Cancer-induced anorexia and malaise are mediated by CGRP neurons in the parabrachial nucleus

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Anorexia is a common manifestation of chronic diseases, including cancer. Here we investigate the contribution to cancer anorexia made by calcitonin gene-related peptide (CGRP) neurons in the parabrachial nucleus (PBN) that transmit anorexic signals. We show that CGRPPBN neurons are activated in mice implanted with Lewis lung carcinoma cells. Inactivation of CGRPPBN neurons before tumor implantation prevents anorexia and loss of lean mass, and their inhibition after symptom onset reverses anorexia. CGRPPBN neurons are also activated in Apcmin/+ mice, which develop intestinal cancer and lose weight despite the absence of reduced food intake. Inactivation of CGRPPBN neurons in Apcmin/+ mice permits hyperphagia that counteracts weight loss, revealing a role for these neurons in a ‘nonanorexic’ cancer model. We also demonstrate that inactivation of CGRPPBN neurons prevents lethargy, anxiety and malaise associated with cancer. These findings establish CGRPPBN neurons as key mediators of cancer-induced appetite suppression and associated behavioral changes.

Illness is associated with behavioral changes (‘sickness behaviors’), which may be adaptive in the acute setting1 but have deleterious consequences if they persist in patients with chronic disease. Notably, anorexia is common in patients with cancer-induced weight loss and, even when overt anorexia is absent, the ability to eat enough to compensate for and counter ongoing weight loss is often lacking. Although available evidence suggests that cancer anorexia arises from signals emanating from the tumor and/or associated inflammatory processes that impinge on neural circuits controlling feeding behavior2, a discrete population of neurons responsible for cancer anorexia has yet to be identified. The absence of this fundamental information may help to explain the ineffectiveness of current treatment options.

In the current work, we focused on the hypothesis that neurons in the external lateral parabrachial nucleus (PBN) that express calcitonin gene-related peptide (CGRP) mediate cancer anorexia, based on both their ability to potently suppress appetite when activated3 and on evidence that they are situated downstream of neural systems activated by various cancer-related signals. Specifically, CGRPPBN neurons are activated by visceral signals that suppress feeding and are inhibited by hunger-promoting hypothalamic agouti-related peptide (AgRP) neurons4,5, neurocircuits that are also implicated in cancer anorexia6–9. Moreover, CGRPPBN neurons are activated by spinally transmitted noxious stimuli and transduce affective–motivational aspects of pain9.

To test this hypothesis, we employed Cre-dependent viruses in transgenic mice to selectively manipulate CGRPPBN neurons in two established murine cancer models: implantable Lewis lung carcinoma (LLC) cells, which induce weight loss and anorexia, and Apcmin/+ mice, which develop intestinal cancer due to an autosomal dominant mutation of the Apc gene10. Although Apcmin/+ mice do not exhibit anorexia, they fail to increase their food intake in the face of weight loss11,12, implying a disruption of the normal adaptive response to negative energy balance. Results of these studies demonstrate that activation of CGRPPBN neurons is required for the effect of cancer to both induce anorexia and malaise (LLC tumor model) and to prevent adaptive increases of food intake that mitigates weight loss (Apcmin/+ mice).

RESULTS

CGRPPBN neurons are activated in LLC tumor-bearing mice

To determine whether CGRPPBN neurons are active during cancer anorexia, we implanted LLC tumor cells in CalcaCreGFP/+ transgenic mice, which express GFP fused to Cre recombinase driven by the CGRP-encoding gene Calca (Fig. 1a). Compared to sham-treated controls (including mice pair-fed to the intake of anorexic mice), tumor-bearing mice exhibited increased Fos expression in the external lateral PBN (Fig. 1b and Supplementary Fig. 1). Approximately 80% of Fos-immunoreactive neurons in tumor-bearing mice corresponded to GFP-labeled CGRPPBN neurons, while 41% of CGRPPBN neurons expressed Fos (Fig. 1c,d), a level comparable to what is observed in healthy mice following a large meal1. As sham-treated mice exhibited Fos in just 3% of CGRPPBN neurons, these data establish that CGRPPBN neurons were inappropriately active in tumor-bearing mice.

Inactivation of CGRPPBN neurons prevents LLC-induced anorexia

To determine whether activation of CGRPPBN neurons is required for cancer anorexia, we injected an adeno-associated virus (AAV) expressing Cre-dependent tetanus toxin (TetTox) light chain (AAV1-DIO-GFP:TetTox) bilaterally into the PBN of CalcaCreGFP/+ mice before LLC tumor implantation (Fig. 2a). Expression of TetTox prevents neurotransmitter release from Cre-expressing neurons13 and therefore selectively and permanently inactivates CGRPPBN neurons. Bilateral TetTox inactivation of CGRPPBN neurons fully prevented

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anorexia in LLC tumor-bearing mice compared to tumor-bearing controls that received PBN injections of AAV1-DIO-GFP control virus (Fig. 2b). Indeed, tumor-bearing mice with bilateral CGRP

neuronal inactivation increased their food intake compared to baseline (before tumor implantation) and compared to sham-treated mice (Fig. 2c). Although LLC tumor-bearing mice with bilateral TetTox inactivation of CGRP

neurons tended to lose less weight than LLC tumor-bearing controls, the effect was not substantial (Supplementary Fig. 2a). Notably, TetTox mice fed ad libitum also developed larger tumors than tumor-bearing controls (Supplementary Fig. 2b).

CGRP

neurons have excitatory axonal projections to the central nucleus of the amygdala (CeA) and oval subnucleus of the bed nucleus of the stria terminalis (ovBNST; Fig. 2d,e),3,9, brain areas involved in affective–motivational responses to stressful stimuli14,15. Cancer anorexia was associated with increased Fos expression in both brain areas, and this effect was also prevented in TetTox mice (Fig. 2f–h) regardless of whether TetTox mice were fed ad libitum or were pair-fed to the intake of tumor-bearing controls (indicating that the outcome cannot be explained by differences of food intake). In addition to demonstrating that activation of CGRP

neurons is required for anorexia in the LLC tumor model, these data support the possibility that projections of these neurons to the CeA and ovBNST contributed to associated behavioral phenotypes.

Inhibiting CGRP

neurons reverses established LLC-induced anorexia

To investigate whether acute inhibition of CGRP

neurons is sufficient to ameliorate established cancer anorexia, we bilaterally transduced these neurons with either AAV1-DIO-hM4Di:mCherry or AAV1-DIO-mCherry control virus (Fig. 3a). With this approach, CGRP

neuronal activity remains intact until administration of clozapine-N-oxide (CNO), an otherwise inert drug that activates the inhibitory hM4Di receptor. To ensure uniform timing of CNO administration relative to anorexia onset, drug administration commenced after the second day of food intake decline. Consistent with our findings in TetTox mice, CNO administration fully reversed anorexia in the hM4Di tumor-bearing group, whereas food intake continued to decline in the CNO-treated mCherry tumor group (Fig. 3b,c and Supplementary Fig. 3a,b).

