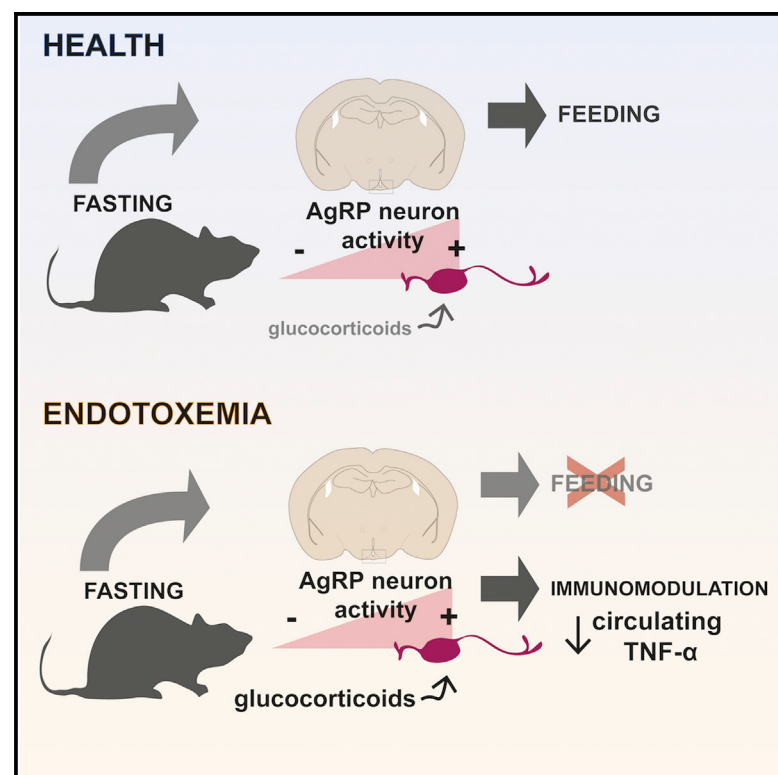


Hypothalamic AgRP neurons exert top-down control on systemic TNF- α release during endotoxemia

Graphical abstract



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In brief

Boutagouga Boudjadja et al. find that fasting-responsive hypothalamic neurons have immunoregulatory functions—an indication that the immunomodulatory effects of fasting and negative energy balance could be mediated, at least in part, by a brain-initiated response and are not completely dependent on nutrient availability per se.

Highlights

- Fasting-induced AgRP neuron activity remains sustained during endotoxemia
- Endotoxemia renders AgRP neurons refractory to inhibitory signals via glucocorticoids
- AgRP neuron activity controls TNF- α release during endotoxemia



Article

Hypothalamic AgRP neurons exert top-down control on systemic TNF- α release during endotoxemia

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<https://doi.org/10.1016/j.cub.2022.09.017>

SUMMARY

Loss of appetite and negative energy balance are common features of endotoxemia in all animals and are thought to have protective roles by reducing nutrient availability to host and pathogen metabolism. Accordingly, fasting and caloric restriction have well-established anti-inflammatory properties. However, in response to reduced nutrient availability at the cellular and organ levels, negative energy balance also recruits distinct energy-sensing brain circuits, but it is not known whether these neuronal systems have a role in its anti-inflammatory effects. Here, we report that hypothalamic AgRP neurons—a critical neuronal population for the central representation of negative energy balance—have parallel immunoregulatory functions. We found that when endotoxemia occurs in fasted mice, the activity of AgRP neurons remains sustained, but this activity does not influence feeding behavior and endotoxemic anorexia. Furthermore, we found that endotoxemia acutely desensitizes AgRP neurons, which also become refractory to inhibitory signals. Mimicking this sustained AgRP neuron activity in fed mice by chemogenetic activation—a manipulation known to recapitulate core behavioral features of fasting—results in reduced acute tumor necrosis factor alpha (TNF- α) release during endotoxemia. Mechanistically, we found that endogenous glucocorticoids play an important role: glucocorticoid receptor deletion from AgRP neurons prevents their endotoxemia-induced desensitization, and importantly, it counteracts the fasting-induced suppression of TNF- α release, resulting in prolonged sickness. Together, these findings provide evidence directly linking AgRP neuron activity to the acute response during endotoxemia, suggesting that these neurons are a functional component of the immunoregulatory effects associated with negative energy balance and catabolic metabolism.

INTRODUCTION

Loss of appetite and negative energy balance are common features of sickness in all animals, and although these responses are thought to have a protective role during acute infection,¹ there is a limited understanding of the mechanistic basis for this notion.

Reduced nutrient levels and the catabolic metabolism associated with negative energy balance are crucial in mediating stress resistance and tissue protection from inflammatory and pathogen-induced damage.^{2–4} However, under physiological conditions, in response to reduced nutrient availability at the cellular and organ levels, negative energy balance recruits distinct brain circuits, whose activity change upon variations in energy balance.⁵

In particular, negative energy balance activates a discrete neuron population in the arcuate nucleus of the hypothalamus

(ARC) defined by the expression of the agouti-related peptide (hereafter AgRP neurons).⁶

Converging evidence indicates that AgRP neurons represent a critical neuronal substrate encoding the central representation of negative energy balance: AgRP neurons are naturally activated by fasting and inhibited during satiety,^{6–8} AgRP neuron ablation causes cessation of feeding and lethal starvation,^{9,10} whereas selective, artificial AgRP neuron activation promotes voracious feeding,^{11,12} behavioral patterns,^{13–15} and neuronal responses in cortical regions¹⁶ similar to those occurring naturally during periods of food deprivation.

Therefore, it is clear that negative energy balance and the neuronal mechanisms encoding its central representation are tightly connected. However, it is not known whether the negative energy balance associated with sickness modulates the immune response exclusively via reduced nutrient availability or also via a



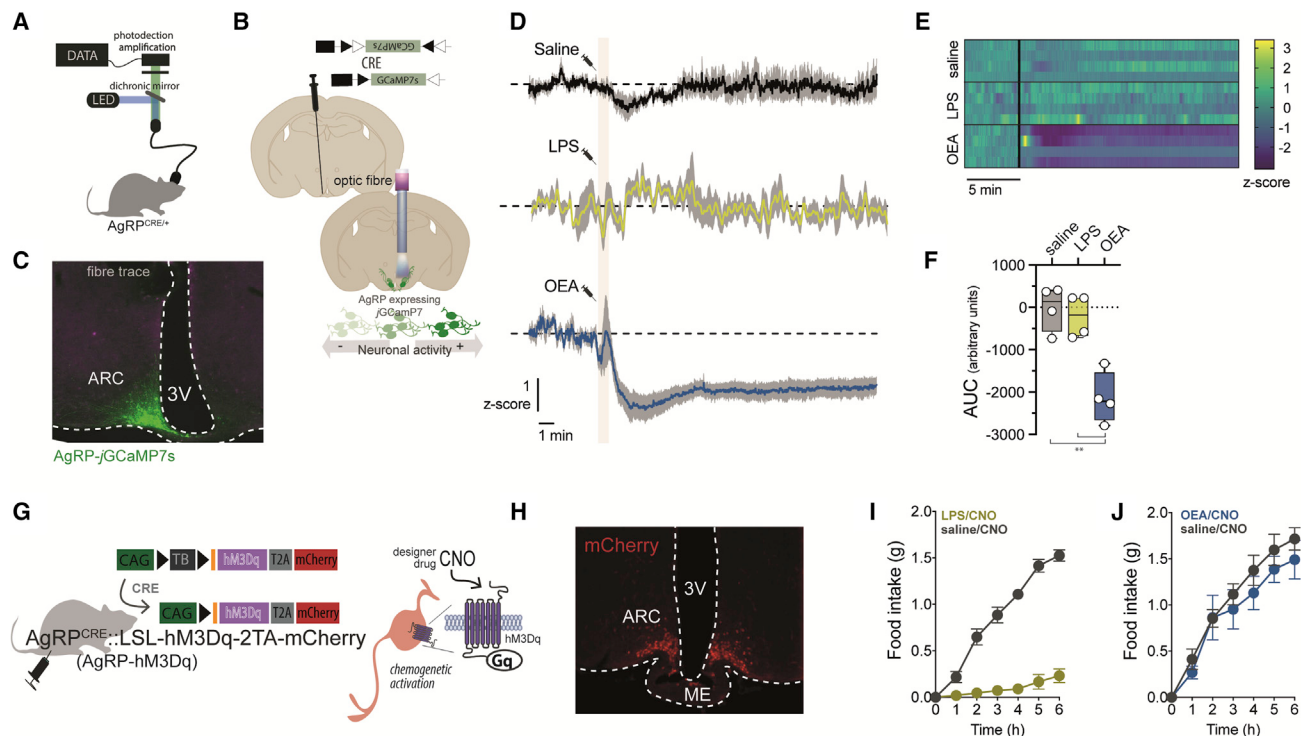


Figure 1. Endotoxin-driven anorexia is independent of AgRP neuron activity

(A and B) Schematics of fiber photometry to measure activity of AgRP neurons using Cre-dependent AAV-expressing *jGCaMP7s* in *AgRP^{CRE/+}* mice.

