MCG101-induced cancer anorexia-cachexia features altered expression of hypothalamic *Nucb2* and *Cartpt* and increased plasma levels of cocaine- and amphetamine-regulated transcript peptides

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Abstract. The aim of the present study was to explore central and peripheral host responses to an anorexia-cachexia producing tumor. We focused on neuroendocrine anorexigenic signals in the hypothalamus, brainstem, pituitary and from the tumor *per se*. Expression of mRNA for corticotropin-releasing hormone (CRH), cocaine- and amphetamine-regulated transcript (CART), nesfatin-1, thyrotropin (TSH) and the TSH receptor were explored. In addition, we examined changes in plasma TSH, CART peptides (CARTp) and serum amyloid P component (SAP). C57BL/6 mice were implanted with MCG101 tumors or sham-treated. A sham-implanted, pair-fed (PF) group was included to delineate between primary tumor and secondary effects from reduced feeding. Food intake and body weight were measured daily. mRNA levels from microdissected mouse brain samples were assayed using qPCR, and plasma levels were determined using ELISA. MCG101 tumors expectedly induced anorexia and loss of body weight. Tumor-bearing (TB) mice exhibited an increase in nesfatin-1 mRNA as well as a decrease in CART mRNA in the paraventricular area (PVN). The CART mRNA response was secondary to reduced caloric intake whereas nesfatin-1 mRNA appeared to be tumor-specifically induced. In the pituitary, CART and TSH mRNA were upregulated in the TB and PF animals compared to the freely fed controls. Plasma levels for CARTp were significantly elevated in TB but not PF mice whereas levels of TSH were unaffected. The plasma CARTp response was correlated to the degree of inflammation represented by SAP. The increase in nesfatin-1 mRNA in the PVN highlights nesfatin-1 as a plausible candidate for causing tumor-induced anorexia. CART mRNA expression in the PVN is likely an adaptation to reduced caloric intake secondary to a cancer anorexia-cachexia syndrome (CACS)-inducing tumor. The MCG101 tumor did not express CART mRNA, thus the elevation of plasma CARTp is host derived and likely driven by inflammation.

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Introduction

In progressive cancer, signals emanating from the tumor will affect the host and result in physiological and behavioral changes that include anorexia, lethargy, fatigue and inflammation. Nearly 80% of patients with progressive cancer are at risk of eventually developing a wasting condition referred to as cancer anorexia-cachexia syndrome (CACS) (1,2). This syndrome is marked by a decrease in nutritional intake and a simultaneous wasting of peripheral tissues, skeletal and adipose tissues primarily, whereas brain and organ tissues appear to be spared (3,4). The specific mechanisms behind CACS are not yet clear, but it is reasonable to suggest that regulatory centers for feeding and homeostasis in the central nervous system (CNS) are involved (5,6).

In the present study, we explored the host response to an anorexia-inducing pro-inflammatory tumor with respect to various feeding regulatory signals present in the hypothalamus and hindbrain. MCG101 is an undifferentiated, endothelial-like, non-metastasizing, solid tumor that produces high amounts of the pro-inflammatory mediator prostaglandin E_2 (7). This mouse model presents with a progressive reduction in *ad libitum* feeding accompanied by peripheral wasting of fat and skeletal muscle masses (8-10), thus resembling CACS in cancer patients. We presently focused on genes coding for corticotropin-releasing hormone (CRH), cocaine- and amphetamine-regulated transcript (CART), nesfatin-1, thyrotropin (TSH) and its receptor (TSHR) as these neuroendocrine factors are able to attenuate food intake at a central level (11-14). These genes are of particular interest since inflammation is a common characteristic of CACS and the hypothalamic expression levels of these substances are responsive to acute inflammation (15-18, data not shown). We recently showed

that acute inflammation produces a robust elevation in plasma CART peptide (CARTp) levels in a prostanoid-independent manner as well (data not shown). Given the pro-inflammatory features of most cancers, it would be reasonable to suspect that a similar plasma CARTp response may manifest in a progressive tumor model as well.

