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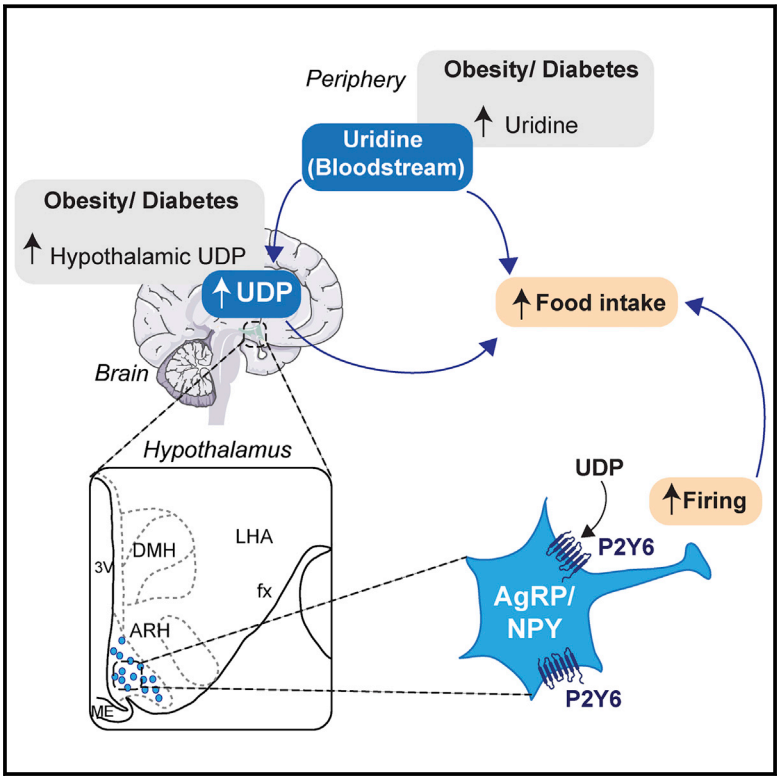
Hypothalamic UDP Increases in Obesity and Promotes Feeding via P2Y6-Dependent Activation of AgRP Neurons

Graphical Abstract



Highlights

- P2Y6 are highly expressed in ARH neurons
- UDP activates orexigenic AgRP neurons through P2Y6-dependent signaling
- UDP activates feeding through activation of AgRP neurons
- Hypothalamic UDP synthesis increases in obesity through elevated circulating uridine supply

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In Brief

The UDP-selective P2Y6 receptor controls orexigenic AgRP neurons and food intake regulation. The pathway is deregulated in obesity, making P2Y6 a potential target for treatment.

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Hypothalamic UDP Increases in Obesity and Promotes Feeding via P2Y6-Dependent Activation of AgRP Neurons

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SUMMARY

Activation of orexigenic AgRP-expressing neurons in the arcuate nucleus of the hypothalamus potently promotes feeding, thus defining new regulators of AgRP neuron activity could uncover potential novel targets for obesity treatment. Here, we demonstrate that AgRP neurons express the purinergic receptor 6 (P2Y6), which is activated by uridine-diphosphate (UDP). In vivo, UDP induces ERK phosphorylation and cFos expression in AgRP neurons and promotes action potential firing of these neurons in brain slice recordings. Consequently, central application of UDP promotes feeding, and this response is abrogated upon pharmacologic or genetic inhibition of P2Y6 as well as upon pharmacogenetic inhibition of AgRP neuron activity. In obese animals, hypothalamic UDP content is elevated as a consequence of increased circulating uridine concentrations. Collectively, these experiments reveal a potential regulatory pathway in obesity, where peripheral uridine increases hypothalamic UDP concentrations, which in turn can promote feeding via PY6-dependent activation of AgRP neurons.

INTRODUCTION

Facing the escalating burden of obesity and type 2 diabetes mellitus (T2DM) (Geiss et al., 2014; WHO, 2006), there is an urge to further define the regulatory principles underlying the control of body weight and glucose homeostasis and to define novel targets for therapeutic intervention. Research over the past decades revealed the critical importance of the central nervous system (CNS) and, more specifically, the hypothalamus in control of homeostatic processes governing energy balance and glycemic control (Schwartz et al., 2013; Sohn et al., 2013). Here, particularly the arcuate nucleus of the hypothalamus (ARH) is of critical importance to coordinate food intake and glycemic control with the energy state of the organism (Sohn et al., 2013; Varela and Horvath, 2012). The ARH contains two main functionally antagonistic neuronal populations: the orexigenic neurons co-expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP) and the anorexigenic neurons that produce proopiomelanocortin (POMC) (Sohn et al., 2013; Varela and Horvath, 2012). Thus, the melanocortin circuitry downstream of AgRP and POMC neurons orchestrates behavioral responses such as feeding and locomotor activity, as well as changes in peripheral glucose metabolism in rodents and in humans (Cone, 2005; Farooqi and O'Rahilly, 2008).

AgRP/NPY and POMC neurons express leptin and insulin receptors, and several studies previously highlighted the critical importance of insulin and leptin action specifically on AgRP/NPY and/or POMC neurons in their anorexigenic and glucoregulatory effects (Belgardt and Brüning, 2010; Könner and Brüning, 2012; Varela and Horvath, 2012; Vogt and Brüning, 2013). However, while leptin and insulin are indisputably critical regulators of AgRP/NPY and POMC neurons, the discovery that obesity and T2DM are associated with the onset of neuronal leptin and insulin resistance in these two neuronal populations limits pharmaceutical interventions targeting these hormonal pathways (Friedman, 2004; Könner and Brüning, 2012; Vogt and Brüning, 2013). Thus, defining alternative signaling pathways in control of the melanocortin circuitry will ultimately expand the panel of putative drugs targets for the treatment of obesity and T2DM.

Among all potential new regulators of the central control of feeding behavior and glucose homeostasis, the family of G-protein-coupled receptors (GPCRs) carries enormous therapeutic potential since, on one hand, they are well known to regulate virtually all core physiological functions and, on the other hand, they are attractive targets for pharmacological intervention due to their high-affinity binding and selectivity (Allen and Roth, 2011). Recently, Ren and coworkers discovered that Gpr17 is expressed on AgRP neurons and that Gpr17 downregulation secondary to AgRP-specific FoxO1 deletion is associated with

decreased body weight and food intake (Ren et al., 2012). Given the putative role of Gpr17 in feeding regulation, we thought of investigating whether related GPCRs could also be expressed on hypothalamic neurons, thereby controlling their function and associated behavioral outcomes. Gpr17 is structurally and phylogenetically related to the purinergic receptors family (P2), more specifically to P2Y receptors activated by uracil nucleotides (Daniele et al., 2011). Interestingly, members of the P2Y family, such as P2Y1 and P2Y14, have previously been associated with homeostatic processes such as food intake and insulin sensitivity (Burnstock et al., 2011; Kittner et al., 2006; Xu et al., 2012). Here, we find that, among uracil nucleotide-sensitive P2Y receptors with so far unknown function in the central control of energy and glucose homeostasis, P2Y6 is the highest expressed in the ARH. Therefore, we decided to directly focus on the putative role of P2Y6 in energy and glucose homeostasis. P2Y6 is a receptor for the nucleotide uridine-diphosphate (UDP), whose synthesis in the CNS depends on the salvage pathway, which is directly controlled by the peripheral supply of the precursor metabolite uridine (Cansev, 2006; Ipata, 2011).

We demonstrate that UDP activates AgRP neurons in a P2Y6-dependent fashion. In accordance with its stimulatory effect on AgRP neurons, centrally administered UDP displays orexigenic effects, which are abrogated upon pharmacologic or genetic inhibition of P2Y6 as well as upon pharmacogenetic inhibition of AgRP neurons. Moreover, hypothalamic UDP content is increased in obesity. Our experiments thus reveal a potential regulatory pathway in obesity, where elevated uridine supply from the periphery increases hypothalamic UDP content to activate AgRP neurons and ultimately promote positive energy balance.

RESULTS

P2Y6 Are Expressed in Neurons of Key Hypothalamic Feeding Regions

In order to identify potential novel GPCR-coupled regulators of energy homeostasis, we assessed the expression of P2Y receptors activated by uracil nucleotides, which have not previously been linked to energy and glucose homeostasis in hypothalami as well as in microdissected ARH. These analyses revealed that, both in hypothalami and in microdissected ARH, the expression level of *P2ry6*-mRNA was ten times higher as compared to the other studied P2Y members (Figures S1A and S1B). To further investigate whether P2Y6 is particularly enriched in the ARH, we next compared the expression levels of *P2ry6*-mRNA in different hypothalamic regions such as the ARH, the paraventricular nucleus of the hypothalamus (PVH), the ventromedial nucleus of the hypothalamus (VMH), the dorsomedial nucleus of the hypothalamus (DMH), and the lateral hypothalamic area (LHA). The greatest mRNA expression level of *P2ry6* was found in the ARH, where it is expressed ≈25 to 40% higher than in other hypothalamic regions (Figure 1A). Since P2Y6 has been described as the receptor for uridine-diphosphate (UDP), we next analyzed whether the expression of P2Y6 occurs in the same regions where UDP is produced. A critical step in neuronal UDP synthesis relies on phosphorylation of uridine, which is mediated by uridine-cytidine kinases (UCK)-1 and -2 (Anderson and Brockman, 1964). Expression analyses of *Uck1* and *Uck2* mRNA revealed an overall similar pattern to that of P2Y6 (Figures 1B and 1C).