(C) Representative microphotograph showing *jGCaMP7s* expression and fiber location.

(D) Photometry traces recorded in food-deprived female mice injected with vehicle, lipopolysaccharide (LPS, 0.1 mg/kg), or oleoylethanolamide (OEA, 10 mg/kg) systemically ($n = 4$). Results were similar when a 10-times higher dose of LPS was used; see Figure S1A.

(E and F) Heatmaps (E) and quantification (F) of AgRP neuron responses (one-way ANOVA: $F_{(2,9)} = 18.30$, $p = 0.0007$; followed by Tukey's multiple comparisons test: ** $p < 0.005$).

(G) Schematics of the chemogenetic strategy.

(H) Representative microphotograph showing mCherry expression in the arcuate nucleus.

(I and J) Effect of LPS or OEA on food intake driven by activation of AgRP neurons in *AgRP-hM3Dq* male mice injected with clozapine-N-oxide (CNO). (I) LPS ($n = 5$), two-way repeated-measures ANOVA; time $\times$ LPS interaction, $F_{(6,48)} = 54.53$, $p < 0.0001$; LPS, $F_{(1,8)} = 153.17$, $p < 0.0001$; time, $F_{(6,48)} = 93.24$, $p < 0.0001$. (J) OEA ($n = 6$), two-way repeated-measures ANOVA; time $\times$ OEA interaction, $F_{(6,60)} = 0.71$, $p = 0.6457$; OEA, $F_{(1,10)} = 0.63$, $p = 0.4442$; time, $F_{(6,60)} = 89.89$, $p < 0.0001$. Data are presented as mean $\pm$ SEM or as individual biological replicates in box-and-whisker plots. 3V, third ventricle; ARC, arcuate nucleus of the hypothalamus; ME, median eminence. See also Figure S1.

centrally mediated response. This question is particularly relevant when considering the long-standing proposal that the central nervous system exerts top-down control of immune and inflammatory responses,^{17–19} as well as current progresses in the neuroimmunology of inflammatory diseases.²⁰

Here, to investigate this knowledge gap, we used the molecularly and anatomically defined AgRP neurons as an entry point to the central representation of negative energy balance. We studied the dynamics and functions of AgRP neurons during acute endotoxemia and found that even though AgRP neuron activity does not affect appetite and nutrient intake, it does influence the inflammatory response.

RESULTS

Endotoxin-driven anorexia is independent of the activity of AgRP neurons

To monitor the activity of AgRP neurons *in vivo* during endotoxemia, we used fiber photometry (Figures 1A–1C). We injected an

adeno-associated virus (AAV), expressing the Cre-inducible calcium indicator *jGCaMP7s*, into the ARC of *AgRP^{CRE/+}* mice and measured real-time, activity-dependent fluorescence via an indwelling optic fiber (Figures 1A–1C). As a reductive and tractable approach to induce endotoxemia, we used lipopolysaccharide (LPS)—a wall component of Gram-negative bacteria—whose immunogenicity is rapid and triggers a well-characterized cytokine response.

LPS does not cause any detectable inhibition of AgRP neurons in food-deprived mice (Figures 1D–1F and S1A), despite being potently anorectic. This indicates that an immunogenic challenge, such as LPS, does not inhibit the activity of AgRP neurons, whose activity remains sustained in food-deprived mice despite profound anorexia. We then tested the anorectic factor oleoylethanolamide (OEA), a lipid messenger released postprandially by small-intestinal enterocytes, which inhibits feeding without inducing immune activation or aversion.^{21,22} Contrary to LPS, an anorectic dose of OEA produces a rapid and stable inhibition of AgRP neurons (Figures 1D–1F). Thus, anorexia induced by sickness or

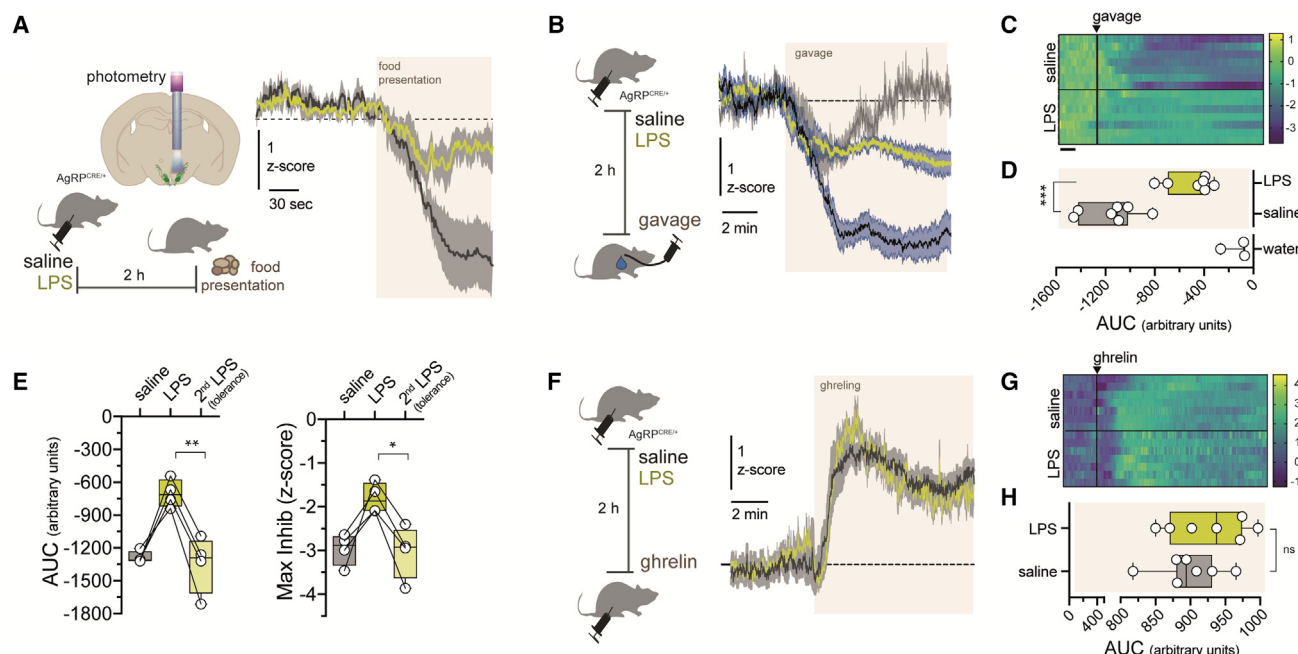


Figure 2. Endotoxemia desensitizes AgRP neurons to inhibition

(A) AgRP neuron responses to food presentation recorded using fiber photometry in mice previously injected with saline or LPS (0.1 mg/kg, intraperitoneally). (B) AgRP neuron responses to an intragastric bolus of glucose in mice previously injected with saline or LPS. (C and D) Heatmaps (C) and quantification (D) of AgRP neuron responses displayed in (B) (saline versus LPS, $t(12) = 6.032$, $***p < 0.0001$). Gray trace in (B) represents AgRP neuron responses to an isovolumetric gavage of water, also presented as AUC in (D). (E) AgRP neuron responses to an intragastric bolus of glucose in a subset of mice challenged with two LPS doses to establish endotoxin tolerance. Data expressed as AUC (one-way repeated-measures ANOVA: $F_{(1,052,3,156)} = 19.81$, $p = 0.0187$; followed by Tukey's multiple comparisons test: $*p < 0.05$, $**p < 0.005$) and maximal inhibition following glucose gavage (one-way repeated-measures ANOVA: $F_{(1,186,3,557)} = 8.587$, $p = 0.0479$; followed by Tukey's multiple comparisons test: $*p < 0.05$). (F) AgRP neuron responses to a systemic injection of ghrelin in mice previously injected with saline or LPS. (G and H) Heatmaps (G) and quantification (H) of AgRP neuron responses displayed in (F). Experiments were performed in female mice. Data are presented as mean $\pm$ SEM or as individual biological replicates in box-and-whisker plots.

physiological signaling seems to differentially impact on the activity of AgRP neurons. To test this further, we artificially increased the activity of AgRP neurons using chemogenetics and tested whether LPS or OEA affects feeding when it is driven by increased AgRP neuron activity. We used mice expressing the stimulatory DREADD allele hM3Dq, together with a mCherry reporter protein, selectively in AgRP neurons (AgRP^{CRE/+}::LSL-hM3Dq-T2A-mCherry; hereafter AgRP-hM3Dq; Figure 1H). As expected, a systemic injection of the hM3Dq actuator, clozapine-N-oxide (CNO), elicits activation of AgRP neurons (Figure S1C) and a robust feeding response in otherwise sated AgRP-hM3Dq mice, whereas it has no effect in littermate controls that do not express hM3Dq (Figure S1D). In line with previous studies,²³ chemogenetic activation of AgRP neurons is not sufficient to counteract the anorexia induced by endotoxemia (Figure 1I). By contrast, OEA fails to suppress feeding when AgRP neurons are artificially activated via chemogenetics (Figure 1J).