In the present study, we thus tested the hypothesis that host mRNA expression of *Crh*, *Cartpt*, *Tshb*, *Tsh* and *Nucb2* in hypothalamic, hindbrain and pituitary tissues are altered as part of the response to MCG101-induced CACS. The possible impact of tumor-induced anorexia vs. food restriction *per se* was also investigated in order to uncover whether changes detected are directly due to tumor factors, or due to a secondary adaptation to reduced caloric intake. Secondly, we aimed to determine whether changes in gene expression are paralleled by altered levels of TSH and CARTp in plasma.

Materials and methods

Animals. Female mice (C57Bl/6JBomTac; Taconic, Denmark) weighing 18-23 g were used in the present study. All animals were kept under controlled ambient conditions: lights on 07:00-19:00 h, temperature 21±1˚C and relative humidity 45-55%. Ethical approval was obtained from the Gothenburg Regional Animal Ethics Committee.

Mice were separated into three weight-matched groups (n=8/group) and housed in pairs. This was carried out to reduce the potential risk of confounding effects of estrus cyclicity, since cage mates tend to synchronize their cycles. Thus, it could be assumed that any random variations in estrogen levels would be equally distributed across groups. The cages were equipped with wire floors, a solid 20-cm square acrylic floor panel and nesting material. Mice were acclimated to the wire-floor cages three days prior to tumor- or sham-implantation.

Mice assigned to the tumor-bearing (TB) group were given bilateral subcutaneous implants of MCG101 tumor pieces (~1-3 mm^3) under isoflurane anesthesia. The freely fed (FF) and pair-fed (PF) control mice underwent sham-implantation.

Feeding and body weight. Food intake and individual body weight were daily recorded at the same time point (07:30-09:30 h). The order in which the cages were checked was performed at random each day. The *ad libitum* feeding was measured for the FF and TB groups. The daily food intake was corrected for food spillage. The PF group was provided with a restricted amount of food equal to that which was consumed by the TB group.

Tissue collection. Fourteen days after tumor implantation, the mice were anesthetized with a mixture of xylazine (5 mg/kg mouse) and ketamine (100 mg/kg mouse) i.p. in a volume of 0.1 ml. EDTA-blood was collected by cardiac puncture and plasma was subsequently isolated by centrifugation (2,000 x g, 10 min, 4˚C). Whole brains and pituitaries were collected for mRNA expression analysis. The whole brains were subdivided into the brainstem and the hypothalamus (defined as caudal to the optic chiasm and rostral to the mammillary bodies). From the hypothalamus, two areas were microdissected using 2 mm biopsy punches. The arcuate nucleus (ARC) was defined as the ventral portion of the third ventricle. The paraventricular area (PVN) was defined as the tissue surrounding the dorsal two-thirds of the third ventricle. All tissues were placed in RNA-Later solution according to the manufacturer's recommendations (Ambion, Life Technologies, The Netherlands) and stored at -20˚C until processing.

Relative expression of mRNA. Tissues were homogenized with a rotor/stator homogenizer in QIAzol lysis reagent supplied with the RNeasy Lipid Tissue Mini kit (Qiagen AB, Sweden). Total RNA was extracted, with a DNase digestion step included, according to the manufacturer's protocol. RNA concentration was determined using a NanoDrop ND-1000 instrument, and RNA integrity was established in a Bioanalyzer 2100 with an Agilent RNA 6000 Nano kit (Agilent Technologies, Germany). The RNA integrity numbers were above 7.5 for all tissue extracts.

