fold change)
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Control (n=7)
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E
Food Intake (g)
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Days after tumor induction
Control (n=7)
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Body weight (g)
380
360
340
320
300
0
0 2 4 6 8 10 12 14 16
Days after tumor induction
G
Food intake (g/day)
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20
15
10
5
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Control (n=7)
PPG-KD (n=7)
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Body weight change (g)
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-10
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Control (n=7)
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Control (n=7)
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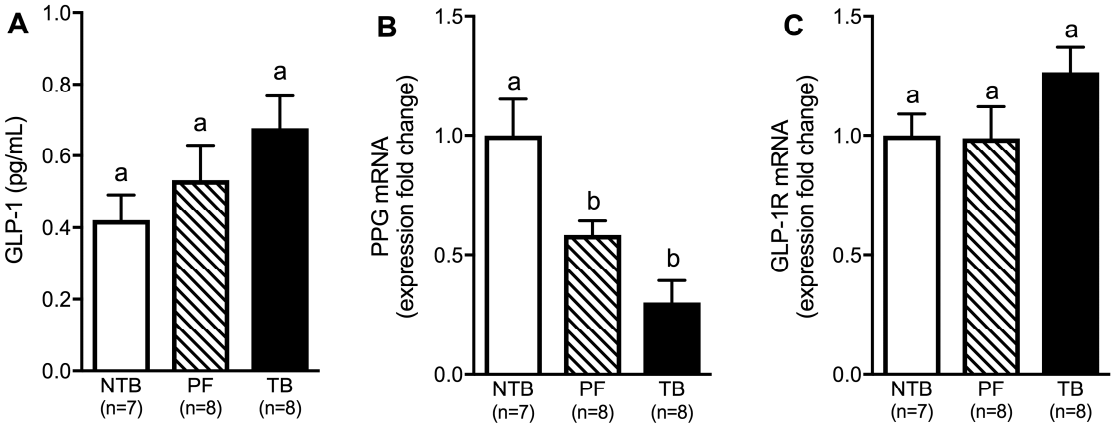

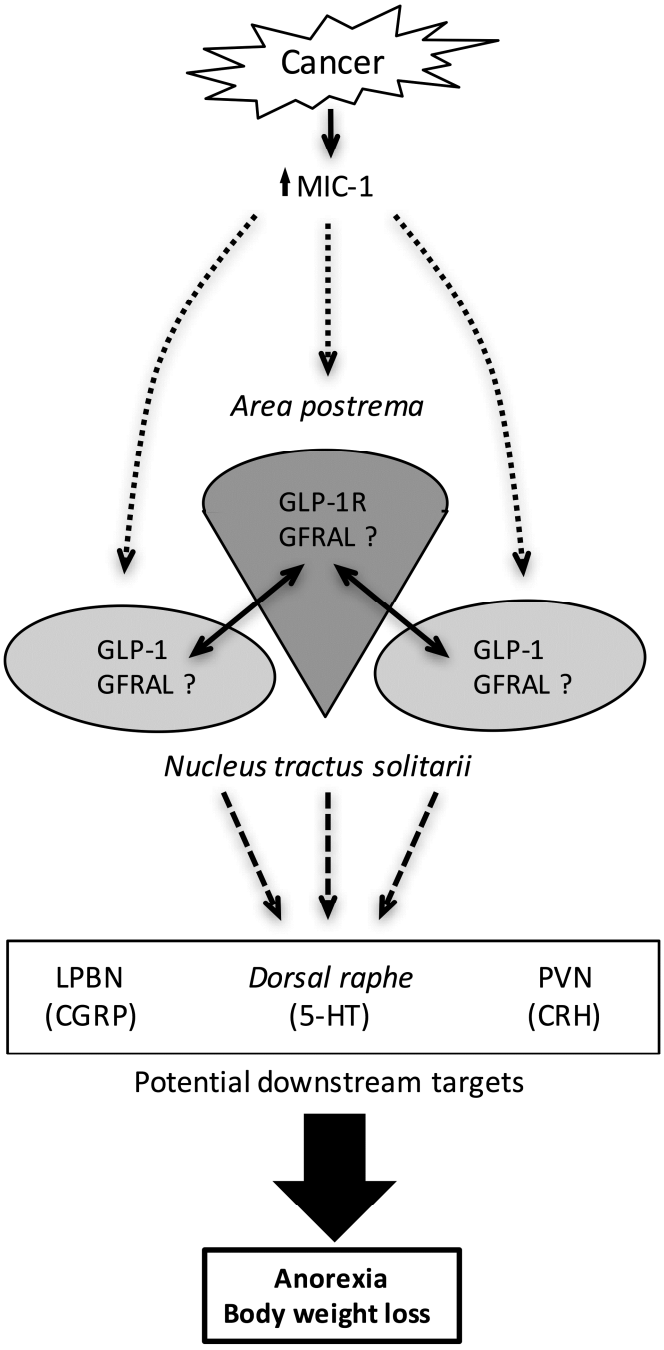

Highlights

- Pharmacological blockade of central GLP-1 receptors attenuates CACS in rats
- GLP-1-ergic neurons of the *nucleus tractus solitarii* mediate CACS
- Central GLP-1 signalling contributes to loss of lean mass and muscle wasting
- GLP-1 receptor antagonists might be therapeutically useful for the treatment of CACS