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Gliotransmission and Brain Glucose Sensing

Critical Role of Endozepines

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Hypothalamic glucose sensing is involved in the control of feeding behavior and peripheral glucose homeostasis, and glial cells are suggested to play an important role in this process. Diazepam-binding inhibitor (DBI) and its processing product the octadecaneuropeptide (ODN), collectively named endozepines, are secreted by astroglia, and ODN is a potent anorexigenic factor. Therefore, we investigated the involvement of endozepines in brain glucose sensing. First, we showed that intracerebroventricular administration of glucose in rats increases DBI expression in hypothalamic glial-like tanycytes. We then demonstrated that glucose stimulates endozepine secretion from hypothalamic explants. Feeding experiments indicate that the anorexigenic effect of central administration of glucose was blunted by coinjection of an ODN antagonist. Conversely, the hyperphagic response elicited by central glucoprivation was suppressed by an ODN agonist. The anorexigenic effects of centrally injected glucose or ODN agonist were suppressed by blockade of the melanocortin-3/4 receptors, suggesting that glucose sensing involves endozepine control of the melanocortin pathway. Finally, we found that brain endozepines modulate blood glucose levels, suggesting their involvement in a feedback loop controlling whole-body glucose homeostasis. Collectively, these data indicate that endozepines are a critical relay in brain glucose sensing and potentially new targets in treatment of metabolic disorders. *Diabetes* 62:801–810, 2013

To regulate energy homeostasis, the brain integrates peripheral signals delivered by the blood, including metabolites and hormones, and generates appropriate responses by modulating food intake and peripheral organ activity (1). The arcuate nucleus of the hypothalamus is a major site for integration of energy status. It possesses two interconnected populations of neurons, one producing the orexigenic neuropeptide Y (NPY), and the other one producing the anorexigenic

peptide α -melanocyte-stimulating hormone (α -MSH), a processing product of proopiomelanocortin (POMC) (2,3). Moreover, agouti-related protein (AgRP), a potent orexigenic peptide, is coexpressed with NPY in most NPYergic arcuate neurons and acts as an endogenous antagonist of the melanocortin (MC) receptors (2).

Direct glucose sensing by the central nervous system has been extensively demonstrated. It is noteworthy that central administration of glucose reduces NPY expression, increases POMC expression, and markedly reduces feeding (4). Conversely, central glucoprivation using 2-deoxyglucose (2-DG) elicits food intake and activates neurohumoral counter-regulatory responses similar to those observed during systemic hypoglycemia (4–7). Importantly, alteration in brain glucose sensing is associated with obesity and diabetes (8,9). The cellular mechanisms underlying central glucose sensing are far from being understood. By analogy to pancreatic β -cells, it may involve GLUT2 and glucokinase (10–12).

Several studies suggest that astroglial cells play an important role in glucose sensing. First, tanycytes, specialized ependymal cells located in the floor of the third ventricle, were found to be glucose-sensitive elements, and stimulation of their cell bodies by glucose evokes calcium waves (13,14). Second, selective destruction of tanycytes impairs feeding and hyperglycemia responses induced by 2-DG (15). Third, genetic inactivation of GLUT2 impairs glucagon secretion induced by hypoglycemia, and re-expression of GLUT2 in glia restores this response (5). Fourth, selective stimulation of glucose metabolism in hypothalamic glia by overexpression of GLUT1 normalizes plasma glucose levels in diabetic rats (16). Together, these studies strengthen the emerging concept that glial cells can detect changes in nutrient availability and interact with neurons to regulate energy homeostasis.

Diazepam-binding inhibitor (DBI) and its peptide fragments, including the octadecaneuropeptide (ODN), which are known to bind benzodiazepine receptors, are collectively termed endozepines (17). These peptides are specifically produced by glial cells in the central nervous system, and hypothalamic astrocytes and tanycytes express high levels of endozepines (18,19). Numerous data indicate that endozepines are secreted from astroglial cells and, in line with well-characterized gliotransmitters, this process is regulated by physiological stimuli (20–22). A role for endozepines in the control of energy homeostasis has been demonstrated by central administration of ODN, or its COOH-terminal octapeptide (OP) fragment, which markedly inhibits food intake and reduces body weight in rodents (23,24). Pharmacological experiments revealed that the anorexigenic effects of ODN and OP are mediated through activation of a metabotropic receptor distinct from benzodiazepine receptors (24).

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Moreover, the intracerebroventricular injection of ODN increases POMC mRNA levels and decreases NPY mRNA levels in the arcuate nucleus (25). Finally, acute food deprivation markedly reduces hypothalamic DBI mRNA levels in mice, indicating that endozepine expression correlates with energy status (18). Altogether, these data led us to hypothesize that endozepines may be released as a function of glucose status and act as a relay in brain glucose sensing.

RESEARCH DESIGN AND METHODS

Animals and surgical procedures. Adult male Wistar rats weighing 300–350 g were housed under constant temperature (22°C) in a 12:12-h light/dark cycle, with free access to standard rat chow and drinking tap water. For intracerebroventricular injections, rats were stereotaxically implanted with a permanent stainless steel cannula into the right lateral ventricle of the brain, as described (26). For intra-arcuate injections, rats were implanted with a 22-gauge single-guide cannula (Plastics One), aimed at the top of the arcuate nucleus using the following stereotaxic coordinates: 3.14 mm posterior to the

bregma, and 0.2 mm lateral and 8.5 mm ventral to the brain surface. The guide cannulas were secured with screws and cranioplastic cement (Dentsply International). To prevent clogging and to reduce the potential for brain infection, sterile obturators were inserted into the guide cannulas. Cannula placement was histologically verified a posteriori by thionin staining of coronal brain sections. Experiments were conducted according to the French and European guidelines for the care and use of laboratory animals (Council Directive 86/609/EEC; license no. 21CAE035).

Materials. D-Glucose, L-glucose, 2-DG, and α-MSH were purchased from Sigma. The metabotropic endozepine receptor agonists ODN (H-Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys-OH) and OP (H-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys-OH), and the metabotropic endozepine receptor antagonist cyclo₁₋₈[DLeu⁵]OP were synthesized as described (27). SHU-9119 was obtained from Polypeptide Laboratories.

Effects of intracerebroventricular glucose injection on hypothalamic DBI and POMC mRNA levels. Animals were divided into three groups of six rats each. In the first and second groups, animals were food-deprived for 13 h, from Zeitgeber time (ZT) 11 to ZT24 (mean glycemia at ZT24: 0.83 ± 0.06 g/L), and received an intracerebroventricular injection of D-glucose (3.5 mg in 7 μL of 0.9% NaCl) or vehicle (7 μL of 0.9% NaCl). A third group was allowed free access to food