Synthesis of cDNA (SuperScript VILO; Life Technologies) was conducted on 0.2-1.0 μg RNA. Resultant cDNA was assayed in triplicate using Power SYBR-Green PCR Master Mix (Life Technologies) in an ABI Prism 7000 HT instrument (Applied Biosystems, Life Technologies). QuantiTect Primer Assay kits for TSHβ (*Tshb*, QT00135303), TSHR (*Tshr*, QT00136955), CART (*Cartpt*, QT00130396), nesfatin (*Nucb2*, QT02281965), CRH (*Crh2*, QT01055789) and GAPDH (*Gapdh*, QT001658692) were purchased from Qiagen Sciences (Germantown, MD, USA). Each 10 μl PCR reaction consisted of 5 μl Master Mix, 1 μl primer assay and 4 μl cDNA diluted in nuclease-free water. All assays underwent 40 thermocycles (10-sec melting at 95˚C and 60-sec annealing/elongation at 60˚C). Gene expression data were calculated relative to *Gapdh* mRNA using the $2^{-\Delta\Delta Ct}$ method.

Plasma assays. Plasma samples were assayed for TSH, CARTp and serum amyloid P component (SAP) levels by ELISA (Kamiya Biomedical Company, Seattle, WA, USA; RayBiotech, Inc., Norcross, GA, USA; R&D Systems, Bio-Techne EMEA, UK). Plasma samples were assayed in duplicate. Plasma aliquots were thawed on ice, briefly vortex-mixed and subsequently prepared according to the respective manufacturer's protocols.

Statistical analysis. Body weights and feeding data were assessed by one-way analysis of variance followed by post hoc Fisher's LSD tests. Between group differences for mRNA and plasma data were evaluated using Kruskal-Wallis analyses followed by pair-wise Mann-Whitney U tests where applicable. Correlations reported were performed on ranked data to obtain Spearman correlation coefficients (ϱ) and P-values. P-values <0.05 were regarded as statistically significant. Data were assessed using SPSS statistics version 20 (IBM Corp., Armonk, NY, USA).

Results

One TB mouse spontaneously died on post-implantation day 11 and was therefore excluded from analysis. Group sizes at the experimental endpoint were TB (n=7), FF (n=8) and PF (n=8). The group sample sizes for ARC relative expression were FF (n=6), TB (n=5), PF (n=7) due to low RNA yield for this tissue.

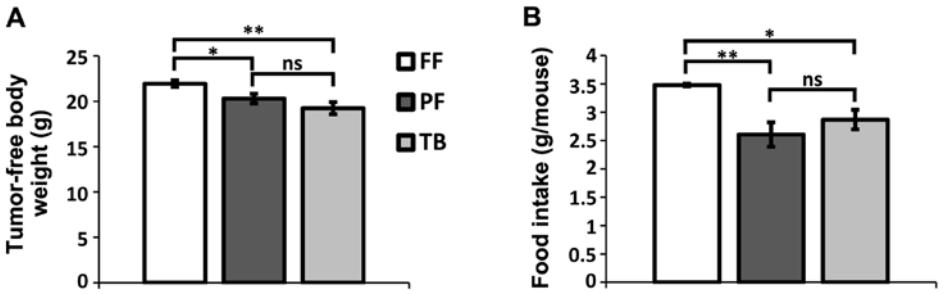

Figure 1. (A) Tumor-free body weight and (B) endpoint food intake of the respective mouse groups presented as the mean ± SEM. The groups are defined as freely fed (FF), tumor bearing (TB) and pair-fed (PF). *P<0.05; **P<0.01. ns, not significant.

