Bacterial sensing via neuronal Nod2 regulates appetite and body temperature

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Abstract: Gut bacteria influence brain functions and metabolism. Here we interrogated whether this influence can be mediated by direct sensing of bacteria cell wall components by brain neurons. We show that bacterial peptidoglycan plays a major role in mediating gut–brain communication via the Nod2 receptor. Peptidoglycan-derived muropeptides reach the brain and alter the activity of a subset of brain neurons that express Nod2. Activation of Nod2 in hypothalamic inhibitory neurons is essential for proper appetite and body temperature control, primarily in female mice. This study reveals that brain neurons directly sense muropeptides via neuronal Nod2 receptor, a microbe-sensing mechanism that regulates feeding behavior and host metabolism.

One-Sentence Summary: Inhibitory hypothalamic neurons sense gut-derived muropeptides via the Nod2 receptor.

Main Text: Brain homeostasis and its downstream effects are sensitive to gut microbiota (*1*). In the absence of microbiota, brain chemistry and metabolism are altered, leading to cognitive and behavioral dysfunction (*2*, *3*). Secreted bacterial compounds, found in the circulation, have been implicated in microbiota–brain communication pathways and have been used or targeted to treat brain–related disorders (*4–6*). During homeostasis, the composition of the gut microbiota changes constantly (*7*), leading to the cyclic release of bacterial compounds into the gut lumen. Some of these compounds can influence metabolism, the immune system and behavior in humans and mice (*2*). One of such compounds, peptidoglycan (PG), is a major component of the bacteria cell wall. Fragments of PG are released upon bacterial growth, replication, or death (*8*). Interestingly, PG fragments, known as muropeptides, have been found in mouse brain (*9*), and studies in *Drosophila* have demonstrated its capacity to influence neuronal activity and plasticity (*10*). Due to their presence in almost all bacteria, and their constant release, muropeptides may serve as important gut-derived signals to the brain.

In mammals, muropeptides are recognized by cytosolic Nod-like receptors (Nod1 and Nod2) and by peptidoglycan recognition proteins (PGRPs) (*11*). Nod1 recognizes meso-diaminopimelic acid (meso-DAP)-containing muramyl tripeptides derived mainly from gram-negative bacteria, whereas Nod2 recognizes muramyl dipeptides (MDP), a motif found in every bacterial PG type (*12*).

Nod2 and its ligands are associated with neurodegeneration and memory functions in mouse models of Parkinson's (*13*) and Alzheimer's diseases (*14*). In humans, variants of *NOD2* are associated with bipolar disorder, schizophrenia and Parkinson's disease (*15–17*). In addition, muropeptides have been implicated in sleep alteration (*18*), and Nod2 and MDP play a role in metabolic regulation (*19*, *20*). Nod2 deficiency leads to metabolic dysfunction in response to diet-induced obesity (*19*), whereas MDP displays a protective role in obesity-induced insulin resistance

(*20*). Thus, Nod2 signaling is involved in both brain and metabolic pathologies. However, it remains unknown whether a gut–brain pathway involving neuronal responses to Nod2 activation is necessary to maintain physiological homeostasis.

Here, we have identified a neuronal and cell-autonomous Nod2 signaling pathway, and explored the impact of this pathway on brain activity and its consequences on behavior and metabolism. We show that Nod2 is expressed by a subset of hypothalamic neurons that respond to MDP from the intestine and regulate food consumption, body temperature, and associated behaviors. This work uncovers a bacteria-driven gut–brain communication modality involved in the control of energy homeostasis.

Results

Brain neurons express Nod2

We first investigated the expression pattern of Nod2 in the central nervous system (CNS) using heterozygote knock-in mice harboring one allele encoding a functional Nod2 receptor and the other allele encoding GFP (*Nod2*^{tm1Jhgt}; here-after named Nod2^{GFP} mice), where GFP served as a reporter for Nod2 expression. We detected GFP expression in several brain regions and by distinct cell types (Fig. 1, A to D, and fig S1, A to C). Neurons expressing GFP, variable in morphology and size, were found mostly in the striatum, thalamus and hypothalamus (Fig. 1, B to D and fig. S1B). No significant neuronal expression was found in the cortex (Fig. 1, A to D, and fig. S1, A and B). In contrast to this selective neuronal expression, microglial and endothelial cells expressing GFP were found in all brain regions (Fig. 1, C and D, and fig. S1C). The neuronal expression of GFP did not extend to the intestine where strong GFP expression was detected in endothelial cells (fig.