Although none of the CNO-treated hM4Di tumor-bearing mice met criteria for euthanasia (Online Methods), they were killed for analysis together with tumor-bearing controls such that study duration and days receiving CNO treatment did not differ between groups (Supplementary Fig. 3c,d). CNO-induced CGRP

neuron inhibition prevented both tumor-induced weight loss (Supplementary Fig. 3e,f) and activation of CGRP

neurons (percent of CGRP

neurons co-expressing Fos ± s.e.m.; 85.2 ± 4.1% mCherry LLC versus 2.2 ± 1.4% hM4Di LLC) and downstream CeA and ovBNST neurons (Fig. 3d–g). Acute inhibition of CGRP

neurons was therefore sufficient to ameliorate cancer anorexia after it was established.

Role of CGRP

neurons in LLC tumor-induced changes of energy expenditure and body composition

Cancer-induced weight loss is often associated with wasting of lean mass (cachexia), which can also contribute to cancer-related mortality16. To determine the role played by activation of CGRP

neurons in cancer-induced wasting of lean mass and associated increase of energy expenditure15, we measured body composition by quantitative magnetic resonance and energy expenditure (by indirect calorimetry) in TetTox mice and GFP controls before and after LLC tumor implantation (Fig. 4a). Although both groups exhibited comparable increases in energy expenditure (Fig. 4b–g) and reductions of body fat mass compared to baseline (Fig. 4h,k), lean body mass was increased in TetTox mice (Fig. 4l) relative to GFP controls (Fig. 4i) in the first body composition measurement (LLC 1). This finding implicates CGRP

neuron activation in LLC-induced loss of lean mass, an effect that is independent of changes in either energy expenditure or fat mass.

A second body-composition analysis (LLC 2) was also performed after the onset of anorexia in GFP control mice (Fig. 4j,m) at time-points that varied according to the time when anorexia became evident in each animal (Supplementary Fig. 4a,b). Even after taking differences of tumor mass into account (LLC 2 minus tumor), TetTox mice did not exhibit the cancer-induced loss of body mass (Fig. 4l and Supplementary Fig. 4f) that was evident in GFP controls (Fig. 4i and Supplementary Fig. 4e). Moreover, whereas loss of fat mass increased between the first and second body-composition measurements in GFP mice (Fig. 4h and Supplementary Fig. 4c), TetTox mice were protected from further fat loss (Fig. 4k and Supplementary Fig. 4d).
TetTox mice developed larger tumors than GFP control mice (Supplementary Fig. 4g). After the final body-composition scan, mice were killed for analysis and Fos staining was again undertaken to confirm efficacy of TetTox-induced CGRPPBN neuron inactivation. As expected, tumor-induced Fos immunoreactivity in the CeA and ovBNST was abolished in TetTox mice (Supplementary Fig. 5a–d). These results collectively indicate that inactivation of CGRPPBN neurons attenuates the loss of both lean and fat mass associated with cancer anorexia.

Involvement of CGRPPBN neurons in ‘nonanorexic’ Apcmin+ mice

A cardinal feature of cancer anorexia is that, unlike in healthy animals and humans, progressive weight loss fails to trigger adaptive increases of food intake. Given the association between cancer and increased energy expenditure18, weight loss in the absence of increased feeding could reflect activation of anorexic neural circuitry that blocks the compensatory increase of feeding normally triggered by energy deficit. To address this issue and to extend our study of CGRP PBN neurons to a genetic cancer model, we used Apcmin/+ mice, which begin to develop intestinal tumors as early as 4 weeks of age and start to lose body weight around 12 weeks of age12,19 despite maintaining food intake at baseline levels.

CalcaCre:GFP/+::Apcmin/+ control mice displayed increased Fos expression in CGRP PBN neurons compared to CalcaCre:GFP/+ littermates at 16 weeks of age (Fig. 5a and Supplementary Fig. 6). To examine

Figure 2  Inactivation of CGRP PBN neurons prevents cancer anorexia. (a) Stereotaxic injections of AAV1-DIO-GFP:TetTox (or AAV1-DIO-GFP control virus) into the PBN of CalcaCre:GFP/+ mice before LLC tumor implantation. (b) Cumulative food intake following tumor implantation (LLC) or sham treatment (sham) in mice with intact (GFP, n = 9 mice), bilateral inactivation (TetTox, n = 8 mice) or unilateral inactivation (TetTox unilat, n = 4 mice) of CGRP PBN neurons (two-way repeated-measures ANOVA; interaction, F42, 350 = 19.99, P < 0.0001; time, F14, 350 = 41.88, P < 0.0001; treatment, F3, 350 = 10.61, P = 0.0001). (c) Average daily food intake at various time epochs (two-way repeated-measures ANOVA; interaction, F3,72 = 7.04, P < 0.0001; time epoch, F3,72 = 24.76, P < 0.0001; treatment, F3,72 = 8.65, P = 0.0005). (d,e) GFP-labeled axonal processes of CGRP PBN neurons in the CeA (d) and ovBNST (e). Yellow dashed outlines represent brain areas that were quantified for Fos expression. (f–g) Quantification of Fos expression in (f) the CeA (one-way ANOVA; F4,38 = 12.79, P < 0.0001) and (g) ovBNST (one-way ANOVA; F4,38 = 25.00, P < 0.0001) of sham or tumor-bearing mice with intact (GFP) or inactivated (TetTox) CGRP PBN neurons. Line graphs show mean ± s.e.m. Box plots show mean (+), median, quartiles (boxes) and range (whiskers) for each treatment. (h) Representative images of data in f and g. *P < 0.05, **P < 0.01, ***P < 0.001; scale bars, 100 µm. See also Supplementary Figure 2.
the effect of inactivating CGRP$^{\text{PBN}}$ neurons on feeding behavior, we bilaterally transduced these neurons with AAV1-DIO-hm4Di:TetTox at 10 weeks of age, before the onset of weight loss. In both male and female Calca$^{\text{Cre}}$:GFP/+:Apc$^{\text{min}}$/+ mice, food intake was increased and weight loss prevented by inactivation of CGRP$^{\text{PBN}}$ neurons compared to either of two control groups (Calca$^{\text{Cre}}$:GFP/+:Apc$^{\text{min}}$/+ and Calca$^{\text{Cre}}$:GFP/+: littermates injected with GFP virus; Fig. 5d–g and Supplementary Fig. 7a–c). The male cohort was killed for analysis at 17 weeks of age for Fos analysis, regardless of body weight differences between groups (some GFP Apc$^{\text{min}}$/+ control mice were euthanized earlier because they met euthanasia criteria). Apc$^{\text{min}}$/+ mice had increased Fos expression (compared to cancer-free littermates in the CeA and ovBNST, which was abolished with TetTox inactivation of CGRP$^{\text{PBN}}$ neurons (Supplementary Fig. 7a–c). The female cohort study continued until mice were euthanized based on the euthanasia criterion of losing 20% or more of peak body weight. Whereas the control group of tumor-bearing females continuously lost weight until criteria for euthanasia were met (average age ± s.e.m., 18.67 ± 0.53 weeks), the body weights of TetTox Apc$^{\text{min}}$/+ mice remained comparable to cancer-free littermates throughout most of their lives (Supplementary Fig. 7e). Weeks later (average age ± s.e.m., 22.50 ± 0.52 weeks), the latter animals exhibited a rapid drop in food intake (Supplementary Fig. 7f,g) and, after being killed for analysis, were found to have signs of gastrointestinal obstruction (stomachs distended with food despite cessation of feeding). Notably, these mice never exhibited signs of distress, which was assessed on a daily basis before they were killed (data not shown), suggesting a role for CGRP$^{\text{PBN}}$ neurons in this outcome as well. Thus, whereas CGRP$^{\text{PBN}}$ neuronal inactivation prolonged life by restoring the ability of Apc$^{\text{min}}$/+ mice to mount an adaptive hyperphagia that prevents weight loss, it did not protect animals from the underlying malignancy.

