The gliotransmitter ACBP controls feeding and energy homeostasis via the melanocortin system

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Gliotransmitters

Gliotransmitters, such as ATP, released by astrocytes, can modulate various functions in the nervous system. They play a crucial role in the formation, activity, and adaptation of neuronal circuits. Astrocytes are key players in the central control of energy balance and the etiology of obesity. They release gliotransmitters, which modulate the activity of neurons and other glial cells.

Introduction

In the central nervous system, the hypothalamus is a key site for the detection and integration of circulating metabolic signals. In turn, the hypothalamus initiates appropriate neuroendocrine and behavioral responses to maintain energy homeostasis. In the arcuate nucleus (ARC) of the hypothalamus, 2 functionally opposing neuronal populations play a critical role in this control, the agouti-related peptide (AgRP) neurons and proopiomelanocortin (POMC) neurons. When activated by signals of energy sufficiency including leptin and insulin, arcuate POMC neurons release α-melanocyte-stimulating hormone that activates the melanocortin-4 receptor (MC4R) and downstream anorectic and catabolic responses (1). The importance of the melanocortin system in the etiology of obesity is underscored by several lines of evidence showing that impairments in metabolic sensing in POMC neurons lead to obesity in rodents (2–6) and that mutation in the genes coding for POMC and MC4R underscores by several lines of evidence showing that impairments in metabolic sensing in POMC neurons lead to obesity in rodents (2–6) and that mutation in the genes coding for POMC and MC4R lead to obesity in rodents (2–6) and that mutation in the genes coding for POMC and MC4R.

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and astrocytes (31–33). Importantly, intracerebroventricular (i.c.v.) administration of ODN reduces food intake (34) and improves glucose tolerance in rodents (33). Despite these findings, the impact of endogenous ACBP-mediated gliotransmission on feeding, energy metabolism, and hypothalamic neuronal activity has not been studied. Here we used multiple gene interventions, Ca^2+ imaging, and electrophysiology to reveal the unique role of ACBP as a gliopeptide in the ARC of the hypothalamus robustly controlling feeding and energy metabolism via the melanocortin pathway.

Results

Acbp gene expression is regulated by fasting but not high-fat feeding in a circadian manner. ACBP is the precursor of the anorectic peptide ODN; thus we tested whether its expression in the hypothalamus was dependent on the time of the day and nutritional status. Acbp mRNA level in ARC microdissections was maximal at zeitgeber time 6 (ZT6; middle of the light cycle) and gradually decreased to its lowest level at ZT18 (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI123454DS1). Acbp expression was decreased by fasting at ZT6 but not ZT18, while pomec levels were reduced at both time points (Supplemental Figure 1, B and C). Finally, acbp gene expression in the ARC was not affected by 3, 7, or 42 days of high-fat feeding (Supplemental Figure 1, D and E). Together, these findings demonstrate that acbp is regulated in a circadian manner by food deprivation but not caloric excess and overweight.

Astrogial ACBP deficiency promotes diet-induced obesity. We then sought to identify the role of astroglial ACBP in energy balance using a cell-specific gene knockout approach. ACBP^fl/flGFAP-Cre (ACBP^GFP KO) mice were generated as we previously described (31). ACBP^GFP KO mice were devoid of ACBP expression in glial fibrillary acidic protein-positive (GFAP) astrocytes and some tanyocytes of the ARC and median eminence as compared with littermate control mice (Supplemental Figure 2A). Moreover, as we previously reported (31), we did not observe ACBP expression in the ependymal layer of the median eminence. Acbp gene expression in ARC microdissections (including the median eminence and ependymal layer) derived from chow- and high fat–fed ACBP^GFP KO and GFAP^cre control mice (ACBP^GFP WT) confirmed acbp gene deletion (Supplemental Figure 2B). Residual acbp expression (10%) likely represents acbp expression in neurons (32) and GFAP-negative astrocytes (Supplemental Figure 2A). Expectedly, acbp expression was reduced by half in ACBP^fl/flGFAP^cre (ACBP^GFP HET) (Supplemental Figure 2B).

Body weight was significantly increased at week 10 while energy expenditure (light phase) was reduced in chow-fed ACBP^GFP KO male mice without changes in cumulative food intake, respiratory exchange ratio (RER), and locomotor activity as compared with controls (Supplemental Figure 2, C–G). Based on accumulating evidence suggesting a key role of hypothalamic astrocytes in feeding in response to leptin (18, 35) and fatty acids (19, 22), we tested whether astroglial ACBP is involved in the anorectic action of these signals. The anorectic response to central leptin was similar in ACBP^GFP KO males and control littermates (Supplemental Figure 2H). In contrast, the anorectic effect of central oleate was absent in ACBP^GFP KO males compared to controls (Supplemental Figure 2I). During a high-fat regimen, astroglial ACBP deficiency considerably enhanced the response to diet-induced obesity in both male and female ACBP^GFP KO mice (Figure 1, A–D). Weight gain and food intake were increased in ACBP^GFP KO male mice as of week 3 of the 16-week high-fat diet (HFD) regimen (Figure 1, A and B). Correspondingly, ACBP^GFP HET male mice showed a less pronounced response to high-fat feeding, suggesting a gene dosage effect. In male and female ACBP^GFP KO mice, weekly food intake was increased before the onset of overweight, suggesting that hyperphagia plays a causal role in the obesity-prone phenotype (insets, Figure 1, B and D). Increased body weight gain in male ACBP^GFP KO mice was not associated with changes in RER or locomotor activity (Supplemental Figure 3, A and B) but was associated with a trend toward reduced energy expenditure after 6 weeks (not shown) or 16 weeks of HFD (Supplemental Figure 3C). In contrast, female ACBP^GFP KO mice had higher RER (Supplemental Figure 3D) without changes in activity and energy expenditure (Supplemental Figure 3, E and F). ACBP^GFP KO mice had greater fat mass (Figure 1E and Supplemental Figure 3G), with subcutaneous fat increased in males (Figure 1F) and intraperitoneal fat increased in females (Supplemental Figure 3G). Increase in fat mass was accompanied by higher plasma leptin levels (Figure 1G). A comparable enhanced weight gain in response to HFD was also observed in female mice on a mixed BL/6–Bom genetic background (Supplemental Figure 3H). Finally, ACBP^GFP KO male mice did not exhibit changes in glucose tolerance (Figure 1, H and I), which could be explained by a compensatory increase in insulin secretion during the glucose tolerance test (Figure 1J) suggestive of an insulin resistance state.