S1D). This pattern of *Nod2* expression in specific brain regions was confirmed using *Nod2* mRNA in situ hybridization (fig. S2).

Microbiota-derived muropeptides are found in the brain

To determine whether Nod2 ligands from the intestine could directly regulate brain neuronal activity, we first assessed whether orally administered muropeptides reach the brain. To this end, mice were gavaged with radiolabelled muropeptides, and tissues collected 4 hours later (fig. S3A). Muropeptides were able to cross the gut barrier, reach the blood circulation, and accumulate in the brain (fig. S3, B and C). Female mice accumulated more muropeptides in the blood than males, but no differences were detected in the brain (fig. S3, B and C). To assess trafficking to the brain of muropeptides released by gut-resident bacteria, mice were colonized with *Escherichia coli* containing radiolabelled PG, and tissues examined after 24 hours (Fig. 1E). Females accumulated more muropeptides in the brain than males, even though similar amounts of muropeptides were detected in the blood in both groups (Fig. 1F, and fig. S3, D to G). These data show that muropeptides can reach the brain from the gut, possibly at different rates in males and females.

Lack of Nod2 on GABAergic neurons leads to metabolic alterations

We next assessed whether a loss in neuronal expression of Nod2 affected brain-controlled metabolism and behavior. Mice were generated that lacked expression of Nod2 in two main classes of CNS neurons: the inhibitory GABAergic ($Vgat^{+}$) neurons and the excitatory $CamKII^{+}$ neurons. To this end, mice encoding floxed alleles of *Nod2* ($Nod2^{flox}$ mice) were crossed to $Vgat^{cre}$ ($Slc32a1^{tm2(cre)Lowl}$) or $Camk2a^{cre}$ mice to generate $Vgat^{\Delta Nod2}$ and $CamKII^{\Delta Nod2}$ mice. Over a period of several months, $Vgat^{\Delta Nod2}$ females gained more weight than $Nod2^{flox}$ and $Vgat^{cre}$ controls (Fig.

2A). Weight difference became significant at around 6 months of age and further increased as mice aged (Fig. 2A). No weight differences were observed in *Vgat*$^{\Delta Nod2}$ males or in *CamKII*$^{\Delta Nod2}$ mice of either sex (Fig. 2, A and B).

In agreement with these findings, an increase in appetite was observed only among older (>6 months) *Vgat*$^{\Delta Nod2}$ female mice (Fig. 2C, and fig. S4, A and B). *Vgat*$^{\Delta Nod2}$ mice ate a higher number of food pellets when compared to *Nod2*^{flox} mice (Fig. 2D). Although control mice ate more frequently, the number of pellets eaten during each meal bout was significantly higher in *Vgat*$^{\Delta Nod2}$ female mice (Fig. 2E). Thus, the absence of Nod2 in inhibitory neurons of older female mice leads to a delay in achieving satiety, revealing a role for Nod2^{+} GABAergic neurons in appetite control. In mice that fully lack Nod2 expression (Nod2^{KO}), both females and males showed increased weight gain with age (fig. S4, C and D), suggesting that additional effects on eating behavior and weight gain are regulated by non-neuronal and intestinal expression of Nod2 (*21*).

To assess whether the Nod2 ligand MDP modulates appetite control, we gavaged *Vgat*$^{\Delta Nod2}$ and *Nod2*^{flox} littermate control mice with MDP (L-D isoform) or an MDP isomer (L-L isoform, hereafter referred to as MDPctr) that does not activate Nod2 (*12*). We observed that control mice treated with MDP ate less than when treated with MDPctr, whereas *Vgat*$^{\Delta Nod2}$ mice ate more (Fig. 2F). Thus, MDP acts as a satiety signal in mice via the Nod2 receptor expressed by inhibitory neurons.