Role of CGRP$^{\text{PBN}}$ neurons in cancer-induced malaise

In humans, loss of appetite resulting from cancer or illness is typically associated with malaise, characterized by feelings of discomfort, anxiety and lack of motivation. To investigate the role played by CGRP$^{\text{PBN}}$ neuronal activation in these responses, we measured sickness behaviors in mice with either intact or TetTox-inactivated CGRP$^{\text{PBN}}$ neurons before and after LLC tumor implantation. To control for differences of food intake between groups, TetTox mice were pair-fed to the intake of tumor-bearing control mice until completion of behavioral studies, at which point the pair-feeding regimen was terminated and ad libitum access to food was restored (Supplementary Fig. 8a).

Lethargy can be assessed in rodents by measuring locomotor activity$^{20}$. Compared to tumor-bearing controls, locomotor activity was increased in TetTox tumor-bearing mice, whether measured as the total distance moved or the average velocity of movement (Fig. 6a–d). To determine whether CGRP$^{\text{PBN}}$ neuronal activation was similarly required for cancer-induced anxiety, we used open-field and elevated-plus-maze tests. Anxiety-like behavior was increased in tumor-bearing controls, and this manifestation of cancer was prevented by inactivation of CGRP$^{\text{PBN}}$ neurons (Fig. 6e,f,h,i). Tumor-bearing control mice also spent more time in a hunched posture (a sign of malaise), and this effect was prevented by CGRP$^{\text{PBN}}$ neuron inactivation (Fig. 6g,j). Nesting behavior offers an additional measure of malaise and lack of motivation in rodents$^{21,22}$. Using a protocol that takes into account...
Figure 4 Role of CGRP<sup>PNB</sup> neurons in cancer-induced cachexia and increased energy expenditure. (a) Time-course of experiments with Calca<sup>Cre/GenF<sup>B</sup></sup>/ mice that previously underwent stereotaxic injections of AAV1-DIO-GFP:TetTox (or AAV1-DIO-GFP control virus) into the PBN. (b–d) Calorimetry measurements from GFP control mice (n = 9) before (baseline) and after tumor implantation (VCO<sub>2</sub>, one-way repeated-measures ANOVA; F<sub>3,35</sub> = 15.62, P < 0.0001; VO<sub>2</sub>, one-way repeated-measures ANOVA; F<sub>3,35</sub> = 15.99, P < 0.0001; kcal per h, one-way repeated-measures ANOVA; F<sub>3,35</sub> = 19.27, P < 0.0001). (e–g) Calorimetry measurements taken from TetTox mice (n = 9) before (baseline or Base) and after tumor implantation (VCO<sub>2</sub>, one-way repeated-measures ANOVA; F<sub>3,35</sub> = 15.29, P < 0.0001; VO<sub>2</sub>, one-way repeated-measures ANOVA; F<sub>3,35</sub> = 18.00, P < 0.0001; kcal per h, one-way repeated-measures ANOVA; F<sub>3,35</sub> = 15.16, P < 0.0001). (h,i) Fat mass and lean body mass measurements of GFP control mice before tumor implantation, 17 d after tumor implantation (LLC1) and after developing anorexia (LLC2); hashtag indicates significant difference (#P < 0.05) between LLC1 and LLC2 (fat mass, one-way repeated-measures ANOVA; F<sub>2,26</sub> = 57.13, P < 0.0001; lean mass, one-way repeated-measures ANOVA; F<sub>3,35</sub> = 5.736, P < 0.01). (j) Average daily food intake of GFP mice before and after tumor implantation (one-way repeated-measures ANOVA; F<sub>3,35</sub> = 10.94, P < 0.0001). (k,l) Fat and lean body mass measurements from TetTox mice before (Base), 17 d after tumor implantation (LLC1) and after developing anorexia (LLC2); fat mass, one-way repeated-measures ANOVA; F<sub>2,26</sub> = 69.74, P < 0.0001; lean mass, one-way repeated-measures ANOVA; F<sub>3,35</sub> = 10.90, P < 0.001). (m) Average daily food intake of TetTox mice before and after tumor implantation (one-way repeated-measures ANOVA; F<sub>3,35</sub> = 3.718, P < 0.05). Box plots show mean (+), median, quartiles (boxes) and range (whiskers) for each treatment. *P < 0.05, **P < 0.01, ***P < 0.001; asterisks indicate significant differences compared to baseline; VCO<sub>2</sub>, carbon dioxide volume; VO<sub>2</sub>, oxygen volume. See also Supplementary Figures 4 and 5.
the rate and quality of nest construction, no differences were detected between GFP and TetTox mice at baseline. Whereas tumor implantation had no effect on nest construction in TetTox mice, control mice failed to finish their nest within 24 h (Fig. 6k,l).

After completion of behavioral studies, tumor-bearing TetTox mice were allowed *ad libitum* access to food. Unlike tumor-bearing controls, which by that point had become moribund and anorexic, TetTox mice nearly doubled their food intake upon termination of the pair-feeding regimen (Fig. 6m). This hyperphagic response reversed cancer-induced weight loss (Supplementary Fig. 8b), which suggests that the ability to eat enough additional food to compensate for weight loss was undermined by cancer anorexia and that this ability was restored by CGRP<sup>PBN</sup> neuronal inactivation. These outcomes cannot be attributed to differences in tumor mass, since tumors tended to be larger in TetTox mice than in controls, although the difference did not reach statistical significance (*P* = 0.09) in this experiment (Supplementary Fig. 8c).

**DISCUSSION**

Insight into neural circuits mediating cancer anorexia is essential for a better understanding of the underlying disease process and for the development of effective treatments. Here using viral and genetic techniques to visualize and selectively manipulate CGRP<sup>PBN</sup> neurons, we established that these neurons were activated in tumor-bearing mice and that this activation was required for cancer-induced appetite suppression and malaise.