Together, these data highlight a fundamental difference between the reductions in feeding caused by endotoxemia and those by physiological signaling, indicating that these two forms of appetite suppression are different in nature. Whereas anorexia induced by the satiation factor OEA occurs with concomitant inhibition of AgRP neurons, anorexia induced by endotoxemia does not and can occur despite the sustained activity of these neurons.

Endotoxemia desensitizes AgRP neurons to inhibition

In physiological conditions, the activity of AgRP neurons induced by food deprivation is rapidly inhibited when food is identified, as well as by post-ingestive nutrient signaling.^{24–26} Thus, we asked how AgRP neurons respond to these inhibitory signals during endotoxemia.

We confirm that AgRP neurons display a rapid inhibition upon sensory detection of food (Figure 2A). However, we found that during endotoxemia this anticipatory inhibition is almost absent (Figure 2A). While the significance of the AgRP neuron inhibition upon food contact remains unresolved,²⁷ it is considered an appetitive response predicting the amount of food consumed in the ensuing meal.^{28,29} Consequently, the AgRP neuron-blunted inhibition in endotoxemic mice could be an epiphenomenon due to a lack of appetitive drive toward food as a result of the endotoxemia-induced inflammatory response. Thus, we asked what the effect of nutrient ingestion on AgRP neuron activity would be if this suppression of appetitive drive is bypassed. To answer this question, we provided mice with nutrients via direct intragastric delivery. We found that while a 0.3-kcal bolus of glucose potently inhibits AgRP neurons in control mice, strikingly, the same bolus is minimally effective in inhibiting these neurons in endotoxemic mice (Figures 2B–2D): an indication that during endotoxemia, AgRP neurons become refractory to

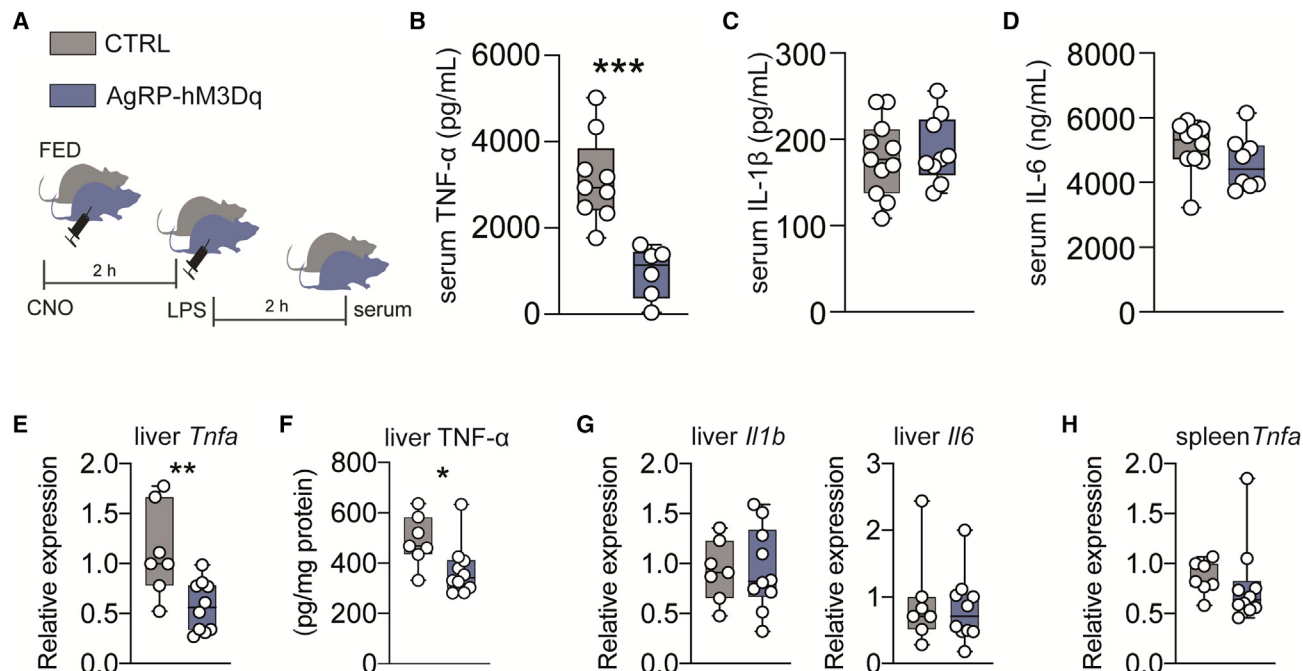


Figure 3. AgRP neuron activity during endotoxemia reduces acute TNF- α secretion

(A) Experimental timeline.

(B–D) Effect of the chemogenetic activation of AgRP neurons on LPS (1 mg/kg, intraperitoneally)-induced circulating levels of TNF- α ($t(13) = 4.705$, $p = 0.0004$), IL-1 β , and IL-6.

(E and F) Effect of the chemogenetic activation of AgRP neurons on LPS-induced liver *Tnf* mRNA ($t(15) = 3.205$, $p = 0.0059$) and protein levels ($t(15) = 2.335$, $p = 0.0338$).

(G) Effect of the chemogenetic activation of AgRP neurons on LPS-induced liver IL-1 β and IL-6 mRNA.

(H) Effect of the chemogenetic activation of AgRP neurons on LPS-induced spleen TNF- α mRNA. Experiments were performed in male mice. Note: chemogenetic activation of AgRP neurons also suppresses TNF- α in female mice (Figure S2B). Data are presented as individual biological replicates in box-and-whisker plots. See also Figures S2 and S3.

inhibition triggered by post-ingestive signals. Furthermore, we found that AgRP neuron inhibition upon intragastric glucose delivery is reverted back to normal if mice are challenged again with a second dose of LPS 3–5 days later (Figure 2E), when LPS fails to trigger systemic inflammation due to the development of endotoxin tolerance.^{30,31} Thus, these data further indicate that the endotoxemia-induced desensitization of AgRP neurons is primarily driven by the acute inflammatory response.

We asked also whether endotoxemia promotes a general impairment of AgRP neuron responsiveness to metabolic signals and tested ghrelin, a gut-released hormone that signals energy deficit and potently activates AgRP neurons. We found, however, that AgRP neurons remain responsive to ghrelin administration and display robust activation both in endotoxemic and control mice (Figures 2F–2H).

Together these data indicate that endotoxemia rapidly desensitizes AgRP neurons to prevent their inhibition but that the neurons remain able to respond to signals of energy deficit.

AgRP neuron activity during endotoxemia reduces acute TNF- α secretion

Our data, so far, provide converging evidence that AgRP neurons remain active when food-deprived mice experience endotoxemia but that their activity does not sustain feeding. Most fundamentally, these observations raise the question as to why

these neurons would remain active during a state of endotoxemia-driven anorexia.

In response to negative energy balance, animals forage for food and take risks, increasing the likelihood of injury and infection. We envision that the occurrence of microbial exposure and immune challenges during a state of negative energy balance might have endowed AgRP neurons with immunoregulatory functions, possibly conferring an evolutionary advantage.

To test this idea, we activated AgRP neurons artificially via chemogenetics in otherwise well-fed mice and assessed the acute inflammatory response to LPS by measuring serum levels of tumor necrosis factor alpha (TNF- α), an early-secreted cytokine and hallmark of endotoxemia that coordinates the immune response and sustains sickness behavior. We found that an acute chemogenetic activation of AgRP neurons in AgRP-hM3Dq mice is sufficient to reduce circulating TNF- α levels in response to LPS (Figures 3B and S2B). This effect seems specific to TNF- α , as AgRP neuron activation does not affect circulating levels of the other early cytokines IL-1 β and IL-6 (Figures 3C and 3D). Notably, several previous studies have established that systemic TNF- α release during endotoxemia can be neurally regulated.^{17–19} We obtained a similar reduction in LPS-induced TNF- α secretion in separate cohorts of AgRP^{CRE/+} mice in which the hM3Dq allele was expressed in AgRP neurons post-developmentally using a Cre-inducible

AAV (Figure S2), ruling out the possibility of developmental expression of the hM3Dq in a cell population other than hypothalamic AgRP neurons. Furthermore, following AgRP neuron activation we found reduced LPS-induced *Tnf* mRNA (Figure 3E) and TNF- α protein (Figure 3F) levels in the liver—a principal source of serum TNF- α during endotoxemia.^{17,32} We found no difference in the spleen (Figure 3H). Thus, the effect of chemogenetic activation of AgRP neurons on acute TNF- α secretion could be mediated, at least in part, by an attenuated hepatic response to endotoxemia.

During negative energy balance, increased AgRP neuron activity can promote energy conservation by reducing thermogenesis, resulting in reduced body temperature.^{14,33} However, chemogenetic activation of AgRP neurons reduces LPS-induced TNF- α levels even under thermoneutral conditions (32°C; Figure S3B), which fully prevent the AgRP neuron-driven reduction of body temperature (Figures S3D and S3E). This indicates that this effect is independent of body temperature. Thermoneutrality also blunts the increase in locomotor activity (a proxy of foraging behavior) that follows chemogenetic activation of AgRP neurons (data not shown), further indicating that the reduction in TNF- α levels is independent of changes in physical activity.