Older *Vgat*$^{\Delta Nod2}$ females also displayed alterations in nest building behavior, shredding less cotton nestlets than *Nod2*^{flox} and *Vgat*^{cre} mice (Fig. 2G and fig. S4E). In rodents, nesting behavior is involved in heat conservation and is strongly influenced by environmental and body temperature (*22*). In agreement with the altered nesting behavior, the daily temperature variation was smaller in *Vgat*$^{\Delta Nod2}$ females than in control mice (Fig. 2H and fig. S4F). Furthermore, female *Vgat*$^{\Delta Nod2}$

mice showed delayed temperature drop in response to fasting (Fig. 2I and fig. S4G). Similarly, beta3-adrenergic agonist injection induced a quick temperature drop in control but not in *Vgat*$^{\Delta Nod2}$ mice (Fig. 2J), altogether showing that *Vgat*$^{\Delta Nod2}$ female mice develop altered body temperature control. Older *Vgat*$^{\Delta Nod2}$ female mice eventually developed a diabetic-like phenotype (fig. S4H), as well as a reduced lifespan (fig. S4I). Thus, Nod2 expression by inhibitory neurons plays an important role in the control of female metabolism.

Sex- and age-dependent MDP-mediated activation of brain neurons

To identify the brain regions affected by MDP, as well as to understand why MDP regulated feeding and body temperature only in older females, younger (2-3 months) females and older (7-8 months) females and males were gavaged with either the Nod2 ligand MDP or MDPctr. An unbiased mapping of Fos expression in the brain revealed that MDP administration induced distinct patterns of neuronal activation among younger and older females and males, as well as a more pronounced effect in older mice, where numerous nuclei were affected (Fig. 3A; Table S1). This age-related difference was not associated with a failure of gut-derived muropeptides to reach the brain of younger females (fig. S5, A to F). Following MDP gavage, only older females showed significant alterations in neuronal activity of the arcuate (ARC) and the dorsomedial (DMH) nuclei of the hypothalamus, as well as in the lateral hypothalamic area, key regions involved in the regulation of feeding behavior and body temperature (Fig. 3, A and B). Thus, older females exhibit higher responsiveness to MDP in regions involved in the regulation of appetite and body temperature (*23*, *24*). Finally, we did not observe significant differences with age and/or sex for neuronal Nod2 expression in the ARC or DMH (fig. S5, G and H).

Hypothalamic GABAergic neurons respond to MDP

To further dissect the MDP-mediated effects on hypothalamic neurons, we analyzed the effect of MDP on GABAergic (inhibitory) neurons of the ARC ($Vgat^{ARC}$). We first confirmed by in situ hybridization that $Vgat^{+}$ and NPY^{+} neurons in this area expressed Nod2 (Fig. 4A and fig. S6A). By contrast, proopiomelanocortin (POMC)-expressing (non-GABAergic) neurons did not express Nod2 (fig. S6B). Next, specific expression of the calcium sensor GCamp in GABAergic neurons allowed for the monitoring of $Vgat^{ARC}$ neuronal activity (Fig. 4, B and C). These neurons are active after a fasting period and rapidly decrease activity upon feeding (*25*) (Fig. 4, D and E). MDP but not MDPctr administration induced a similar long-lasting decrease in the spontaneous activity of $Vgat^{ARC}$ neurons in control mice, but not in *Vgat*$^{\Delta Nod2}$ mice (Fig. 4, D to G and fig. S6, C to I). Thus, MDP decreases $Vgat^{ARC}$ neuronal activity via the Nod2 receptor and influences appetite control by modulating hypothalamic circuits.

To demonstrate cell-autonomous regulation of $Vgat^{ARC}$ neurons by MDP, we performed ex vivo patch-clamp recordings on brain slices from *Vgat*^{cre}*Nod2*^{GFP} mice injected with a Cre-dependent reporter virus to target $Vgat^{+}Nod2^{+}$ neurons and confirm their identity post-recording (fig. S6J). We characterized cell excitability after infusion of MDP or MDPctr into individual neurons. Thirty minutes after infusion, MDP, but not MDPctr, induced a strong decrease in the number of action potentials elicited in Nod2-expressing neurons (Fig. 4, H and I, and fig. S6K). This reduction in cell excitability did not result from changes in cell membrane-intrinsic properties, as no significant differences were observed in input membrane resistance (Fig. 4J), nor in the threshold to trigger firing activity (Fig. 4K). Thus, MDP decreases the $Vgat^{ARC}$ neuronal activity in a cell-autonomous manner.