Because of its key role in energy homeostasis, the hypothalamic arcuate nucleus has been a prominent focus of prior work in this field. In healthy animals, weight loss activates AgRP neurons<sup>23</sup>, which stimulates appetite<sup>24</sup>, reduces metabolic rate<sup>25</sup> and inhibits neural circuits that suppress appetite, including circuits involving both POMC-expressing neurons<sup>26</sup> and CGRP<sup>PBN</sup> neurons<sup>4</sup>. One hypothesis invoked to explain cancer anorexia proposes that, despite weight loss and appropriate changes in circulating hormones<sup>27-28</sup>, inflammation-mediated activation of POMC neurons increases downstream melanocortin signaling via the melanocortin-4 receptor (MC4R) and thereby inhibits food intake despite ongoing weight loss<sup>29</sup>. The same mechanism is proposed to simultaneously block activation of AgRP neurons<sup>8</sup>, which functionally oppose POMC neurons in part by releasing AgRP, an endogenous MC4R antagonist<sup>31</sup>. Indeed, cancer anorexia can be attenuated by central administration of MC4R antagonists<sup>32-33</sup>. Since CGRP<sup>PBN</sup> neurons express MC4R<sup>34</sup> and are supplied with inhibitory projections from AgRP neurons<sup>4</sup>, they are potential mediators of cancer anorexia lying downstream of the arcuate nucleus.

However, cancer anorexia is not always ameliorated by central injection of MC4R antagonists<sup>35</sup>, and cancer anorexia can be associated with seemingly appropriate responses of arcuate nucleus neurons to weight loss<sup>8,27-36</sup>. Furthermore, neurocircuits additional to the arcuate hypothalamus are implicated in other models of inflammatory anorexia<sup>39</sup>. For example, anorexia following administration of lipopolysaccharide, an endotoxin that induces systemic inflammation and anorexia, cannot be reversed by activation of AgRP neurons<sup>40</sup> but is ameliorated by inhibiting CGRP<sup>PBN</sup> neurons<sup>3</sup>. Mechanisms underlying cancer anorexia may therefore vary depending on the type of cancer and/or severity of cancer-induced inflammation, and they likely involve extra-hypothalamic anorexigenic circuits.

These considerations support a model in which cancer-associated inflammatory cytokines induce anorexia in part by activating the
‘parabrachial–amygdala threat circuit’\(^9\), potentially via effects on upstream vagal and spinal sensory pathways, in addition to effects on arcuate nucleus neurons. In support of this hypothesis, inactivation of CGRP\(^{PBN}\) neurons in mice with implanted LLC tumors prevented the onset of cancer anorexia, and chemogenetic inhibition of these neurons reversed established anorexia in these mice, a previously

**Figure 6** Inactivation of CGRP\(^{PBN}\) neurons attenuates sickness behaviors in LLC cancer model. (a,b) Hourly locomotor activity of mice with intact (GFP, \(n = 8\)) or inactivated (TetTox, \(n = 7\)) CGRP\(^{PBN}\) neurons, (a) before tumor implantation (pre-LLC) and (b) 13 d after implantation (pre-LLC, two-way repeated-measures ANOVA; interaction, \(F_{22, 286} = 1.12, P = 0.3222\); time, \(F_{22, 286} = 39.31, P < 0.0001\); LLC, two-way repeated-measures ANOVA; interaction, \(F_{1, 286} = 4.48, P < 0.0001\); time, \(F_{22, 286} = 15.91, P < 0.0001\); treatment, \(F_{1, 286} = 14.73, P = 0.0021\)). Grey-shaded region on x axis represents dark cycle. (c,d) Total distance traveled and average velocity of locomotor activity during 23-h recording period of GFP and TetTox mice before and after tumor implantation (total distance, two-way repeated-measures ANOVA; interaction, \(F_{1,13} = 8\); or inactivated (TetTox, \(F_{1,13} = 16.74, P = 0.0013\); distance moved, two-way repeated-measures ANOVA; interaction, \(F_{1,13} = 1.59, P = 0.2299\); time, \(F_{1,13} = 1.02, P = 0.3310\); treatment, \(F_{1,13} = 0.28, P = 0.6045\); time in hunched posture, two-way repeated-measures ANOVA; interaction, \(F_{1,13} = 9.82, P = 0.0079\); time, \(F_{1,13} = 5.92, P = 0.0302\); treatment, \(F_{1,13} = 16.26, P = 0.0014\)). (k,l) Quantification and representative images (24-h timepoint) of nesting behavior before (pre) and 16 d after tumor implantation (two-way ANOVA; interaction, \(F_{15, 156} = 3.01, P = 0.0003\); time, \(F_{15, 156} = 22.80, P < 0.0001\); treatment, \(F_{2, 156} = 44.61, P < 0.001\)). (m) Daily food intake of GFP and TetTox mice after tumor implantation (two-tailed Student’s t test on day 17, \(t_{13} = 6.36, P < 0.0001\)). TetTox mice were pair-fed to the intake of GFP mice until day 16, after which TetTox mice were allowed *ad libitum* access to food. Line graphs show mean ± s.e.m. Box plots show mean (+), median, quartiles (boxes) and range (whiskers) for each treatment. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\). See also Supplementary Figure 8.
unreported and potentially clinically relevant finding. CGRP\textsuperscript{PBN} neuron inactivation also enabled animals to increase food intake to counteract cancer-induced weight loss. In \textit{Apc\textsuperscript{min/+}} mice, this effect prevented cancer-induced weight loss, and in LLC tumor-bearing mice, it enabled robust hyperphagia after being pair-fed to the intake of LLC tumor-bearing controls.

That CGRP\textsuperscript{PBN} neuron inactivation conferred the capacity of tumor-bearing mice to increase food intake in response to weight loss implies that the brains of tumor-bearing mice did in fact receive appropriate energy-deficit signals but that activation of CGRP\textsuperscript{PBN} neurons blocked their transduction into an adaptive feeding response. Our data suggest that the CeA and BNST lie downstream of CGRP\textsuperscript{PBN} neurons in this anorexigenic pathway. Although CGRP\textsuperscript{PBN} neurons do not have direct axonal projections to the arcuate nucleus, the CeA and BNST have extensive hypothalamic connections that may ultimately be integrated with hypothalamic homeostatic signals that regulate appetite\textsuperscript{41}.

We selected the LLC and \textit{Apc\textsuperscript{min/+}} cancer models for our studies because they differ in several key aspects, including their effect on feeding behavior (anorexia versus failure to escalate food intake to compensate), time-course of weight loss (weeks versus months), source of tumor (implantable versus genetic mutation) and tumor location (subcutaneous versus gastrointestinal). Given these differences, we suspect that CGRP\textsuperscript{PBN} neurons represent a common node for suppression of appetite by many cancers, and additional studies are warranted to test this hypothesis. We also note that since activation of CGRP\textsuperscript{PBN} neurons can prevent the expected compensatory increase in food intake during weight loss, this mechanism may contribute to weight loss even when food intake is not obviously affected.