Together, these data indicate that AgRP neuron activity inhibits TNF- α production during the early-phase inflammatory response to endotoxemia. Therefore, the desensitization of AgRP neurons that we report to occur acutely during endotoxemia can be adaptive in nature, as the resulting sustained tone of the neurons might contribute to controlling TNF- α levels. Notably, our findings that AgRP neurons have anti-inflammatory properties also concur with a previous report showing that AgRP-specific knockdown of the key cellular metabolic sensor Sirtuin 1—which impairs AgRP neuron excitability—results in a pro-inflammatory state and disease susceptibility.³⁴

Endogenous glucocorticoids mediate endotoxemia-induced desensitization of AgRP neurons and enable their anti-inflammatory effects

Next, we sought to understand the mechanism underlying the endotoxemia-induced AgRP neuron desensitization. It is well established that endogenous glucocorticoid levels increase acutely during endotoxemia.³⁵ AgRP neurons express the *Nr3c1* gene that encodes the glucocorticoid receptor (GR) whose activation results in an increased AgRP tone.^{36–38} Thus, we hypothesized that increased glucocorticoid levels could be one of the mechanisms driving the observed endotoxemia-dependent AgRP neuron desensitization. To test this hypothesis, we used *AgRP^{CRE/+}::Nr3c1^{flx/flx}* mice (hereafter AgRP^{GR-KO}) that bear conditional deletion of the *Nr3c1* gene in AgRP neurons,³⁷ and we transduced these neurons with the Cre-inducible calcium indicator jRCaMP7s to perform photometry recordings (Figures 4A–4C). As described above, we provided food-deprived mice with a 0.3-kcal bolus of glucose via intragastric delivery during normal or endotoxemic conditions. Strikingly, we found that in AgRP^{GR-KO} mice, glucose gavage inhibits AgRP neurons to the same extent following either saline or LPS injection (Figures 4D–4F). Thus, GR expression in AgRP neurons is necessary for the endotoxemia-dependent desensitization, revealing endogenous glucocorticoid action on these neurons as an important underlying mechanism.

Finally, we capitalized on the AgRP^{GR-KO} mice and sought to test the hypothesis that the endotoxemia-induced desensitization of AgRP neurons could be adaptive in nature, providing a mechanism to sustain fasting-induced AgRP neuron tone and contribute to controlling TNF- α levels. We reasoned that since the endotoxemia-dependent desensitization of AgRP neurons is absent in AgRP^{GR-KO} mice, they should display an impaired fasting-induced suppression of TNF- α secretion during endotoxemia. To test this, we measured LPS-induced circulating TNF- α levels in fed and fasted AgRP^{GR-KO} mice and littermate controls. As expected, we found that fasting significantly reduces TNF- α levels in response to endotoxemia in wild-type controls (Figure 4H). AgRP^{GR-KO} mice, however, have similar TNF- α levels during both fed and fasted conditions (Figure 4H). Circulating levels of IL-1 β and IL-6 do not differ significantly (Figure 4I). Furthermore, we found that AgRP^{GR-KO} mice display a longer endotoxemia-induced anorexia when challenged with LPS (Figure S4A). There is no difference, however, between AgRP^{GR-KO} mice and controls in spontaneous or fasting-induced feeding under physiological conditions (Figures S4B and S4C), indicating that glucocorticoid action is dispensable for the appetite-promoting functions of AgRP neurons.³⁹ Therefore, the prolonged endotoxemia-induced anorexia in AgRP^{GR-KO} mice likely reflects an increased sickness response due to the loss of endogenous glucocorticoid signaling in AgRP neurons. These data add further support to the hypothesis that glucocorticoid signaling sustains the anti-inflammatory effect of AgRP neurons, and they might suggest a possible additional bottom-up mechanism through which glucocorticoids exert their pleiotropic effects on inflammation.

DISCUSSION

Here, we report that AgRP neuron activity remains sustained when endotoxemia occurs during a state of negative energy balance, such as fasting, and that AgRP neuron activity does not influence feeding behavior. We further found that systemic inflammation rapidly desensitizes AgRP neurons to inhibitory signals preventing their inhibition. Functionally, mimicking fasting-induced AgRP neuron activity by selectively increasing the activity of these neurons via chemogenetic is sufficient to reduce circulating TNF- α in otherwise fed mice, but the same manipulation does not reverse endotoxemic anorexia. Our data further indicate that endogenous glucocorticoids play an important role in maintaining AgRP neuron activity during endotoxemia and, importantly, that the loss of glucocorticoid signaling in these neurons reduces the anti-inflammatory effect of fasting, resulting in an increased TNF- α secretion and an overall prolonged sickness response.

Multiple lines of evidence have highlighted the impact of nutritional state on peripheral immunity, with food deprivation and caloric restriction exerting well-established anti-inflammatory adaptations.⁴⁰ These effects were first observed and particularly prominent in the context of endotoxemia following exposure to bacterial products,^{2–4} as experimentally mimicked in our studies using the LPS. However, even though the anti-inflammatory effects of fasting and the adaptive nature of negative energy balance during sickness responses have been generally attributed to reduced nutrient availability and its impact on host and

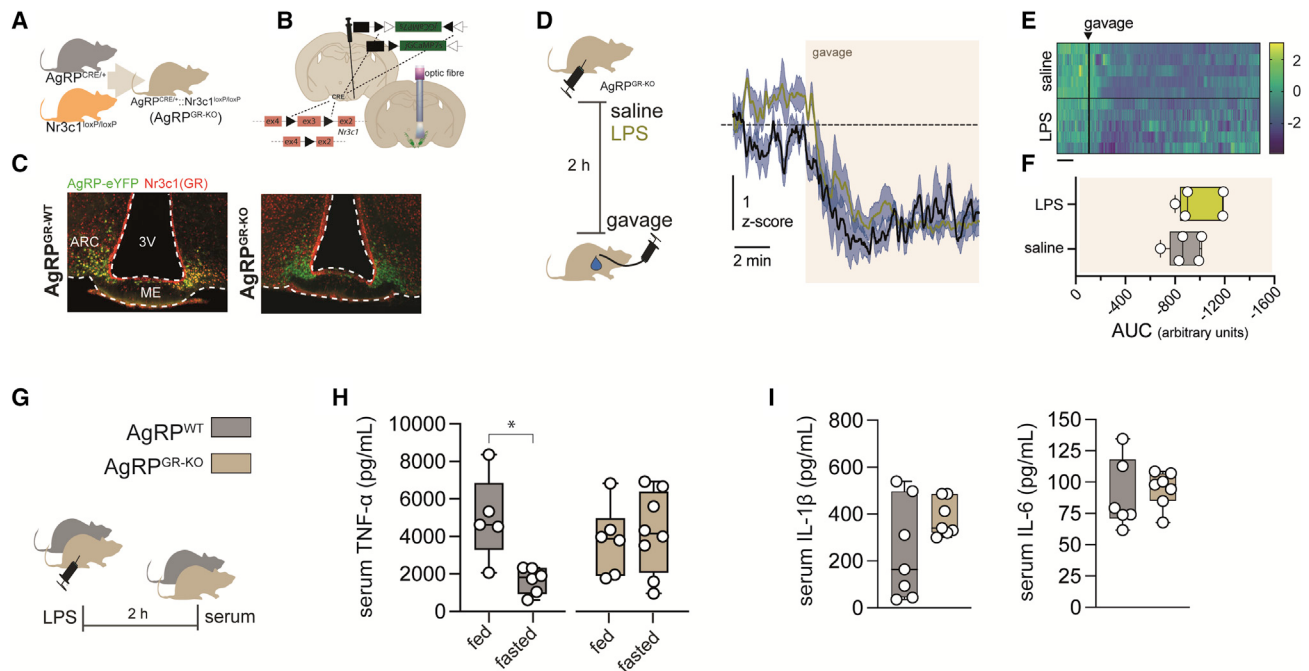


Figure 4. Endogenous glucocorticoids mediate endotoxemia-induced desensitization of AgRP neurons and enable their anti-inflammatory effects

(A and B) Schematics of the strategy to generate mice lacking glucocorticoid receptor (GR) in AgRP neurons (AgRP^{GR-KO}).
(C) Representative microphotographs of immunohistochemical detection of GR in AgRP^{GR-WT} and AgRP^{GR-KO} mice. AgRP neurons are identified as eYFP positive cells.
(D) AgRP neuron responses to an intragastric bolus of glucose in AgRP^{GR-KO} female mice previously injected with saline or LPS (0.1 mg/kg, intraperitoneally).
(E and F) Heatmaps (E) and quantification (F) of AgRP neuron responses (saline versus LPS, $t(8) = 1.136$, $p = 0.2889$).
(G) Experimental timeline used to assess the acute inflammatory response to LPS (1 mg/kg, intraperitoneally) in AgRP^{GR-WT} and AgRP^{GR-KO} male mice.
(H) LPS-induced circulating level of TNF- α in fed and fasted AgRP^{GR-WT} and AgRP^{GR-KO} mice. Two-way ANOVA; genotype, $F_{(1,21)} = 0.78$, $p = 0.387$; feeding status, $F_{(1,21)} = 3.71$, $p = 0.069$; interaction, $F_{(1,21)} = 6.11$, $p = 0.022$. * $p < 0.05$, Tukey's multiple comparisons test.
(I) LPS-induced circulating levels of IL-1 β and IL-6 in fasted AgRP^{GR-WT} and AgRP^{GR-KO} mice. Data are presented as mean $\pm$ SEM or as individual biological replicates in box-and-whisker plots. 3V, third ventricle; ARC, arcuate nucleus of the hypothalamus; ME, median eminence.
See also Figure S4.

pathogen metabolism, our results suggest that these effects are at least in part a result of a central, AgRP neuron-driven mechanism and not completely dependent on nutrient availability per se.