Next, we aimed to verify P2Y6 expression in hypothalamic nuclei through the analyses of GFP immunohistochemistry in mice, which express GFP from the endogenous *P2ry6* locus (P2Y6-EGFP mice) (Bar et al., 2008). Consistent with the pattern of *P2ry6* mRNA expression, these immunohistochemical analyses revealed a very specific regional distribution of P2Y6-dependent GFP expression most prominently in the ARH and to a minor extent in the PVH, while the VMH, the DMH, and LHA exhibited only a minor proportion of cells positive for GFP expression (Figures 1D and S1C). We also compared the expression pattern of P2Y6-dependent GFP expression with that of the endogenous *P2ry6* mRNA available through the Allen Brain Atlas, which revealed a similar distribution of *P2ry6* expression in the hypothalamus (http://mouse.brain-map.org/experiment/show?id=1800). Collectively, these analyses revealed a predominant enrichment of P2Y6 expression in the ARH.

To further investigate which cell type in the ARH expresses P2Y6, we next performed co-immunohistochemical staining for GFP in P2Y6-EGFP mice and for antibodies directed to either CD11b (microglia) or GFAP (astrocytes) or using fluorescent Nissl staining NeuroTrace (neurons). There was no overlap between cells expressing GFAP or CD11b and those expressing GFP from the *P2ry6* locus (Figure 1E). In contrast, there was a clear overlap of immunoreactivity for P2Y6-dependent GFP expression in the NeuroTrace-positive neurons in the ARH (Figure 1E). Taken together, these experiments clearly indicate that the UDP receptor P2Y6 is highly expressed in neurons of the ARH.

The P2Y6 Agonist UDP Activates Neurons in the ARH

Having identified a restricted domain of P2Y6 expression in neurons of the ARH, we next investigated whether intracerebroventricular (icv) application of the P2Y6 agonist UDP modulates activation of ARH neurons. Following icv administration of either vehicle (i.e., saline) or 30 μM UDP, quantitative assessment of cFos immunoreactive cells revealed a 2.5-fold increase in cFos immunoreactivity in the ARH (Figure 2A). Similarly, the VMH, DMH, and LHA exhibited a significant increase in cFos-immunoreactive cells following icv UDP application (Figure S2A). In contrast, cFos immunoreactivity in the PVH was only increased to a minor extent (Figure S2A). Since cFos activation can occur either directly in the respective brain area or indirectly via *trans*-synaptic activation of cells located in projecting areas of directly activated neurons, we sought to investigate the direct effect of UDP on its P2Y6. In the periphery, UDP action on P2Y6 can lead to tyrosine phosphorylation of MAP kinases ERK-1 and ERK-2 (pERK) (Bar et al., 2008). Using a hypothalamic cell line, we found that, indeed, UDP activated ERK-phosphorylation also in these cells (Figure S2B). Therefore, we used pERK as a direct readout of UDP action on P2Y6. Quantification of pERK immunoreactivity revealed that UDP significantly increased the number of pERK-positive cells in the ARH (Figure 2B). Taken together, these analyses revealed that P2Y6 are expressed on ARH neurons and that they are functional, as UDP directly activates signaling in these neurons.

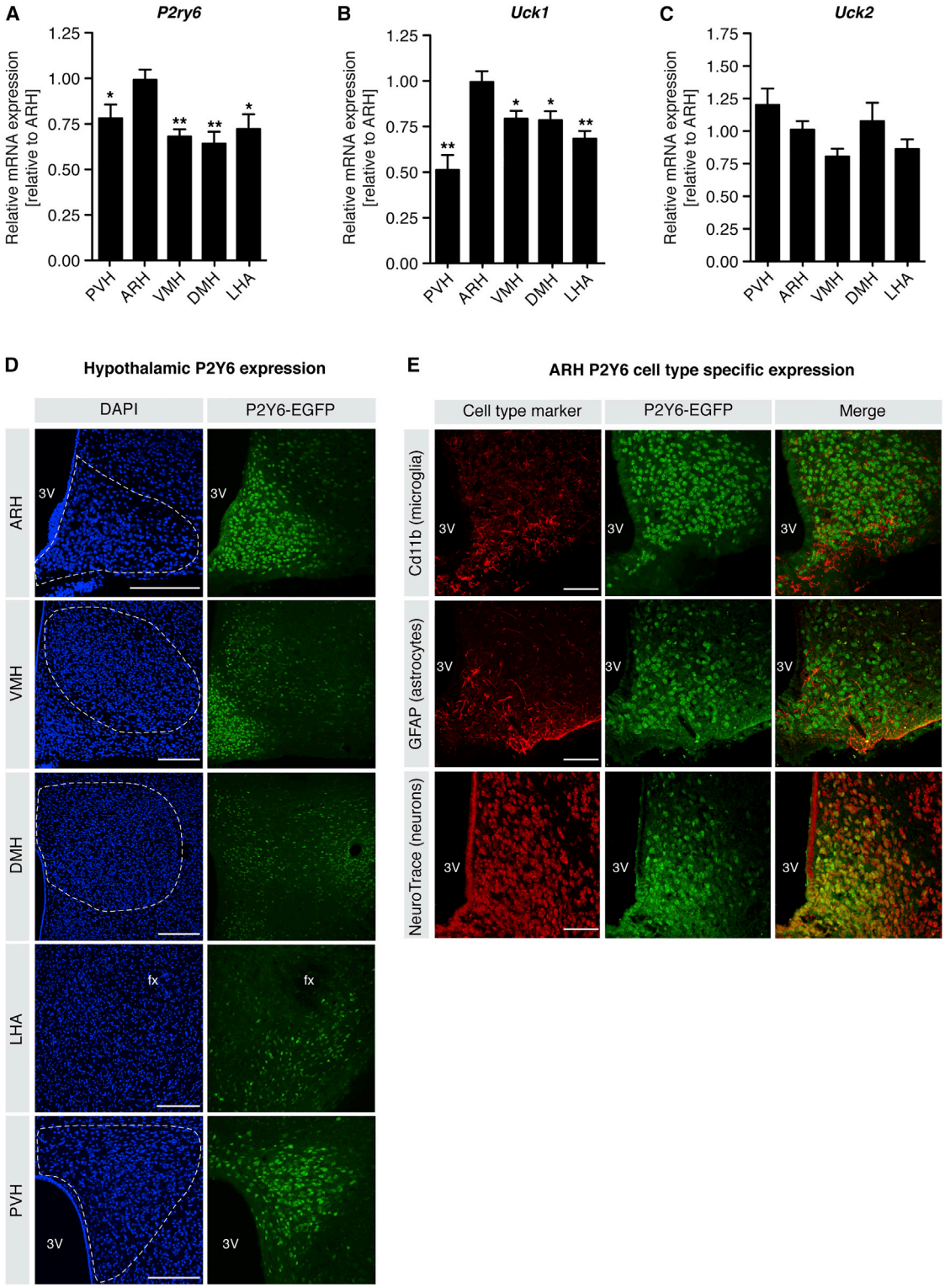

Figure 1. P2Y6 Is Highly Expressed in Neurons of the Arcuate Nucleus

(A–C) Quantitative real-time PCR analysis of (**A**) *pyrimidinergic receptor P2Y*, *G protein coupled*, *6* (*P2yr6*) (n = 18–20 samples per region), (**B**) *uridine-cytidine kinase 1* (*Uck1*) (n = 18–20 samples per region), and (**C**) *uridine-cytidine kinase 2* (*Uck2*) (n = 17–19 samples per region) mRNA expression in key microdissected hypothalamic regions: the paraventricular nucleus of the hypothalamus (PVH), the arcuate nucleus of the hypothalamus (ARH), the ventromedial nucleus of the hypothalamus (VMH), the dorsomedial nucleus of the hypothalamus (DMH), and the lateral hypothalamic area (LHA).

