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Administration of IL-1 β to the 4th ventricle causes anorexia that is blocked by agouti-related peptide and that coincides with activation of tyrosine-hydroxylase neurons in the nucleus of the solitary tract

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Abstract

Inflammation-associated cachexia is associated with multiple chronic diseases and involves activation of appetite regulating centers in the arcuate nucleus of the hypothalamus (ARH). The nucleus of the solitary tract (NTS) in the brainstem has also been implicated as an important nucleus involved in appetite regulation. We set out to determine whether the NTS may be involved in inflammation-associated anorexia by injecting IL-1 β into the 4th ventricle and assessing food intake and NTS neuronal activation. Injection of IL-1 β produced a decrease in food intake at 3 and 12 h after injection which was ameliorated at the 12 h time point by a sub-threshold dose of agouti-related peptide (AgRP). Investigation into neuron types in the NTS revealed that IL-1 β injection was associated with an increase in c-Fos activity in NTS neurons expressing tyrosine hydroxylase (TH). Additionally, injection of IL-1 β into the 4th ventricle did not produce c-Fos activation of neurons expressing pro-opiomelanocortin (POMC) in the ARH, cells known to be involved in producing anorexia in response to systemic inflammation. Double-label *in situ* hybridization revealed that TH neurons did not express IL-1 receptor I (IL1-RI) transcript, demonstrating that c-Fos activation of TH neurons in this setting was not via direct stimulation of IL-1 β on TH neurons themselves. We conclude that IL-1 β injection into the 4th ventricle produces anorexia and is accompanied by an increase in activation in TH neurons in the NTS. This provides evidence that the brainstem may be an important mediator of anorexia in the setting of inflammation.

Keywords

Tyrosine hydroxylase; Brainstem; Appetite regulation; IL-1 β ; Cytokines; AgRP

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1. Introduction

Inflammation is a common link to cachexia caused by a variety of underlying conditions [42]. These processes, including cancer, renal failure and heart disease, involve up-regulation of pro-inflammatory cytokines and are associated with anorexia, loss of lean body mass and increased resting energy expenditure [8]. Prior experiments in our lab and others have implicated a role for melanocortin neurons in the hypothalamus in producing these symptoms, with marked activation of pro-opiomelanocortin (POMC) neurons and a co-incident decrease in feeding behavior following peripheral injection of lipopolysaccharide (LPS) or intracerebroventricular (ICV) injection of IL-1 β into the 3rd ventricle [6,21,24,25,38]. We have also shown that POMC neurons in the arcuate nucleus of the hypothalamus (ARH) express IL-1 receptor (IL1-R) and that there is an increase in c-Fos activation in POMC neurons following ICV injection of IL-1 β into the lateral ventricles [38]. Conversely, ICV injection of agouti-related protein (AgRP, an endogenous antagonist of α -MSH action at the melanocortin-3 and 4 receptors [MC3-R and MC4-R]) in widespread nuclei overcomes the anorexic effects of cachexia and results in an increase in feeding behavior [3,24,25].

POMC is also expressed in the nucleus of the solitary tract (NTS) in the brainstem, a nucleus that is involved in several processes important to feeding behavior, including taste reception, gastric motility, and response to fasting and has been further implicated in the response to cancer cachexia [14,15,30,37,47]. In the NTS POMC expression is decreased in response to fasting and POMC neurons in the NTS have been shown to be activated by cholecystokinin (CCK), leptin [9,11], and PYY [2]. Additionally, the NTS is located adjacent to the dorsal motor nucleus of the vagus (DMN) and receives afferent inputs from stretch receptors in the stomach and intestines and sends efferent signals affecting intestinal motility [15,34]. The NTS is also situated near the area postrema, a circumventricular organ on the floor of the 4th ventricle that provides exposure to circulating factors such as cytokines. Interestingly, in previous studies using IL-1 β injections to the lateral ventricles of the brain we did not note any sign of activation of POMC neurons in the brainstem but did note a significant increase in c-Fos activation of unidentified neurons in the NTS [38].

Another class of neurons present in the NTS expresses tyrosine hydroxylase (TH). These neurons include A2 neurons involved in noradrenergic output. There are multiple reasons to suspect that TH neurons in the NTS would be involved in appetite regulation. These neurons express bombesin, an appetite-suppressing substance and are in close approximation to neurons expressing melanin-concentrating hormone, an orexigenic peptide [23,46]. Expression of TH in the NTS is altered with fasting [17], and TH-expressing neurons are involved in glucose regulation [16,35]. A recent report identified these neurons as being activated during the anorexia produced by administration of serotonin-receptor agonists [20]. Finally, TH-expressing neurons have been shown to be involved in taste sensation, an important component of appetite [5], and they participate in gastric motility via esophageal–gastric reflex [10,36].