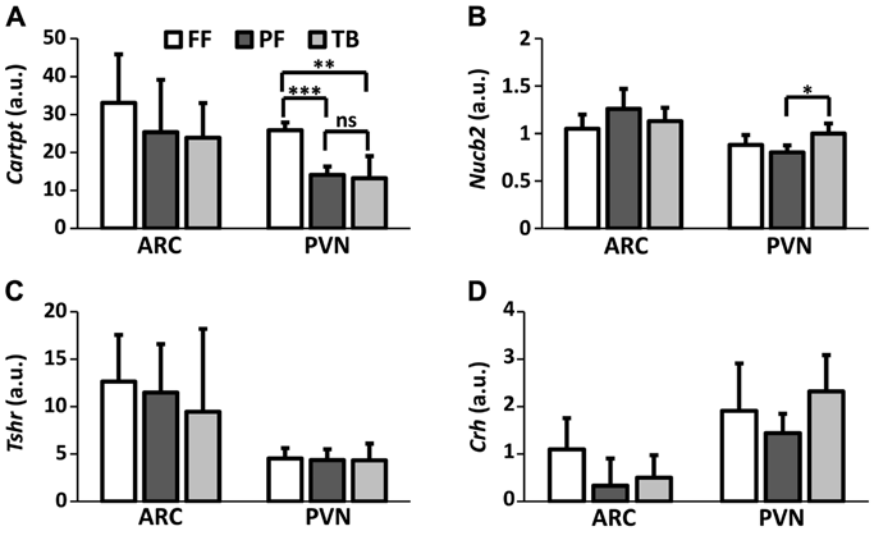

Figure 2. Relative mRNA expression of (A) *Cartpt*, (B) *Nucb2*, (C) *Tshr* and (D) *Crh* [median with interquartile deviation, arbitrary units (a.u.)] in the hypothalamic arcuate nucleus (ARC) and paraventricular area (PVN) of freely fed (FF), tumor-bearing (TB) and pair-fed (PF) mice. *P<0.05; **P<0.01; ***P<0.001. ns, not significant.

Body weight and feeding. There was no difference in baseline body weight between the groups ($F_{2,20}$=0.38; P=0.69), but at the experimental endpoint there was a significant difference in tumor-free body weight among the groups (Fig. 1A; $F_{2,20}$=6.81; P=0.006). Both TB and PF mouse groups had lower average body weights than the FF control group (P=0.002 and P=0.033, respectively). There was no difference between the TB and PF mice with respect to tumor-free body weight (Fig. 1A; P=0.17).

Baseline food intake was defined as the average food intake/mouse during the first three post-implantation days. There was no difference in basal feeding between groups ($F_{2,9}$=0.34; P=0.72). The endpoint food intake was similarly defined as the average food intake during the final three observation days. There was a significant difference in endpoint food consumption (Fig. 1B; $F_{2,9}$=7.90; P=0.01). Post hoc comparisons confirmed that the TB and PF mouse groups did not differ in food intake (P=0.28) and that both groups consumed less food than the FF group (P=0.02 and P=0.004, respectively).

Relative expression of mRNA. In the PVN, there was an overall effect on *Cartpt* (χ^2=13.12; df=2; P=0.001) and *Nucb2* (χ^2=7.10; df=2; P=0.03) mRNA expression. *Cartpt* mRNA expression was significantly reduced in both the TB (P<0.01) and PF (P<0.001) groups compared to FF controls (Fig. 2A). The reduced *Cartpt* mRNA expression was similar between the TB and PF groups (P=0.69). The *Nucb2* mRNA expression in the PVN differed between the TB and PF groups (P<0.05) with the FF group having intermediate expression (Fig. 2B). Neither chronic tumor burden nor food restriction affected gene expression in the ARC (Fig. 2) and brainstem (Fig. 3) with respect to *Cartpt*, *Nucb2*, *Tshr* and *Crh* mRNA.

There was an overall effect on pituitary *Cartpt* (χ^2=12.73; df=2; P=0.002) and *Tshb* (χ^2=15.89; df=2; P<0.001) mRNA expression (Fig. 4). Subsequent post hoc tests indicated that CART mRNA expression was greater in the TB (P<0.001) and PF (P<0.05) mice compared to the FF controls (Fig. 4). *Tshb* mRNA expression, which was only detectable in the pituitary, was significantly higher in the TB compared to both the FF (P<0.001) and PF (P<0.01) groups (Fig. 4). Food restriction in the PF group was capable of elevating pituitary *Tshb* mRNA (P<0.05) but not to the extent of the TB condition.