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UDP Activates AgRP/NPY-Expressing Neurons in the ARH

Given the expression of the UDP receptor P2Y6 in the ARH, as well as the clear activation of both cFos-expression and ERK phosphorylation in the ARH in response to icv UDP application, we next aimed to define the molecular identity of the P2Y6-expressing, UDP-responsive neurons in this region. Interestingly, UDP-induced pERK immunoreactive cells displayed a specific anatomical distribution mainly in the ventromedial region of the ARH (Figure 2B). Given that the ventromedial part of the ARH mainly contains AgRP/NPY-coexpressing neurons (Chronwall, 1985), we next directly investigated the specific effect of P2Y6/UDP on those neurons. For this purpose, we repeated the UDP-induced pERK experiments in mice, which express GFP under control of the NPY promotor (NPY-GFP mice). This analysis revealed that, indeed, the number of NPY-expressing neurons exhibiting pERK immunoreactivity doubled following icv UDP application, as compared to vehicle-treated controls (Figure 2C). Similar results were found using cFos as a readout for NPY neuron activation (Figure S2C). In contrast, UDP failed to significantly increase pERK immunoreactivity in GFP-expressing anorexigenic POMC neurons of POMC-GFP mice (Figure 2D). Collectively, these experiments indicate that UDP specifically evokes ERK phosphorylation immunoreactivity in orexigenic AgRP/NPY neurons, but not in POMC neurons.

Based on the restricted action of UDP on AgRP/NPY neurons in the ARH, we specifically focused on the role of UDP/P2Y6 on this neuronal population. We first investigated the expression of P2Y6 on AgRP/NPY neurons. To this end, we performed a co-immunohistochemistry for GFP expression on ARH sections of P2Y6-EGFP mice together with antisera directed against AgRP. This analysis revealed co-expression of P2Y6 and endogenous AgRP (Figures 3A and S3A). In addition, we performed co-staining of endogenous P2Y6 in transgenic mice that express the red tomato protein under control of the AgRP promoter through Cre-loxP-mediated recombination in $AgRP^{tdTomato}$ mice (Figures 3B, S3B, and S3C). Using this approach, we also found that genetically marked AgRP neurons express endogenous P2Y6 receptors.

Next, we aimed to further directly support the notion that the P2Y6 agonist UDP can modulate the activity of AgRP-expressing neurons in the ARH. Therefore, we performed perforated patch-clamp recordings from $AgRP^{tdTomato}$ neurons (Figures 3C–3F). Here, we also found that >60% of AgRP neurons are sensitive to UDP (Figure 3E), as application of 3 μM UDP resulted in an increased action potential frequency of these cells (Figures 3C–3F). In contrast, when we conducted similar recordings in brain slices of POMC-GFP mice, we found that only a small proportion of POMC neurons are responsive to UDP and that UDP does not significantly affect POMC neuron action potential firing on a population basis (Figures S3D and S3E). Collectively, these experiments indicate that orexigenic AgRP/NPY-coexpressing neurons in the ARH not only express the UDP receptor P2Y6, but that they are also directly activated following UDP application both in vitro and in vivo.

UDP Promotes Feeding via P2Y6-Dependent Activation of AgRP Neurons

In light of the pivotal role of AgRP neuron activation in the stimulation of food intake, we investigated whether UDP was indeed capable of promoting an increase in feeding. Therefore, control mice were icv injected with increasing doses of UDP before the onset of the dark period, i.e., when spontaneous feeding occurs in mice. Indeed, central application of UDP acutely enhanced spontaneous food intake in a dose-dependent manner (Figure 4A). Importantly, the same dose of centrally applied UDP (30 μM), which evoked the strongest activation of neurons in the ARH, also elicited the strongest stimulatory effect on food intake by increasing food intake by 45% compared to animals subjected to vehicle injection (Figures 4A and S4A–S4C).

To directly assess whether the ability of centrally applied UDP to activate feeding depends on AgRP neuron activation, we investigated the effect of centrally administered UDP in mice, which allow for pharmacogenetic inhibition of AgRP neurons. To this end, we injected $AgRP^{Cre}$ mice bilaterally into the ARH with an AAV, which co-expresses in a Cre-dependent manner the inhibitory DREADD channel hM4D and mCherry. Immunostaining for m-Cherry revealed successful bilateral targeting of the ARH in these mice (Figure 4B). While in Cre-negative control animals bilateral injection of the AAV and intraperitoneal CNO application had no effect on UDP's ability to increase food intake, UDP's food intake-stimulatory effect was completely abolished upon CNO-mediated inhibition of AgRP neurons of AAV-injected $AgRP^{Cre}$ mice (Figure 4C). While AgRP neuron activity is required for UDP's orexigenic effect, mRNA expression levels of *Agrp*, *Npy*, or *Pomc* are not affected over time by icv UDP application (Figures S4D–S4F). Taken together, these experiments clearly revealed that centrally applied UDP enhances food intake and that this effect is abolished when activation of AgRP neurons is specifically inhibited.

To further elucidate the specific contribution of P2Y6-mediated signaling in UDP-dependent activation of food intake, we took both a pharmacological and a genetic approach. We first compared the orexigenic effect of centrally applied UDP in either the absence or presence of the well-characterized P2Y6 antagonist MRS 2578. Again, while UDP injection increased food intake by ≈50% also in these independent sets of experiments, this response was completely abrogated upon co-treatment with the P2Y6 antagonist (Figure 5A). Importantly, for this experiment, we used a dose of MRS 2578 that does not modulate food intake

(**D**) Representative microphotographs of GFP immunostaining (P2Y6-EGFP, green) and of corresponding nuclear counterstaining (DAPI, blue) in the ARH, the VMH, the DMH, the LHA, and the PVH of P2Y6-EGFP mice.

(**E**) Representative microphotographs of ARH co-immunostaining of P2Y6-EGFP (P2Y6-EGFP, green) and of: (top row) integrin αM (Cd11b, microglia, red), (middle row) glial fibrillary acicid protein (GFAP, astrocytes, red), and (bottom row) fluorescent Nissl staining (NeuroTrace, neurons, red).

Data are presented as mean ± SEM. Scale bars: D, 100 μm and E, 50 μm. V3, third ventricle. *$p < 0.05$ and **$p < 0.01$, as determined by one-way ANOVA followed by Newman-Keuls posthoc test. See also Figure S1.

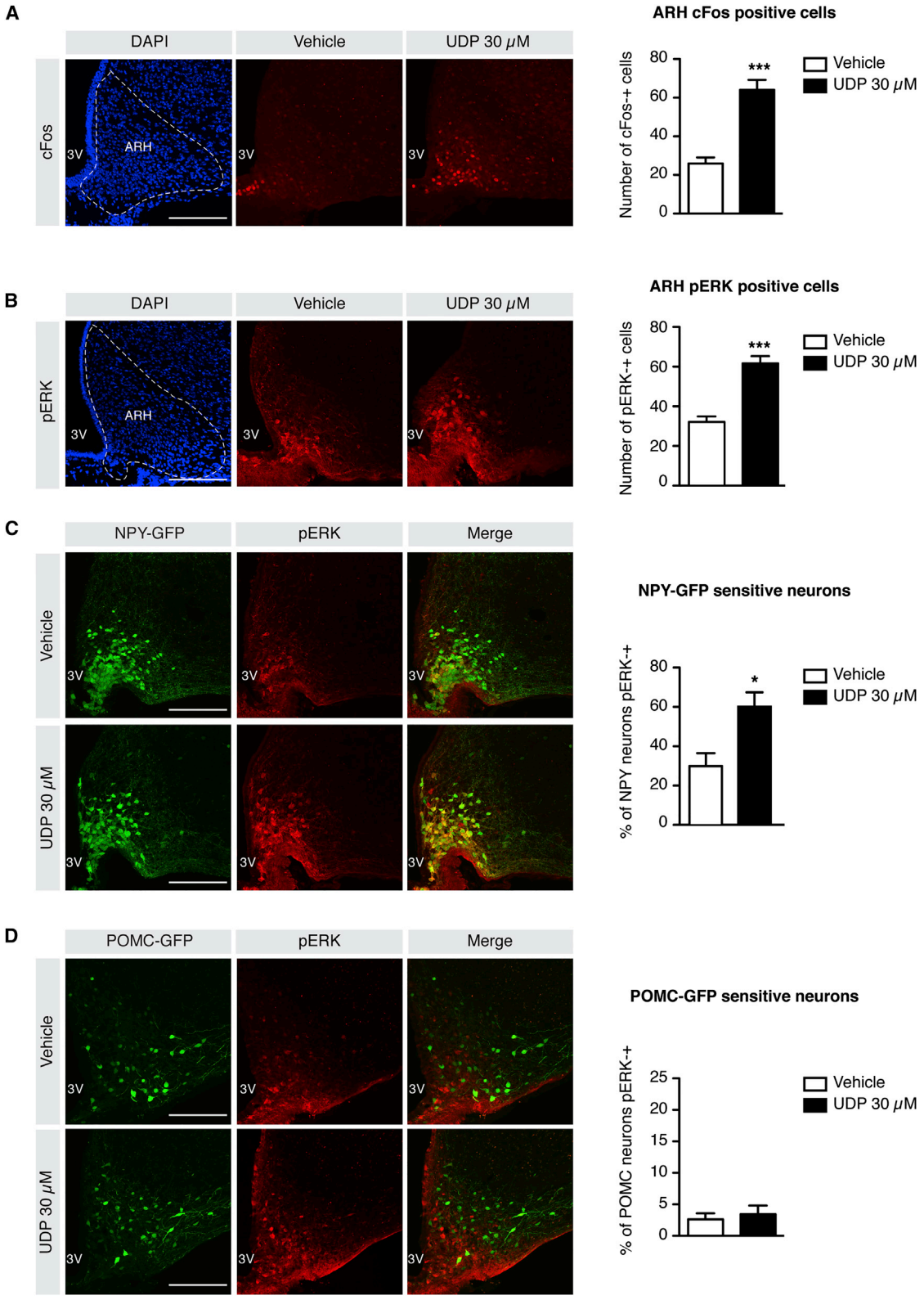

Figure 2. UDP Triggers pERK Activation in Orexigenic AgRP Neurons, but Not in Anorexigenic POMC Neurons