TH neurons in the NTS have not been shown to project directly to the ARH, but project to the locus coeruleus and parabrachial nucleus, both of which are heavily innervated by fibers from POMC-expressing neurons in the arcuate [1,27,28,40,43]. Given the known appetite-regulating roles of the NTS, we set out to investigate the effects of a local inflammatory stimulus—IL-1 β injected to the 4th ventricle—on feeding behavior as well as the response of POMC and TH neurons to inflammation.

2. Methods

2.1. Animals and surgical procedures

Male Sprague–Dawley rats (300–350 g; Charles River Laboratories, Wilmington, DE) were maintained on a normal 12:12 light/dark cycle with *ad libitum* access to food (Purina rodent diet 5001; Purina Mills) and water. Experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committees of Oregon Health and Science University.

2.2. ICV cannulation and injections

Rats were anesthetized with a ketamine cocktail (25 mg/mL Ketamine, 5 mg/mL Xylazine, 1 mg/mL Acepromazine, and sterile water, administered at 0.15 mL/100 g of rat body weight) and placed in a stereotaxic apparatus (Cartesian Instruments). A sterile guide cannula with obturator stylet (Plastics One, Roanoke, VA) was stereotactically implanted into the fourth ventricle (on the midline, 2.5 mm anterior to occipital suture line, and 5.2 mm below the surface of the skull). Rats receiving lateral ventricle cannulations underwent the same procedure with the guide cannula stereotactically implanted into the lateral ventricle (1.0 mm posterior to bregma, 0.5 mm lateral to midline, and 2.3 mm below the surface of the skull). These cannulae were then fixed in place with dental cement. The animals were individually housed after surgery for a minimum of 1 week with daily handling, prior to receiving daily ICV injections of 1 μ L of commercial artificial cerebrospinal fluid (aCSF, Harvard Apparatus, Holliston, MA).

To confirm cannula placement in the 4th ventricle, 1 week after cannula placement rats underwent injection with 5-thioglucoase, described previously [44]. Briefly, 5TG is a non-metabolizable glucose isomer that causes NTS neurons to induce counter-regulatory responses to increase plasma glucose levels. Rats had blood glucose measured by tail vein sampling prior to ICV injection of 5-deoxyglucose (Sigma–Aldrich, St. Louis, MO) 210 μ g in 2 μ L aCSF. Follow-up blood glucose sampling was then performed 45–60 min later, and a rise in blood glucose of at least 80% was used to confirm placement of cannula in the 4th ventricle. Initial 5TG injection was performed using an injector that extended 2 mm below the level of the cannula. Rats that did not have an adequate rise in blood glucose had the 5TG injection repeated at least 3 days later with a cannula injector of different length, first 2.5 mm below the cannula, then 1.5 mm, then 3.0 mm, then 1.0 mm. The animals were then grouped by injector length and subsequent injections (including experimental treatments and bromophenol blue injection at time of sacrifice) were all performed using these injector lengths.

For food intake experiments, rats were pre-treated 1 h before lights out with 1 μ L of either aCSF or 1 nmol of AgRP dissolved in 1 μ L aCSF. Then, just prior to lights out rats were treated with either 2 μ L of aCSF or 10 ng of rat IL-1 β (R&D Systems, Inc., Minneapolis, MN) dissolved in aCSF. Food was weighed just prior to lights out and again at 3 h after lights out and 12 h after lights out, and food intake was calculated. Individual rats were measured for food intake on more than one occasion if a minimum of 4 days had passed from the prior testing.

2.3. Perfusion for immunohistochemistry

Two hours prior to perfusion, rats were given ICV injections of either 1 nmol AgRP in 1 μ L aCSF or an injection of 1 μ L of aCSF alone. One hour prior to perfusion, rats received ICV injections of 10 ng rat IL-1 β dissolved in 2 μ L aCSF or aCSF alone. At time of perfusion, rats were deeply anesthetized and perfused transcardially with 0.9% saline followed by ice-cold 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS). Following perfusion, 0.5 μ L of bromophenol blue was injected in the cannula to further confirm 4th ventricle placement. Brains were post-fixed for overnight in fixative and then stored 2–3 days in 20% sucrose in PBS as a cryoprotectant before being frozen at –80 °C until use.