To the best of our knowledge, our findings provide the first evidence directly linking AgRP neuron activity to the peripheral inflammatory response, pointing to AgRP neurons as a critical functional component of the anti-inflammatory effects associated with negative energy balance and catabolic metabolism.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cub.2022.09.017>.

ACKNOWLEDGMENTS

The authors acknowledge with gratitude Peter Morgan (University of Aberdeen, UK) for insightful discussions and support in the early stages of this project. pGP-AAV-syn-FLEX-jGCaMP7s-WPRE was a gift from Douglas Kim & GENIE Project (Janelia Research Campus, Virginia, USA). DREADD plasmids and vectors were a gift from Bryan Roth (University of North Carolina at Chapel Hill, North Carolina, USA). This work was funded by an MRC Career Development Award (MR/P009824/1 and MR/P009824/2), European Crohn's and Colitis Organization (ECCO) grant to G.D., MRC grant (MR/W004623/1) to G.D. and M.H., BBSRC grant (BB/V016318/1) to G.D. and S.M.L., and a Tenovus Scotland grant to G.A. The original line for conditional expression of hm3Dq

was generated with the support of ISSF Wellcome Trust (105625/Z/14/Z) to G.D. The A.W. laboratory is supported by the Fitzgerald and Mawer endowment funds. G.C.D.P. was supported in part by a travel grant from Capes/PDSE (process 88881.190595/2018-01; Brazil). Funders had no involvement in the writing of the manuscript and the decision to submit it for publication.

AUTHOR CONTRIBUTIONS

G.A. and G.D. conceived the project and supervised the experiments and their analyses. M.B.B., I.C., J.H., G.C.D.P., J.P.C.-d.-A., E.H., G.A., and G.D. performed experiments and data analysis. M.B.B. generated codes for data analysis. S.M.L., M.H., and A.W. provided reagents and materials. G.A. and G.D. wrote the manuscript, with inputs from S.M.L., M.H., and A.W. All authors contributed to data interpretation and the editing of the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: May 16, 2022

Revised: July 21, 2022

Accepted: September 8, 2022

Published: September 30, 2022

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-c-Fos Antibody	Abcam	Cat# ab190289; RRID: AB_2737414
Anti-GFP Antibody	Abcam	Cat# ab13970; RRID: AB_300798
Donkey Anti-Goat IgG H&L (Alexa Fluor 594)	Abcam	Cat# ab150132; RRID: AB_2810222
Donkey Anti-Rabbit IgG H&L (Alexa Fluor 594)	Abcam	Cat# ab150076; RRID: AB_2782993
Goat polyclonal antibody to mCherry	Sicgen	Cat# ab0040-200; RRID: AB_2333093
Glucocorticoid receptor Antibody	Proteintech	Cat# CL488-24050; RRID: AB_2919198
Goat Anti-Chicken IgY H&L (Alexa Fluor 488)	Abcam	Cat# ab150169; RRID: AB_2636803
Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-11039; RRID: AB_2534096
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	Thermo Fisher Scientific	Cat# A-11037; RRID: AB_2534095
Bacterial and virus strains		
pGP-AAV-syn-FLEX-jGCaMP7s-WPRE	Addgene	Addgene 104491-AAV1; RRID: Addgene_104491
pAAV-hSyn-DIO-hM3D(Gq)-mCherry	Addgene	Addgene 44361-AAV8; RRID: Addgene_44361
pAAV-hSyn-DIO-mCherry	Addgene	Addgene 50459-AAV8; RRID: Addgene_50459
Chemicals, peptides, and recombinant proteins		
Clozapine-N-oxide (CNO)	Tocris	Cat# 4936
D-Glucose	Sigma-Aldrich	Cat# 346351
Formalin, 10%	Sigma-Aldrich	N/A
Ghrelin	Tocris	Cat# 1465/1
Lipopolysaccharide (LPS)	Sigma-Aldrich	Cat# L4391
Oleylethanolamide	Sigma-Aldrich	Cat# O0383
TRI-Reagent	Sigma-Aldrich	Cat# 15596026
Critical commercial assays		
High-capacity cDNA reverse transcription kits	Thermo Fisher Scientific	Cat# 4368814
Mouse IL-1 beta/IL-1F2 DuoSet ELISA	R&D system	Cat# DY401
Mouse IL-6 DuoSet ELISA	R&D system	Cat# DY406
Mouse TNF-alpha DuoSet ELISA	R&D system	Cat# DY410
PowerTrack SYBR Green Master Mix	Thermo Fisher Scientific	Cat# A46109
RNeasy Mini Kit	QIAGEN	Cat# 74104
Experimental models: Organisms/strains		
Mouse: Agrp ^{tm1(cre)Low/J}	The Jackson Laboratory	RRID: IMSR_JAX: 012899
Mouse: B6.129S4-Gt(ROSA)26Sor ^{tm1(FLP1)Dym/RainJ}	The Jackson Laboratory	RRID: IMSR_JAX:009086
Mouse: B6.129P2-Nr3c1 ^{tm2Gsc/leg}	European Mouse Mutant Archive	EM:02124
Mouse: B6.129X1-Gt(ROSA)26Sor ^{tm1(EYFP)Cos/J}	The Jackson Laboratory	RRID: IMSR_JAX:006148
Mouse: FSF-LSL-hM3Dq-T2A-mCherry	This paper	N/A
Oligonucleotides		
Primers for rt-PCR see Table S1	N/A	N/A
Software and algorithms		
Doric Neuroscience Studio	Doric Lenses	RRID: SCR_018569; https://neuro.doriclenses.com/products/doric-neuroscience-studio
FLIR ResearchIR	Teledyne FLIR LLC	RRID: SCR_016330; https://www.testco.uk/flir-researchir-standard-software

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Prism 8.0	Graphpad	RRID: SCR_002798; https://www.graphpad.com/
ImageJ	National Institutes of Health (NIH)	https://imagej.net/software/imagej/
MatLab	MathWorks	RRID: SCR_001622; https://uk.mathworks.com/products/matlab.html
Other		
3M RelyX Unicem 2 Automix Self-Adhesive Resin Cement Value	3M	https://www.3m.com/3M/en_US/p/d/b00007450/
Nanoject II Auto Nanoliter Injector	Drummond Scientific Company	Cat# 3-000-204
Thermal camera FLIR A655sc	Teledyne FLIR LLC	RRID: SCR_016330: Model: A655sc, FOV 15°

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Giuseppe D'Agostino (giuseppe.dagostino@manchester.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

AgRP^{Cre} (*AgRP^{tm1(cre)Low/J}*, strain #012899) mice were obtained from Jackson Laboratories. LSL-hM3Dq-T2A-mCherry mice were generated using the TARGATT technology at Applied StemCell (Milpitas, CA, USA). Briefly, an integration cocktail was microinjected into the pronuclei of heterozygous embryos harvested from B6 mice previously engineered with three tandem attP sequences into mouse H11 locus. The integration cocktail consisted of a plasmid donor DNA vector containing frt-STOP-frt-loxP-STOP-loxP-hM3Dq-T2A-mCherry flanked by two attB sites and *in vitro* transcribed ϕ C31 integrase mRNA. Transgenic FSF-LSL-hM3Dq-T2A-mCherry founders were identified by PCR-based genotyping. Mice were further crossed with a pan FLPe-expressing mouse line (B6.129S4-Gt(ROSA)26Sor^{tm1(FLP1)Dym}/RainJ, strain #:009086) to remove the frt-STOP-frt cassette and generate the parent Cre-inducible LSL-hM3Dq-T2A-mCherry line. *Nr3c1^{flox/flox}* (B6.129P2-Nr3c1tm2Gcs/leg EMMA strain #02124) were crossed with *AgRP^{Cre}* mice to obtain *AgRP^{Cre/+}::Nr3c1^{flox/flox}* mice (*AgRP^{GR-KO}*). This line was described and validated previously.³⁷ *AgRP^{GR-KO}* mice were further crossed with R26-stop-eYFP mice (B6.129X1-GT(ROSA)26Sor^{tm1(EYFP)Cos}/J strain #006148) were obtained from Jackson Laboratories and were also previously described.³⁷ Females and males mice were used for experiments. All mice were provided with standard mouse chow and water *ad libitum* and housed at 22–24°C with a 12-hour light/12-hour dark cycle, unless otherwise noted. All experimental procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and local ethical review.