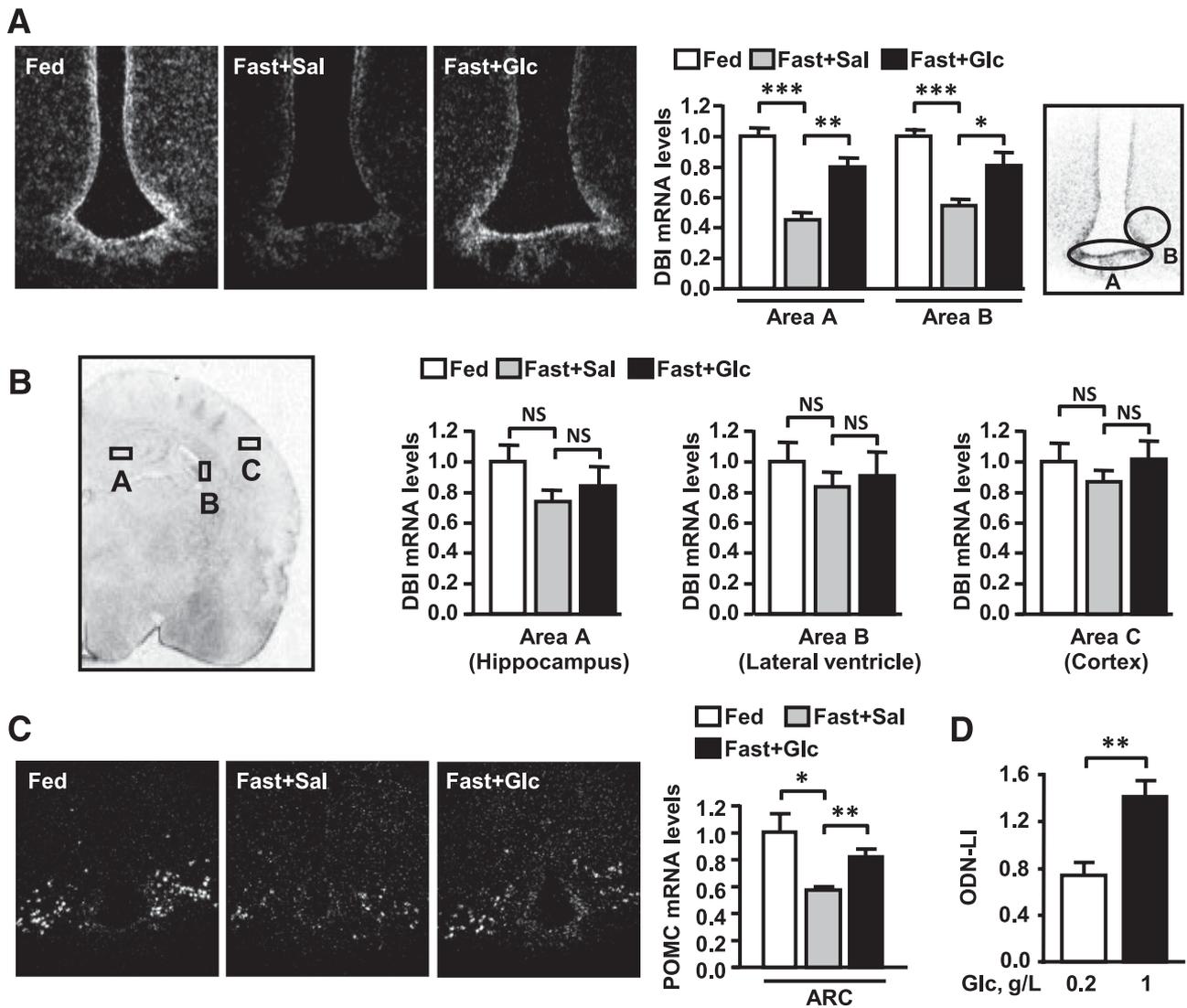


FIG. 1. Glucose stimulates the expression of endozepines in the rat hypothalamus. **A:** Hypothalamic DBI mRNA levels from normally fed (Fed) and overnight-fasted saline-injected (0.9% NaCl, Fast+Sal) or glucose-injected (Fast+Glc) animals. Quantitation was performed in area A (floor of the third ventricle) and area B (infralateral wall of the third ventricle). Values are normalized to those obtained with fed animals (*n* = 6). **B:** Extrahypothalamic DBI mRNA levels from Fed, Fast+Sal (0.9% NaCl), or Fast+Glc animals. Quantitation was performed in hippocampal CA1 area (Area A), in the cells lining the lateral ventricle (Area B), and in the cortex (Area C). Values are normalized to those obtained with fed animals (*n* = 6). **C:** POMC mRNA levels in the arcuate nucleus (ARC) from Fed, Fast+Sal, or Fast+Glc animals. Values are normalized to those obtained with fed animals (*n* = 5). **D:** Hypothalamic explants were preincubated in 0.2 g/L glucose (Glc) and then incubated in 0.2 or 1 g/L glucose. ODN-like immunoreactivity (ODN-LI) released during the incubation in 0.2 or 1 g/L was normalized to that released during the preincubation period (*n* = 6–8). Data are expressed as mean ± SEM. Unpaired *t* test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS, not statistically different.

throughout the experiment, and vehicle was administered intracerebroventricularly at ZT24 (mean glycemia at ZT24: 1.13 ± 0.03 g/L). Rats were anesthetized 3 h after injection (ZT27) and perfused transcardially with 4% paraformaldehyde. Brains were removed and processed for *in situ* hybridization, as previously described (18), with rat DBI (GenBank NM_031853, nucleotides 1 to 526) and mouse POMC (GenBank NM_008895, nucleotides 98 to 384) probes. To assess the specificity of the hybridization signals, consecutive sections were alternatively hybridized with sense and antisense probes. The optical density of the hybridization signal measured in each specific region was corrected for the average background signal. Quantitative data are expressed as mean \pm SEM ($n = 6$ rats per group).

Immunohistochemistry. Rat brain coronal sections (5 μ m) mounted on coated glass slides (Menzel-Gläser) were incubated at room temperature for 20 min in PBS containing 1% normal goat serum, 1% BSA, and 0.3% Triton X-100, and then incubated at 4°C for 18 h with rabbit antiserum against rat ODN (1:100 dilution [28]) and mouse monoclonal antibody directed against dopamine- and cAMP-regulated phosphoprotein-32 (1:250 dilution; BD Biosciences) or mouse

monoclonal antibody directed against vimentin (1:1,000 dilution; clone V9; DAKO) in PBS/Triton X-100/BSA. Sections were rinsed in PBS and incubated at room temperature for 90 min with the secondary antibodies (AlexaFluor 488-conjugated goat anti-mouse and AlexaFluor 594-conjugated goat anti-rabbit antibodies, 1:100 each; Molecular Probes, Thermo Fisher Scientific) in PBS/Triton X-100/BSA. After washes in PBS, tissue slices were mounted in PBS-glycerol (1:1) and examined using a confocal laser scanning microscope (Leica TCS SP2 AOBSS; Leica Microsystems). Previous data suggest that the antiserum against rat ODN also recognizes precursor forms that may include full-length DBI (28).