2.4. Immunohistochemistry for c-Fos co-labeling

Dual-immunofluorescence histochemistry was performed as previously described [38]. Briefly, free-floating sections were cut in 30 μm -thick sections from perfused brains using a sliding microtome. Three separate sets of sections were generated from the brainstem and hypothalamus of each brain such that each set was composed of every-third section. Brainstem sections were collected from the spinal cord rostrally through the facial nucleus [29]. Hypothalamic sections were collected from the diagonal band of Broca caudally through the mammillary bodies. Brainstem sections were incubated for 1 h at room temperature in blocking reagent (5% normal donkey serum in 0.01 M PBS and 0.3% Triton X-100). After the initial blocking step, the sections were incubated in rabbit anti-c-Fos antibody (PC38, EMD Biosciences, Inc., San Diego, CA) diluted 1:75,000 in blocking reagent for 48 h at 4 $^{\circ}\text{C}$, followed by incubation in 1:500 donkey anti-rabbit Alexa Fluor 594 (Molecular Probes, Inc., Eugene, OR) for 1 h at room temperature. Brainstem sections were then incubated in sheep anti-TH 1:5000 (Chemicon International, Temecula, CA) overnight before being incubated in donkey anti-sheep Alexa Fluor 488 (Molecular Probes) diluted 1:4000 in blocking reagent for 1 h at room temperature. Between each stage the sections were washed thoroughly with 0.01 M PBS. Incubating the sections in the absence of primary antisera was used to ensure specificity of the secondary antibodies. Sections were mounted onto gelatin-coated slides, coverslipped using Vectashield mounting media (Vector Laboratories, Burlingame, CA) and viewed under a fluorescence microscope (Leica 4000 DM; Leica Microsystems, Bannockburn, IL).

Tissue sections were incubated for 1 h at room temperature in blocking reagent (5% normal donkey serum in 0.01 M PBS and 0.3% Triton X-100). After the initial blocking step, the sections were incubated in sheep anti-c-Fos antibody (Millipore, Inc.) diluted 1:50,000 in blocking reagent for 48 h at 4 $^{\circ}\text{C}$, followed by incubation in 1:500 donkey anti-sheep Alexa Fluor 594 (Molecular Probes, Inc., Eugene, OR) for 1 h at room temperature. Hypothalamic sections were then incubated in rabbit anti-POMC-precursor (Phoenix, Inc. Burlingame, CA) overnight before being incubated in donkey anti-rabbit Alexa Fluor 488 (Molecular Probes) diluted 1:4000 in blocking reagent for 1 h at room temperature. Sections were then washed and mounted as described above.

2.5. Cell counting

The number of c-Fos immunoreactive cells and double-labeled cells were manually counted in sections representing the ARC and the NTS by investigators blinded to the treatments. Results were expressed as the number of cells per section as well as the percentage double-labeled. Each set of ARC sections contained 7–9 sections expressing immunopositive cells and each set of caudal brainstem sections contained 5–7 caudal brainstem sections expressing immunopositive cells. A cell was determined to be single labeled when visible only under the fluorescence filter corresponding to the emission wavelength of the primary/secondary antibody complex used (e.g. 594 nm and not 488 nm for c-Fos). When the cell/cell nucleus were visible at both 594- and 488-nm filters, it was deemed to be double-labeled. Double-labeled cells were examined at multiple focal planes within the section and at multiple magnifications to ensure that the cell was indeed representative of a single cell labeled with both antibody complexes and not two single-labeled cells in close proximity within different levels of the optical section. The cells were also examined under a third wavelength (350 nm) not corresponding to the emission wavelength of either of the secondary antibodies to ensure that the immunoreactivity was specific.