Plasma TSH, CARTp and SAP levels. Kruskal-Wallis analysis revealed that there was an overall effect on plasma CARTp levels (Table I). Mann-Whitney U tests further showed that TB mice had a significantly higher plasma CARTp concentrations than the FF (P<0.01) or PF (P<0.01) groups. Plasma CARTp

Table I. Plasma concentrations of TSH, CARTp and SAP in freely fed (FF), tumor-bearing (TB) or pair-fed (PF) mice presented as medians (interquartile range).

	FF n=8	TB n=7	PF n=8	χ^2	P-value
TSH pg/ml	172 (82)	302 (318)	243 (206)	0.85	0.653
CARTp pg/ml	190 (373)	1,661 (1,834)[a]	512 (370)[c]	11.72	**0.003**
SAP μg/ml	1.9 (0.8)	55 (40)[b]	1.5 (0.6)[d]	14.35	**<0.001**

Kruskal-Wallis χ^2 and P-values are reported, and subsequent post hoc levels of significance are indicated with superscripts. [a]P<0.01, [b]P<0.001 vs. FF; [c]P<0.01, [d]P<0.001 vs. TB. TSH, thyrotropin; CART, cocaine- and amphetamine-regulated transcript; CARTp, CART peptides; SAP, serum amyloid P component.

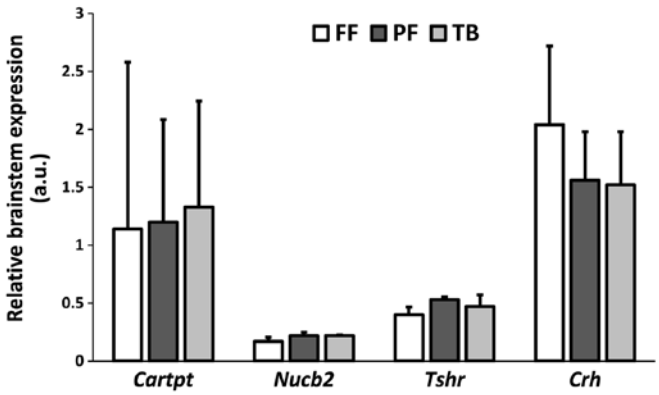

Figure 3. Relative mRNA expression (median with interquartile deviation, arbitrary units) from brainstems of freely fed (FF), tumor-bearing (TB) and pair-fed (PF) mice.

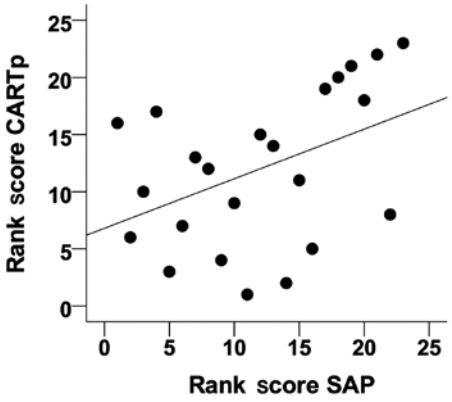

Figure 5. Positive Spearman correlation between plasma SAP and CARTp levels in the mice. Spearman ϱ=0.43; P=0.04; n=23.

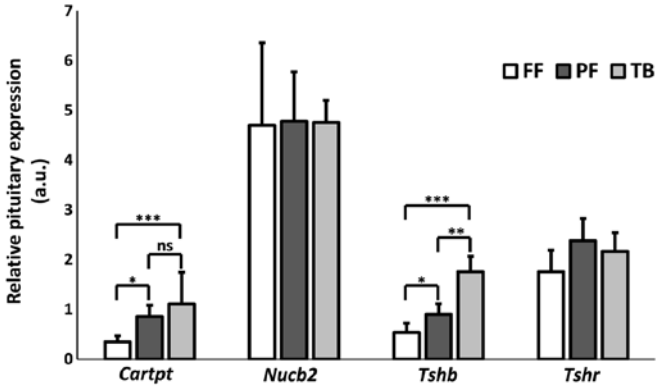

Figure 4. Relative mRNA expression [median with interquartile deviation, arbitrary units (a.u.)] from pituitaries of freely fed (FF), tumor-bearing (TB) and pair-fed (PF) mice. *P<0.05; **P<0.01; ***P<0.001. ns, not significant.

appeared on average to be elevated in the PF group but the difference was not significantly different from the FF controls (P=0.08). Given the elevated plasma CARTp in TB animals, we hypothesized that the MCG101 tumors *per se* were a source of circulating CARTp. However, we found *Cartpt* mRNA to be below the limit of detection in the tumors (data not shown).