Expression of Nod2 in ARC and DMH neurons regulates body weight and temperature

Finally, we assessed whether Nod2-expressing hypothalamic neurons regulated metabolism in "steady state" control female mice. In such mice, the ARC and DMH neurons showed different levels of activity, as measured by Fos expression, when compared to *Vgat*$^{\Delta Nod2}$ mice (Fig. 5, A and B; Table S2). We observed in those areas Nod2^{+} and Nod2^{+}Vgat^{+} neurons (Fig. 5, C to F). To confirm the causal implication of these neurons in the control of metabolism in older females, Nod2 expression was locally abrogated by the injection of a Cre-expressing virus into the hypothalamus of *Nod2*^{flox} mice (Fig. 5, G and H). Cre-injected *Nod2*^{flox} females gained more weight, consumed more food, and showed a smaller variation in body temperature than Cre-injected controls (Fig. 5, I to K). These mice also developed a tendency to decreased nest building (Fig. 5L). Thus, the expression of Nod2 in ARC and DMH hypothalamic neurons is necessary to regulate feeding behavior and body temperature in older female mice.

In order to show that microbiota-derived Nod2-ligands are involved in this regulation, *Nod2*^{flox} and control female mice were injected with Cre-expressing virus and treated with broad spectrum antibiotics (ABX) for 13 weeks. ABX treatment has been shown to effectively reduce the amount of muropeptides in the blood (*26*). No difference in weight gain or food consumption was observed between *Nod2*^{flox} and control mice injected with the Cre-expressing virus during ABX treatment (Fig. 5, M and N, and fig. S7A). By contrast, ABX removal led to increased weight gain in Nod2^{flox} mice (Fig. 5M) and to a decrease in food consumption only in control mice (Fig. 5N), that was associated with a normalization of the intestinal microbiota (fig. S7, B and C). These data indicate that the microbiota plays a role in the production of Nod2 ligands and the regulation of appetite by Nod2-expressing hypothalamic neurons.

Discussion

This work unveils a gut–brain communication pathway, in which the expression of the Nod2 receptor in hypothalamic inhibitory neurons regulates appetite and body temperature in response to bacterial–derived muropeptides.

Using mutant mice and virus-induced gene deletion, we have identified the role of Nod2 in inhibitory neurons in the control of body temperature and appetite. Diverse mechanisms have been proposed for the bacterial influence on host appetite, involving microbial metabolites such as short-chain fatty acids (*27*) and the *E. coli* protein ClpB (*28*). Here we describe another mechanism by which gut bacteria muropeptides may control host feeding behavior. As the transient postprandial increase in the gut microbial population (*7*) may lead to an increased release in cell wall-derived muropeptides, the host may use this bacterial signal to limit feeding as well as bacterial expansion. Alternatively, the bacterial microbiota may modulate the host's feeding behavior to stabilize its intestinal niche.

Previous studies, using loss of function approaches, have also reported diverse functions of PG in gut–brain crosstalk. Lack of proper PG cleavage by the host (due to the absence of Pglyrp2) leads to several behavioral impairments including anxiety-like behavior (*29*). Moreover, the specific deletion of the Nod1 receptor in epithelial cells increases the susceptibility of mice to stress-induced anxiety-like behavior and cognitive impairment (*30*). In our hands, no changes in anxiety-like behavior were observed in $Vgat^{\Delta Nod2}$ mice or in $CamKII^{\Delta Nod2}$ female mice (fig. S8, A and B). By contrast, total $Nod2^{KO}$ females over 6 months of age developed stronger anxiety-like behavior when compared to their wild-type controls (fig. S8C). Given the widespread expression of Nod2 in different cell types and locations, the differences observed between $Vgat^{\Delta Nod2}$ and $Nod2^{KO}$ mice suggest that additional PG-dependent alterations may arise from mechanisms involving non-

neuronal cell types. It is also possible that, endogenous Nod2 ligands, besides bacteria-derived muropeptides, play a role in the phenotypes we observed. Although its relevance in vivo or in neurons has not been demonstrated yet, in vitro studies have revealed that Nod2 may respond to endogenous sphingosine-1-phosphate released under cellular stress conditions (*31*).