2.6. In situ hybridization

Rats were treated with intraperitoneal injections of saline or 500 $\mu\text{g}/\text{kg}$ lipopolysaccharide (LPS *Escherichia coli* 055:B5, Sigma–Aldrich, St. Louis, MO) 8 h before sacrifice. Brains were removed and snap frozen and stored at -80°C . Coronal brainstem sections (20 μm) were

cut on a cryostat and thaw-mounted onto Superfrost Plus slides (VWR Scientific, West Chester, PA). Sections were collected in a 1:6 series rostrally from the spinal cord through the facial nucleus [29]. Antisense ³³P-labeled rat TH (corresponding to bases 721–1168 of rat TH; GenBank accession number L22651.1) (conc. 0.025 pmol/mL), POMC (corresponding to bases 49–644 of rat pro-opiomelanocortin; GenBank accession number AF510391) (concentration 0.1 pmol/mL), or rat cocaine and amphetamine regulated transcript (CART) (corresponding to bases 54–726 of rat CART; GenBank accession number NM 07110) (conc. 0.025 pmol/mL) were denatured, dissolved in hybridization buffer along with tRNA (1.7 mg/mL), and applied to slides. Controls used to establish the specificity of the IL-1R riboprobe included slides incubated with an equivalent concentration of radiolabeled sense IL-1R riboprobe, or radiolabeled antisense probe in the presence of excess (1000×) unlabeled antisense probe. Slides were covered with glass coverslips, placed in a humid chamber, and incubated overnight at 55 °C. The following day, slides were treated with RNase A and washed under conditions of increasing stringency. Slides were dipped in 100% ethanol, air dried, and then dipped in NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY). Slides were developed 5–7 days later and coverslipped. Determination of relative expression of transcript was determined by counting silver grains clusters corresponding to cells in the region of the NTS, as described previously [4].

2.7. Double-label ISH

Simultaneous visualization of TH and IL-1R mRNA in the rat brain ($n = 4$) was performed as previously reported [4], with slight modifications. Coronal sections (20 μm) were cut on a cryostat and thaw-mounted onto Superfrost Plus slides (VWR Scientific, West Chester, PA). Brainstem sections were collected in a 1:6 series from the facial nucleus (Bregma –10.00 mm) caudally through the spinal cord (30). Antisense ³³P-labeled rat IL-1R riboprobe (corresponding to bases 207–930 of rat interleukin-1 receptor type I; GenBank accession number M95578) (0.2 pmol/mL) and antisense digoxigeninlabeled rat TH riboprobe (corresponding to bases 721–1168 of rat TH; GenBank accession number L22651.1) (concentration determined empirically) were denatured, dissolved in hybridization buffer along with tRNA (1.7 mg/mL), and applied to slides. Controls used to establish the specificity of the IL-1R riboprobe included slides incubated with an equivalent concentration of radiolabeled sense IL-1R riboprobe, or radiolabeled antisense probe in the presence of excess (1000×) unlabeled antisense probe. Slides were covered with glass coverslips, placed in a humid chamber, and incubated overnight at 55 °C. The following day, slides were treated with RNase A and washed under conditions of increasing stringency. The sections were incubated in blocking buffer and then in Tris buffer containing antidigoxigenin fragments conjugated to alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN), diluted 1:250, 3 h at room temperature. TH cells were visualized with Vector Red substrate (SK-5100; Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Slides were dipped in 100% ethanol, air dried, and then dipped in NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY).

Slides were developed 8 days later and coverslipped. Determination of cells expressing both IL-1R and TH mRNA was performed using criteria previously described [4]. Briefly, TH-mRNA-containing cells were identified under fluorescent illumination, and custom-designed software was used to count the silver grains (corresponding to radiolabeled IL-1R mRNA) over each cell. Signal-to-background ratios (SBRs) for individual cells were calculated; an individual cell was considered to be double-labeled if it had an SBR of 2.5 or more. For each animal, the amount of double-labeling was calculated as a percentage of the total number of POMC-mRNA-expressing cells and then averaged across animals to produce mean ± SEM.

3. Results

3.1. Injection of AgRP into the 4th ventricle overcomes IL-1 β -induced anorexia by 12 h

Animals that received ICV injections of aCSF/IL-1 β or AgRP/IL-1 β had a significant decrease in food intake at 3 h after lights out compared to either animals receiving aCSF/aCSF or AgRP/aCSF (aCSF/aCSF 7.62 ± 0.43 , $n = 31$; aCSF/IL-1 β 2.04 ± 0.29 , $n = 19$ [$p < 0.001$ vs. aCSF/aCSF and AgRP/aCSF]; AgRP/IL-1 β 3.04 ± 0.50 , $n = 17$ [$p < 0.001$ vs. aCSF/aCSF and AgRP/aCSF]; AgRP/aCSF 8.20 ± 1.05 , $n = 5$) (Fig. 1A). There was no significant difference in food intake between the aCSF/aCSF and AgRP/aCSF groups.