Cancer anorexia, cachexia and tumor growth

Loss of lean body mass is a major contributor to cancer-related mortalities\textsuperscript{16} that can potentially arise from circulating factors (released by either the tumor or the animal) acting on muscle tissue, combined with increased energy expenditure, metabolic dysregulation and reduced food intake\textsuperscript{16}. Using the LLC tumor model, we provided evidence implicating activation of CGRP\textsuperscript{PBN} neurons in this wasting process, since cancer-induced loss of lean body mass was ameliorated by inactivation of these neurons and prevention of anorexia. From this observation, one might predict that provision of sufficient calories would prevent or reverse weight loss in cancer patients. In the clinic, however, this is not typically observed, presumably because of an ongoing catabolic state that is not remedied by providing nutritional support\textsuperscript{42}. This observation has diverted attention from the problem of anorexia because it implies that restoring normal food intake will not suffice to prevent cachexia, a problem compounded by the fact that conventional therapeutic agents used to stimulate appetite in cancer patients have glucocorticoid-like effects\textsuperscript{43} that may exacerbate muscle wasting\textsuperscript{44,45}. In this context, our finding that inhibition of CGRP\textsuperscript{PBN} neurons protected against loss of lean body mass raises the possibility that these neurons influence processes involved in cachexia independently of their effect on food intake. Future studies to examine the role of these neurons in cancer cachexia are therefore warranted.

Although our studies were not designed to investigate the roles of either CGRP\textsuperscript{PBN} neurons or anorexia on tumor growth, we observed larger tumors in two separate experiments in which mice with inactive CGRP\textsuperscript{PBN} neurons had \textit{ad libitum} access to food. That this increase in tumor growth depends on increased food intake is consistent with evidence that mice fed the same amount of food as tumor-bearing control mice did not develop larger tumors. It remains possible, however, that activation of CGRP\textsuperscript{PBN} neurons also elicited food intake-independent responses that limit tumor growth. Recent studies suggest that activation of CGRP\textsuperscript{PBN} neurons contributes to chemotherapy-induced anorexia and weight loss\textsuperscript{46}. Examination of tumor growth in mice receiving chemotherapy treatment will be needed to examine the potential clinical utility of inhibiting CGRP\textsuperscript{PBN} neurons in cancer patients.

Cancer-induced malaise

Cancer patients often experience profound deterioration in quality of life as their disease progresses, marked by feelings of malaise and associated ‘sickness behaviours’. Although these behavioral consequences of cancer likely have a complex, multifactorial etiology, the fact that tumor-bearing mice exhibit similar responses indicates that at least some of the underlying mechanisms are shared across species. We found that mice with implanted LLC tumors exhibited behavioral phenotypes suggestive of lethargy, anxiety and malaise. The observation that these behaviors were ameliorated by inactivation of CGRP\textsuperscript{PBN} neurons suggests that in addition to suppressing appetite, cancer-induced activation of CGRP\textsuperscript{PBN} neurons contributed to the associated affective–motivational state. These findings are consistent with an emerging view that the external lateral PBN (including CGRP neurons) plays a critical role in coordinating behavioral, affective and neuroendocrine responses to noxious stimuli\textsuperscript{47}.

Future dissection of downstream CGRP\textsuperscript{PBN} neuronal pathways may assist in determining whether anorexia and associated sickness behaviors involve separate or overlapping neural pathways. Because our behavioral studies were performed using a pair-feeding regimen, to prevent potentially confounding differences of intake between groups, the possibility can be considered that increased hunger (induced by pair-feeding) contributed to the effect of CGRP\textsuperscript{PBN} neuron inactivation to alleviate sickness behaviors. We view this explanation as unlikely, however, since chronic food restriction in healthy mice does not increase homecage locomotor activity\textsuperscript{48} and because anorexia and decreased locomotor activity are separable features of illness\textsuperscript{20,33}. Although anxiety-like behaviors are sensitive to perceived energy deficits\textsuperscript{49,50}, we note that even in sated mice, experimental activation of CGRP\textsuperscript{PBN} neurons is sufficient to elicit behavioral stress responses\textsuperscript{9}.