Culture of hypothalamic explants. Half-rat hypothalami were equilibrated in artificial cerebrospinal fluid medium (26 mmol/L NaHCO₃, 125 mmol/L NaCl, 1.2 mmol/L Na₂HPO₄, 3 mmol/L KCl, 2 mmol/L CaCl₂, 1.2 mmol/L MgSO₄, 0.2 g/L glucose, 1.8 mg/mL ascorbic acid, and 100 μ g/L aprotinin) at 37°C for 1 h under constant bubbling of 95% O₂ and 5% CO₂. The explants were then preincubated for 45 min with fresh medium containing 0.2 g/L glucose and incubated 45 min in medium containing 0.2 or 1 g/L glucose. Measurement of

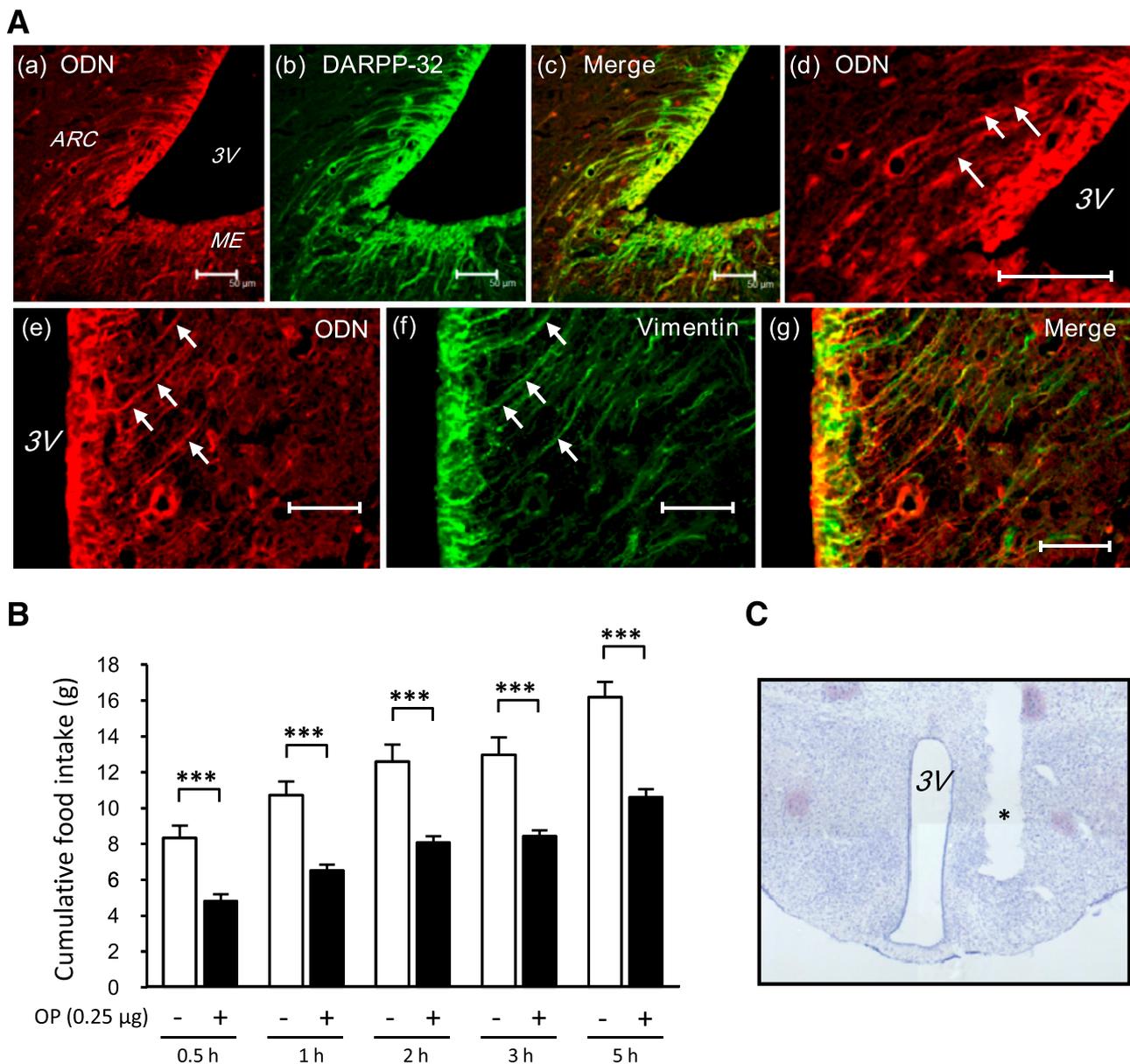


FIG. 2. Arcuate endopeptides regulate feeding behavior. *A, top row:* Hypothalamic section labeled with ODN (*a, c, and d*) and dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32) (*b and c*) antibodies. *d:* Higher magnification view of panel *a*. *A, bottom row:* Hypothalamic section labeled with ODN (*e and g*) and vimentin (*f and g*) antibodies. Arrows show tanyocyte processes that extend into the parenchyma. *ARC*, arcuate nucleus; *ME*, median eminence; *3V*, third ventricle. Scale bars = 50 μ m. *B:* Rats fasted for 18 h (from ZT8 to ZT26) received a single unilateral injection of OP (+) or vehicle (-) in the arcuate nucleus. Access to food was restored 45 min later, and cumulative food intake was measured at the indicated time points ($n = 7-8$). Data are expressed as mean \pm SEM. Unpaired *t* test: *** $P < 0.001$. *C:* Verification of cannula placement (*). Photomicrograph of a thionin-stained coronal section from an animal in which cannula has been inserted at the top of the arcuate nucleus. (A high-quality digital representation of this figure is available in the online issue.)