At 12 h, there was a normalization of food intake among animals treated with AgRP/IL-1 β vs. aCSF/IL-1 β (aCSF/aCSF 21.22 ± 0.63 , $n = 31$; aCSF/IL-1 β 11.81 ± 0.80 , $n = 19$ [$p < 0.001$ vs. aCSF/aCSF]; AgRP/IL-1 β 19.99 ± 0.94 , $n = 17$ [$p < 0.001$ vs. aCSF/aCSF, AgRP/IL-1 β and AgRP/aCSF]; AgRP/aCSF 23.89 ± 2.10 , $n = 5$) (Fig. 1B). Again, there was no significant difference in food intake between animals receiving aCSF/aCSF vs. AgRP/aCSF.

3.2. Injection of IL-1 β into the 4th ventricle produces c-Fos activation of tyrosine-hydroxylase neurons in NTS

Animals that received 4th ventricular injections with aCSF/IL-1 β had a significant increase in c-Fos activation in NTS neurons expressing tyrosine hydroxylase as compared to animals receiving aCSF/aCSF (aCSF/aCSF $36.9 \pm 3.4\%$ of all TH+ cells that were c-Fos positive, $n = 8$ animals; aCSF/IL-1 β $52.8 \pm 3.4\%$, $n = 9$ [$p < 0.05$ vs. aCSF/aCSF]; AgRP/IL-1 β 52.8 ± 3.2 , $n = 10$; (Fig. 2). There was not a significant difference in c-Fos activation among animals receiving AgRP/IL-1 β compared to those receiving aCSF/IL-1 β ($p > 0.05$). Our lab had previously demonstrated a lack of c-Fos activation in the NTS following lateral ventricle injection of IL-1 β [38].

3.3. Lack of activation of TH neurons in the AP following 4th ventricle injection of IL-1 β

There was a nearly complete absence of c-Fos activation in TH neurons in the area postrema among any of the treatments studied (aCSF/aCSF $4.2\% \pm 2.2$, $n = 6$; aCSF/IL-1 β $5.2\% \pm 2.1$, $n = 6$; AgRP/IL-1 β $3.5\% \pm 2.0$, $n = 6$) (Fig. 3). There was no significant difference between any of the groups ($p > 0.05$).

3.4. Lack of POMC activation in ARH following 4th ventricular injection of IL-1 β

Animals that received 4th ventricle injections of IL-1 β did not have an increase in c-Fos activation of POMC neurons in the ARH at the experimental time point that we investigated (4th V aCSF/aCSF $6.38\% \pm 0.85$, $n = 5$; 4th V aCSF/IL-1 β 2.33 ± 0.46 , $n = 5$; 4th V AgRP/IL-1 β $2.34\% \pm 0.3$, $n = 5$; Lat V aCSF/aCSF 12.65 ± 4.5 , $n = 4$ [$p < 0.01$ vs. 4th V aCSF/IL-1 β and 4th V AgRP/IL-1 β]; Lat V aCSF/IL-1 β 26.6 ± 01.6 , $n = 3$ [$p < 0.001$ vs. all other groups]) (Fig. 4A–M).

Similarly, there was not an increase in the total number of c-Fos (+) nuclei in the ARH per section counted among animals receiving 4th ventricle IL-1 β vs. aCSF, though there was an IL-1 β induced increase in c-Fos activation in the ARH among animals receiving lateral ventricle injections (4th V aCSF/aCSF $15.13 \pm 2.08\%$ of all POMC+ neurons that were c-Fos +, $n = 5$ animals; 4th V aCSF/IL-1 β 9.23 ± 1.26 , $n = 5$ [$p < 0.05$ vs. aCSF/aCSF]; 4th V AgRP/IL-1 β 6.60 ± 0.32 , $n = 5$; Lat V aCSF/aCSF 24.78 ± 7.24 , $n = 4$ [$p < 0.05$ vs. 4th V aCSF/IL-1 β , $p < 0.01$ vs. 4th V AgRP/IL-1 β]; Lat V aCSF/IL-1 β 33.38 ± 4.47 , $n = 3$ [$p < 0.05$ vs. 4th V aCSF/aCSF, $p < 0.01$ Ket/aCSF/IL-1 β , $p < 0.001$ vs. 4th V aCSF/IL-1 β and 4th V AgRP/IL-1 β]) (Fig. 4N).