(A and B) Confocal images and quantification comparison of (**A**) cFos- and (**B**) pERK-immunoreactive cells in the arcuate nucleus (ARH) of mice after intracerebroventricular administration of vehicle (saline) or 30 μM UDP (n_{cFos} = 7 versus 9; n_{pERK} = 14 versus 16).

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by itself (Figure 5A). To investigate whether the inhibition of UDP-induced feeding by central administration of MRS 2578 might stem from unspecific side effects of the antagonist, we also tested the effect of MRS 2578 on the ability of centrally applied ghrelin to increase feeding. Here, icv application of 2 μg ghrelin increased feeding to a comparable extent as observed upon UDP injection (Figure 5B). However, co-application of the P2Y6 antagonist MRS 2578 had no effect on ghrelin's ability to increase food intake (Figure 5B). These findings clearly provided the first evidence that UDP's ability to increase feeding is specifically mediated via P2Y6-dependent signal transduction.

To further support this notion, we compared the ability of centrally applied UDP to increase food intake in control and in P2Y6-deficient mice ($P2Y6^{\Delta/\Delta}$). While UDP clearly increased feeding in control mice, it completely failed to increase food intake in litter mates lacking functional P2Y6 expression (Figure 5C). In contrast, P2Y6-deficient mice exhibited a comparable ability to increase food intake in response to central ghrelin application, as compared to their litter mate controls (Figure 5D). Collectively, both pharmacological and genetic inhibition of P2Y6 specifically abrogate the acute orexigenic action of centrally applied UDP, but not of ghrelin, clearly demonstrating that UDP specifically engages P2Y6 signaling to increase food intake.

Having defined that the food-intake-promoting activity of UDP is mediated through P2Y6-dependent signaling, we aimed to assess whether UDP's ability to increase AgRP neuron firing depends on functional P2Y6 signaling. To this end, we performed electrophysiological recordings from genetically identified AgRP neurons in $AgRP^{tdTomato}$ mice. MRS 2578 preincubation abrogated UDP's ability to increase action potential firing of all but a single recorded AgRP neuron (Figures 5E and 5F). Thus, not only UDP-induced activation of feeding, but also UDP-stimulated activation of AgRP neurons critically depends on functional P2Y6 signaling.

Hypothalamic UDP Concentrations Are Increased in Obesity

Having identified a regulatory role for UDP-evoked P2Y6-dependent signaling in control of AgRP neuron activity and feeding, we assessed whether P2Y6 expression or UDP concentrations might be altered in the hypothalamus in obesity. Therefore, we compared the hypothalamic mRNA expression of *P2ry6* in control mice and diet-induced obese animals as well as in control mice and mice carrying a mutation in the leptin receptor gene (*db*/*db* mice). This analysis revealed unaltered expression of *P2ry6* in the hypothalamus of diet-induced and genetically obese animals (Figures 6A and 6B). In contrast, ultra-performance liquid chromatography (UPLC)-based assessment of hypothalamic UDP concentrations revealed a significant increase in hypothalamic UDP content in both obese mouse models (Figures 6C and 6D). Moreover, hypothalamic UDP concentrations positively correlated both with body weight and markers of impaired glucose homeostasis such as fasting glycemia and HOMA-IR in obese mice (Figures 6E and 6F). Collectively, these experiments revealed that, under conditions of obesity, hypothalamic concentrations of the P2Y6 ligand UDP are increased in the absence of alterations in *P2ry6* mRNA expression.

Circulating Uridine Concentrations Are Increased in Obesity and Can Promote Hypothalamic UDP Synthesis

Since we detected elevated hypothalamic UDP concentrations in both diet-induced and genetically determined obesity, we further investigated potential mechanisms underlying this phenomenon. Thus, we monitored the mRNA expression of key enzymes of UDP synthesis and conversion (Figure S5). However, there was no consistent alteration in hypothalamic mRNA expression for the key regulatory gene products in UDP synthesis or conversion detectable in the hypothalamus of high-fat-diet-fed or *db*/*db* mice (Figures 7A and 7B).

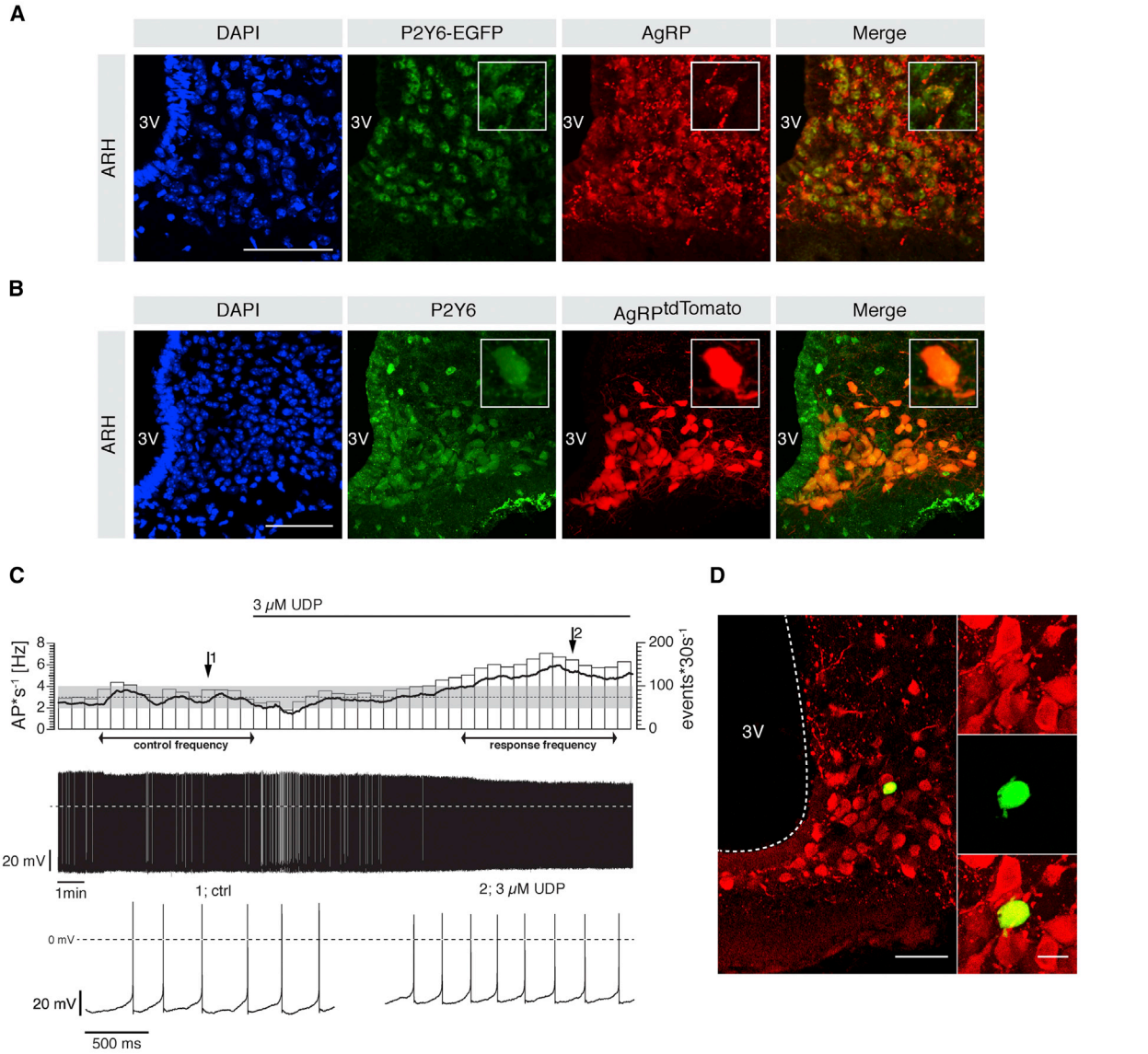
Neuronal UDP synthesis critically depends on the availability of uridine, which reaches the brain via transporter-mediated uptake (Cansev, 2006; Ipata, 2011). Therefore, we compared the expression of known transporters for pyrimidine and pyrimidine metabolites in the hypothalamus of control mice and diet-induced obese animals, as well as *db*/*db* mice. However, there was no consistent alteration in the mRNA expression of *Slc28a* and *Slc29a* family members in diet-induced or genetically determined obesity (Figures 7C and 7D).