CONCLUSIONS

In summary, our data indicate that activation of CGRP\textsuperscript{PBN} neurons is both necessary and sufficient to explain major affective–motivational features of cancer, including anorexia, lethargy, anxiety and malaise. Translation of these findings to the clinic may ultimately assist in the identification of therapeutics with the potential to substantially improve the quality of life of patients with cancer.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
C.A.C., B.E.W., R.D.P. and M.W.S. conceived and designed the study. C.A.C. and A.J.B. performed and analyzed histological and feeding experiments. C.A.C., A.J.B. and S.H. performed and analyzed sickness behavior tests. R.D.P. and M.W.S. provided equipment and reagents. C.A.C. and M.W.S. wrote the manuscript with input from all authors.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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1. Hart, B.L. Basic principles of the behavior of sick animals. Neurosci. Biobehav. Rev. 12, 123–137 (1988).
2. Ezeoke, C.C. & Morley, J.E. Pathophysiology of anorexia in the cancer cachexia syndrome. J. Cachexia Sarcopenia Muscle 6, 287–302 (2015).
3. Carter, M.E., Soden, M.E., Zweifel, L.S. & Pimpler, R.D. Genetic identification of a brain circuit that suppresses appetite. Nature 503, 111–114 (2013).
4. Campos, C.A., Bowen, A.J., Schwartz, M.W. & Pimpler, R.D. Parabrachial GGRP neurons control the regulation of food intake. Cell Metab. 23, 811–820 (2016).
5. Roman, C.W., Derkach, V.A. & Pimpler, R.D. Genetically and functionally defined NTS to PBN brain circuits mediating anorexia. Nat. Commun. 7, 11905 (2016).
6. Tsai, V.W. et al. The anorectic actions of the TGFβ cytokine MIG-1GD15 is an intact brainstem area postrema and nucleus of the solitary tract. PLoS One 9, e100370 (2014).
7. Ruud, J. & Blomqvist, A. Identification of rat brainstem neuronal structures activated during cancer-induced anorexia. J. Comp. Neurol. 504, 275–286 (2007).
8. Scarlett, J.M. et al. Regulation of agouti-related protein messenger ribonucleic acid transcription and peptide secretion by acute and chronic inflammation. Endocrinology 149, 4837–4845 (2008).
9. Han, S., Soleiman, M.T., Soden, M.E., Zweifel, L.S. & Pimpler, R.D. Elucidating an effective pain circuit that creates a threat memory. Cell 162, 363–374 (2015).
10. Moser, A.R., Pitot, H.C. & Dove, W.F. A dominant mutation that predisposes to folic acid deficiency in mice. J. Comp. Neurol. 235, 123–137 (1988).
11. Mehli, K.A., Davis, J.M., Berger, F.G. & Carson, J.A. Myofiber degeneration/regeneration is induced in the cachectic ApcMin/+ mouse. J. Appl. Physiol. 99, 2379–2387 (2005).
12. Puppa, M.J. et al. Gut barrier dysfunction in the ApoCIII+/- mouse model of colon cancer cachexia. Biochem. Biophys. Acta 1812, 1601–1606 (2011).
13. Kim, J.C. et al. Linking genetically defined neurons to behavior through a broadly applicable silencing allele. Neuron 63, 305–315 (2009).
14. Davis, M., Walker, D.L., Miles, L. & Grillon, C. Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. Neuropsychopharmacology 35, 105–135 (2010).
15. Neugebauer, V., Li, W., Bird, G.C. & Han, J.S. The amygdala and persistent pain. Neuroscientist 10, 221–234 (2004).
16. Fearon, K., Arends, J. & Baracos, V. Understanding the mechanisms and treatment options in cancer cachexia. Nat. Rev. Clin. Oncol. 10, 90–99 (2013).
17. Kir, S. et al. Tumour-derived PTH-related protein triggers adipose tissue browning and cancer cachexia. Nature 513, 100–104 (2014).
18. Brooks, S.L., Neville, A.M., Rothwell, N.J., Stock, M.J. & Wilson, S. Sympathetic activation of brown-adipose-tissue thermogenesis in cachexia. Biosci. Rep. 1, 509–517 (1981).
19. Tucker, J.M. et al. Response to 5-fluorouracil chemotherapy is modified by dietary folic acid deficiency in ApcMin+/- mice. Cancer Lett. 187, 153–162 (2002).
20. Grossberg, A.J. et al. Inflammation-induced lethargy is mediated by suppression of orexin neuron activity. J. Neurosci. 31, 11376–11386 (2011).
21. Gaskill, B.N., Karas, A.Z., Garner, J.P. & Pritchett-Conring, K.R. Nest building as an indicator of health and welfare in laboratory mice. J. Vis. Exp. 82, 51012 (2013).
22. Szczypta, M.S. et al. Dopamine production in the caudate putamen restores feeding in dopamine-deficient mice. Neuron 30, 819–828 (2001).
23. Morton, G.J., Meek, T.H. & Schwartz, M.W. Neurobiology of food intake in health and disease. Nat. Rev. Neurosci. 15, 367–378 (2014).
24. Aponte, Y., Atasoy, D. & Sternson, S.M. AgRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. Nat. Neurosci. 14, 351–355 (2011).
25. Krashe, M.J. et al. Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. J. Clin. Invest. 121, 1424–1428 (2011).
26. Atasoy, D., Betley, J.N., Su, H., & Sternson, S.M. Deconstruction of a neural circuit for hunger. Nature 488, 172–177 (2012).
27. Bing, C., Taylor, S., Tisdale, M.J. & Williams, G. Cachexia in MAC16 adencocarcinoma suppression of hunger despite normal regulation of leptin, insulin and hypothalamic neuropeptide Y. J. Neurochem. 79, 1004–1012 (2001).
28. Shimizu, Y. et al. Increased plasma ghrelin level in lung cancer cachexia. Clin. Cancer Res. 9, 774–778 (2003).
29. Grossberg, A.J. et al. Arcuate nucleus proopiomelanocortin neurons mediate the acute anorectic actions of leukemia inhibitory factor via gp130. Endocrinology 151, 606–616 (2010).
30. Marks, D.L., Ling, N. & Cone, R.D. Role of the central melanocortin system in cancer cachexia. Cancer Res. 61, 1432–1438 (2001).
31. Paes, J., Mackerlova, L. & Blomqvist, A. Expression of melanocortin-4 receptor by parabrachial neurons responsive to immune and aversive stimuli. Neuroscience 141, 287–297 (2006).
32. Chance, W.T., Sheriff, S., Dayal, R. & Balasubramaniam, A. refractory hypothalamic alpha-mSH safety and AGRP feeding systems in rats bearing MCA sarcomas. Peptides 24, 1909–1919 (2003).
33. Wisse, B.E., Schwartz, M.W. & Cummings, D.E. Reversal of cancer anorexia by blockade of central melanocortin receptors in rats. Endocrinology 142, 2014–2020 (2001).
34. Paes, J., Mackerlova, L. & Blomqvist, A. Expression of melanocortin-4 receptor by parabrachial neurons responsive to immune and aversive stimuli. Neuroscience 141, 287–297 (2006).
35. Reyes, T.M. & Sawchenko, P.E. Involvement of the arcuate nucleus of the hypothalamus in interleukin-1-induced anorexia. J. Neurosci. 22, 5091–5099 (2002).
36. Liu, Y. et al. Lipopolysaccharide rapidly and completely suppresses Agrp neuron-mediated food intake in male mice. Endocrinology 157, 2380–2392 (2016).
37. Liao, Y. et al. The amygdaloid complex: anatomy and physiology. Physiol. Rev. 83, 803–834 (2003).
38. Nixen, D.W. et al. Hyperalimentation of the cancer patient with protein-calorie undernutrition. Cancer Res. 41, 2038–2045 (1981).
39. Mann, M. et al. Agouti-related protein activity of megestrol. A summary of Food and Drug Administration experience and a review of the literature. Arch. Intern. Med. 157, 1651–1656 (1997).
40. Simons, J.P. et al. Effects of medroxyprogesterone acetate on food intake, body composition, and resting energy expenditure in patients with advanced, non-hormone-sensitive cancer: a randomized, placebo-controlled trial. Cancer 82, 553–560 (1998).
41. Schramm, O., Kalista, S., Barbé, C., Loumaye, A. & Thissen, J.P. Agouti-related skeletal muscle atrophy. Int. J. Biochem. Cell Biol. 45, 2163–2172 (2013).
42. Alhadeff, A.L., Holland, R.A., Nelson, A., Grill, H.J. & De Jonghe, B.C. Glutamate receptors in the central nucleus of the amygdala mediate cisplatin-induced malaise and energy balance dysregulation through directed hindbrain projections. J. Neurosci. 35, 11094–11104 (2015).
43. Salep, C.B. The house alarm. Cell Metab. 23, 754–755 (2016).
44. Yamamoto, K. et al. Changes in behavior and gene expression induced by caloric restriction in C57BL/6 mice. Physiol. Genomics 39, 227–235 (2009).
45. Dietrich, M.O., Zimmer, M.R., Bober, J. & Horvath, L.T. Hypothalamic Agp neurons drive stereotypic behaviors beyond feeding. Cell 160, 1222–1232 (2015).
46. Padilla, M.L. et al. Agouti-related peptide nervous circuits mediate adaptive behaviors in the starved state. Nat. Neurosci. 19, 734–741 (2016).
ONLINE METHODS

Animals. CalcaCreGFP/+ mice (C57Bl/6 background) were generated and maintained as previously described. Male CalcaCreGFP/+ mice were used for all behavioral and immunohistochemical Fos studies involving LLC tumor implantation. Both genders were used for experiments involving CalcaCreGFP+/−/Apoc3−/−, double-transgenic mice. Each experiment involved mice from at least six different litters. Mice (ranging from 3–6 months of age) were assigned into each group to counterbalance for differences in body weight and age. Investigators were not blinded to treatment conditions. Following stereotaxic surgery, mice were singly housed for at least 3 weeks before and during experimentation, with ad libitum access (unless noted otherwise) to standard chow diet (LabDiet 5053) in temperature- and humidity-controlled facilities with 12/12-h light/dark cycles. Behavioral experiments were conducted during the light cycle, unless stated otherwise. The criteria for euthanasia was a cumulative score of 8 from the grading rubric (Supplementary Table 1), which involves body weight and subjective observations of distress-like symptoms. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington.