We previously reported ACBP expression in discrete ARC neurons. In addition, ACBP is highly expressed in ependymocytes and tanyocytes (Supplemental Figure 2A and refs. 32, 33), both of which are targeted by ACBP ablation in our KO model (Supplemental Figure 2A and ref. 31). Thus, it is possible that ACBP loss of function in ependymocytes and/or tanyocytes may contribute to the observed phenotype. To verify this, ACBP^GFP mice were crossed with Nkx2.1^Cre mice (ACBP^Nkx2.1 KO), in which Cre is driven by Nkx2.1, a promoter expressed in hypothalamic ependymocytes, tanyocytes, and neurons (36, 37). As expected, ACBP protein expression was reduced in cells lining the third ventricle (Supplemental Figure 4A), and acbp mRNA decreased by 64% in ARC microdissections (Supplemental Figure 4B). However, both male and female ACBP^Nkx2.1 KO mice on an HFD had similar body weight gain and cumulative food intake compared with control littermates (Supplemental Figure 4, C and D), suggesting that ACBP deficiency in hypothalamic ependymocytes, tanyocytes, and neurons does not influence energy balance in obeseologic conditions. Together, these results imply that pan-brain astroglial ACBP deficiency increases the susceptibility to overweight in chow-fed mice and to diet-induced hyperphagia and obesity.

Acbp gene rescue in ARC astrocytes prevents diet-induced obesity. Our results suggest that astroglial ACBP plays an important role in high-fat feeding and body weight regulation, yet the pan-astroglial KO model does not permit identification of the brain region(s) involved. Based on a previous report showing that administration of the ODN C-terminal octapeptide in the ARC exerts anorectic effects similar to those of i.c.v. administration (33) and on the strong ACBP expression in the ARC (32, 33), we designed an adeno-
Figure 1. Pan-brain astroglial ACBP deficiency promotes diet-induced obesity. (A–D) Body weight and cumulative food intake of male (A and B) and female (C and D) ACBPGFAP WT, HET, and KO mice fed with an HFD during 16 weeks. Insets in B and D represent average weekly food intake. (E–G) Fat mass (E), fat depot weights (F), and fasting plasma leptin levels (G). (H and I) Intraperitoneal glucose tolerance test (IPGTT; 1.5 g/kg) (H) and area under the curve (AUC) (I). (J) Plasma insulin levels during the IPGTT. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control littermates, 2-way ANOVA with Bonferroni post hoc test (A–D and J). *P < 0.05, **P < 0.01, ***P < 0.001 compared with controls, 1-way ANOVA with Bonferroni post hoc test (E and F). **P < 0.01 compared with controls, Student’s t test (G). n = 8–15 for male mice (A, B, and E–J) and 6–7 for female mice (C and D).
Expression of ACBP was partially restored in ARC astrocytes of KO-ARCACBP mice as compared with KO-ARCGFP mice, but not in ependymocytes and tanycytes (Figure 2B). This partial rescue of \( \text{acbp} \) in the ARC (Figure 2C) prevented the decrease in associated virus (AAV) to rescue ACBP expression selectively in GFAP\(^{+}\) astrocytes of the ARC of ACBP\(^{GFAP}\) KO mice (KO-ARCACBP) (38). Control mice (GFAP\(^{Cre}\) and ACBP\(^{GFAP}\) KO) were injected with a GFP-expressing AAV (Figure 2, A and B) (WT-ARC GFP and KO-ARC\(^{GFAP}\), respectively). Expression of ACBP was partially restored in ARC astrocytes of KO-ARCACBP mice as compared with KO-ARCGFP mice, but not in ependymocytes and tanycytes (Figure 2B). This partial rescue of \( \text{acbp} \) in the ARC (Figure 2C) prevented the decrease in