3.5. Lack of increased transcription of TH, POMC and CART following LPS inflammatory stimulus

Intraperitoneal injections of LPS did not produce an increase in tyrosine hydroxylase transcript amount in the NTS by 2 h, as measured by single-label *in situ* hybridization (saline 77.5 grains/cell $\pm$ 2.5, n = 6; LPS 70.8 $\pm$ 2.6, n = 6 [NS]) (Fig. 5A). Similarly, there was no change in POMC transcript amount in the NTS (saline 84.1 grains/cell $\pm$ 3.3, n = 6; LPS 84.7 $\pm$ 3.0, n = 6 [NS]) (Fig. 5B), nor was there a change in CART transcript in the NTS following LPS injection (saline 60.7 grains/cell $\pm$ 2.1, n = 6; LPS 54.8 $\pm$ 3.1, n = 6 [NS]) (Fig. 5C).

3.6. Double-label ISH

Brainstem sections from rats were processed for double-label *in situ* hybridization for TH and IL-1-RI. Less than or equal to 2% of TH mRNA-expressing neurons in the NTS, represented by cell bodies filled with fluorescent red precipitate, had overlying clusters of silver grains, signifying a lack of co-expression of TH and IL-1-RI mRNA (Fig. 6). Semiquantitative image analysis revealed an overall signal-to-background ratio of 1.3 $\pm$ 0.7 (56 $\pm$ 10 cells/animal, n = 4 animals). Using a signal-to-background ratio of 2.5 as the threshold for neurons to be considered double-labeled, 2% + 1% of the digoxigenin-labeled TH neurons co-expressed IL-1R mRNA.

4. Discussion

We have demonstrated a potential role for the brainstem in the production of inflammation-associated anorexia, such as is seen in the syndrome of cachexia [7], and this effect is ameliorated via melanocortin antagonism with AgRP. Our rats, after receiving IL-1 β injections into the region of the NTS, showed short-term (3 h) and long-term (12 h) suppression of food intake, and the long-term suppression of food intake was overcome by treatment with a sub-threshold dose of AgRP. This decrease in feeding behavior is similar to the anorexia seen when IL-1 β is administered into the lateral or 3rd ventricle, suggesting one possible mechanism for producing these effects is back-flow of IL-1 β from the brainstem back to the hypothalamus. However, activation of hypothalamic POMC neurons does not appear to occur in these experiments, since there was no significant difference in c-Fos activity in the ARH between animals given IL-1 β or aCSF (Fig. 4). This was in contrast to animals given IL-1 β into the lateral ventricles, which had a significant increase in c-Fos activity in POMC neurons in the ARH and had been previously shown to have decreased feeding behavior [38]. Together these data demonstrate that the decrease in feeding behavior was likely due to a brainstem-specific effect of IL-1 β administered to the 4th ventricle.

We initially suspected that the small group of neurons expressing POMC in the NTS would play a causative role in inflammation-induced anorexia. However, in previous experiments injecting IL-1 β into the lateral ventricle, we saw an increase in c-Fos activation in POMC neurons in the ARH but did not observe a similar increase in c-Fos activation among POMC neurons in the NTS [38]. In the current set of experiments, we did not see an increase in POMC transcript, as had been observed among POMC neurons in the arcuate [32].

Despite the lack of involvement of POMC neurons in the NTS, we continued to suspect a role for the NTS in anorexia, since previous experiments had shown extensive c-Fos activation of other non-POMC neurons in the NTS [38]. Our further investigations revealed that the decrease in feeding behavior that we observed following injection of IL-1 β into the 4th ventricle was accompanied by an increase in c-Fos immunoreactivity in tyrosine-hydroxylase neurons in the NTS. This is consistent with data that demonstrates that TH neurons are involved in multiple aspects of appetite regulation, including taste sensation, intestinal motility, response to cholecystokinin (CCK) and other peptides involved in feeding behavior [5,17,23,33,36,46].

Though we cannot say definitively that the anorexigenic effect of IL-1 β in the brainstem is due to this observed activation of TH neurons, their involvement is highly suggestive of TH neurons playing a causative role.

We additionally looked at expression levels of TH transcript following a generalized inflammation (lipopolysaccharide, also called bacterial endotoxin) and did not observe a change in TH transcription in this setting. Obviously, changes in neuronal activation and synaptic transmission can occur without obvious changes in the expression of biosynthetic enzymes. Alternate explanations include increases in TH activity brought about by some other form of modification of the transcript or the enzyme itself. We also did not see changes in transcript levels for POMC or cocaine and amphetamine regulated transcript in the NTS following the same inflammatory stimulus, two known anorexigenic peptides expressed in the NTS. Given that we only analyzed a single time point after LPS injection (8 h), it remains possible that inflammation can alter the expression of these genes in the NTS at earlier or later time points.