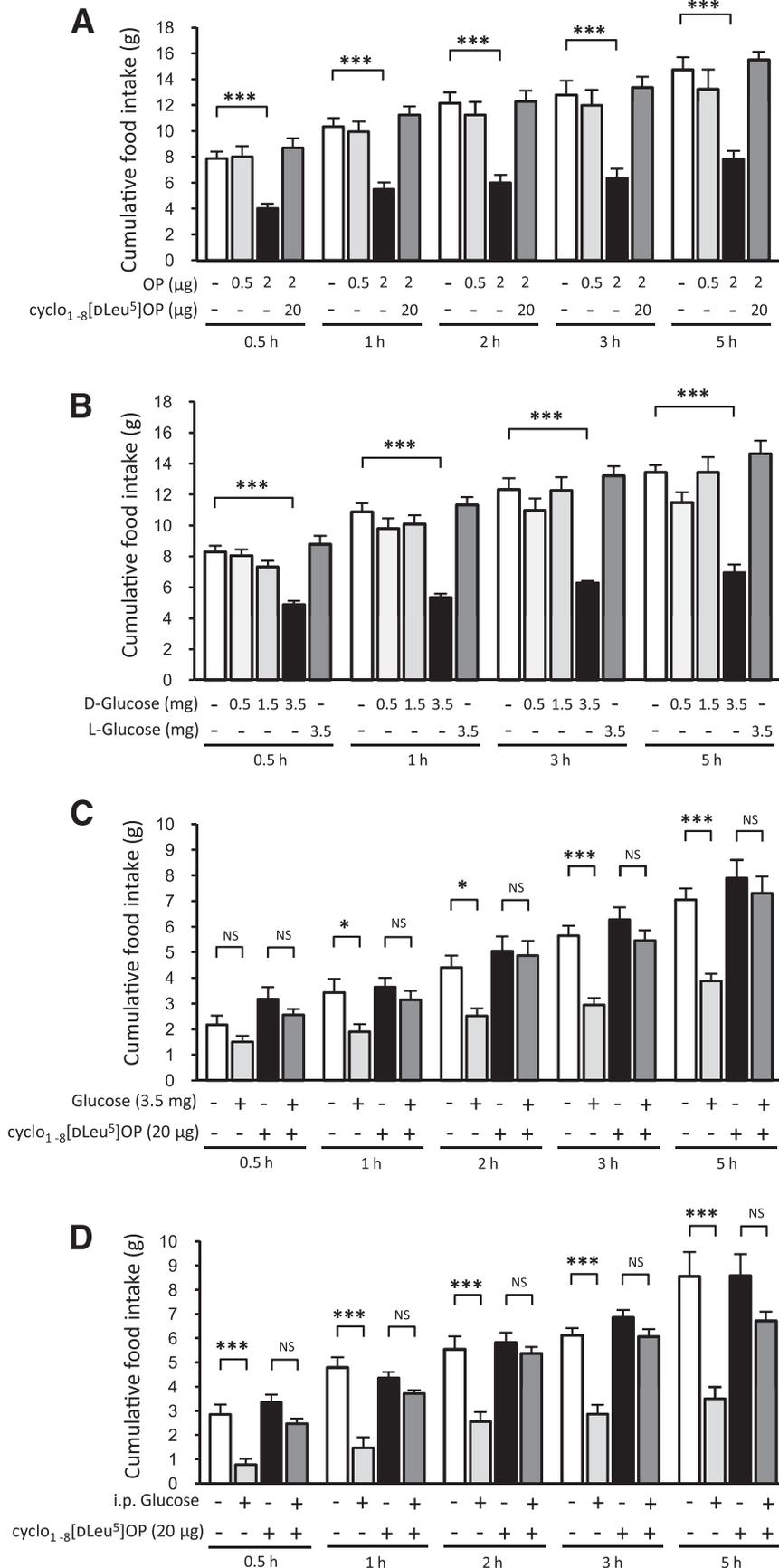


FIG. 3. The anorexigenic effect of glucose is suppressed by an ODN antagonist. *A, B, and C:* Rats fasted for 18 h (from ZT8 to ZT26) received a single intracerebroventricular injection containing the indicated substances diluted in 0.9% NaCl. Access to food was restored 45 min later, and cumulative food intake was measured at the indicated time points ($n = 6-8$). *D:* Rats fasted for 18 h (from ZT8 to ZT26) received an

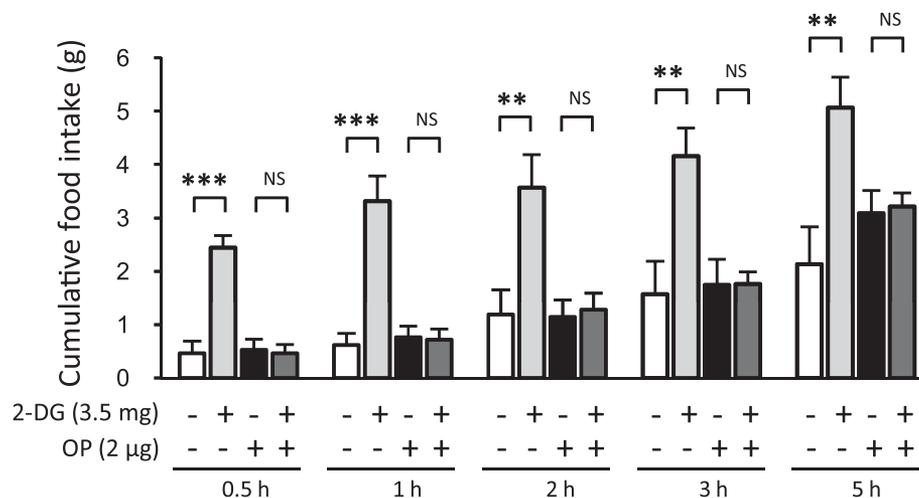


FIG. 4. The hyperphagic response to 2-DG is suppressed by an ODN agonist. Normally fed rats received a single intracerebroventricular injection at ZT5 containing the indicated substances diluted in 0.9% NaCl. Access to food was restored 45 min later, and cumulative food intake was measured at the indicated time points ($n = 7-8$). Data are expressed as mean \pm SEM. One-way ANOVA, followed by a post hoc multiple comparison Student-Newman-Keuls test: ** $P < 0.01$; *** $P < 0.001$; NS, not statistically different.

ODN-like immunoreactivity released in media was performed by radioimmunoassay (19).

Food intake experiments. All intracerebroventricular injections were performed in a final volume of 7 μ L 0.9% NaCl. The doses of intracerebroventricularly injected ODN, OP, and cyclo_{1-s}[DLeu⁵]OP were selected according to previous feeding experiments performed in mice (24). Intracerebral injections were performed in a final volume of 0.2 μ L artificial cerebral spinal fluid through the implanted guide cannula using an inner cannula (28-gauge stainless steel; Plastics One) that was custom-cut to extend 0.5 mm beyond the tip of the guide cannula. Intraperitoneal injection of glucose (1 g/kg body weight) was performed using a 40% D-glucose solution prepared in 0.9% NaCl. Rats had access to a weighed food pellet (20 g) 45 min after injections. Cumulative food intake was measured by briefly (<20 s) removing and weighing the pellet at the indicated time points.

Measurement of glycemia. Glucose concentration was measured from blood samples obtained from the tail of the animals, using ACCU-CHEK Performa glucose meter (Roche).

Glucose tolerance test. Rats were food-deprived for 19 h (from ZT10 to ZT29) and intracerebroventricularly injected with saline (7 μ L of 0.9% NaCl) or ODN (2 μ g in 7 μ L of 0.9% NaCl). Glucose (1 g/kg body weight) was intraperitoneally administered 30 min later. Glycemia was measured immediately before and 20, 40, 60, 90, 120, and 180 min after the intraperitoneal glucose load.

Statistical analysis. Statistical analysis was performed using Prism 5 software (GraphPad). All data are expressed as mean \pm SEM. For each test, a value of $P \leq 0.05$ was considered statistically significant.

RESULTS

Glucose increases the expression of endozepines in rat hypothalamus. To examine whether glucose regulates the expression of DBI in the rat hypothalamus, glucose or vehicle were injected intracerebroventricularly in fasted animals, and DBI mRNA levels were measured by in situ hybridization. The control group consisted of rats with free access to food throughout the experiment. In fed rats, brain sections hybridized with the DBI probe exhibited strong labeling in the periventricular region (Fig. 1A). A 16-h fasting markedly reduced periventricular labeling (Fig. 1A). Central administration of glucose at the end of the

fasting period partially restored DBI mRNA levels in cells lining the floor and infralateral walls of the third ventricle (Fig. 1A). To exclude any nonspecific effect of the treatments, analyses of DBI mRNA levels were also performed in hippocampus (CA1 area), in cells lining the lateral ventricle, and in the cortex (Fig. 1B). Our data show that neither fasting nor intracerebroventricular glucose injection are able to modulate DBI mRNA levels in these areas (Fig. 1B). In agreement with previous results obtained in mice (4), fasting reduced POMC mRNA levels in the arcuate nucleus and intracerebroventricular injection of glucose partially restored POMC mRNA levels (Fig. 1C). We next tested the effect of glucose on endozepine release from rat hypothalamic explants. An increase of glucose concentration in the culture medium stimulated endozepine release from the explants (Fig. 1D). **Arcuate endozepines regulate feeding behavior.** Intracerebroventricular administration of ODN or its COOH-terminal OP has been shown to exert a potent anorexigenic effect in rodents (23,24). However, functional evidence for a role of hypothalamic endozepines in the regulation of feeding was still missing. We first assessed the identity of the endozepine-expressing cells in the hypothalamus by using immunohistochemistry. ODN immunoreactivity was mainly detected in the periventricular area and in thin cytoplasmic processes extending into the parenchyma, a labeling pattern characteristic of hypothalamic tanycytes as revealed by colocalization of ODN with dopamine- and cAMP-regulated phosphoprotein-32 and vimentin (Fig. 2A). We next tested the effect of a unilateral injection of the ODN agonist OP or vehicle directly into the arcuate nucleus. A marked inhibition of food intake was observed in rats receiving OP (Fig. 2B). This anorexigenic effect was observed at 30 min, and cumulative food consumption remained significantly reduced during the entire 5-h test period (Fig. 2B). Together, these data support the

intracerebroventricular injection of cyclo_{1-s}[DLeu⁵]OP (+) or vehicle (-), immediately followed by an intraperitoneal administration of glucose (+; 1 g/kg body weight) or vehicle (-). Access to food was restored 45 min later, and cumulative food intake was measured at the indicated time points ($n = 7-8$). Data are expressed as mean \pm SEM. One-way ANOVA, followed by a post hoc multiple comparison Student-Newman-Keuls test: * $P < 0.05$; *** $P < 0.001$; NS, not statistically different.