The lack of Nod2 in inhibitory neurons had particularly strong effects on appetite in females over 6 months of age. A variety of brain- and metabolism-related diseases have shown sex- and age-dependent phenotypes (*32–35*). Thus, understanding the mechanisms behind such biases may lead to more specific and efficient therapeutic approaches. To explore the mechanisms behind such sex and age-specificity we evaluated different parameters: 1) Nod2 expression by hypothalamic nuclei; 2) brain neuronal response to MDP; and 3) PG-pharmacokinetics. Among these factors, only the increased accumulation of muropeptides in the female brain, when compared to males, agreed with the sex-specific hypothalamic neuronal activation in response to MDP. Previous studies have also described age and sex biases in the context of gut–brain communication (*29*, *36*). For example, microglial responses to bacterial metabolites are stronger in males during the embryonic phase, whereas female microglia are more responsive in adulthood (*36*). Moreover, the absence of Pglyrp2 influences brain development and behavior, leading eventually to sex- and age-dependent alterations (*9*, *29*). Many additional factors showing age and sex differences, such as hormonal status and intestinal and blood brain barrier permeability, as well as microbiota composition, are likely to play a role in our observations (*32*, *37–39*).

On an evolutionary perspective, it is possible to speculate that female-specific appetite control systems were positively selected given the importance of energy balance in females for sexual maturity and pregnancy (*40*). In our study, the differences in weight became significant around 6 months of age and increased with time. This time period correlates with hormonal changes in female mice associated with pre-menopausal states in humans (*41*), such as the significant decrease

in estradiol production. This hormone plays an important role not only in sexual maturity but also in energy balance. Specific deletion of estradiol receptor α in POMC cells leads to body weight increases only in female mice. Estradiol activate POMC neurons and consequently inhibit NPY neurons, leading to a decrease in food intake (*42*). Here we show that MDP effects on food intake are mediated via the GABAergic neurons in the ARC, which includes mostly NPY neurons. Therefore, estradiol and MDP may exert their effects trough similar pathways, as estradiol-induced effects requires NPY neurons (*42*). To explain the appearance of this phenotype in older females alone, we speculate that, in the presence of higher levels of estradiol in younger females, the MDP effect on food intake is masked as estradiol may be a more potent anorexia inducer. As the levels of estradiol begin to decrease (as observed in pre-menopausal and menopausal states), this control of food intake would be attenuated and MDP anorexic effects via Nod2 hypothalamic neurons would play a more prominent role in appetite control.

In addition to the well-studied control of host immune response by Nod2, our work highlights the need to consider the effects of Nod2 activation on brain neuronal activity in the context of using Nod2 ligands as a potential therapeutic tool. As PG-derived metabolites are already used in clinics for cancer therapies and planned for other applications (*43*), a better understanding of the physiological roles of Nod2 and its ligands is therefore extremely important. Our work reveals a sex- and age-dependent pathway of gut–brain crosstalk that may open up additional avenues for the treatment of neurological and metabolic disorders.

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Supplementary Materials:

Materials and Methods

Fig S1 to S8

Table S1 and S2

References (44-53)

Legends to Figures

Figure 1. Nod2 and Nod2-ligands in the brain. (**A, B, D**) Immunofluorescence of *Nod2*^{GFP} mouse brain slices. Representative images of (A) brain sagittal slices stained for Nod2 (GFP; white) and (B) of a coronal slice stained for neurons (NeuN; red), microglia (Iba1; white), Nod2 (GFP; green) and cell nuclei (DAPI; blue), highlighting cortex, thalamus, striatum, and hypothalamus; scale bars=150 μm. (**C**) Quantification of neurons (Neun^{+}); microglia (Iba^{+}) and their co-localization with Nod2 (GFP^{+}) in the cortex, striatum, thalamus, and hypothalamus. (D) Co-localization of Nod2 (green) with Iba1 (white) or NeuN (red) in the cortex, thalamus, striatum and hypothalamus. Yellow arrows indicate Iba1^{+}Nod2^{+} cells; white arrows indicate NeuN^{+}Nod2^{+} cells; scale bars=10 μm. (**E**) PG tracking experiment scheme. (**F**) Radioactivity detected 24 hours after gavage with ^{3}H-labeled *E. coli* in the blood and brain of older females and males (7-8 months; n=6 per group). Controls were gavaged with non-labeled *E. coli* (n=4). Data were analyzed by unpaired *t* test (F); shown as average ± SEM; **P*≤0.05. Abbreviations: Caudate putamen (CPu); hypothalamus (Hyp); inferior colliculus (IC); lateral geniculate nucleus (LGN); medial geniculate nucleus (MGN); nucleus accumbens (Acc); olfactory tubercles (OT); posterior thalamic nucleus (Po); preoptic area (POA); substantia nigra (SN); ventral medial nucleus of the thalamus (VM); ventral tegmental area (VTA) and peptidoglycan (PG).