As expected, the presence of the PGE_2-producing tumor resulted in a stark elevation of plasma SAP (Table I) compared to both the FF (P<0.001) and PF (P<0.001) animals. Plasma CARTp and SAP concentrations had a positive correlation (Fig. 5; ϱ=0.43; P=0.04; n=23). Plasma TSH concentrations did not differ significantly among the groups (Table I).

Discussion

In the present experiment, we investigated whether expression of various neuroendocrine signals that negatively regulate food intake in the CNS in the healthy state may be altered in response to CACS. We used the MCG101 mouse model which displays CACS to answer this question. In the present study, we showed that *Nucb2* was elevated in the PVN of TB animals in contrast to the PF controls (Fig. 2B). The bioactive *Nucb2* peptide product, nesfatin-1, inhibits food intake at a central level (14,19,20). Our finding is noteworthy since it may suggest that induction of *Nucb2* in the PVN could play a primary role in promoting the tumor-induced anorexia response of the host. To the best of our knowledge, there are no clinical studies regarding any possible central role of nesfatin-1 in man, although circulating nesfatin-1 is reported to be affected in patients with CACS (21). Progressive cancer in CACS has a major impact on the organism and it is reasonable to assume that physiological stress mechanisms will be recruited, for example HPA axis activation. Notably, there is an overlap of nesfatin-1- and *CRH*-containing cells within a small subset of neurons in the PVN of the rat (22). In contrast to *Nucb2*, we could not detect

any significant change in *Crh* mRNA expression in the PVN in either the TB or PF animals, however (Fig. 2D). In fact, the lack of any differences in *Crh* mRNA levels between groups in the PVN may imply that chronic tumor exposure does not lead to an activation of the HPA axis by *CRH*. Since *Crh* mRNA remained unaltered, our conclusion is that the MCG101 tumor induces a CRH-independent increase in *Nucb2* mRNA in the PVN. Our data further raises the possibility that nesfatin-1 could participate in the tumor-induced reduction of caloric intake set point observed in CACS.

Besides nesfatin-1 and CRH as possible central-acting anorexigens of relevance in CACS, we investigated changes in *Cartpt* mRNA expression in the PVN and ARC as well. CARTp have potent anorexigenic effects and are proposed as endogenous satiety signals (12). It was shown in the rat that CACS induced by LC-6-JCK tumors can lead to lowered *Cartpt* expression in the ARC (23). This cited study did not, however, incorporate a pair-fed control group. Whereas we found no effect on *Cartpt* mRNA in the ARC, there was a strong suppression of *Cartpt* in the PVN in the TB animals vs. FF controls. This finding on its own would have led us to believe that tumor signals would directly cause suppression of *Cartpt* mRNA in the PVN. However, *Cartpt* was suppressed to a similar degree in animals pair-fed vs. the TB group (Fig. 2A). These findings suggest that the suppression of *Cartpt* in the PVN is a phenomenon secondary to the reduced caloric intake which results from tumor-induced anorexia. Thus, CART suppression in the PVN appears to be adaptive rather than causal with respect to CACS. The reduction of hypothalamic CART in PF animals vs. FF controls (Fig. 2A) corresponds to some previous studies in food-deprived vs. post-prandial rats (12), cf (24). Our results are also consistent with a study in rats carrying an MNU-induced mammary tumor, wherein CACS animals were unresponsive to intraventricular CARTp and CART antiserum, and they displayed weaker immunostaining in hypothalamic structures suggestive of reduced CARTp activity (25). Together, our results clearly support that decreased hypothalamic *Cartpt* expression is a compensatory mechanism, likely aimed at defending caloric intake. This compensatory reduction in hypothalamic *Cartpt* expression was a result of the anorexia *per se* rather than a direct response to tumor factor impact. In conjunction with previous studies (23,25), our data further support that such an adaptive CARTp-mechanism may be common feature in CACS, since it is neither restricted to one single species, nor to a specific tumor type.