METHOD DETAILS

Drugs, surgeries and viral vectors

Oleylethanolamide (10mg/kg, Cat. No.00383, Sigma-Aldrich, UK) was dissolved in sterile saline with 5% Tween-80 and of 1% DMSO. Ghrelin (60μg/mouse, Cat. No.1465/1, Tocris, UK), Clozapine-N-oxide (CNO; 3mg/kg in *AgRP*-hM3Dq mice and 1mg/kg in mice transduced via hM3Dq via site specific AVVs delivery; Cat. No.4936, Tocris, UK), LPS (Cat. No L4391, Sigma-Aldrich, UK) were dissolved in sterile saline and administered intraperitoneally (IP). For main photometry experiments and feeding studies the dose of LPS was 0.1mg/Kg. We confirmed the lack of *AgRP* neuron inhibition in an additional experiment using LPS at 1mg/Kg. The LPS tolerance experiment was performed by administering a second dose of LPS (0.1mg/Kg) 3–5 days following the first

exposure. For assessing cytokines and gene expression the dose of LPS was 1mg/Kg. D-Glucose (Cat. No. 346351, Sigma-Aldrich, UK) was dissolved in water at a concentration of 0.9g/ml and 0.3mL/mouse administered via oral gavage.

AAVs were injected into mice anaesthetized with isoflurane (2–3% in oxygen) and placed in a stereotaxic frame. The skull was exposed, and holes drilled at the site of injection. AAVs were delivered unilaterally or bilaterally to the ARC via a glass micropipette affixed to a Nanoject II Auto Nanoliter Injector (Drummond Scientific Company, PA) using co-ordinates, -1.5 mm A/P, ± 0.2 mm M/L, -5.7 mm D/V from bregma (300 nl; 1×10^{13} vg/mL). AAV-syn-FLEX-jGCaMP7s-WPRE (Cat. No.104491-AAV1), AAV-hSyn-DIO-hM3D(Gq)-mCherry (Cat. No. 44361-AAV8) and AAV-hSyn-DIO-mCherry (Cat. No. 50459-AAV8) were obtained from Addgene (Watertown, MA, US). For photometry recordings, an optic fiber was implanted after AAVs injection, during the same surgical procedure. Optical fibers (fiber core = 400 μ m; 0.5 NA; 1.25 mm $\varnothing$ metal ferrule) were implanted over the ARC (Bregma: AP: -1.5 mm, L: $+0.3$ mm, DV: 5.7 mm from skull surface) and fixed to the skull surface using dental cement (Relyx unicem 2, 3M, UK). Experiments were performed at least 3 weeks after surgery to allow for recovery and viral expression.

Dual wavelength fiber photometry

A 2-channel LED Driver (LEDD_2; Doric Lenses) was used to modulate blue (470 nm) and purple (405 nm) LED light sources at 211 Hz and 511 Hz, respectively. Light was sent to a Fluorescence Mini Cube (FMC4_AE(405)_E(460-490)_F(500-550)_S; Doric Lenses) through low autofluorescence optical fiber patch cords (MFP_400/430/LWMJ-0.48_1m_FCM-FCM_T0.05; Doric Lenses). A pigtailed rotary joint (FRJ_1x1_PT_400/430/LWMJ-0.57_1m_FCM_0.06m_FCM; Doric Lenses) was used to allow for free movement of the mice. 470 nm (Ca^{2+} -dependent fluorescence) and 405 nm (Ca^{2+} -independent isosbestic fluorescence) signals were collected through the same optic fiber cable, spectrally separated by a dichroic mirror, amplified and quantified using a Doric Fluorescence Detector (Doric Lenses). Data was collected via the Doric Neuroscience Studio Software (Doric Lenses) and demodulated using a lock-in detection algorithm. The 470 and 405 nm signals were processed and normalized to baseline signals independently to define $\Delta F/F$ ($F - F_{\text{baseline}}/F_{\text{baseline}}$) using MATLAB (MathWorks). F_{baseline} was the mean of the fluorescence detected during the pre-stimulus period. Isosbestic $\Delta F/F$ was subtracted from the 470 nm $\Delta F/F$ and data were down-sampled to 1 Hz in MATLAB (MathWorks). Mice used for photometry recording were validated before the initiation of the full experimental sequence. Presentation of food to a fasted mouse results in a rapid and substantial inhibition of AgRP neuron activity and only the subjects that exhibited this response were used for further testing (inclusion criteria: detection of spontaneous neuronal activity before and at least 10% inhibition after food contact/consumption). Post-hoc validation of transgene expression and fiber placement was performed at the end of each study histologically.

Food intake and body temperature

Food intake was measured either manually or recorded automatically using the Promethion Core System (Sable System International, Germany). For post-fasting re-feeding studies, mice were fasted overnight and food returned the following morning. Contact-free body temperature was measured using videos recorded with a thermal camera FLIR A655sc High Resolution LWIR (Teledyne FLIR LLC). For each mouse the maximal temperature registered in each frame was extracted and averaged using the FLIR ResearchIR Standard Software (Teledyne FLIR LLC).

Immunofluorescent staining

For immunofluorescent staining, mice were transcardially perfused with phosphate buffered saline (PBS) followed by 10% neutral buffered formalin (SigmaAldrich). Brains were extracted, post-fixed in 10% neutral buffered formalin at 4 °C, cryoprotected in 20% sucrose at 4 °C, and then 25 μ m-thick coronal sections cut on a freezing sliding microtome. Sections were washed in PBS before blocking in 0.5% BSA/0.25% Triton X-100 in PBS for 1 h at room temperature. Tissue was incubated overnight in blocking buffer containing the primary antibodies: rabbit anti-FOS (Abcam, Cat. No. ab190289; diluted 1/1000), chicken anti-GFP (Abcam, Cat. No. ab13970; diluted 1/1000), goat anti-mCherry (Sicgen, Cat. No. ab0040-200; diluted 1/5000). The next day, sections were washed in PBS and then incubated in blocking buffer containing secondary antibodies: Donkey anti-rabbit (Abcam, Cat. No. ab150076, Alexa Fluor 594; diluted 1/1000); Goat anti-chicken (Abcam, Cat. No. ab150169, Alexa Fluor 488; diluted 1/1000); Donkey anti-goat (Abcam, Cat. No. ab150132, Alexa Fluor 594; diluted 1/1000) for 2 hr. For GR staining, free-floating sections were incubated in 1% SDS then blocked in 5% goat serum with 0.5% BSA and 0.25% Triton-X for 1 h at room temperature. Sections were co-incubated with 1:5000 anti-GFP (Abcam, ab13970) and 1:300 Anti-GR (Proteintech, 24050-I-AP) primary antibodies for 3h at room temperature followed by overnight at 4 °C. After PBS washes, sections were incubated with 1:400 goat anti-rabbit 594 (Life Technologies, A11037) and 1:400 goat anti-chicken 488 (Life Technologies, A11039) secondary antibodies for 2 hours at room temperature. Sections were then washed in PBS and mounted on slides, cover slipped and visualized on a Zeiss Axiomanager.D2 upright microscope (Zeiss) and images captured using a Coolsnap HQ1 camera (Photometrics, AZ) through Micromanager software v1.4.23 (<https://imagej.net/Micro-Manager>). Specific band pass filter sets for DAPI, FITC and Texas Red were used to prevent bleed through from one channel to the next. All images were processed and analyzed in Fiji ImageJ (<https://fiji.sc/>).

Serum cytokine measurement

Whole blood was collected from mice by cardiac puncture in terminally anaesthetized mice and serum isolated. Serum TNF- α (Cat. No. DY410), IL-6 (Cat. No. DY406), and IL-1 β (Cat. No. DY401) concentrations were assayed by sandwich ELISA following manufacturer instructions (R&D system).

RNA extraction & real time PCR

RNA isolation and purification from spleen and liver samples was performed using TRI-Reagent (Sigma, 15596026) and QIAGEN RNeasy Mini Kit (74104), according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from RNA templates using High capacity cDNA reverse transcription kits (ThermoFisher, 4368814) and GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). Real-time PCR experiments were carried out using a 7500 Fast Real-time PCR System (Applied Biosystems) using the QuantiFast SYBR green detection method (3). The following forward (F) and reverse (R) primers were used and are provided as 5'-3' sequence: *Gapdh*, (F) TTGTCTCCTGCGACTTCAACA, (R) GGTCTGGGATGGAAATTGTGAG; *Tnf α* , (F) TCTGTCTACTGAACCTCGGGGTG, (R) ACTTGGTGGTTTGCTACGACG; *Il1b*, (F) CAGTTGTCTAATGGGAACGTCA, (R) GCACCTTCTTTTCCTTCATCTTT; *Il6*, (F) GACTTCATCCAGTTGCCCTTCTTGG, (R) CCAGTTTGGTAGCATCCATCATTCT.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using Prism 8.0 (Graphpad Software). Data were analyzed using t test, one-way or two-way ANOVA with post hoc comparisons, where appropriate. Data are presented as mean $\pm$ SEM and/or as individual biological replicates and statistical significance was set at $p < 0.05$.