Virus production. AAV1-CA-BD-DIO-GFP: TetTox, AAV1-EF1a-DIO-GFP, AAV1-EF1a-DIO-hM4Di:mCherry and AAV1-EF1a-DIO-mCherry viral vectors were produced by transfecting HEK cells and then purifying cell extracts by pelleting through sucrose and by CsCl-gradient ultracentrifugation. Final pellets were suspended in 0.1 M PBS.

Stereotaxic surgery. All mice were anesthetized with 1.5 to 2.0% isoflurane (mixed with 0.8 L per min of oxygen) for stereotaxic surgeries and virus injections. Virus (0.3 µL per side) was injected using a glass capillary attached to a Nanoject (Drummond, catalog #3-00-204) using the following coordinates: −4.65 mm posterior to bregma, −1.30 mm lateral from midline and −2.90 mm ventral to dura. These coordinates were verified with Fluoro-Gold (Sigma-Aldrich, catalog #39286) injections in nonexperimental mice, which produced Fluoro-Gold labeling that was restricted to the lateral PBN.

Tumor cell culturing and implantation. LLC cells were cultured in DMEM with 10% FBS (FBS) as recommended by the supplier (American Type Culture Collection; catalog #CRL-1642; lot #62996849), harvested and stored in 1.7-mL aliquots (10% glycerol in culture media) in liquid nitrogen for long-term storage. The cell line was authenticated by the supplier, but subcultures were not authenticated before tumor implantation. At the beginning of each experiment, cells were defrosted, cultured, harvested, pelleted and suspended in fresh DMEM with 10% FBS. Approximately 1 × 10⁶ cells (in 0.2 mL of DMEM solution) were injected subcutaneously into the left flank of mice.

Body composition analysis. Measures of fat and lean body mass were determined in live mice using quantitative magnetic resonance (QMR) spectroscopy (EchoMRI 3-in-1, Echo MRI) QM. QMR measures of fat content were validated by scanning a calibration holder containing a known amount of fat. Because tumors are detected as lean body mass by the QMR machine (verified with isolated tumor samples), the tumor mass was subtracted from the lean body mass measurement for final analysis of body composition in tumor-bearing mice.

Indirect calorimetry. Mice were acclimated to metabolic cages before measurement of energy expenditure using a computer-controlled indirect calorimetry system (Prometheus, Sable Systems) made available through the University of Washington Nutrition Obesity Research Center (NORC) Energy Balance and Glucose Metabolism (EBGM) Core. Calorimeter cages (similar to home cages with bedding) were equipped with water bottles and housed in a temperature in a temperature- and humidity-controlled cabinet (Caron Products and Services). O₂ consumption and CO₂ production was measured for each animal for 1 min at a time at 10-min intervals as described. The respiratory quotient (RQ) was calculated as the ratio CO₂ production and O₂ consumption. Energy expenditure was calculated using the Weir equation. To control for the influence of body size variation on total energy expenditure, group comparisons were adjusted for total body mass using analysis of covariance. Data acquisition and analysis were coordinated by MetaScreen v. 1.6.2., and the raw data was processed using ExpeData v. 1.4.3. (Sable Systems). For further details regarding analysis of energy expenditure, see the “Experimental designs” subsection below.

Behavioral measures. Food intake and bodyweight measurements. Food intake and body weights were monitored manually on a daily basis, including during an acclimation period (about 3 d) before tumor implantation.

Locomotor activity. Mice were housed in a home cage Phenotyper (Noldus) apparatus equipped with infrared video tracking, made available through the University of Washington Center on Human Development and Disability (CHDD) Mouse Behavior Lab. Video files were analyzed with EthoVision software (v. XT 10, Noldus). Mice were housed in these cages for 3 d before tumor implantation (starting on day −8) and for an additional 3 d after tumor implantation (starting on day 10). Data were analyzed from the third day (for both before and after tumor implantation), after mice had acclimated to the cage.

Open-field test. Mice were placed in the center of a 40 × 40-cm square open-field arena with nontransparent white Plexiglas. The total distance moved and time spent in the center (20 × 20-cm imaginary square), during the 10-min trial, were analyzed with video-tracking software (EthoVision XT 10, Noldus). Hunched posture was defined as ≤35% of the maximum body elongation length using a three-point detection setting that detects the distance between the nose, midsection and base of the tail.

Elevated-plus-maze test. The maze used in this study has two closed arms (5.1 × 30 cm) surrounded by 20-cm high nontransparent walls and two open arms (5.1 × 30 cm). The total distance moved and time in closed and open arms (during the 10 min trial) was analyzed with EthoVision.

Nest construction. To examine nesting behavior, mice were transferred to a new home cage with clean bedding and new nest-building material, a 5 × 5 cm square of compressed cotton (Nestlets, Ancare). Nest building was scored 2, 4, 6, 8 and 24 h later, as described.

Experimental designs. LLC cohort 1 (Fig. 1). Tumor-bearing and sham-treated mice (including those pair-fed to the intake of tumor-bearing mice) were killed for analysis after a statistical difference was observed between the food intake of tumor-bearing mice and sham-treated mice with ad libitum food access. Because CGRPRBD neurons are activated after consuming a large meal, brains from tumor-bearing mice and healthy sham-treated controls were obtained during the light cycle (when food intake is minimal), 4 h after food removal. These mice were killed for analysis 14 d after tumor implantation and Fos immunoreactivity was analyzed in the PBN.

LLC cohort 2 (Fig. 2). Five experimental groups were examined for this study; each received bilateral PBN injections of either AAV1-DIO-GFP or AAV1-DIO-GFP:TetTox before tumor implantation or sham treatment; GFP LLC, TetTox LLC, pair-fed TetTox LLC, GFP sham and pair-fed GFP sham. A sixth group, TetTox unlat LLC, was added after post hoc confirmation of (nonpurposeful) unilateral viral transduction. To acclimate mice to testing procedures, food intake and body weights were recorded manually on a daily basis (1 h before the start of the dark cycle) several days before tumor implantation. Mice had ad libitum access to food, with the exception of pair-fed control groups, which were fed the same amount of food that their tumor-bearing GFP counterparts were consuming after tumor implantation (individually matched before tumor implantation based on bodyweight). Fourteen days after tumor implantation, food was withheld for 4 h during the light cycle, after which mice were killed for analysis for immunohistochemical detection of Fos in the CeA and oVBNST.