In addition to examining putative hypothalamic effects in CACS, we also investigated such responses in the pituitary and brainstem which are of importance in mediating central-peripheral signaling. In contrast to the PVN, *Cartpt* mRNA in the pituitary (Fig. 4) was elevated in response to chronic MCG101 exposure or to food restriction. The pituitary *Cartpt* mRNA levels did not differ between the TB and PF groups. Whereas there was no effect on pituitary *Tshr* or *Nucb2* mRNA expression in any of the groups, there was a slight but significant increase in *Tshb* mRNA in response to food restriction. Notably, however, this expression was effectively doubled in the TB animals. Thusly, the TB condition gave rise to a *Tshb* response which can only be partially explained by reduced caloric intake (Fig. 4). The present change in *Tshb* gene expression was not paralleled by any changes in plasma TSH levels (Fig. 4 and Table I). The plasma TSH data are consistent with previous studies in cancer cachexia patients and experimental MCG101-bearing mice (26,27). In these cited studies, patients and animals with CACS showed normal circulating TSH levels but apparent hypothyroidism, which was also not completely explained by nutritional status alone. The increase in pituitary *Tshb* mRNA in the TB group seen in this study, is therefore likely a synergistic effect of a response to tumor signals in combination with a response to the reduced caloric intake. With regard to the brainstem, we found no significant effects on any of the investigated genes (Fig. 3). In contrast to the hypothalamic tissue, we did not micro-dissect specific brainstem nuclei. Therefore, we cannot completely exclude the possibility of reciprocal gene responses, cancelling out any otherwise detectable changes in mRNA expression within the different brainstem substructures.

Circulating CARTp levels are reported to be elevated in conditions of progressive cancer, and CART expression in neuroendocrine tumors is associated with worse survival (28-30). An interesting finding of the present study was that plasma concentrations of CARTp were significantly higher in tumor-bearing animals. In order to address whether the plasma CARTp was tumor- or host-derived, we thus analyzed the possible presence of *Cartpt* mRNA in MCG101 tumors. *Cartpt* was below the limit of detection in the tumor samples (data not shown). Although we cannot identify the originating tissue from which the CARTp was released, we can say with certainty that the elevation in plasma can be entirely attributed to a host response. While previous studies have suggested CARTp as a candidate biomarker for cancer (28-30), our current data supporting plasma CARTp as a host response challenges the usefulness of CARTp as a diagnostic biomarker for cancers. We further found plasma CARTp levels to be correlated to plasma SAP levels (Fig. 5). This correlation is similar to our own previous findings where lipopolysaccharide induced a robust increase in plasma CARTp, which was also associated with the degree of inflammation (data not shown). The physiological role for circulating CARTp remains to be conclusively determined. Although acutely administered doses of CARTp in the periphery do not appear to influence feeding or gastric function (31-34), the long-term chronic effects of circulating CARTp on food intake or metabolism have yet to be elucidated.

In conclusion, *Nucb2* mRNA was increased in the PVN of MCG101 tumor-bearing mice compared to pair-fed mice, which may suggest a role for hypothalamic nesfatin-1 in the etiology of CACS. *Cartpt* expression- in the PVN and the pituitary are also altered in response to CACS, but these changes appear to be adaptive to reduced caloric intake rather than caused by tumor-specific signals. The present findings in conjunction with previous reports suggest that increased concentration of CARTp in plasma is a peripheral host response to cancer anorexia-cachexia. Since the levels of plasma CARTp correlate to plasma levels of SAP, it is possible that the increase in circulating CART is driven by tumor-induced inflammation.

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