Current Biology, Volume 32

Supplemental Information

Hypothalamic AgRP neurons

exert top-down control on systemic

TNF- α release during endotoxemia

Mehdi Boutagougua Boudjadja, Isabella Culotta, Gabriela C. De Paula, Erika Harno, Jenna Hunter, João Paulo Cavalcanti-de-Albuquerque, Simon M. Luckman, Matthew Hepworth, Anne White, Gabriella Aviello, and Giuseppe D'Agostino

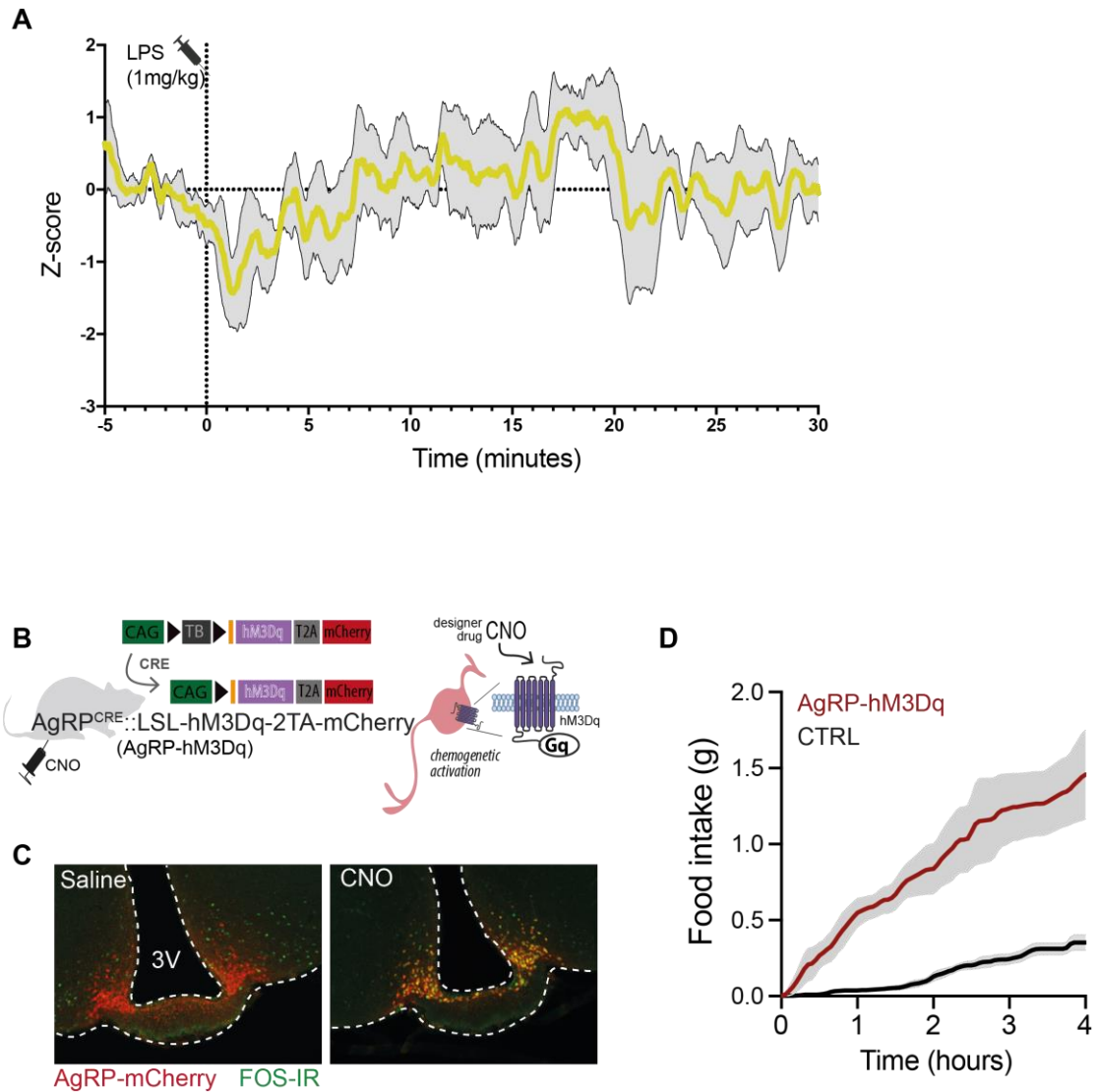


Figure S1. Endotoxin-driven anorexia is independent of AgRP neuron activity.

Related to Figure 1.

(A) Photometry recoding in food-deprived female mice injected with lipopolysaccharide (LPS, 1 mg/kg) systemically (n=4). **(B)** Schematics of the chemogenetic strategy. **(C)** Representative microphotograph showing mCherry expression in the arcuate nucleus and immunostaining for FOS – a marker a neuronal activation – in AgRP-hM3Dq injected with saline (left panel) or CNO (3mg/kg, IP; right panel). Note, almost all AgRP-mCherry express FOS upon CNO treatment. **(D)** CNO injection triggers food consumption in AgRP-hM3Dq mice (n=4),

but not in littermate controls (n=6). Two-way ANOVA: Interaction: $F_{(81,574)} = 3.68$, $p < 0.0001$; genotype: $F_{(1,574)} = 1495.58$, $p < 0.0001$; Time: $F_{(81,574)} = 11.10$, $p < 0.0001$. Data are presented as mean $\pm$ SEM. 3V, third ventricle.

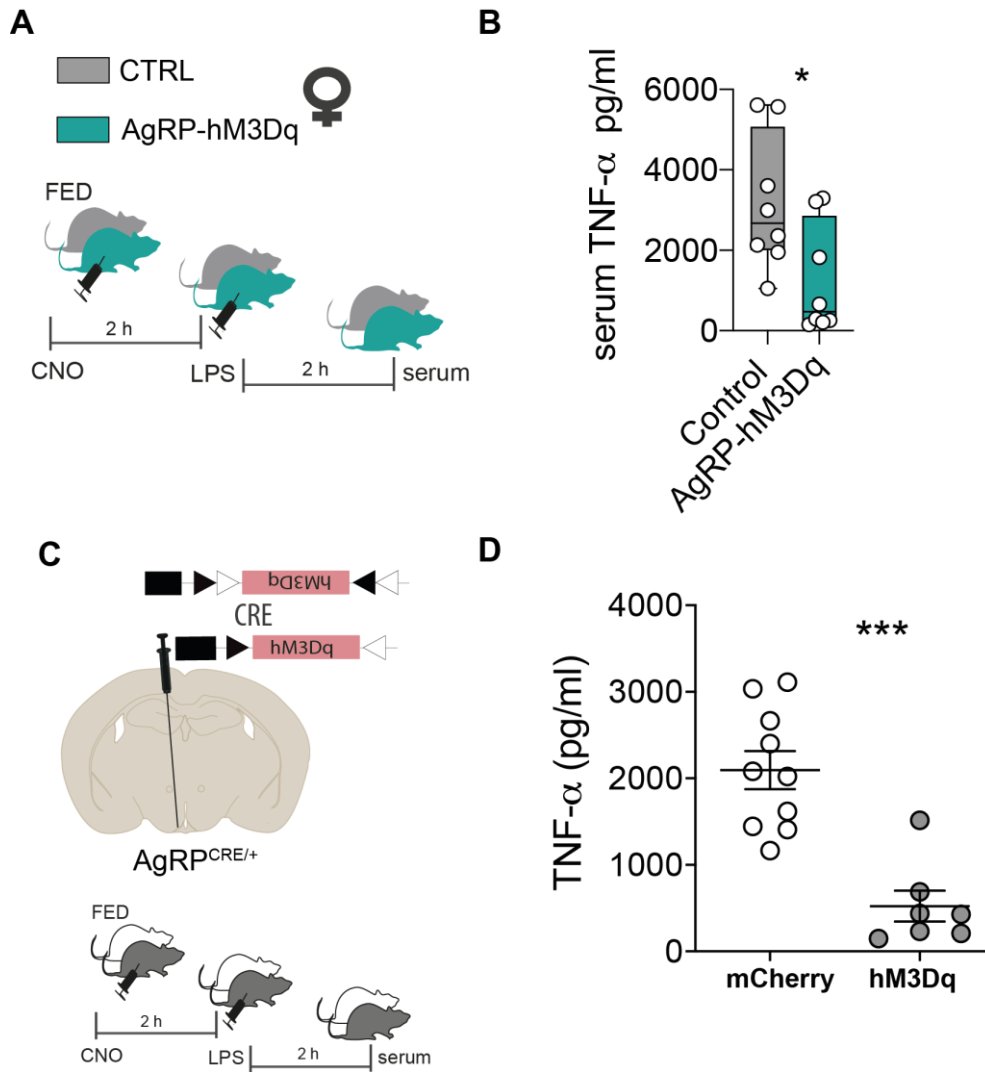


Figure S2. AgRP neuron activity during endotoxemia reduces acute TNF- α secretion. Related to Figure 3.