Figure 2. Genetic rescue of ACBP in GFAP\(^{+}\) astrocytes of the ARC prevents diet-induced obesity. (A and B) Immunostaining of GFAP (red) and GFP (green) in GFAP-Cre mice injected with AAV expressing GFP under the control of the GFAP promoter in the ARC (A) and ACBP (red) and GFP (green) in AAV\(^{GFAP}\) KO mice injected with AAV expressing GFP (left, KO-ARCGFP) or ACBP (right, KO-ARCACBP) in the ARC (B). White arrowheads indicate cells coexpressing GFAP and GFP (A) and cells coexpressing ACBP and GFP (B). Scale bars: 100 \( \mu \text{m} \) in top panels and 50 \( \mu \text{m} \) in zoomed panels (bottom). Representative images from 3 different mice. 3v, third ventricle. (C–E) \( \text{Acbp} \) expression measured by quantitative PCR in ARC and VMH microdissections (C), and \( \text{pomc} \) (D) and \( \text{agrp} \) (E) mRNA levels in ARC microdissections. *\( P < 0.05 \), ***\( P < 0.001 \), ****\( P < 0.0001 \) compared with WT-ARCGFP, 1-way ANOVA with Bonferroni post hoc test, \( n = 6–9 \). (F and G) Body weight (F) and cumulative food intake (G) in animals fed with an HFD during 12 weeks. *\( P < 0.05 \), **\( P < 0.01 \) KO-ARCACBP compared with KO-ARC\(^{GFAP}\), 2-way ANOVA with Bonferroni post hoc test, \( n = 6–9 \).
ACBP+ astrocytes were in close proximity to POMC neurons (Figure 3A). Second, using patch-clamp electrophysiological recordings in brain slices from POMC-eGFP mice, we found that ODN considerably increased the action potential (AP) frequency of all POMC neurons tested without affecting the firing rate of neighboring non-POMC neurons within the ARC (Figure 3, B–E).

To determine whether the anorectic and metabolic effects of central ODN are dependent on the melanocortin system, ODN was administered i.c.v. in obese MC4R-KO mice and control WT mice. The dose of ODN was chosen based on a previous study in mice (34). Intracerebroventricular ODN decreased food intake in WT pomc mRNA expression without affecting agrp mRNA levels (Figure 2, D and E) and the diet-induced obesity and hyperphagia phenotype (Figure 2, F and G). These findings strongly suggest that ACBP in ARC astrocytes, but not in tanycytes, ependymocytes, or extra-ARC astrocytes, is important for controlling energy balance.

Central effects of ODN on energy homeostasis rely on the melanocortin system. Our findings that astroglial ACBP in the ARC modulates high-fat feeding and body weight and a report that the anorectic effect of ODN can be offset by a melanocortin-3/4 receptor antagonist (33) suggest that the catabolic effects of ACBP could rely on the melanocortin system. First, we observed that several ACBP+ astrocytes were in close proximity to POMC neurons (Figure 3A). Second, using patch-clamp electrophysiological recordings in brain slices from POMC-eGFP mice, we found that ODN considerably increased the action potential (AP) frequency of all POMC neurons tested without affecting the firing rate of neighboring non-POMC neurons within the ARC (Figure 3, B–E).

Figure 3. ODN selectively activates POMC neurons in the ARC. (A) Immunostaining of ACBP+ astrocytes (red) in close proximity to ARC POMC-eGFP neurons (green). Boxed area is represented with orthogonal projections. Scale bar: 100 μm in left panel and 25 μm in right panel. Representative images from 3 different mice. (B–E) Representative trace and quantification of action potential (AP) frequency in ARC POMC (B and C; n = 8 neurons from 7 mice) or non-POMC (D and E; n = 12 neurons from 7 mice) neurons in the presence or absence of 1 nM ODN. **P < 0.001 compared with control and #P < 0.05 compared with ODN, 1-way ANOVA with repeated measures with Bonferroni post hoc test.
of ACBP and its derived peptide ODN are mediated via the ARC toward increased mRNA levels (Figure 4K) and was sufficiently compared with WT-ARC-GFP, Student’s t test. (J and K) Agrp expression measured by quantitative PCR in ARC (n = 11-14) and VMH (n = 4-5) microdissections (J) and pomc in ARC microdissections (n = 8) (K) from C57BL/6 WT male mice injected bilaterally in the ARC with AAV expressing GFP (WT-ARC<sup>GFP</sup>) or ACBP (WT-ARC<sup>ACBP</sup>) under the control of the GFAP promoter. ***P < 0.001 compared with WT-ARC<sup>GFP</sup>, Student’s t test. (L and M) Body weight gain (L) and cumulative food intake (M) in WT-ARC<sup>GFP</sup> and WT-ARC<sup>ACBP</sup> mice (n = 12-15). *P < 0.05, **P < 0.01 compared with WT-ARC<sup>GFP</sup>, 2-way ANOVA with Bonferroni post hoc test. (J and K) Agrp expression measured by quantitative PCR in ARC (n = 11-14) and VMH (n = 4-5) microdissections (J) and pomc in ARC microdissections (n = 8) (K) from C57BL/6 WT male mice injected bilaterally in the ARC with AAV expressing GFP (WT-ARC<sup>GFP</sup>) or ACBP (WT-ARC<sup>ACBP</sup>) under the control of the GFAP promoter. ***P < 0.001 compared with WT-ARC<sup>GFP</sup>, Student’s t test. (L and M) Body weight gain (L) and cumulative food intake (M) in WT-ARC<sup>GFP</sup> and WT-ARC<sup>ACBP</sup> mice (n = 12-15). *P < 0.05, **P < 0.01 compared with WT-ARC<sup>GFP</sup>, 2-way ANOVA with Bonferroni post hoc test.