In contrast, when we assessed circulating serum uridine concentrations, there was a significant increase in serum uridine concentrations in obese mice (Figure 7E). Moreover, we detected a positive correlation between serum uridine concentrations and hypothalamic UDP content in these animals (Figure 7F). Collectively, these data indicate that, in obesity, serum uridine concentrations increase and this subsequently may lead to increased hypothalamic UDP synthesis.

To directly address whether elevation of serum uridine concentrations—as observed in obesity—can lead to increased hypothalamic UDP synthesis, we next injected control animals intraperitoneally with uridine. The acute i.p. injection of 50 mg/kgBW uridine resulted in a rapid increase of serum uridine concentrations 60 min post injection and serum levels returned to baseline concentrations 90 min after injection (Figure 7G). This increase in circulating uridine concentrations resulted in subsequent elevation of hypothalamic UDP content as early as 90 min after peripheral application of uridine (Figure 7H). Finally, consistent with the notion that increased hypothalamic UDP concentrations can enhance feeding, animals injected intraperitoneally with uridine increased food intake significantly 4 hr after peripheral application of uridine (Figure 7I). Taken together, our experiments revealed that, in obesity, circulating uridine concentrations are increased, providing enhanced substrate availability for hypothalamic UDP synthesis and ultimately promoting feeding via UDP-induced P2Y6 signaling in the CNS (Figure S6).

(C and D) Confocal images and quantitative comparison of (**C**) NPY-GFP and (**D**) POMC-GFP expressing neurons pERK immunoreactive after intracerebroventricular administration of vehicle (saline) or 30 μM UDP ($n_{NPY\text{-}GFP}$ = 4 versus 5; $n_{POMC\text{-}GFP}$ = 5 versus 5). Scale bar, 100 μm. V3, third ventricle.

Data are presented as mean ± SEM. **p < 0.01, ***p < 0.001, as determined by unpaired Student's t test. See also Figure S2.



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DISCUSSION

Better defining the regulatory processes underlying the central control of food intake and metabolism is key to the development of novel drugs targeting the current obesity and diabetes epidemic (Geiss et al., 2014; WHO, 2006). Here, we have unraveled a pathway that regulates feeding behavior as we uncover an unappreciated role for the UDP-selective P2Y6 in control of AgRP neuron activity and their associated orexigenic effects. Moreover, we reveal that this UDP/P2Y6 pathway is activated in response to raising uridine supply from the periphery of the organism, as observed in obesity, where both circulating uridine concentrations and hypothalamic UDP content are increased (Figure S6). While currently little information is available on the physiological regulation of circulating uridine or hypothalamic UDP concentrations in relation to acute changes in energy state, some studies have recently described that circulating plasma uridine concentrations are increased in humans suffering from T2DM and correlate with the degree of insulin resistance also in rodents, consistent with the increase of circulating uridine in *db/db* mice reported here (Dudzinska et al., 2013; Hamada et al., 2007; Hawkins et al., 1997; Urasaki et al., 2014; Yamamoto et al., 2010). Given that AgRP neurons are not only critical for the control of feeding, but also for control of hepatic glucose production (Könner et al., 2007), altered uridine/UDP-mediated control of these cells may not only contribute to obesity development, but also to the pathogenesis of altered glucose homeostasis. Clearly, future studies on P2Y6-deficient mice will have to delineate the role of UDP/P2Y6-dependent signaling in the development of obesity and T2DM, as well as states of negative energy balance or weight loss.

Extracellular nucleotides act as signaling molecules to mediate a wide range of cellular and physiological responses by acting on cell-surface purinergic receptors (P2) (Abbracchio et al., 2009; Burnstock et al., 2011). A striking example of the importance of purinergic signaling was the discovery that ATP acts as a neurotransmitter regulating core functions, such as circadian rhythm, sensory processes, and nociception, as well as higher-order cognitive functions, including motivation, learning, and memory (Burnstock, 2006; Burnstock et al., 2011). Surprisingly, despite indisputable recognition of the critical importance of purine and pyrimidine signaling as a privileged route for cell-to-cell communication in numerous physiological processes, very little is known about the role of purinergic signaling in the CNS-dependent control of energy and glucose homeostasis. Within the several members of the P2 family, we particularly focused on the P2 receptors activated by uracil nucleodites and derived sugar nucleotides based on their structural and phylogenetic similarity to Gpr17 (Daniele et al., 2011), which was recently identified as a novel FoxO1-dependent regulator of AgRP neuron activity (Ren et al., 2012). Among the uracil nucleotides and derivates-binding members with unknown function in the CNS-dependent control of metabolism, P2Y6 stands out by its high expression levels in the ARH. As opposed to the hippocampus, where P2Y6 colocalizes with activated microglia (Koizumi et al., 2007), in the ARH of lean mice, we identify P2Y6 as being specifically expressed in neurons, providing evidence for a region-dependent, cell-type-specific expression pattern of P2Y6 in the CNS. Guided by the microglial expression of P2Y6, previous studies had mostly focused on the microglial role of P2Y6, where P2Y6 signaling was demonstrated to activate microglia and to promote phagocytosis (Koizumi et al., 2007). Here, we show that, in contrast, P2Y6 directly activates AgRP neurons in the ARH to potently promote feeding, while it only has minor effects on the activation of anorexigenic POMC neurons. This notion is consistent with the robust activation of feeding upon optogenetic or pharmacogenetic activation of AgRP neurons (Aponte et al., 2011; Krashes et al., 2011) and their previously defined crucial role for the maintenance of feeding, as evidenced by toxin-mediated ablation of these cells in adult mice (Gropp et al., 2005; Luquet et al., 2005).

Besides our demonstration that AgRP neurons express P2Y6 and that AgRP neuron activity is required to promote feeding in response to centrally applied UDP, we reveal that UDP triggers pERK phosphorylation and increased action potential firing in a substantial proportion of AgRP neurons. Excitatory effects of UDP have previously been described in cultured sympathetic neurons in which UDP-induced depolarization evoked noradrenaline release (Nörenberg et al., 2000; von Kügelgen et al., 1997). Accordingly, similar mechanisms may occur in AgRP neurons, where UDP induces increased firing of action potentials to subsequently release GABA and the characteristic orexigenic neuropeptides produced by these cells, i.e., NPY and AgRP. Recently, Krashes and coworkers described the differential functions of neuromediators released by AgRP neurons in controlling temporally distinct phases of eating behavior. Here, GABA and NPY are

Figure 3. AgRP Neurons Express P2Y6 Whose Activation by UDP Directly Increases Firing Rate

(A and B) Representative microphotographs of co-immunostaining of (**A**) P2Y6-EGFP (P2Y6-EGFP, green) and of AgRP (AgRP, red) and (**B**) P2Y6 (P2Y6, green) and $AgRP^{tdTomato}$ ($AgRP^{tdTomato}$, red) in the arcuate nucleus of the hypothalamus (ARH).

(**C**) UDP effect on a single AgRP neuron. Action potential frequency (black line) and respective rate histogram (30 s bins, top), original recording (middle), and traces in higher resolution (bottom) of an AgRP neuron increasing firing frequency in response to the application of 3 μM UDP. Shaded gray background indicates ± 3 × SD and the dotted line the respective mean of the control calculated from 12 bins of 30 s each. Arrows beneath the bins indicate the bins used to calculate means and SD for control and effect, respectively. The increase in action potential frequency is larger than 3 × SD of the control, thus defining this neuron as a responsive neuron.

(**D**) Recorded neuron, identified by antibody staining against tdTomato (red) and a biocytin fill of the recorded neuron (green).

(**E**) Overall responsiveness to 3 μM UDP of the recorded AgRP neuron population (n = 11 in total: 4 non-responsive neurons versus 7 responsive).

(**F**) Quantification of action potential (AP) frequency (n = 11, responsive and non-responsive pooled). Gray circles mark single recordings responding with a significant increase in AP frequency; open circles are non-responsive neurons. All recordings have been conducted in synaptically isolated neurons.

Scale bars: A and B, 50 μm and D, 40 μm and 10 μm in the magnifications, respectively. V3, third ventricle. Data are represented as boxplots generated according to the Tukey method (mean, "+"; median, horizontal line). **$p < 0.01$, as determined by paired Student's t test, including all recorded 11 neurons. See also Figure S3.