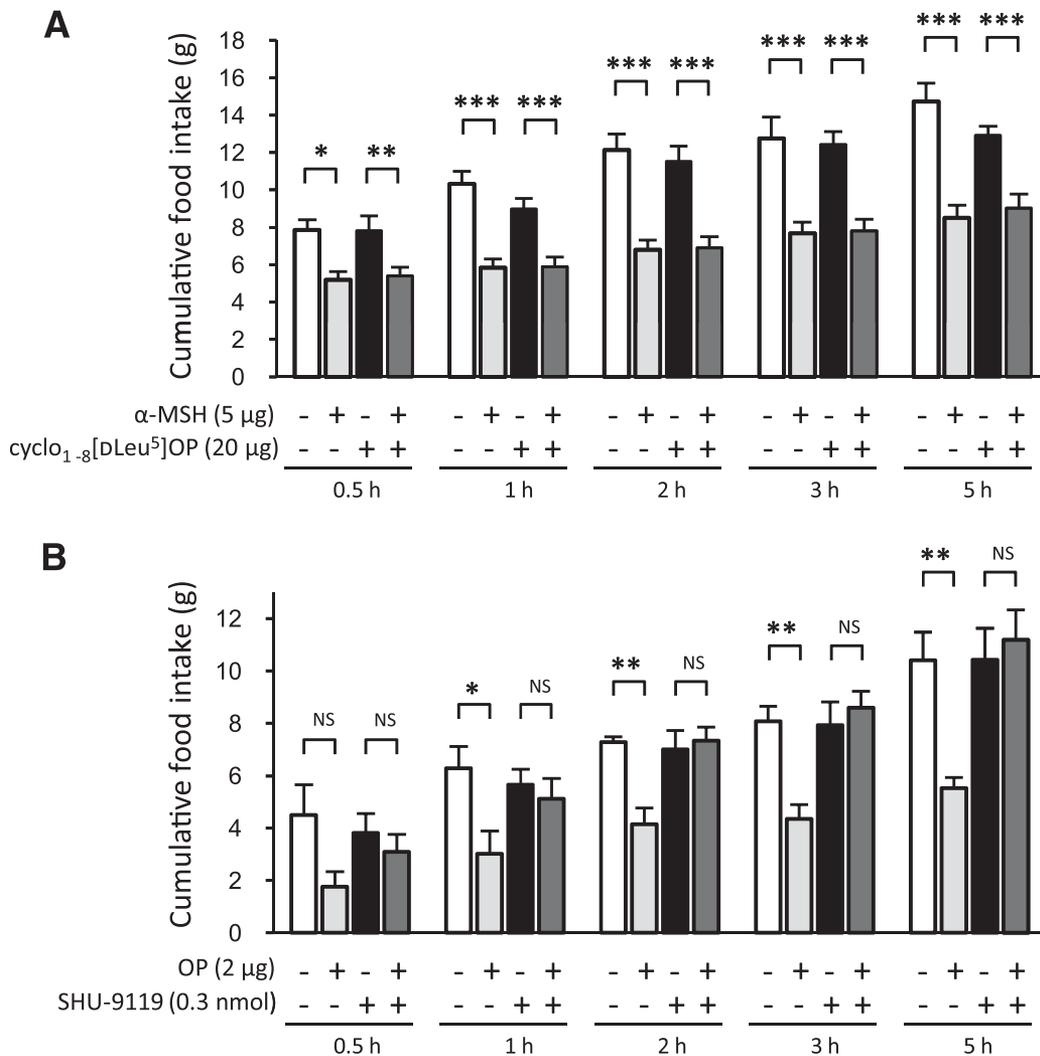


FIG. 5. MC3/4-Rs relay the anorexic effect of endozepines. **A** and **B**: Rats fasted for 18 h (from ZT8 to ZT26) received a single intracerebroventricular injection containing the indicated substances diluted in 0.9% NaCl. Access to food was restored 45 min later, and cumulative food intake was measured at the indicated time points. Data are expressed as mean \pm SEM ($n = 7-8$ for data presented in **A**; $n = 5-6$ for data presented in **B**). One-way ANOVA, followed by a post hoc multiple comparison Student-Newman-Keuls test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not statistically different.

view that endozepines produced by tanycytes may control arcuate neurons involved in feeding behavior.

Central administration of an ODN antagonist suppresses the anorexic effect of glucose. We have previously shown in mice that the potent anorexic effect of endozepines was abolished by the analog cyclo₁₋₈[DLeu⁵]OP (24), a selective antagonist of the metabotropic receptor (27). In a preliminary step, we verified that the antagonist had a similar action in rats. The anorexic effect induced by an intracerebroventricular injection of the ODN agonist OP (2 μ g) was totally blocked by a coinjection of cyclo₁₋₈[DLeu⁵]OP at a dose of 20 (Fig. 3A) or 5 μ g (data not shown). To evaluate the role of endozepines in central glucose sensing, we tested whether the anorexic effect of central administration of glucose could be affected by the antagonist. Food intake was markedly reduced in fasted rats receiving an intracerebroventricular injection of D-glucose at a dose 3.5 mg compared with rats receiving saline solution (Fig. 3B). An identical dose of L-glucose, used as an osmotic control, was without effect on food intake (Fig. 3B). We noted that the relatively high dose

of D-glucose necessary to reduce feeding is similar to that reported in previous studies (29,30) and may reflect the high level of clearance of this molecule in the cerebrospinal fluid after a single bolus injection (31). As shown in Fig. 3C, intracerebroventricular injection of the ODN antagonist cyclo₁₋₈[DLeu⁵]OP, which did not alter food intake by itself, totally blocked the anorexic effect of D-glucose. Parallel experiments were performed with an intraperitoneal injection of D-glucose. We found that reduction of food intake induced by the intraperitoneal administration of D-glucose was blunted by the intracerebroventricular injection of cyclo₁₋₈[DLeu⁵]OP (Fig. 3D).