Figure 2. Nod2 expression by inhibitory neurons affects feeding and temperature in older female mice. (**A, B**) Mouse body weights up to 12 months of age. (A) *Vgat*$^{\Delta Nod2}$ females (n=13-30) and males (n=6-29). (B) *CamKII*$^{\Delta Nod2}$ females (n=3-14) and males (n=7-9). (**C-F**) *Vgat*$^{\Delta Nod2}$ female mice eating behavior. (C) Food eaten in 24 hours by older (7-8 months; n=7-9) and younger mice (3-4 months; n=10-19). (D-F) Food eaten measured with an automated system during the dark period (age >6 months). (D) Number of food pellets eaten. (E) Meal bouts and food pellets eaten per meal (n=8 per group). (F) Food pellets eaten 4 hours after MDP or MDPctr gavage (n=5-6). Data normalized by amount eaten after MDPctr gavage. (**G**) Nest building test. Amount of cotton used to build the nest (unrolled cotton) is shown for younger (3-4 months; n=20-29) and older mice (7-8 months; n=13-15). (**H-J**) Body temperature measurements using telemetric probes for *Vgat*$^{\Delta Nod2}$ females (age >6 months). (H) Daily variation in temperature at steady state. Daily difference between maximum and minimum temperature (delta) per mouse and, mean maximum and minimum reached over several days (n=7-9). (I) Body temperature response to fasting (n=4-6) and (J) before and after beta3-adrenergic agonist (CL316,243) injection (n=5 per group). Data analyzed by two-way ANOVA (A to C, I and J); unpaired *t* test (D to F and H for 3 variables, delta, max and min); Fisher's exact test (G); shown as average ± SEM; **P*≤0.05. Abbreviations: older females (OF); younger females (YF); muramyl dipeptide (MDP); MDP isomer (MDPctr).

Figure 3. MDP affects neuronal activation in a sex- and age-dependent manner. (**A and B**) Analysis of brain neurons Fos expression in wild type mice 3 hours post MDP or MDPctr gavage; younger females (2-3 months); older females (7-8 months) and older males (7-8 months) (n=4 per group). (A) Volcano plots from the automated analysis of Fos$^+$ cells distribution in the brain. (B) Raw data, heatmaps and *P*-values maps of Fos expression in the hypothalamus, highlighting DMH, ARC, and LH. Shown are regions with higher (red) or lower (green) number of Fos$^+$ neurons in

the MDP group as compared to the MDPctr group; scale bars=200 μm. Data were analyzed by unpaired *t* test; **P*≤0.05. Abbreviations: Arcuate hypothalamic nucleus (ARC); Dorsomedial nucleus of the hypothalamus (DMH); Dorsal part of the lateral geniculate complex (LGd); Fasciola cinereal (FC); Flocculus (FL); Gigantocellular reticular nucleus (GRN); Globus pallidus external segment (GPe); Globus pallidus internal segment (GPi); Hippocampal formation (HPF); Inferior olivary complex (IO); Lateral dorsal nucleus of thalamus (LD); Lateral hypothalamic area (LH); Medial geniculate complex (MG); Medial septal nucleus (MS); middle cerebellar peduncle (mcp); Nucleus of the lateral lemniscus (NLL); Paragigantocellular reticular nucleus (PGRN); Parasubthalamic nucleus (PSTN); Postsubiculum (POS); Superior olivary complex (SOC); and muramyl dipeptide (MDP); MDP inactive isomer (MDPctr = CTR).