In these conditions, ODN was still able to increase action potential in obese ob/ob mice, in which the melanocortin system is functional. Similarly to what we observed in WT mice, i.c.v. ODN reduced feeding and increased RER in ob/ob mice compared with controls (Figure 4, G–I). To validate the regional specificity of ODN anorectic action, we used a viral strategy to selectively overexpress ACBP in ARC GFAP<sup>+</sup> astrocytes of C57BL/6 WT mice (WT-ARC<sup>ACBP</sup>; Figure 4J). In a consistent manner, we found that ACBP overexpression in GFAP<sup>+</sup> astrocytes of the ARC (Figure 4J) led to a trend toward increased pomc mRNA levels (Figure 4K) and was sufficient to reduce body weight gain and cumulative food intake over 10 weeks in Chow-fed mice (Figure 4, L and M). Together, these findings strongly suggest that the anorectic and metabolic effects of ACBP and its derived peptide ODN are mediated via the ARC melanocortin system.

**ODN activates POMC neurons through a GABA<sub>a</sub>-independent but ODN GPCR-dependent mechanism.** ODN has been shown to act as a negative allosteric modulator of the GABA<sub>a</sub> receptor (39). Importantly, POMC neurons of the ARC receive strong inhibitory GABAergic inputs from neighboring neurons (40, 41), suggesting that ODN-induced POMC neuron activation could be due to inhibition of GABAergic inputs. Thus, the frequency and amplitude of spontaneous inhibitory postsynaptic currents (sIPSC) were measured on POMC and non-POMC neurons in brain slices from POMC-eGFP mice (42). ODN significantly decreased sIPSC frequency on POMC and non-POMC neurons (Figure 5, A and B) without affecting sIPSC amplitude (Figure 5C), showing that ODN inhibits GABAergic inputs on ARC neurons. Importantly, these findings suggest that the decrease in GABA input is not sufficient to increase neuron activity and, thus, that the selective activation of POMC neurons by ODN (Figure 3, B–E) is independent of the GABA<sub>a</sub> receptor. To confirm this, brain slices were pretreated with GABA<sub>a</sub> inhibitors to block inhibitory inputs onto POMC neurons. In these conditions, ODN was still able to increase action potential frequency of POMC neurons, suggesting that ODN activates these neurons independently of its action on inhibitory inputs and thus implicates another receptor (Figure 5, D and E).

The second potential mechanism of action of ODN implicates the ODN G protein–coupled receptor (GPCR) coupled to phospholipases C and Ca<sup>2+</sup> (43, 44). Although the ODN GPCR remains unidentified, cyclic analogs of ODN were designed based on the peptide sequence of ODN and selected for their agonist or antagonist properties (44). Application of the antagonist of ODN GPCR (cLLOP) suggested that the anorectic action of i.c.v. ODN is mediated through the unidentified GPCR (34). Thus, we tested whether activation of the ODN GPCR was sufficient to activate POMC neurons and reduce feeding. First, we found that treatment with the ODN GPCR agonist (cOP) increased the firing activity of POMC neurons (Figure 5F and Supplemental Figure 5A), without affecting the firing rate of neighboring non-POMC neurons in the ARC (Supplemental Figure 5, B and C). Ca<sup>2+</sup> imaging was performed in freshly dissociated hypothalamic neurons in culture, which are isolated from each other (no dendrites and axons) (Supplemental Figure 5D), ruling out GABAergic inputs (45). Using this model, we observed that ODN increased intracellular Ca<sup>2+</sup> oscillations in approximately 10% of the neurons tested (9.5% ± 1.5%; Supplemental Figure 5E), a percentage compatible with the proportion of POMC neurons in mediobasal hypothalamic culture. Importantly, the ODN GPCR antagonist cLLOP reduced both the number of ODN-responsive neurons (5.5% ± 1.7%, P < 0.05, Student’s t test) and the amplitude of ODN response (Supplemental Figure 5, E–G), suggesting that ODN-induced neuronal activation is dependent on the ODN GPCR. Importantly, this was confirmed by electrophysiological recordings showing that the activation of POMC neurons by ODN in the presence of GABA<sub>a</sub> inhibitors was reversed by the ODN GPCR antagonist cLLOP (Supplemental Figure 5, H and I). Next, we observed that i.c.v. injection of the ODN GPCR agonist cOP decreased food intake after a fast (Figure 5G). Finally, daily i.c.v. administration of the ODN GPCR agonist reduced feeding and body weight in ob/ob mice (Figure 5, H and I). These results strongly suggest that ODN-induced POMC neurons’ activation and anorectic responses are mediated by the unidentified ODN GPCR and that activation of the receptor promotes weight loss in obese mice.

**Discussion**

Astrocytes not only play a central role in the energy requirements of the brain but also produce and release gliotransmitters that modulate neural communication and play key roles in cognitive function (46) and behavior (47).