LLC cohort 3 (Fig. 3). Four experimental groups were examined for this study; each received bilateral PBN injections of either AAV1-DIO-mCherry or AAV1-DIO-hM4Di:mCherry before tumor implantation or sham treatment; mCherry LLC, hM4Di LLC, mCherry sham and hM4Di sham. After an acclimation period of daily food intake and body weight monitoring, mice were implanted with LLC cells (or sham-treated) and had ad libitum food access for the entirety of the study. After approximately 2 d of decreased food intake, tumor-bearing mice received twice-daily injections of CNO (2 mg/kg i.p., Sigma) and two-bottle choice of water or CNO-containing water (0.03 mg/mL, NIMH Chemical Synthesis and Drug Supply Program). The criteria for euthanasia involved a cumulative score of 8 from the grading rubric (Supplementary Table 1), which includes body weight and subjective observations of distress-like symptoms. None of the sham-treated or hM4Di tumor-bearing mice reached the criteria for euthanasia, but they were matched with mCherry tumor-bearing mice (based on when CNO
Images were acquired at 10× and 20× magnification of 8; criteria for euthanasia (loss of 20% peak body weight or cumulative distress score).

Apcmin/+ cohorts (Fig. 5). For PBN Fos analysis, the first group of Calca−/− mice (male and female) were killed for analysis at 16 weeks of age. For feeding behavior analysis, Calca−/− mice received bilateral PBN injections of either AAV1-DIO-GFP or AAV1-DIO-GFP:TetTox at 10 weeks of age, and food intake and body weights were monitored starting at 12 weeks of age. Calca−/− littermate controls were used as controls and received bilateral PBN injections of AAV1-DIO-GFP. The male cohort of animals were killed for analysis at 17 weeks of age (regardless of body weight); some were euthanized earlier if they met the pre-established criteria for euthanasia (loss of 20% peak body weight or cumulative distress score of 8; Supplementary Table 1). Brains from the male cohort were processed for immunohistochemical analysis of Fos in the CeA and ovBNST. The female cohort of mice was euthanized based on the pre-established criteria for euthanasia (loss of 20% peak body weight or cumulative distress score of 8; Supplementary Table 1).

LLC cohort 5 (Fig. 6). For sickness-behavioral analyses of tumor-bearing mice with intact or inactivated CGRP+/− neurons, we used a within-subjects experimental design involving GFP and TetTox mice before and after tumor implantation. TetTox mice were pair-fed during days 5–16 after tumor implantation to control for differences in food intake. We conducted anxiety tests 4 d before and 15 d after tumor implantation. Nesting behavior was analyzed 3 d before and 16 d after tumor implantation. Locomotor activity was analyzed from data collected 5 d before and 13 d after tumor implantation.

Immunohistochemistry. For all immunohistochemical experiments, mice were anesthetized (Behaasana, 320 mg/kg delivered i.p.) and intracardially perfused with 0.1 M PBS followed by 4% paraformaldehyde. Brains were then extracted, postfixed in 4% paraformaldehyde overnight and cryoprotected in 0.1 M PBS followed by 4% paraformaldehyde. Coronal cryostat sections 30-µm thick were collected, and every third section of the PBN and BNST, or every fourth section of the CeA, were processed for immunolabeling and quantification. For co-labeling of GFP and Fos, sections were incubated for 16 h at room temperature (20–22 °C) in chicken anti-GFP (1:10,000, catalog # ab13970, lot #GR236651-4, Abcam) and goat anti-Fos (1:700, catalog # sc-52-G, lot #F1615, Santa Cruz Biotechnology). Because we ran out of our original Fos antibody, tissue in Figure 4 was stained with goat anti-Fos from a different lot (1:700, catalog # sc-52-G, lot #F1616 Santa Cruz Biotechnology). The sections were then washed and incubated for 2 h at room temperature in Alexa Fluor 488-conjugated donkey anti-chicken (1:400, Jackson ImmunoResearch) and CY5-conjugated donkey anti-goat (1:400, Jackson ImmunoResearch).

Microscopy and Fos analysis. Images were acquired at 10× and 20× magnification for figure presentation and quantification, respectively, using a laser-scanning confocal microscope (FV1200, Olympus). Fos and GFP colocalization counts were performed using the Cell Counter feature on ImageJ (v. 1.48). Cell counts were obtained from two sections for each brain region corresponding to the following coordinates56: −5.15 mm and −5.25 mm from bregma, PBN (unilateral, left PBN); −1.55 mm and −1.75 mm from bregma, CeA (bilateral); 0.15 mm and 0.25 mm from bregma, BNST (bilateral).

Statistics. Data were analyzed using Prism 5.0 (GraphPad Software). Sample sizes were estimated based on prior experience and expected variability in behaviors between tumor-bearing animals. We excluded an animal from data analysis if post hoc histological analysis showed no viral transduction, as indicated by an absence of GFP or mCherry fluorescence. For graphs comparing two experimental conditions, we used unpaired two-tailed Student’s tests. For graphs comparing multiple treatments (single timepoints), we used one-way repeated-measures ANOVA with Tukey’s post hoc multiple comparisons tests. Datasets with multiple treatments and timepoints were analyzed with two-way repeated-measures ANOVA (time-repeated factor) followed by Bonferroni’s post hoc tests. For analysis of Apcmin/+ experiments, which involved euthanasia of mice during the study, we used regular (not repeated-measures) two-way ANOVA (time-repeated factor). Normality and variance were examined with Shapiro–Wilk tests and Bartlett’s tests, respectively. A Supplementary Methods Checklist is available.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

51. Taicher, G.Z., Tinsley, F.C., Redereman, A. & Heiman, M.L. Quantitative magnetic resonance (QMR) method for bone and whole-body-composition analysis. Anal. Bioanal. Chem. 377, 990–1002 (2003).
52. Kaiyala, K.J. et al. Acutely decreased thermoregulatory energy expenditure or decreased activity energy expenditure both acutely reduce food intake in mice. PLoS One 7, e41473 (2012).
53. Weir, J.B.D. New methods for calculating metabolic rate with special reference to protein metabolism. J. Physiol. (Lond.) 109, 1–9 (1949).
54. Butler, A.A. & Kozak, L.P. A recurring problem with the analysis of energy expenditure in genetic models expressing lean and obese phenotypes. Diabetes 59, 323–329 (2010).
55. Kaiyala, K.J. et al. Identification of body fat mass as a major determinant of metabolic rate in mice. Diabetes 59, 1657–1666 (2010).
56. Han, S., Tai, C., Jones, C.J., Scheuer, T. & Catterall, W.A. Enhancement of inhibitory neurotransmission by GABAA receptors having α2,3-subunits ameliorates behavioral deficits in a mouse model of autism. Neuron 81, 1282–1289 (2014).
57. Deacon, R.M. Assessing nest building in mice. Nat. Protoc. 1, 1117–1119 (2006).
58. Dong, H.W. Allen Reference Atlas: A Digital Color Brain Atlas of the C57Black/6J Male Mouse (Wiley, 2008).