Figure 4. MDP decreases $Vgat^{ARC}$ neuronal activity. (**A**) Representative images of RNAscope® with *Nod2* (green) and *scl32a1* (*Vgat*; red) probes and nuclear staining (DAPI; blue) in the ARC. Co-localization of *Nod2* and *scl32a1* can be seen, with $Nod2^{KO}$ mice used as a negative control; scale bars= 50 μm. (**B to G**) In vivo fiber photometry (females >6 months). (B) Scheme of calcium activity recording scheme for $Vgat^{ARC}$ neurons. (C) Representative immunofluorescence image of the brain of a mouse subjected to fiber photometry, GCaMP (GFP; green) and cell nucleus (DAPI; blue); scale bars= 200 μm. Injection site and canula track (dashed lines) are highlighted. (D) Representative traces of GCaMP6f fluorescence signals from $Vgat^{ARC}$ neurons of fasted mice. (E) Number of spontaneous events before (baseline) and after food presentation (food) for fasted mice. Lines connect each individual (n=4). (F) Number of spontaneous events after MDPctr or MDP gavage for fasted $Vgat^{cre}$ mice (n=6), and (G) without fasting for $Vgat^{cre}$ (n=7) and $Vgat^{\Delta Nod2}$ (n=5) mice. Lines connect each individual. Data normalized by baseline obtained before gavage. (**H to K**) Ex vivo patch-clamp recordings in $Nod2^{+}$ $Vgat^{ARC}$ neurons (females >6 months; n=4 mice;

nine MDPctr and seven MDP cells). (H) Representative traces of triggered action potentials from cells treated with MDPctr or MDP at T0 and T30 (minutes). (I) Maximum number of spikes, (J) membrane resistance and (K) rehobase measurements along the recording. Data analyzed by paired *t* test (E and F); two-way ANOVA (G, I to K); shown as average ± SEM; **P*≤0.05. Abbreviations: Third ventricle (3rd V); muramyl dipeptide (MDP); MDP inactive isomer (MDPctr).

Figure 5. Hypothalamic Nod2^{+} neurons are required for weight and temperature control. (**A and B**) Fos expression in female *Vgat*$^{\Delta Nod2}$ mice and controls at steady-state (7-8 months; n=4 per group). (A) Volcano plots of brain Fos^{+} cells distribution in the brain. (B) Heatmaps and *P*-values maps of Fos expression in the hypothalamus, highlighting DMH and ARC. Shown are regions with higher (red) or lower (green) number of Fos^{+} neurons in the *Vgat*$^{\Delta Nod2}$ group as compared to the control group; scale bar=200 µm. (**C and D**) Representative immunofluorescence image of (C) ARC and (D) DMH in *Nod2*^{GFP} mice; Nod2 (GFP; green) and cell nucleus (DAPI; blue). (**E**) Representative immunofluorescence image of *Vgat*^{cre}*Nod2*^{GFP} mouse brains injected with AAV9.CAG.FLEX.Tdtomato virus in DMH and ARC. Co-localization of Nod2 (green) with Tdtomato (red) is highlighted; scale bars=100 µm. (**F**) Frequency of Tdtomato^{+} cells expressing GFP among total Tdtomato^{+} cells (n=4 mice). (**G**) Viral injection scheme. (**H**) Representative immunofluorescence image of *Rosa*^{YFP} mouse brain injected with AAV9.hSyn.Cre virus in DMH and ARC; YFP (GFP; green) and cell nucleus (DAPI; blue). (**I**) Body weight weekly measures post-viral injection (females, 2-3 months at week 0; n=8-11). (**J**) Food eaten in 40 hours (n=5-6). (**K**) Body temperature variation in 24 hours (n=4-5). (**L**) Nest building test. Amount of cotton used to build the nest (unrolled cotton) is shown (n=9-10). (**M**) Virus-injected mice treated with ABX. Body weight weekly measures during and post-ABX treatment (females, 2-4 months at week 0; n=13 per group). (**N**) Food eaten in 40 hours at week 13 (during ABX treatment) and 24 (post-

ABX treatment) (n=7 per group). Data analyzed by unpaired *t* test (A, J and K); two-way ANOVA (I, M); Fischer's exact test (L); repeated measures ANOVA (N); shown as average ± SEM; **P*≤0.05. Abbreviations: Arcuate nucleus of the hypothalamus (ARC); Dorsomedial nucleus of the hypothalamus (DMH); Perihypoglossal nuclei (PHY) and antibiotics (ABX).

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