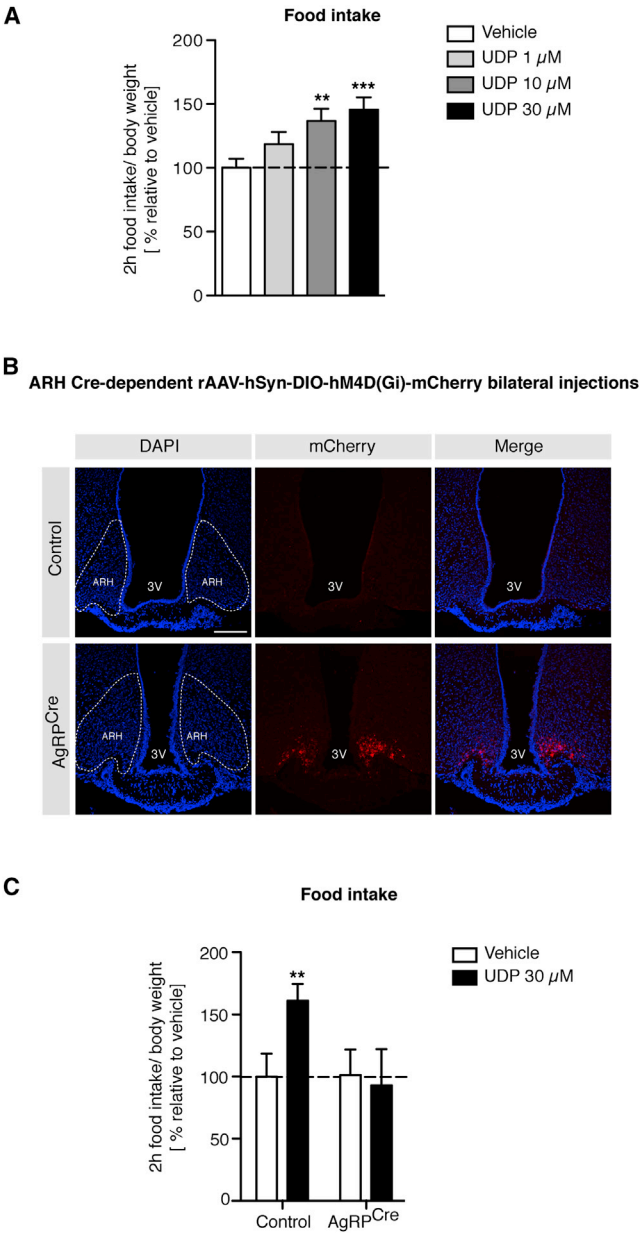

Figure 4. Central UDP Dose Dependently Increases Food Intake, and Its Orexigenic Effects Require AgRP Neuron Activity

(**A**) 2 hr food intake measurement (depicted as food intake to body weight) after intracereborventricular (icv) administration of increasing doses of UDP (1 μM, 10 μM, and 30 μM) or vehicle (saline) (n = 26 versus 13 versus 11 versus 14).

(**B**) Representative microphotographs of mCherry immunostainng in control and AgRP^{Cre} mice injected bilaterally in the arcuate nucleus (ARH) with Cre-dependent rAAV-hSyn-DIO-hM4D(Gi)-mCherry (mCherry, red; DAPI counterstaining, blue). Scale bar, 100 μm. V3, third ventricle.

(**C**) 2 hr food intake measurement in virus-injected AgRP^{Cre} mice or control wild-type litter mates. Mice received CNO injections (0.3 mg/kgBW) 15 min prior to icv administration of 30 μM UDP or vehicle (saline) (n = 6 versus 8 versus 5 versus 5).

Data are presented as mean ± SEM. **p < 0.01, ***p < 0.001, as compared to vehicle as determined by one-way ANOVA followed by Newman-Keuls post-hoc test (A) or unpaired Student's t test (C). See also Figure S4.

promoting food intake in the short term, whereas AgRP is responsible for the long-term stimulation of eating (Krashes et al., 2013). This study and the specific short-term effect of UDP on food intake suggest that the orexigenic effect of UDP may depend on GABA- and/or NPY-mediated signals originating from AgRP/NPY co-expressing neurons rather than on AgRP. This notion is further substantiated through the notion that UDP acutely promotes feeding without obvious changes in the transcription of *Agrp* and *Npy* mRNAs. However, this assumption will have to be directly addressed in future studies.

In accordance with the ability of UDP to increase Ca^{2+} levels in neurons, it is well described that activation of P2Y6 by UDP leads to activation of various Ca^{2+}-dependent signaling pathways, including IP_3 accumulation and an increase in cytoplasmic free Ca^{2+} from intracellular Ca^{2+} stores (Brunschweiger and Müller, 2006). In addition, P2Y6 shares convergent signaling pathways with the receptor for ghrelin, which also exerts its orexigenic effects predominantly through the activation of AgRP neurons (Tschöp et al., 2000 and for review, Andrews, 2011). First, as for ghrelin (Steculorum et al., 2015), we found that UDP activates MAPK/ERK signaling in AgRP neurons both in vitro and in vivo. Second, several peripheral effects of P2Y6 directly rely on AMPK activation (Balasubramanian et al., 2013, 2014), and AMPK is a critical element in mediating the orexigenic effects of ghrelin through activation of AgRP/NPY neurons (Andrews, 2011). Moreover, consistent with a critical role for AMPK regulation in AgRP neuron activity, AgRP cell-specific AMPK-deficient mice develop a lean phenotype (Claret et al., 2007), further supporting the idea that the orexigenic effect of UDP may indeed be associated with a P2Y6-dependent activation of AMPK in these cells. Clearly, the downstream signaling pathway(s) of P2Y6 in AgRP/NPY neurons will have to be further characterized in detail.

Another key aspect of the results presented here is our demonstration that the orexigenic effect of UDP completely relies on functional P2Y6 signaling, as evidenced by pharmacologic and genetic inhibition of P2Y6 signaling. This rules out the possibility that UDP is used as a precursor metabolite by membrane-bound or soluble enzymes since P2Y6 is the exclusive receptor for UDP and not for its metabolites, such as UDP glucose or UTP, which, respectively, act through P2Y14 and P2Y2/P2Y4 (Brunschweiger and Müller, 2006). This point is of critical importance, as it was previously shown that UDP glucose, when co-injected icv with leukotrienes, increases food intake (Ren et al., 2012). Our experiments thus clearly identify specifically P2Y6 as a target for inhibiting the food-intake-promoting actions of increased hypothalamic UDP in obesity.

In addition to expression of P2Y6, we reveal that critical components of enzymatic machinery for the biosynthesis of UDP—UCK-1 and -2, which catalyze the phosphorylation of uridine to UMP and represent the rate-limiting step for the UDP production (Anderson and Brockman, 1964)—are highly expressed in the ARH. The exact nature of UDP-synthesizing and -releasing cells, as well as a potential autocrine versus paracrine mode of UDP action on P2Y6 expressed on AgRP neurons, still remains to be investigated. CNS UDP biosynthesis is critically dependent on uridine supply and therefore directly relies on uridine uptake from the periphery (Cansev, 2006; Ipata, 2011). Irrespective of