(A) Experimental timeline. (B) Effect of the chemogenetic activation of AgRP neurons on LPS-induced circulating levels of TNF- α in female mice ($t(14)=2.521$, $p=0.0245$). (C) Targeted delivery of AAVs to the ARC of AgRPCRE/+ adult mice and experimental timeline. AAV8-hSyn-DIO-hM3D(Gq)-mCherry (hM3Dq; $n=7$) or AAV8-hSyn-DIO-mCherry (mCherry; $n=10$). (D) Effect of the chemogenetic activation of AgRP neurons on LPS-induced circulating levels of TNF- α in male mice ($t(15)=5.183$, $p=0.0001$).

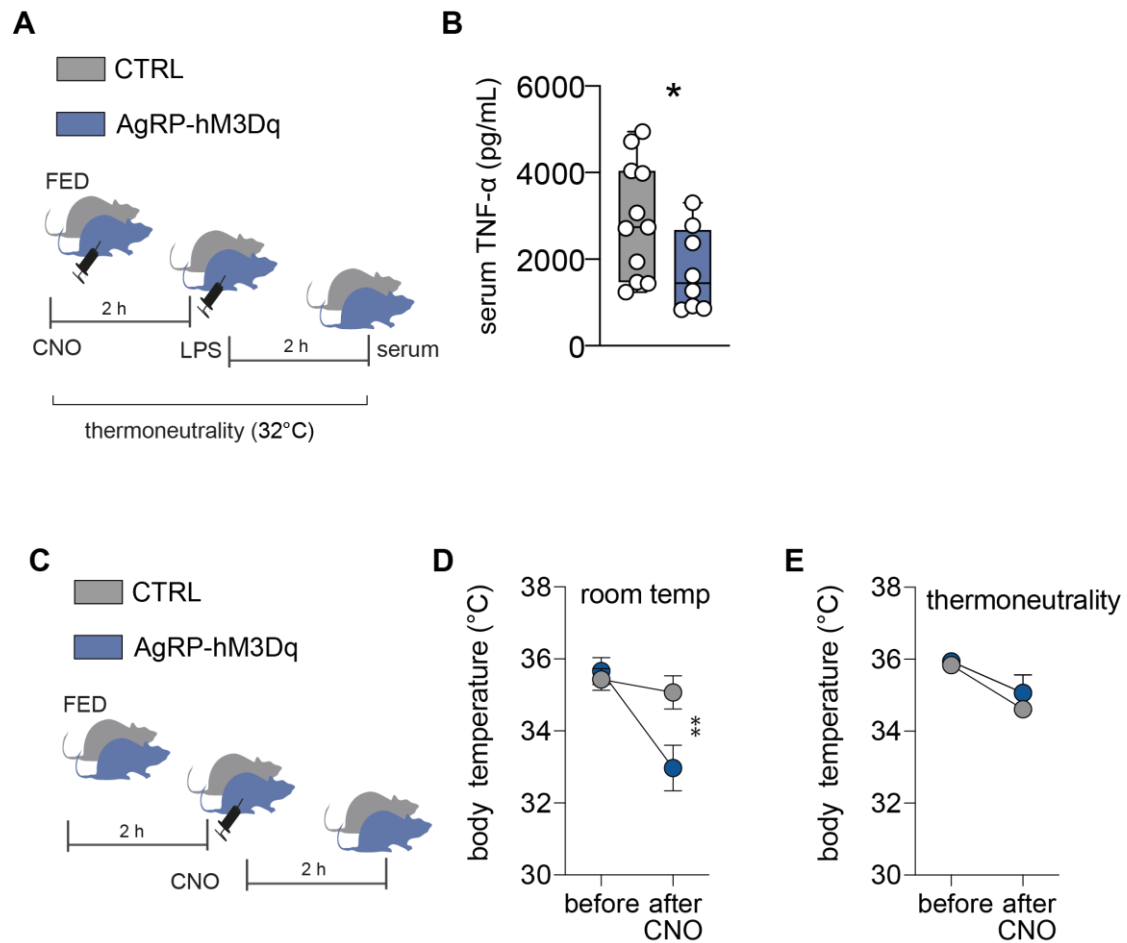


Figure S3. AgRP neuron activity during endotoxemia reduces acute TNF-α secretion independently of thermoregulation. Related to Figure 3.

(A) Experimental timeline. (B) Effect of the chemogenetic activation of AgRP neurons on LPS-induced circulating levels of TNF-α ($t(15)=2.335$, $p=0.0338$). (C) Experimental timeline. (D) Reduction in body temperature upon a CNO injection (3mg/kg, IP) in AgRP-hM3Dq mice ($n=6$), but not in littermate controls ($n=9$), kept at regular housing temperature. Two-way RM ANOVA: CNO x genotype interaction $F_{(1,13)}=12.91$, $p=0.0033$; CNO $F_{(1,13)}=22.30$, $p=0.0004$; genotype $F_{(1,13)}=2.849$, $p=0.1152$; followed by Sidak's multiple comparisons test, $**p<0.005$. (E) Thermoneutral conditions prevent the reduction in body temperature upon AgRP neuron chemogenetic activation. AgRP-hM3Dq mice ($n=8$), but not in littermate controls ($n=12$). Two-way RM ANOVA: CNO x genotype interaction $F_{(1,18)}=1.01$, $p=0.3282$; CNO $F_{(1,18)}=36.37$, $p<0.0001$; genotype $F_{(1,18)}=0.64$, $p=0.4328$.

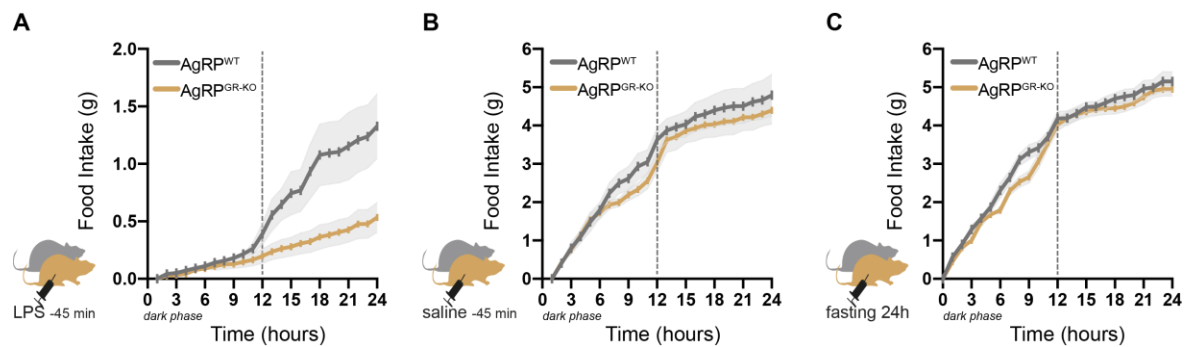


Figure S4. Food intake in *AgRP^{GR-WT}* and *AgRP^{GR-KO}* mice during endotoxemic and physiological conditions. Related to Figure 4.

(A) Food intake recording over a 24-hour period in *AgRP^{GR-WT}* and *AgRP^{GR-KO}* mice injected with LPS (0.1 mg/kg, IP) 45 minutes before the onset of the dark phase (n=8-9). Two-way ANOVA: time x genotype interaction $F_{(24,360)}=6.255$, $p<0.0001$; time $F_{(1,249,18.73)}=27.70$, $p<0.0001$; genotype $F_{(1,15)}=6.351$, $p=0.0236$. (B) Food intake recorded over a 24-hour period in *AgRP^{GR-WT}* and *AgRP^{GR-KO}* mice injected with saline 45 minutes before the onset of the dark phase (n=7). (C) Food intake recorded over a 24-hour period in *AgRP^{GR-WT}* and *AgRP^{GR-KO}* mice following 24 hours food deprivation (n=6).

Target	Bank Number	Forward (5'-3')	Reverse (5'-3')
Gapdh	NM_001289726.1	TTGTCTCCTGCGACTTCAACA	GGTCTGGGATGGAAATTGTGAG
Il1b	NM_008361.4	CAGTTGTCTAATGGGAACGTCA	GCACCTTCTTTTCCTTCATCTTT
Il6	NM_031168.2	GACTTCCATCCAGTTGCCTTCTTGG	CCAGTTTGGTAGCATCCATCATTTCT
Tnfa	NM_013693.3	TCTGTCTACTGAACTTCGGGGTG	ACTTGGTGGTTTGCTACGACG

Table S1. Primer Sequences for qRT-PCR, Related to STAR Methods