The present study identified the gliopeptide ACBP and its product ODN, commonly referred to as endozepines, as important hypothalamic regulators of energy balance via direct modulation of the melanocortin system. ACBP ablation in astrocytes led to increased susceptibility to diet-induced hyperphagia and obesity, while viral-mediated restoration of ACBP in ARC GFAP<sup>+</sup> astrocytes was sufficient to prevent this effect. Our results further show that the anorectic action of endozepines is mediated by direct activation of POMC neurons and the downstream melanocortin pathway via the ODN GPCR, whose activation reduced body weight and feeding in obese mice. Collectively, our results suggest that GPCR-mediated activation of POMC neu-
Figure 5. ODN activates POMC neurons through a GABA <sub>A</sub>-independent but ODN GPCR-dependent mechanism. (A) Representative voltage-clamp whole-cell recording of a POMC neuron with or without 1 nM ODN. (B and C) Quantification of sIPSC frequency (B) and amplitude (C) of POMC and non-POMC neurons before and during ODN application. *P < 0.05 compared with control, paired Student’s t test, n = 5–6 neurons from 5–6 mice. (D) Representative cell-attached recording of a POMC neuron in the presence of bicuculline, picrotoxin, cyanoxinal, and APV before and during ODN application (1 nM). (E) Quantification of AP frequency in POMC neurons with or without the inhibitors and ODN (1 nM). *P < 0.05 compared with inhibitors (PTX/BCC), 1-way ANOVA repeated measures with Bonferroni post hoc test. n = 10 neurons from 10 mice. (F) Quantification of AP frequency of POMC neurons before and during COP (2 nM) application. *P < 0.05 compared with control, paired Student’s t test, n = 6 neurons from 4 mice. (G and H) Cumulative food intake in overnight-fasted (16 hours) C57BL/6 WT male mice following i.c.v. administration of 50 ng of COP or saline (n = 9–10). (H and I) Cumulative food intake and percent body weight change (I) following daily i.c.v. administration of 50 ng of COP or saline in ad libitum-fed ob/ob mice (n = 6–8). (G–I) *P < 0.05, **P < 0.01, ***P < 0.001 compared with saline, 2-way ANOVA with Bonferroni post hoc test.

Our findings highlight novel aspects of endozepine signaling in POMC neurons. ODN activates POMC neurons in a GABA <sub>A</sub>-independent manner, suggesting that endozepine signaling involves an unidentified ODN GPCR. This concept is supported by both our Ca<sup>2+</sup> imaging and electrophysiology data showing that cDNaP antagonist of the GPCR, decreased the number of ODN-responsive neurons and the intensity of Ca<sup>2+</sup> responses in dissociated hypothalamic neurons, and reversed the activation of POMC neurons by ODN in brain slices. The notion of direct and selective activation of POMC neurons has to be taken cautiously, since we cannot rule out that ODN may affect different neuronal populations within other nuclei of the hypothalamus or extrahypothalamic areas that project onto and activate POMC neurons. Nonetheless, our data at the neuronal level are consistent with a study showing that the central anorectic effect of ODN is not affected by a GABA<sub>A</sub> antagonist (34). Together, these findings demonstrate that ODN stimulation of the melanocortin system and inhibition of feeding are GABA<sub>A</sub>-independent. However, ODN decreased GABAergic inputs in all the neurons we recorded (POMC and non-POMC), suggesting a broad impact in ARC neurons that may affect the excitability of other neuronal population(s). Further investigations will be required to characterize more precisely the effect of ODN on GABA<sub>A</sub> currents and its impact on other neocircuits in the hypothalamus.

Consistent with previous findings (34), administration of the ODN GPCR agonist COP centrally decreased feeding in WT mice in a manner similar to i.c.v. ODN. In addition, we found that daily administration of the agonist lowered feeding and promoted body weight loss in obese ob/ob mice. Together, our results suggest that chronic stimulation of endozepine signaling, virally (Figure 2) or pharmacologically (Figure 5, H and I), exerts potent anorectic effects in mouse models of obesity.

Our findings suggest that endozepines mostly influence energy balance by reducing food intake, while increasing locomotor activity and RER. These effects are consistent with the activation of the melanocortin system (5); however, one would have expected ACBP or ODN to promote energy expenditure. It is possible that endozepines activate only a subset of POMC neurons or that higher doses may be required to affect energy expenditure.

ACBP may well exert a dual action in non-neuronal cells, as both a neurotransmitter and regulator of intracellular fatty acid metabolism. We recently showed that ACBP deficiency impairs the intracellular metabolism of unsaturated fatty acids in astrocytes (32). For this reason we cannot rule out that the unresponsiveness to the anorectic effect of central oleate and/or the obesity-prone phenotype observed in ACBP<sup>GFP</sup> KO mice may involve alterations of astrocyte fatty acid metabolism. However, the hyperphagia induced by ACBP deficiency in astrocytes is consistent with the anorectic effects induced by both i.c.v. ODN (34) (Figure 4, A and G) and viral-mediated expression of ACBP in ARC astrocytes (Figure 4, L and M). Together, these findings provide compelling evidence that arcuate ACBP and its product ODN are anorectic glycoproteins. These findings raise the question of whether and which circulating metabolic signals stimulate the release of hypothalamic ACBP. It has been reported that glucose increases ACBP secretion in hypothalamic explants ex vivo (33). Our findings in vivo show that ACBP<sup>GFP</sup> KO mice have a normal decrease in feeding in response to leptin but a dampened anorectic response to central oleate. This carbohydrate utilization. In addition, our results strongly suggest that ODN-induced POMC neuronal activation is independent of GAB<sub>A</sub> and involves the unidentified ODN GPCR. This concept is supported by both our Ca<sup>2+</sup> imaging and electrophysiology data showing that cDNaP, an antagonist of the GPCR, decreased the number of ODN-responsive neurons and the intensity of Ca<sup>2+</sup> responses in dissociated hypothalamic neurons, and reversed the activation of POMC neurons by ODN in brain slices. The notion of direct and selective activation of POMC neurons has to be taken cautiously, since we cannot rule out that ODN may affect different neuronal populations within other nuclei of the hypothalamus or extrahypothalamic areas that project onto and activate POMC neurons. Nonetheless, our data at the neuronal level are consistent with a study showing that the central anorectic effect of ODN is not affected by a GABA<sub>A</sub> antagonist (34). Together, these findings demonstrate that ODN stimulation of the melanocortin system and inhibition of feeding are GABA<sub>A</sub>-independent. However, ODN decreased GABAergic inputs in all the neurons we recorded (POMC and non-POMC), suggesting a broad impact in ARC neurons that may affect the excitability of other neuronal population(s). Further investigations will be required to characterize more precisely the effect of ODN on GABA<sub>A</sub> currents and its impact on other neocircuits in the hypothalamus.