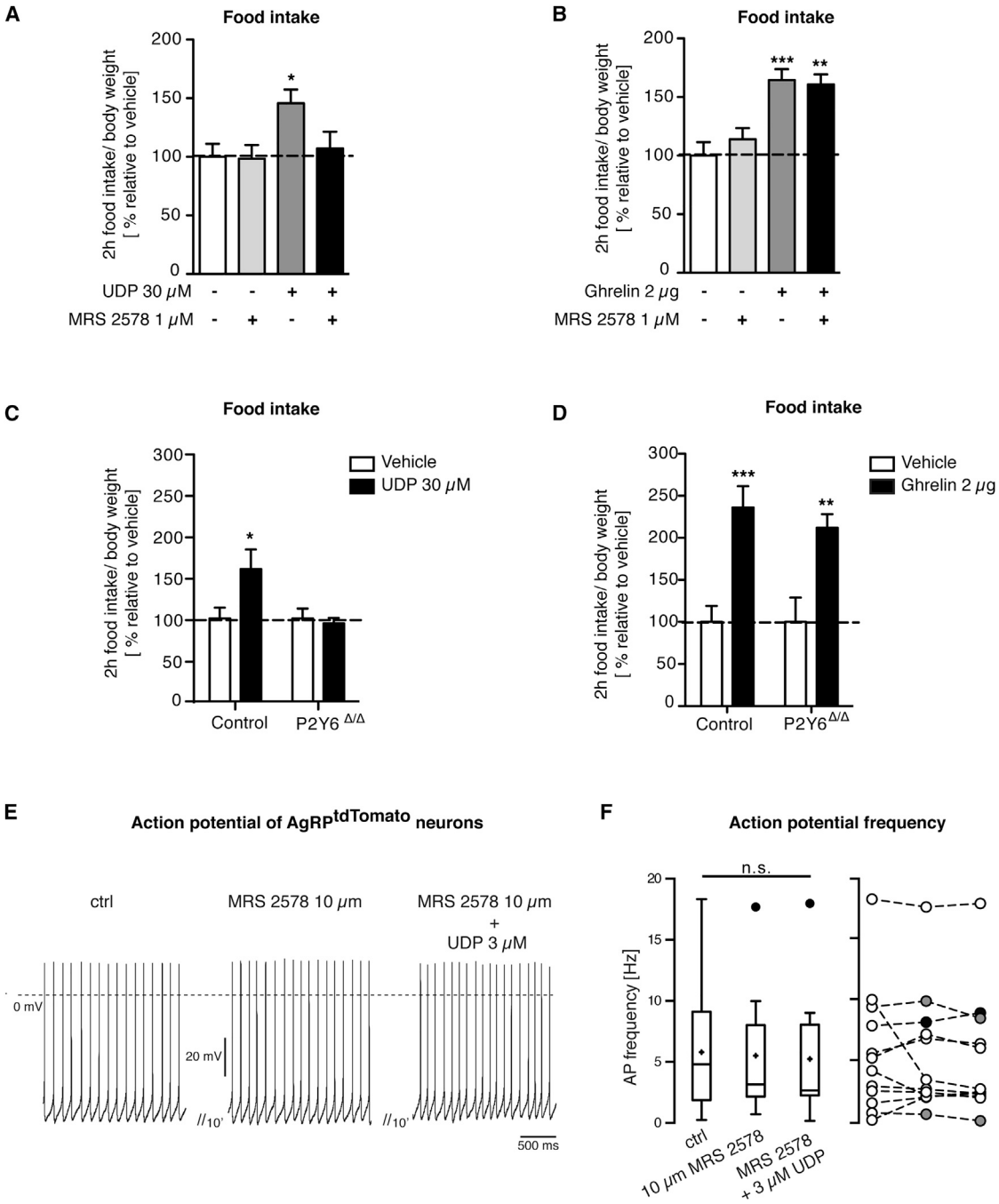

Figure 5. UDP Promotes Feeding and Activates AgRP Neurons via P2Y6-Dependent Signaling

(A–D) 2 hr food intake measurement after intracerebroventricular (icv) administration of (**A**) vehicle (0.1% DMSO), 1 μM of the P2Y6-specific antagonist MRS 2578, 30 μM UDP or co-icv of 30 μM UDP, and 1 μM MRS 2578 (n = 10 versus 11 versus 14 versus 12) or (**B**) vehicle (0.1% DMSO), 1 μM MRS 2578, 2 μg ghrelin or co-icv of 2 μg ghrelin, and 1 μM MRS 2578 (n = 13 versus 13 versus 14 versus 14). 2 hr food intake measurement after icv administration of (**C**) vehicle (saline) or 30 μM UDP or (**D**) vehicle (saline) or 2 μg ghrelin in P2Y6-deficient mice ($P2Y6^{\Delta/\Delta}$) or control littermates (n_C = 10 versus 6 versus 9 versus 6, n_D = 9 versus 9 versus 5 versus 6).

(**E**) Original traces showing the action potential firing during the application of MRS 2578 and during the application of 3 μM UDP in the presence of MRS 2578.

(**F**) Quantification of action potential frequency (AP) (n = 12). One neuron significantly increased (black circle) and two decreased (gray circles) action potential frequency upon application of 3 μM UDP in the presence of MRS 2578.

Data are presented as mean ± SEM. *p < 0.05 (A–D) and as boxplots generated according to the Tukey method (mean, "+"; median, horizontal line) (F). *p < 0.05, **p < 0.01, ***p < 0.001, as compared to vehicle or control as determined by one-way ANOVA followed by Newman-Keuls posthoc test (A, B, F) or unpaired Student's t test (C and D).

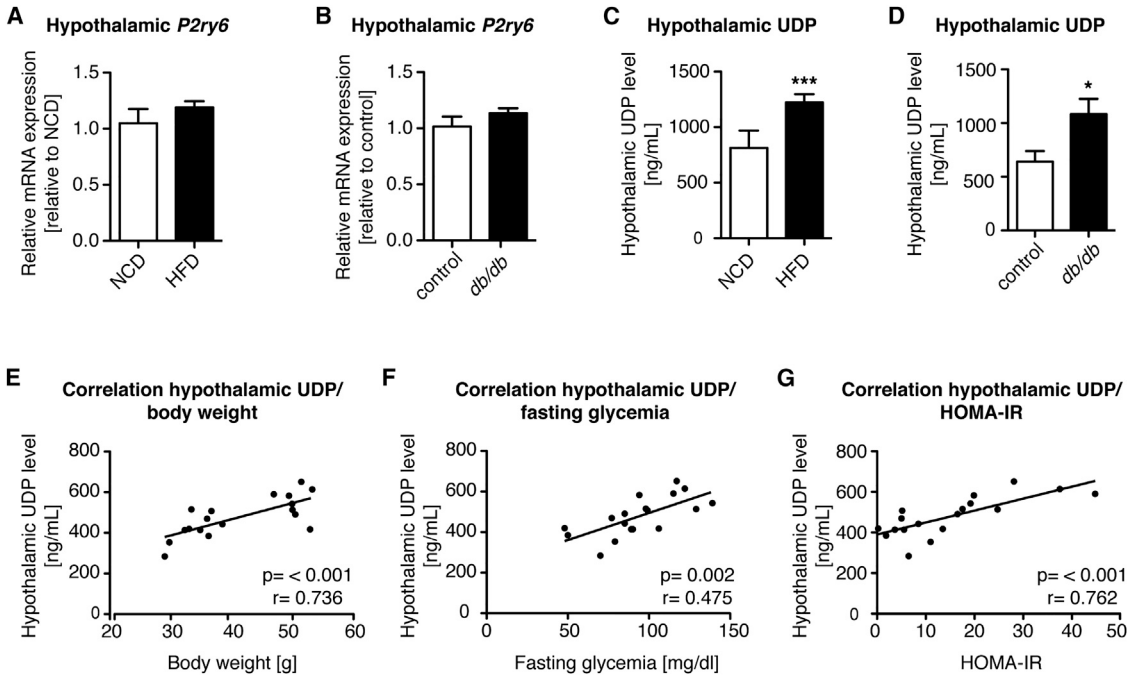

Figure 6. Hypothalamic UDP Content Is Increased in Dietary and Genetically Obese Mice

(A and B) Quantitative real-time PCR analysis of *pyrimidinergic receptor P2Y, G protein coupled, 6* (*P2yr6*) in hypothalamus of (**A**) diet-induced obese animals fed a high-fat diet (HFD) or control animals receiving a normal chow diet (NCD) (n = 7 versus 8) and (**B**) *db/db* and their control littermates (n = 5 versus 7).

(C–F) Hypothalamic contents of (**C**) HFD versus NCD mice (n = 10 versus 9) and (**D**) in db/db and control mice (n = 7 versus 7). Correlation of hypothalamic UDP and (**E**) body weight, (**F**) fasting glycemia, and (**G**) the homeostatic model assessment index of insulin resistance (HOMA-IR) in NCD and HFD mice (n = 18) (r, Pearson's r).

Data are presented as mean ± SEM. **p < 0.01, ***p < 0.001, as determined by unpaired Student's t test (A–D) or Pearson's correlation (E and F). See also Figure S5.

whether UCK-1 and UCK-2 expression in the ARH are restricted to neurons (autocrine regulation) or other cell types to promote paracrine UDP/P2Y6 signaling in AgRP neurons, our results clearly show that increased hypothalamic UDP concentrations, as observed in obese mice, and the hyperphagia secondary to elevated central UDP can be mimicked by peripheral application of uridine. Notably, insulin resistance in humans correlates with increased circulating uridine concentrations (Dudzinska et al., 2013; Hamada et al., 2007). While the predominant source of uridine in lean subjects is the liver (Dobolyi et al., 2011), future studies will have to unravel the mechanisms and tissues responsible for increased uridine synthesis in metabolic disorders.

In summary, the present study identifies the UDP/P2Y6 axis as a regulator of AgRP neuron activity and feeding behavior. This offers the unique opportunity to pursue agonists and antagonists for this GPCR as targets for the treatment of diseases associated with negative or positive energy balance.

EXPERIMENTAL PROCEDURES

Animal Care

All animal procedures were conducted in compliance with protocols approved by local government authorities (Bezirksregierung Köln, Cologne, Germany) and were in accordance with National Institutes of Health guidelines. Unless otherwise stated, animals were fed normal chow diet. Diet-induced obese mice were fed a high-fat diet containing 26.2% carbohydrates, 26.3% protein, and 34.9% fat (60% of calories from fat) or corresponding normal chow control diet. C57Bl/6N were purchased from Charles Rivers. *db/db* mice and their control litter mates were purchased from The Jackson Laboratories. All experiments have been performed in adult mice.