Central administration of an ODN agonist suppresses the hyperphagic response induced by glucoprivation. Central injection of the glucose analog 2-DG, an inhibitor of glycolysis, mimics hypoglycemia and acutely stimulates feeding (6,7). As expected, the intracerebroventricular injection of 2-DG stimulated feeding in normally fed rats (Fig. 4). A marked stimulation was observed at 30 min, and cumulative food consumption remained significantly

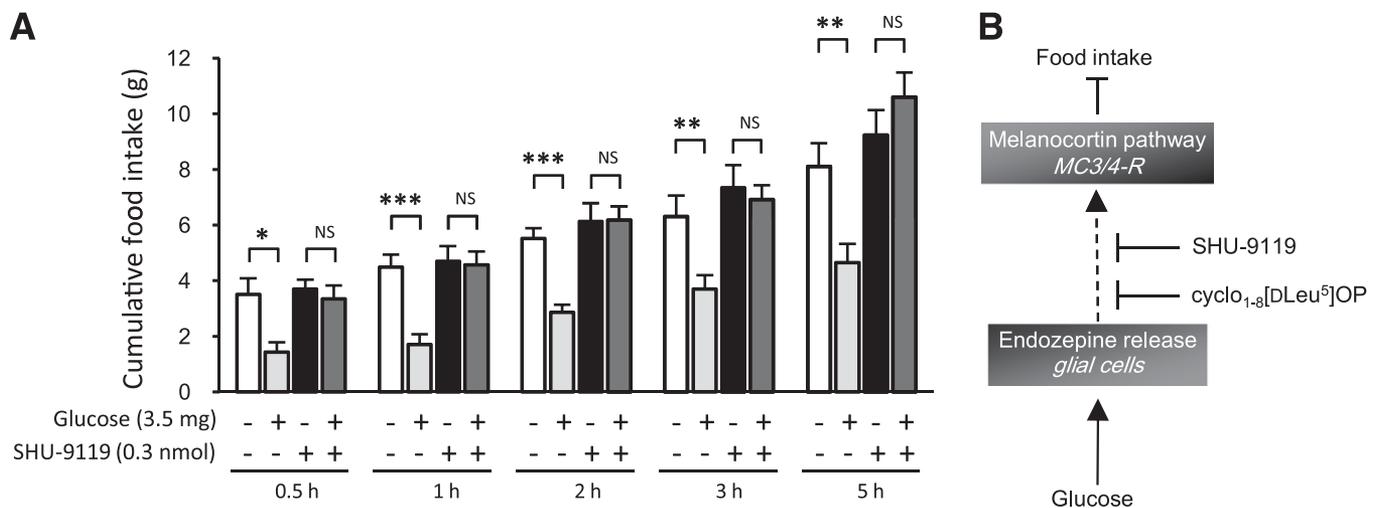


FIG. 6. MC3/4-Rs relay the anorexigenic effect of glucose. **A:** Rats fasted for 18 h (from ZT8 to ZT26) received a single intracerebroventricular injection containing the indicated substances diluted in 0.9% NaCl. Access to food was restored 45 min later, and cumulative food intake was measured at the indicated time points ($n = 6-7$). Data are expressed as mean \pm SEM. One-way ANOVA, followed by a post hoc multiple comparison Student-Newman-Keuls test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not statistically different. **B:** Model summarizing the role of endozepines in the regulation of central glucose sensing.

increased during the entire 5-h test period. This stimulation of food intake by 2-DG was totally suppressed by coinjection of the ODN agonist OP (Fig. 4).

The anorexigenic effects of endozepines and glucose are relayed by activation of the MC 3/4 receptors. The central MC pathway, via activation of the MC 3/4 receptors (MC3/4-R), is a major regulator of feeding behavior, and its involvement in brain glucose sensing has already been suggested (8). Therefore, we investigated whether endozepines exert their anorexigenic effect upstream or downstream of MC3/4-R. As shown in Fig. 5A, the potent anorexigenic effect of a central injection of α -MSH was not affected by coinjection of the ODN antagonist cyclo₁₋₈[DLeu⁵]OP, indicating that endozepines do not participate in downstream events elicited by activation of the MC3/4-Rs. We next used SHU-9119, a selective antagonist of the MC3/4-R (32). Intracerebroventricular administration SHU-9119, which did not alter food intake by itself, totally blocked the anorexigenic effect of OP (Fig. 5B).

Although central injection of glucose has been shown to regulate arcuate POMC and AgRP mRNA levels (4,30), functional evidence for a role of the MC pathway in the anorexigenic effect of glucose was still missing. We showed that reduction of food intake induced by the intracerebroventricular injection of glucose was blunted by coinjection of SHU-9119 (Fig. 6A). Altogether, our data indicate that activation of the MC3/4-R is an obligate relay in the anorexigenic effects of both endozepines and glucose (Fig. 6B).

Central endozepine signaling regulates blood glucose levels. Brain glucose sensing was demonstrated to play a key role in the regulation of blood glucose levels (33,34). Therefore, we tested the possible involvement of central endozepine signaling in this process. We first assessed the effects of ODN agonist and antagonist on basal glycemia in normally fed rats. Central injection of the agonist did not have any significant effect (Fig. 7A). However, the antagonist cyclo₁₋₈[DLeu⁵]OP increased blood glucose levels, with a maximal effect observed 20 min postinjection (Fig. 7A), suggesting the existence of an endozepine tone reducing glycemia. Because the

endogenous level of endozepines is expected to be already high in normally fed rats, the effect of the agonist was studied during a glucose tolerance test, after an overnight fast. Rats received an acute intracerebroventricular injection of ODN or vehicle and were challenged 30 min later with an intraperitoneal glucose load. Elevation of glycemia induced by the intraperitoneal glucose injection was markedly reduced in ODN-treated animals (Fig. 7B).

DISCUSSION

In this report, we demonstrated that acute pharmacological modulation of the central endozepine tone totally disrupted feeding responses to glucose status. The current study also identified endozepines as new players in brain regulation of whole-body glucose homeostasis.

Endozepines and brain glucose sensing. Several studies support the hypothesis that glial cells are key elements in brain glucose sensing, but their exact role in this process remained elusive. Here, we demonstrate that glucose stimulates endozepine expression in hypothalamic tanyocytes as well as endozepine release from hypothalamic explants. Moreover, we show that the feeding behaviors elicited by glucose or 2-DG were reversed by the intracerebroventricular injection of an ODN antagonist or agonist, respectively. Together, these data support the view that regulation of feeding by glucose involves endozepine production by glia.

In situ hybridization experiments indicated that central injection of glucose in fasted rats significantly increases DBI mRNA levels in the periventricular zone of the hypothalamus. In agreement with previous data (19), immunohistochemical labeling revealed that endozepines are highly expressed in tanyocytes, a population of specialized ependymal cells lining the third ventricle. Anatomical studies had long suggested that tanyocytes, which contact the cerebrospinal fluid and send long processes to specific hypothalamic nuclei, are poised to sense and transmit metabolic signals to neurons. In fact, recent findings indicate a key role of tanyocytes in hypothalamic glucose sensing. Tanyocytes are directly glucose-sensitive (13,14),