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suggests that the release of astroglial ACBP could be stimulated by oleate to in turn inhibit feeding. Additional studies will be needed to assess this hypothesis and determine whether other metabolic signals modulate ACBP release.

At the gene level, the reduced expression of acbp in the ARC in response to fasting is in agreement with recent in situ hybridization data in rats (33) and is consistent with its anorectic action. Interestingly, the diurnal expression pattern of acbp is similar to that of fatty acid–binding protein 7 (FABP7) (53), a brain-specific isoform of FABP strongly expressed in hypothalamic astrocytes (54).

While our study demonstrates the importance of ACBP in the hypothalamus, recent findings show that ACBP is expressed in glial cells of the rat brainstem, including the nucleus tractus solitarius (55), in which POMC neurons are also located. In addition, i.c.v. injection of ODN in the fourth ventricle reduces food intake (55). This raises the possibility that brainstem ACBP may reduce feeding behavior by activating POMC neurons in the nucleus tractus solitarius. However, our viral approaches targeting ARC astrocytes suggest that hypothalamic ACBP is sufficient to reduce food intake (Figure 2G and Figure 4M). Nonetheless, the contribution of ACBP in different brain regions in short- versus long-term regulation of food intake and the underlying mechanisms in extra-hypothalamic regions await further investigations.

More generally, it is important to mention that ACBP is expressed in several brain regions (e.g., amygdala, hippocampus) that are not commonly associated with the control of energy balance. Although the role of ACBP in these regions is still unclear, studies suggest that endogenous ACBP may play a role in social (56) and learning behavior (57). Importantly, we recently reported that astroglial ACBP deficiency does not affect anxiety in mice (31), ruling out the possibility that susceptibility to diet-induced obesity in ACBPGFAP KO mice is confounded by changes in anxiety-like behavior.

Altogether, our studies demonstrate that astroglial endozenepines play a key role in the hypothalamic control of energy balance. Our findings, along with a study showing that acyl-CoA binding domain–containing 7 (ACBD7, a paralog gene of ACBP) is expressed in ARC neurons and regulates feeding (58), suggest that endozenepines and endozenepine-like peptides are key modulators of the neurocircuits regulating energy homeostasis. These findings suggest that targeting endozenepine signaling may represent a novel therapeutic avenue for obesity. More generally, our results support the emerging concept that hypothalamic astrocytes and astrocyte-derived signals play an important role in the regulation of energy balance. Undoubtedly, additional work will be required to identify the signals and pathways modulating endozenepine secretion in hypothalamic astrocytes and to identify the ODN GPCR.

Methods

Animals

Experimental animals were bred under specific pathogen–free conditions on a 12-hour light/12-hour dark cycle (dark from 6:00 am to 6:00 pm). Housing temperature was maintained at 21°C (70°F) with free access to water and standard chow diet. Cages and water were autoclaved, and regular chow diet was irradiated. Cages were supplemented with nesting materials, and cages were changed every 2 weeks. Health status was monitored via a sentinel mouse exposed to feces from the same rack.

After genotyping (4 weeks of age), experimental mice were moved to an experimental housing room on a reverse light/dark cycle (dark cycle from 10:00 am to 10:00 pm). Mice were maintained in groups with 2–4 mice per cage until they were allocated to their experimental groups. Purchased animals were maintained in a reverse light/dark cycle for at least 10 days before starting the experimentation.

For all studies, age- and sex-matched littersmates were used and individually housed in a reverse light/dark cycle unless otherwise specified. Genotype, sex, age, and number of mice are indicated for each experiment in the appropriate figure legends or section of Methods. Upon completion of the studies, mice were anesthetized with ketamine/xylazine, and blood was collected via cardiac puncture when necessary. Mice were then euthanized by decapitation before tissue collection. All mice were treatment-naïve at the time of study.

ACBPfl/fl mice were donated by Susanne Mandrup (University of Southern Denmark, Odense, Denmark) (31, 59) and were backcrossed at least 8 generations on the C57BL/6J genetic background (C57BL/6J, 000664). Female ACBPfl/fl mice on the C57BL/6J background were bred with male mice expressing Cre recombinase under the mouse glial fibrillary acidic protein (GFAP) promoter [B6.Cg-Tg (Gfap-cre)73.12Mvs/J, 012886], obtained from The Jackson Laboratory. ACBPfl/fl;Cre (WT), ACBPfl/fl;Cre (HET), and ACBPfl/fl;Cre (KO) were obtained by breeding of female ACBPfl/fl with male ACBPfl/fl;Cre to obtain littersmates of all genotypes. Some studies (Supplemental Figures) were performed on ACBP-KO animals (on a mixed C57BL/6J and Bom background) obtained by breeding of ACBPfl/fl mice, on the original C57BL/6 Bom genetic background, with GFAP-Cre or Nkx2.1-Cre mice [C57BL/6J-Tg(Nkx2-1-cre)2Sand/J, 008661].