The means by which TH neurons might produce this effect is unclear at this time. We noted that the effects of 4th ventricular IL-1 β injection on feeding behavior were ameliorated at the 12-h time point by a sub-threshold dose of AgRP (which did not increase food intake when given with an injection of aCSF instead of IL-1 β , Fig. 1). Because AgRP acts as an antagonist of MC3-R and MC4-R receptors, this amelioration of IL-1 β anorexia by AgRP raises the question of whether activation of TH neurons decreases feeding behavior by acting either directly or indirectly on POMC neurons. If TH neurons produce their effect via activation of POMC neurons, one would expect AgRP to inhibit the effects of TH stimulation by blocking α -MSH at the level of the second order neurons in the hypothalamus and brainstem. The potential exists for TH neurons in the NTS to act via modulation of POMC neuronal output, given other roles for TH neurons in appetite-related processes and given the close proximity between TH and POMC neurons in the NTS. Additionally it has been well documented that TH neurons in the NTS project to the parabrachial nucleus, which is also a site for projection from neurons from the arcuate nucleus [21-24].

Nevertheless, the arguments against TH neurons acting via POMC neurons are stronger, because (1) we do not see other signs of POMC neuron activation in the NTS or ARH at the same time point when we see TH activity and (2) we do not observe amelioration of IL-1 β -induced activity in the short-term, despite injecting AgRP 1 h prior to IL-1 β . A more likely explanation is that AgRP improves food intake by acting through a parallel pathway in the brainstem, potentially by decreasing melanocortin tone on second-order neurons expressing MC4-R as suggested by similar 4th ventricle administration [41,45]. Additionally, AgRP has been shown to have long-term effects that appear to be separate from short-term modulation of melanocortin activity [13,31] and there has been some suggestion that AgRP may act independently of its effect on melanocortin receptors [12]. Thus, there is potential that the appetite stimulation that we observed is related to these long-term MC4-R-independent effects.

Our studies reported here are preliminary in that we have not performed experiments to block action of TH neurons to see if this ameliorates the anorexigenic effects of IL-1 β administered to the brainstem. We also have not delineated the exact mechanism by which TH neurons are activated by IL-1 β , though double-label *in situ* hybridization revealed that TH neurons do not express IL1-R1 receptor, eliminating direct activation of IL-1 β on TH neurons as a mechanism (Fig. 6). Alternate means of IL-1 β activation of TH neurons include relayed messages from other neurons or glial cells. Other researchers have recently shown that exposure to PGE₂—a downstream product of IL-1 β stimulation in glial cells—modulated neuronal electrical activity in the NTS, suggesting that glial cell production of PGE₂ in response to IL-1 β may be the

means by which TH neurons are activated [19,26]. Further experiments will be required to investigate whether TH neurons are activated by PGE₂.

Another means by which TH neurons could be activated during inflammation is via serotonin, which is increased in some portions of the brain during inflammation [22,39] and acts on the serotonin receptor 5-HT₁ in the brainstem. A recent report demonstrated c-Fos activation of tyrosine-hydroxylase neurons in the NTS associated with anorexia following peripheral infusion of the serotonin agonist m-chlorophenylpiperazine (mCPP) [20], and though their study—like ours—did not establish causality, a previous study had demonstrated that the anorexigenic effects of mCPP were blocked via 4th ventricle infusion of the 5-HT₁ antagonist mesulergine [18]. Interestingly, the recent report of TH neuron activation following serotonin infusion also established a lack of response of CART neurons in the region, similar to our observations [20]. Clearly, more work is needed to establish whether the serotonin system may serve as an important role in IL-1 β -induced activation of TH-neurons in the NTS, as well as to discover the means by which AgRP ameliorates the resultant anorexia.

In conclusion, these experiments identify TH neurons in the NTS as having c-Fos activation that coincides with inhibition of feeding behavior following injection of IL-1 β into the brainstem. This suppression of feeding behavior occurs in the absence of c-Fos activity in the arcuate nucleus of the hypothalamus, a center known to be involved in the inhibition of feeding behavior. These findings add support to our knowledge of the NTS as an important center involved in feeding behavior.

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Fig. 1.