Genetic Mouse Models

All mice used in this study were previously published and have been described in the respective paper: P2Y6-EGFP and P2Y6-deficient mice (Bar et al., 2008), NPY-GFP mice (B6.FVB-Tg(Npy-hrGFP)1Lowl/J) (van den Pol et al., 2009), POMC-GFP mice (C57BL/6J-Tg(Pomc-EGFP)1Low/J) (Cowley et al., 2001), AgRP-IRES-Cre mice (AgRP^{Cre}) (Tong et al., 2008), and tdTomato reporter mice (B6;129S6-Gt(ROSA) 26Sor$^{tm9(CAG-tdTomato)Hze}$/J) (Madisen et al., 2010).

Food Intake Measurement

For all food intake studies, mice received administration of the compound prior to the onset of the dark phase, and food intake measurement was performed during the dark phase using chow diet.

Intracerebroventricular Injections

Intracerebroventricular (icv) cannulations were performed as described elsewhere (Klöckener et al., 2011). Mice received icv administration of 2 μl of each coumpounds.

AAVs Stereotaxic Injections and CNO Administration

Bilateral injections of AAVs into the ARH were performed as described elsewhere (Betley et al., 2013). rAAV5/hsyn-DIO-hM4D(Gi)-mCherry was obtained from the UNC Gene Therapy Vector Core. Animals received intraperitoneal injections of clozapine N-oxide (CNO, 0.3 mg/kgBW; Sigma) 15 min prior to icv administration of UDP, as described above.

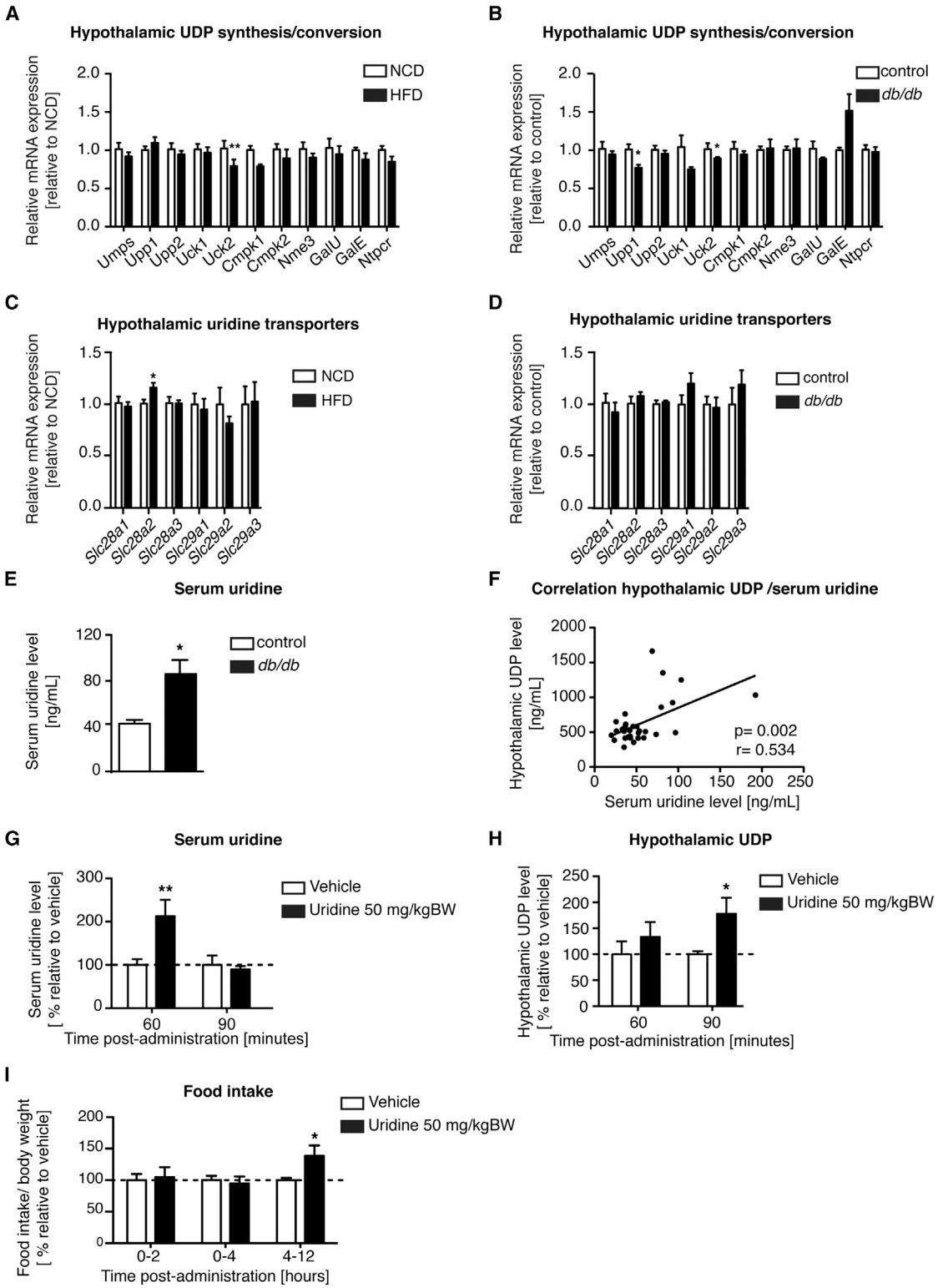

Figure 7. Hypothalamic UDP Is Increased in Obesity as a Consequence of Elevated Peripheral Uridine Supply

(A and B) Quantitative real-time PCR analysis of mRNA expression levels of enzymes involved in UDP synthesis and conversion in (**A**) diet-induced obese mice fed a high-fat diet (HFD) or a normal chow diet (NCD) (n = 5 versus 5) and (**B**) in db/db and their control litter mates (n = 5 versus 6).

(C and D) mRNA expression levels of transporters of uridine and uridine-associated nucleotides in (**C**) HFD and NCD mice (n = 8 versus 8) and in (**D**) *db/db* and their control litter mates (n = 5 versus 7).

(legend continued on next page)

Intraperitoneal Administration of Uridine

12-week-old C57Bl6/N male mice received 50 mg/kgBW of uridine (i.p.; 10 μl/g; Sigma) or vehicle (saline).

Ultra-Performance Liquid Chromatography

See the Supplemental Experimental Procedures for additional details.

Immunohistochemistry

Mice were perfused transcardially with 4% paraformaldehyde, and brains were processed as descrided previously (Steculorum et al., 2015). References and dilution used for each antibody are included in the Supplemental Experimental Procedures. Pictures were acquired using a confocal Leica TCS SP-8-X microscope equipped with a 20× objective.

Electrophysiology

Perforated patch recordings were performed on brain slices from $AgRP^{tdTomato}$ or POMC-GFP mice. For detailed information, see the Supplemental Experimental Procedures.

Gene Expression Analysis, Cell Culture, Western Blot, and Analytical Procedures

For basic proccedures, see the Supplemental Experimental Procedures.

Statistics

All values were expressed as the means ± SEM. Statistical analyses were conducted using GraphPad PRISM (version 5.0a). Data sets with only two independent groups were analyzed for statistical significance using unpaired two-tailed Student's t test. Data sets comparing food intake in the same animal subjected to two different treatments were analyzed for statistical significance using paired two-tailed Student's t test. Data sets with more than two groups were analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls posthoc test. For correlation analysis, the Pearson product-moment correlation (Pearson's r) was used and reported in the corresponding figure. All p values below 0.05 were considered significant. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.08.032.

AUTHOR CONTRIBUTIONS

J.C.B. and S.M.S. conceived the project, designed the experiments, and wrote the manuscript. S.M.S. performed experiments and analyzed data. N.E. provided technical assistance. L.P., S.B., and P.K. performed and analyzed electrophysiological recordings. Y.H. conducted UPLC analysis, and M.I. provided the P2Y6-EGFP and P2Y6-deficient mice.

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(**E**) Serum uridine levels of control and *db*/*db* mice (n = 9 versus 10).
(**F–I**) (**F**) Correlation of hypothalamic UDP and serum uridine levels (n = 29) (r, Pearson's r). Effects of intraperitoneal injection of uridine (50 mg/kgBW) or vehicle (saline) in C57Bl6/N mice on (**G**) serum uridine (n = 4–5 per group) and on (**H**) hypothalamic UDP contents (n = 5 per group) 60 and 90 min post-injection as well on (**I**) food intake (n = 15 per group).
Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, as determined by unpaired Student's t test (A–E and G–I) or Pearson's correlation (F). See also Figure S6.

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