Male MC4R-KO and control wild-type (WT) mice (B6; 129S4-Mec4mmtlom/J, 006414), POMC-eGFP mice [C57BL/6J-Tg(Pomc-eGFP);1Low/J, 009593], and ob/ob (B6.Cg-Lep+/J, 000632) mice were purchased from The Jackson Laboratory (6–10 weeks old). Male POMC-eGFP hemizygous mice were bred with C57BL/6J WT females from the same genetic background to produce experimental animals.

Astrocyte-specific overexpression of ACBP

Ten- to twelve-week-old male C57BL/6J WT mice were injected bilaterally in the arcuate nucleus (ARC) as previously described (38) according to stereotaxic coordinates (from bregma: –1.5 mm anteroposterior, 0.15 mm lateral, and –5.9 mm dorsoventral from the dura) with 400 nl per side of either control [AAV5-GFAP(0.7)-GFP, Vector Biolabs] or overexpressor [AAV5-GFAP(0.7)-mACBP-IRESC-GFP-WPRE, Vector Biolabs] virus at a concentration of 2.6 × 10^9 genome copies (GC)/μL (1.04 × 10^9 GC per side) to generate WT-ARCACBP and WT-ARCACBP mice. Mice were allowed to recover for 1 week before the beginning of the study. Placement and efficacy of viral expression of ACBP were measured by quantitative PCR (qPCR) on ARC and ventromedial hypothalamus (VMH) microdissections. Mice that did not show at least a 10% increase in ACBP expression in the ARC compared with WT-ARCACBP controls were excluded from the study.

Astrocyte-specific rescue of ACBP

Ten- to twelve-week-old male ACBPGFAP WT (GFAP-Cre) and ACBPGFAP KO mice were injected bilaterally in the ARC according to stereotaxic coordinates (from bregma: –1.5 mm anteroposterior, 0.15 mm lateral,
and –5.9 mm dorsoventral from the dura) with 400 nl per side of either control [AAV5-GFAP(0.7)-GFP, Vector Biolabs] or overexpressor [AAV5-GFAP(0.7)-mACBP-IRE5-GFP-WPRE, Vector Biolabs] virus at a concentration of 2.6 × 10^9 GC/μl (1.04 × 10^9 GC per side) to generate WT-ARC:

KO-ARC:

KO-ARCACBP mice. Mice were allowed to recover for 3 weeks before the onset of the study. Placement and efficacy of viral expression of ACBP were measured by qPCR on ARC and VMH microdissections. Mice that did not show at least a 10% increase in ACBP expression in the ARC compared with KO-ARC controls were excluded from the study.

In vivo studies

High-fat diet studies. Five- to six-week old mice (ACBP:

KO, HET, and control littermates) were individually housed and fed either chow during 12 weeks or a high-fat diet (HFD) (Modified AIN-93G purified rodent diet with 50% kcal from fat derived from palm oil; Dyets) during 16 weeks. Five- to six-week-old mice on a mixed BL/6-Bom background (ACBP:

KO, HET, and control ACBP:

littermates) were individually housed and fed with an HFD (F3282, 60% kcal from fat, Bioserv) during 12 weeks. Body weight and food intake were measured weekly from 9:00 am to 10:00 am at the end of the light cycle. WT-ARC:

and WT-ARCACBP mice were individually housed after surgery and fed on chow. Food intake was measured weekly from 9:00 am to 10:00 am during 10 weeks starting 1 week after the surgery. WT-ARC:

KO-ARC:

and KO-ARCACBP mice were individually housed following surgery and fed with the HFD. Food intake was measured weekly from 9:00 am to 10:00 am during 12 weeks starting 3 weeks after surgery.

Metabolic cages. Respiratory exchange ratio (RER), energy expenditure, and locomotor activity were measured using indirect calorimetry in Comprehensive Lab Animal Monitoring System metabolic cages (CLAMS, Columbus Instruments International). Animals were single-housed in CLAMS apparatus at 21°C (70°F) in a dark/light cycle matching their housing conditions during 24 hours for acclimation followed by 48 hours of measurement. Energy expenditure was normalized by lean mass.

Glucose tolerance. Experimental mice were food-deprived during 5 hours with ad libitum access to water. A bolus of glucose (1.5 g/kg) was administered via an intraperitoneal injection, and glycemia was measured from blood sampled at the tail vein using an Accu-chek Performa glucometer at T0 (before injection), 15, 30, 60, and 90 minutes. Tail vein blood samples were collected via a capillary for insulin assays.

Body composition analysis. Total fat and lean mass were assessed using a nuclear echo MRI whole-body composition analyzer. Intraperitoneal (perigonadal) and subcutaneous (inguinal) fat pads were collected and weighed using an analytical scale (Sartorius).