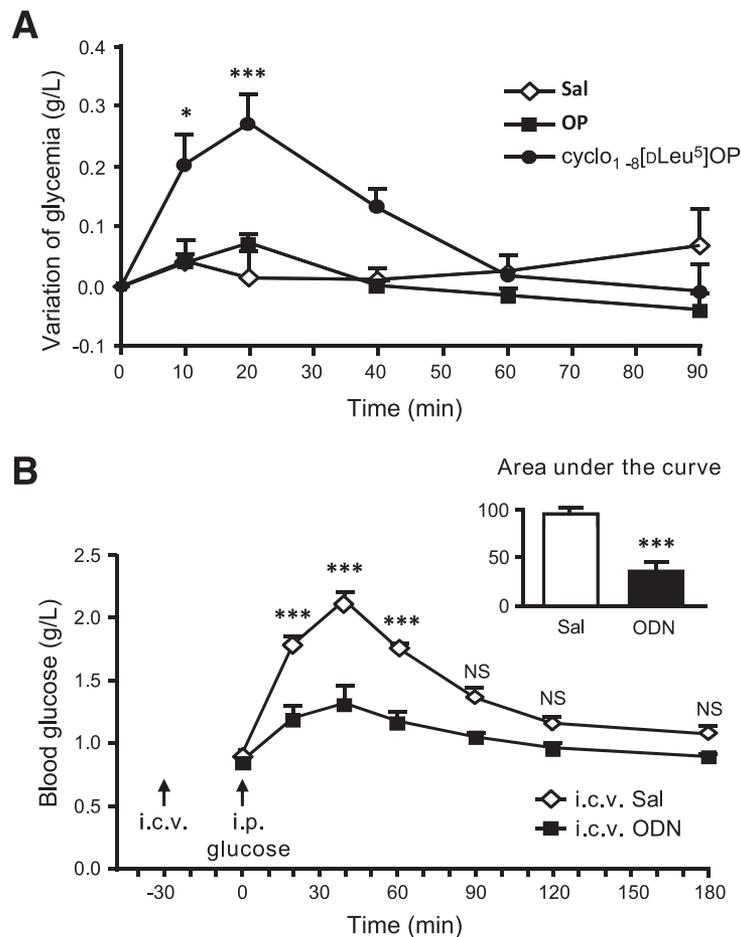


FIG. 7. Peripheral blood glucose levels are regulated by central endozepines. **A:** Variation of glycemia in normally fed rats after an intracerebroventricular (i.c.v.) injection at ZT2 of OP (2 µg), cyclo₁₋₈[dLeu⁵]OP (20 µg), or saline vehicle (Sal). Data are expressed as mean ± SEM (n = 4–6). Two-way ANOVA, followed by a post hoc Bonferroni test: *P < 0.05; ***P < 0.001 vs. intracerebroventricular vehicle-injected animals. Glycemia (mean ± SEM) measured in each group just before intracerebroventricular injections (time 0) was as follows: OP, 1.23 ± 0.03 g/L; cyclo₁₋₈[dLeu⁵]OP, 1.26 ± 0.03 g/L; vehicle, 1.26 ± 0.06 g/L. **B:** Glucose tolerance test on fasted rats after intracerebroventricular injection of ODN (2 µg) or Sal. Data are expressed as mean ± SEM (n = 5). Two-way ANOVA, followed by a post hoc Bonferroni test: ***P < 0.001; NS, not statistically different vs. intracerebroventricular vehicle-injected animals.

and their selective destruction by alloxan impairs the feeding response induced by 2-DG (15). Collectively, these studies and data presented here underscore the importance of a close functional coupling between tanycytes and neurons for glucose regulation of feeding.

The precise mechanism by which endozepines regulate feeding behavior is still unclear and will deserve further investigations. The intracerebroventricular injection of ODN was previously found to increase arcuate POMC mRNA levels while decreasing NPY mRNA levels (25). We found here that activation of the MC3/4-R is a necessary step in the anorexigenic effect of endozepines. We can speculate from these data that endozepines could exert their action by stimulating α-MSH release and/or by inhibiting the release of AgRP, an endogenous antagonist of the MCRs. Moreover, models of arcuate neuron interactions include projections of NPY/AgRP cells to POMC neurons (35,36) as well as POMC projections to NPY/AgRP neurons (37,38). Identification of the primary neurons targeted by endozepines will therefore require patch-clamp recordings on green fluorescent protein-tagged POMC or NPY/AgRP neurons in the presence of presynaptic input blockers. Finally, we cannot exclude from the present data that the effect of endozepines may be relayed, at least in

part, by brainstem MCRs that are also involved in the regulation of energy homeostasis (39,40). Maekawa et al. (11) have shown that ependymal cells bordering the fourth ventricle express GLUT2 and glucokinase and may therefore have glucose-sensing properties. The potential involvement of endozepines in brainstem glucose sensing deserves further studies.

Implications for whole-body glucose homeostasis.

Central glucose sensing, through changes in autonomic outputs, was demonstrated to play a key role in the regulation of peripheral glucose metabolism (15,33,34). The present data show that central injection of the antagonist cyclo₁₋₈[dLeu⁵]OP in normally fed rats significantly increased, by itself, blood glucose levels, suggesting that endogenous endozepines tonically reduce glycemia. Consistent with this, endozepines have already been demonstrated to exert a tonic inhibitory effect on food consumption (24). In contrast, the absence of effect of OP suggests that the endozepine tone is high at the beginning of the day in normally fed rats and cannot be further increased by the intracerebroventricular injection of the agonist. Accordingly, we observed a marked effect of ODN on glucose tolerance in animals fasted during the night, a condition that reduces the level of endogenous

endozepines. These data provide an important baseline for further studies aimed at identifying the mechanisms engaged by endozepines in the control of blood glucose levels. Several facets of glucose homeostasis will be explored, including modulation of insulin and/or glucagon secretions as well as regulation of peripheral insulin sensitivity. We noted that, at the central level, the MC pathway might possibly act as a relay in the hypoglycemic action of endozepines because it has been shown to regulate glucose homeostasis through modulation of hepatic insulin sensitivity (8,41).

Altogether, the present data strongly support the notion that glial cells play an important role in glucose sensing and may regulate energy balance through the controlled production of endozepines.

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D.L. researched data and contributed to discussion. S.A., J.O., J.-L.D.R., J.L., B.L., C.B., B.M.-R., and G.P. researched data. V.C., H.C., and H.V. contributed to discussion and reviewed and edited the manuscript. D.R. contributed to discussion. M.-C.T. contributed to discussion and wrote the manuscript. F.M. researched data and wrote the manuscript. F.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