Injection of IL-1 β to brainstem induces anorexia. Rats were administered 4th ventricle injections of aCSF or 1 nmol AgRP 1 h prior to lights out followed by aCSF or 10 ng IL-1 β just prior to lights out. Food was weighed at lights out and 3 and 12 h later. *** $p < 0.001$.

Fig. 2.

Injection of IL-1 β into the 4th ventricle causes c-Fos activation of tyrosine-hydroxylase cells in the NTS. Rats were administered 4th ventricle injections of aCSF/1 nmol AgRP 3 h prior to euthanizing and aCSF/10 ng IL-1 β injections 2 h prior to euthanizing. Animals were perfused with formaldehyde and brains were sectioned prior to double-label immunohistochemistry for tyrosine hydroxylase (488 nm secondary antibody [green fluorescence]) and c-Fos (594 nm secondary antibody [red fluorescence]). (A–L) Representative sections from animals treated with aCSF/aCSF (A–C), AgRP/IL-1 β (D–F) and aCSF/IL-1 β (G–L), with views at 488 nm fluorescence (A, D, G, and J), 594 nm (B, E, H, and K) and combined (C, F, I, and L). A–I are at 10 $\times$ magnification; J–L are 20 $\times$ magnifications from G–I. Arrows in L indicate TH neurons with c-Fos labeling. Sections were counted by blinded investigator for the number of TH

neurons having c-Fos (+) nuclei (M) and the total number of c-Fos nuclei per section (N). $*p < 0.05$; $**p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 3.

Injection of IL-1 β into the 4th ventricle does not cause c-Fos activation of tyrosine-hydroxylase cells in the area postrema. Rats were administered 4th ventricle injections of aCSF/1 nmol AgRP 3 h prior to sacrifice and aCSF/10 ng IL-1 β injections 2 h prior to sacrifice. Animals were perfused with formaldehyde and brains were sectioned prior to double-label immunohistochemistry for tyrosine hydroxylase (488 nm secondary antibody [green fluorescence]) and c-Fos (594 nm secondary antibody [red fluorescence]). (A–C) Representative sections from animals treated with aCSF/IL-1 β with views at 488 nm fluorescence (A), 594 nm (B) and combined (C). Sections were counted by blinded investigator for the number of TH neurons in the AP having c-Fos (+) nuclei (D). NS = not significant ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 4.

Injection of IL-1 β into the lateral but not the 4th ventricle causes c-Fos activation of POMC neurons in the ARH. Rats were administered 4th ventricle injections of aCSF/1 nmol AgRP 3 h prior to euthanizing and aCSF/10 ng IL-1 β injections 2 h prior to euthanizing or lateral ventricle injections of aCSF/10 ng IL-1 β 2 h prior to euthanizing. Animals were perfused with formaldehyde and brains were sectioned prior to double-label immunohistochemistry for POMC (488 nm secondary antibody [green fluorescence]) and c-Fos (594 nm secondary antibody [red fluorescence]). (A–L) Representative sections from animals treated with 4th ventricle aCSF/aCSF (A–C), 4th ventricle aCSF/IL-1 β (D–F) and lateral ventricle IL-1 β (G–L), with views at 488 nm fluorescence (A, D, G, and J), 594 nm (B, E, H, and K) and combined

(C, F, I, and L). A–I are at 10× magnification; J–L are 20× magnifications from G–I. Arrows in L indicate POMC neurons with c-Fos labeling. Sections were counted by blinded investigator for the number of TH neurons having c-Fos (+) nuclei (M) and the total number of c-Fos nuclei per section (N). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, NS = not significant ($p > 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 5.

Lack of increased mRNA expression of TH, POMC and CART following LPS treatment. Rats received intraperitoneal injections of saline or 500 $\mu\text{g/kg}$ lipopolysaccharide. Brains were snap frozen and sectioned prior to *in situ* hybridization with ^{33}P -labeled riboprobes to TH (A), POMC (B) and CART (C). Following emulsion exposure and development, silver grain clusters in the NTS were counted by blinded investigator. NS = not significant.

Fig. 6.

Lack of co-expression of TH and IL1-RI. Rats underwent double-label *in situ* hybridization using a digoxigenin-labeled riboprobe to TH (cells labeled by fluorescent red precipitate, wide arrow) and a ^{33}P -labeled riboprobe to IL1-RI (white silver-grain clusters, narrow arrow), revealing that TH-expressing cells in the NTS do not express IL1-RI.