Intracerebroventricular cannula implantation. Male mice were anesthetized with isoflurane and placed on a stereotaxic apparatus (Kopf Instruments). Animals were implanted with a guide cannula (Plastics One) into the right lateral ventricle according to stereotaxic coordinates (from bregma: –0.5 mm anteroposterior, +1 mm lateral, and –2.1 mm dorsoventral from the dura). Cannulated mice were allowed to recover for a week before i.c.v. administration of angiotensin II (40 ng in 2 μl) to prevent POMC neuron overexcitation (Figure 5). For the measurement of spontaneous inhibitory postsynaptic currents (siPCs) under whole-cell voltage-clamp recordings, pipettes were filled with a cesium chloride solution containing (in mM): 140 CsCl, 3.6 NaCl,
Changes in intracellular calcium levels (\([\text{Ca}^{++}\])_i\) were monitored in
nerve cells using excitation at 340 and 380 nm and emissions (420–600 nm) with
Fura-2 fluorescence images were acquired every 10 seconds by alter-
atively exciting the cells at 340 and 380 nm. Changes in calcium levels were quantified by calculation
of the 340 /380 nm fluorescence ratio, repres-
entative of \([\text{Ca}^{2+}]_i\), were obtained after correction for background
fluorescence values. Changes in \([\text{Ca}^{2+}]_i\) were quantified by calculation
of the integrated area under the curve (AUC) of each ODN response with
the TILL Photonics software. Neurons were considered as
ODN-responsive neurons if the increase in \([\text{Ca}^{2+}]_i\), occurred between 2
and 10 minutes of treatment, had an amplitude greater than 0.2 \((\text{Aratio}
340/380)\), lasted at least 30 seconds, and was transient. At the end of each recording, neuronal excitability was verified by measurement of \([\text{Ca}^{2+}]_i\) response to 50 mM KCl. Neurons not responding to KCl were
excluded from the analysis. Analysis of each experiment was obtained
from at least 3 independent cultures prepared from at least 2 animals.

Immunofluorescence. Male mice were perfused intracardially with
4% paraformaldehyde under ketamine/xylazine anesthesia. The brains
were postfixed 3 hours in 4% paraformaldehyde, cryopreserved in
20% sucrose, and cryosectioned at 30 \(\mu\)m using a sliding microtome
(SM 2000R Leica). Sections were blocked and incubated with primary antibodies overnight at 4°C followed by 2-hour incubation at 22°C with
secondary antibodies. Sections were mounted and imaged with a Zeiss
fluorescence microscope (Carl Zeiss AG). Primary antibodies used were
anti-ACBP/DBI (1:600; DBI-Rb-Af300, Frontier Institute), anti-ACBP
(1:200; polyclonal antibody; gift of J. Knudsen and S. Mandrup, Univer-
sity of Southern Denmark, Odense, Denmark), and anti-glial fibillary
acidic protein (1:1000; Mab360, Millipore Corp.). Secondary antibod-
ies were Alexa Fluor 546–goat anti-rabbit IgG (A-11035) and Alexa
Fluor 488–goat anti-mouse IgG (A-11001) (1:1000; Life Technologies).

Real-time PCR. Real-time PCR was performed as previously
described (32). Fresh ARC microdissections that include the median
eminence and the ependymal layer, or VMH microdissections, were
immediately frozen on dry ice before RNA extraction using the TRIzol
method (Life Technologies). RNA concentration was quantified spect-
rophotometrically using a NanoDrop 2000 (Thermo Fisher Scientific),
and 1 \(\mu\)g of total RNA was reverse-transcribed by M-MuLV reverse
transcriptase (Life Technologies) with random hexamers following
the manufacturer’s conditions. The reaction mix was then diluted
5-fold before use. Quantitative gene expression was measured from
1:10 cDNA dilutions. Real-time PCR was performed using the Quant-
Fast SYBR Green PCR kit (Qiagen) according to the manufacturer’s
guidelines on a Corbett Rotor-Gene 6000. Data were analyzed using the
standard curve method and normalized to actin, cyclophilin, or 18S
RNA expression levels.

Blood chemistry. Plasma insulin and leptin levels were measured in
blood samples collected at sacrifice or during the glucose tolerance test in
chow- or HFD-fed ACBP\textsuperscript{Cre}\textsuperscript{WT and ACBP\textsuperscript{Cre}\textsuperscript{KO}} male mice. Insu-
lin and leptin assays were performed by the core metabolic phenotyping
platform of the CRCHUM using commercially available ELISA kits.

Statistics. All statistical analyses were performed using GraphPad Prism soft-
ware. Intergroup comparisons were performed by ANOVA with Bon-
ferroni post hoc tests or Student’s t test (2-tailed) as described in the
figure legends. P less than 0.05 was considered significant. Data are
expressed as means ± SEM.

Study approval. All procedures using animals were reviewed and approved by the institu-
tional animal care and use committee (Comité Institutionnel de Protec-
tion des Animaux, protocol CM16007TAs) of Centre de Recherche du Cen-
tre Hospitalier de l’Université de Montréal (CRCHUM) and the French
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de Bourgogne (C2EA105) and Université de Bordeaux (C2EA 50).

Author contributions. KB, BT, and LB helped with colony management and mouse
model validation and performed feeding, metabolic, qPCR, and
immunofluorescence studies. CC performed \([\text{Ca}^{2+}]_i\) imaging. DR
performed colony genotyping, glucose tolerance testing, and
i.c.v. and AAV injections. DC and ZH performed AAV injections in
POMC-Cre mice. FL, HM, and XF performed electrophysiological
recordings. EB synthesized the agonist and antagonist. SF and LP
contributed to conceptualization and results interpretation. KB,
XF, SF, and TA analyzed results and prepared the manuscript.

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