- Blouet C, Schwartz GJ. Hypothalamic nutrient sensing in the control of energy homeostasis. *Behav Brain Res* 2010;209:1–12
- Rossi M, Kim MS, Morgan DG, et al. A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of α -melanocyte stimulating hormone in vivo. *Endocrinology* 1998;139:4428–4431
- Stanley BG, Leibowitz SF. Neuropeptide Y injected in the paraventricular hypothalamus: a powerful stimulant of feeding behavior. *Proc Natl Acad Sci U S A* 1985;82:3940–3943
- Bady I, Marty N, Dallaporta M, et al. Evidence from *glut2*-null mice that glucose is a critical physiological regulator of feeding. *Diabetes* 2006;55:988–995
- Marty N, Dallaporta M, Foretz M, et al. Regulation of glucagon secretion by glucose transporter type 2 (*glut2*) and astrocyte-dependent glucose sensors. *J Clin Invest* 2005;115:3545–3553
- Miselis RR, Epstein AN. Feeding induced by intracerebroventricular 2-deoxy-D-glucose in the rat. *Am J Physiol* 1975;229:1438–1447
- Smith GP, Epstein AN. Increased feeding in response to decreased glucose utilization in the rat and monkey. *Am J Physiol* 1969;217:1083–1087
- Parton LE, Ye CP, Coppari R, et al. Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity. *Nature* 2007;449:228–232
- Vidarsdottir S, Smeets PA, Eichelsheim DL, et al. Glucose ingestion fails to inhibit hypothalamic neuronal activity in patients with type 2 diabetes. *Diabetes* 2007;56:2547–2550
- García MA, Millán C, Balmaceda-Aguilera C, et al. Hypothalamic ependymal glial cells express the glucose transporter GLUT2, a protein involved in glucose sensing. *J Neurochem* 2003;86:709–724
- Maekawa F, Toyoda Y, Torii N, et al. Localization of glucokinase-like immunoreactivity in the rat lower brain stem: for possible location of brain glucose-sensing mechanisms. *Endocrinology* 2000;141:375–384
- Millán C, Martínez F, Cortés-Campos C, et al. Glial glucokinase expression in adult and post-natal development of the hypothalamic region. *ASN Neuro* 2010;2:e00035
- Frayling C, Britton R, Dale N. ATP-mediated glucosensing by hypothalamic tanyocytes. *J Physiol* 2011;589:2275–2286
- Orellana JA, Sáez PJ, Cortés-Campos C, et al. Glucose increases intracellular free Ca^{2+} in tanyocytes via ATP released through connexin 43 hemichannels. *Glia* 2012;60:53–68
- Sanders NM, Dunn-Meynell AA, Levin BE. Third ventricular alloxan reversibly impairs glucose counterregulatory responses. *Diabetes* 2004;53:1230–1236
- Chari M, Yang CS, Lam CK, et al. Glucose transporter-1 in the hypothalamic glial cells mediates glucose sensing to regulate glucose production in vivo. *Diabetes* 2011;60:1901–1906
- Tonon MC, Leprince J, Gandolfo P, et al. Endozepines. In *Handbook of Biologically Active Peptides*. Kastin AJ, Ed. Oxford, U.K., Elsevier, 2006, p 813–819
- Compère V, Lanfray D, Castel H, et al. Acute food deprivation reduces expression of diazepam-binding inhibitor, the precursor of the anorexic octadecaneuropeptide ODN, in mouse glial cells. *J Mol Endocrinol* 2010;44:295–299
- Tonon MC, Désy L, Nicolas P, Vaudry H, Pelletier G. Immunocytochemical localization of the endogenous benzodiazepine ligand octadecaneuropeptide (ODN) in the rat brain. *Neuropeptides* 1990;15:17–24
- Loomis WF, Behrens MM, Williams ME, Anjard C. Pregnenolone sulfate and cortisol induce secretion of acyl-CoA-binding protein and its conversion into endozepines from astrocytes. *J Biol Chem* 2010;285:21359–21365
- Masmoudi O, Gandolfo P, Leprince J, et al. Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates endozepine release from cultured rat astrocytes via a PKA-dependent mechanism. *FASEB J* 2003;17:17–27
- Patte C, Gandolfo P, Leprince J, et al. GABA inhibits endozepine release from cultured rat astrocytes. *Glia* 1999;25:404–411
- de Mateos-Verchere JG, Leprince J, Tonon MC, Vaudry H, Costentin J. The octadecaneuropeptide [diazepam-binding inhibitor (33-50)] exerts potent anorexic effects in rodents. *Eur J Pharmacol* 2001;414:225–231
- do Rego JC, Orta MH, Leprince J, Tonon MC, Vaudry H, Costentin J. Pharmacological characterization of the receptor mediating the anorexic action of the octadecaneuropeptide: evidence for an endozepinergic tone regulating food intake. *Neuropsychopharmacology* 2007;32:1641–1648
- Compère V, Li S, Leprince J, Tonon MC, Vaudry H, Pelletier G. Effect of intracerebroventricular administration of the octadecaneuropeptide on the expression of pro-opiomelanocortin, neuropeptide Y and corticotropin-releasing hormone mRNAs in rat hypothalamus. *J Neuroendocrinol* 2003;15:197–203
- Lectez B, Jeandel L, El-Yamani FZ, et al. The orexigenic activity of the hypothalamic neuropeptide 26Rfa is mediated by the neuropeptide Y and proopiomelanocortin neurons of the arcuate nucleus. *Endocrinology* 2009;150:2342–2350
- Leprince J, Oulyadi H, Vaudry D, et al. Synthesis, conformational analysis and biological activity of cyclic analogs of the octadecaneuropeptide ODN. Design of a potent endozepine antagonist. *Eur J Biochem* 2001;268:6045–6057
- Malagon M, Vaudry H, Van Strien F, Pelletier G, Gracia-Navarro F, Tonon MC. Ontogeny of diazepam-binding inhibitor-related peptides (endozepines) in the rat brain. *Neuroscience* 1993;57:777–786
- Kurata K, Fujimoto K, Sakata T, Etou H, Fukagawa K. D-glucose suppression of eating after intra-third ventricle infusion in rat. *Physiol Behav* 1986;37:615–620
- Cha SH, Wolfgang M, Tokutake Y, Chohan S, Lane MD. Differential effects of central fructose and glucose on hypothalamic malonyl-CoA and food intake. *Proc Natl Acad Sci U S A* 2008;105:16871–16875
- Fujita N, Sakamaki H, Uotani S, et al. Intracerebroventricular administration of insulin and glucose inhibits the anorectic action of leptin in rats. *Exp Biol Med* (Maywood) 2003;228:1156–1161
- Hruby VJ, Lu D, Sharma SD, et al. Cyclic lactam alpha-melanotropin analogues of Ac-Nle4-cyclo[Asp5, D-Phe7,Lys10] alpha-melanocyte-stimulating hormone-(4-10)-NH2 with bulky aromatic amino acids at position 7 show high antagonist potency and selectivity at specific melanocortin receptors. *J Med Chem* 1995;38:3454–3461
- Borg WP, Sherwin RS, During MJ, Borg MA, Shulman GI. Local ventromedial hypothalamus glucopenia triggers counterregulatory hormone release. *Diabetes* 1995;44:180–184
- Lam TK, Gutierrez-Juarez R, Pocaí A, Rossetti L. Regulation of blood glucose by hypothalamic pyruvate metabolism. *Science* 2005;309:943–947

35. Cowley MA, Smart JL, Rubinstein M, et al. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 2001;411:480–484
36. Tong Q, Ye CP, Jones JE, Elmquist JK, Lowell BB. Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance. *Nat Neurosci* 2008;11:998–1000
37. Barsh GS, Schwartz MW. Genetic approaches to studying energy balance: perception and integration. *Nat Rev Genet* 2002;3:589–600
38. Seeley RJ, Drazen DL, Clegg DJ. The critical role of the melanocortin system in the control of energy balance. *Annu Rev Nutr* 2004;24:133–149
39. Grill HJ, Ginsberg AB, Seeley RJ, Kaplan JM. Brainstem application of melanocortin receptor ligands produces long-lasting effects on feeding and body weight. *J Neurosci* 1998;18:10128–10135
40. Li G, Zhang Y, Rodrigues E, et al. Melanocortin activation of nucleus of the solitary tract avoids anorectic tachyphylaxis and induces prolonged weight loss. *Am J Physiol Endocrinol Metab* 2007;293:E252–E258
41. Coppari R, Ichinose M, Lee CE, et al. The hypothalamic arcuate nucleus: a key site for mediating leptin's effects on glucose homeostasis and locomotor activity. *Cell Metab* 2005;1:63–72