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A role for the CREB co-activator CRTC2 in the hypothalamic mechanisms linking glucose sensing with gene regulation

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Within the central nervous system (CNS), the hypothalamus senses and integrates information on the nutrient state of the body. However, the molecular mechanisms translating nutrient sensing into changes in gene expression and, ultimately, nutrient intake remain unclear. A crucial function for the cyclic AMP-response element binding protein (CREB) co-activator CREB-regulated transcription co-activator 2 (CRTC2) in maintaining glucose homeostasis has been shown in the liver. Here, we report CRTC2 expression in distinct areas of the CNS, including hypothalamic neurons. We show that hypothalamic CRTC2 phosphorylation and subcellular localization is altered by nutrient state. Specifically, glucose regulates hypothalamic CRTC2 activity via AMP-activated protein kinase (AMPK)-mediated phosphorylation of CRTC2. Hypothalamic AMPK controls the expression of the cAMP response element (CRE) gene, insulin receptor substrate 2 (*Irs2*), by regulating CRTC2 occupancy of the *Irs2* promoter. Indeed, CRTC2 is required for the appropriate expression of specific hypothalamic CRE genes. Our data identify CRTC2 as a new hypothalamic AMPK target and highlight a role for CRTC2 in the mechanisms linking hypothalamic glucose sensing with CRE gene regulation.

Keywords: CRTC2; CREB; hypothalamus; glucose sensing; AMPK

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INTRODUCTION

Within the central nervous system (CNS), the hypothalamus is central to the sensing and regulation of nutrient state. However, although the importance of hypothalamic glucose-sensing neurons in the maintenance of nutrient homeostasis is clear (Parton *et al*, 2007), the neuronal mechanisms linking glucose sensing to changes in gene expression and, ultimately, nutrient intake remain poorly defined.

Cyclic AMP-response element binding protein (CREB)-regulated transcription co-activators (CRTCs; Conkright *et al*, 2003; Iourgenko *et al*, 2003) are a new family of CREB co-activators. Although phosphorylated CRTC is sequestered in the cytoplasm under basal conditions, it is dephosphorylated in response to calcium and cAMP signals, and translocates to the nucleus. There, association with CREB activates cAMP-response element (CRE)-mediated gene transcription (Bittinger *et al*, 2004; Screaton *et al*, 2004). The family member CRTC2 has a crucial function in the maintenance of glucose homeostasis; in response to fasting, CRTC2 activation initiates the gluconeogenic programme in the liver (Koo *et al*, 2005; Dentin *et al*, 2007). In this setting, AMP-activated protein kinase (AMPK) and the AMPK family members salt-inducible kinase (SIK)1 and SIK2 can phosphorylate CRTC2 at Ser 171 and thereby inhibit CRTC2 activity (Koo *et al*, 2005; Dentin *et al*, 2007).

Considering (i) the importance of calcium and cAMP second messenger pathways in neurons, and (ii) the important function of hypothalamic AMPK in regulating food intake (Minokoshi *et al*, 2004), we hypothesized that CRTC2 might be a downstream target of AMPK in neurons. Hypothalamic CRTC2 might, therefore, constitute a molecular link between cellular energy sensing and CRE-mediated gene transcription.

RESULTS

CRTC2 expression in distinct CNS structures

Immunohistochemistry (anti-TORC2-1cKSCN; Bittinger *et al*, 2004) identified CRTC2 protein in several distinct areas of the CNS of fed mice, including the hippocampus, red, trigeminal and hypoglossal nuclei (Fig 1A–G), and several nuclei of the hypothalamus (Fig 1B). Co-localization with NeuN confirmed that CRTC2 expression was restricted to neurons (data not shown). CRTC2 subcellular localization varied across different areas of the CNS (Fig 1A–G) and within the hypothalamus: CRTC2 localized to the nucleus in the arcuate (ARC), ventromedial (VMH) and paraventricular hypothalamic (PVH) nuclei, whereas expression was cytoplasmic in the lateral hypothalamic area (LHA; Figs 1B, 2A).

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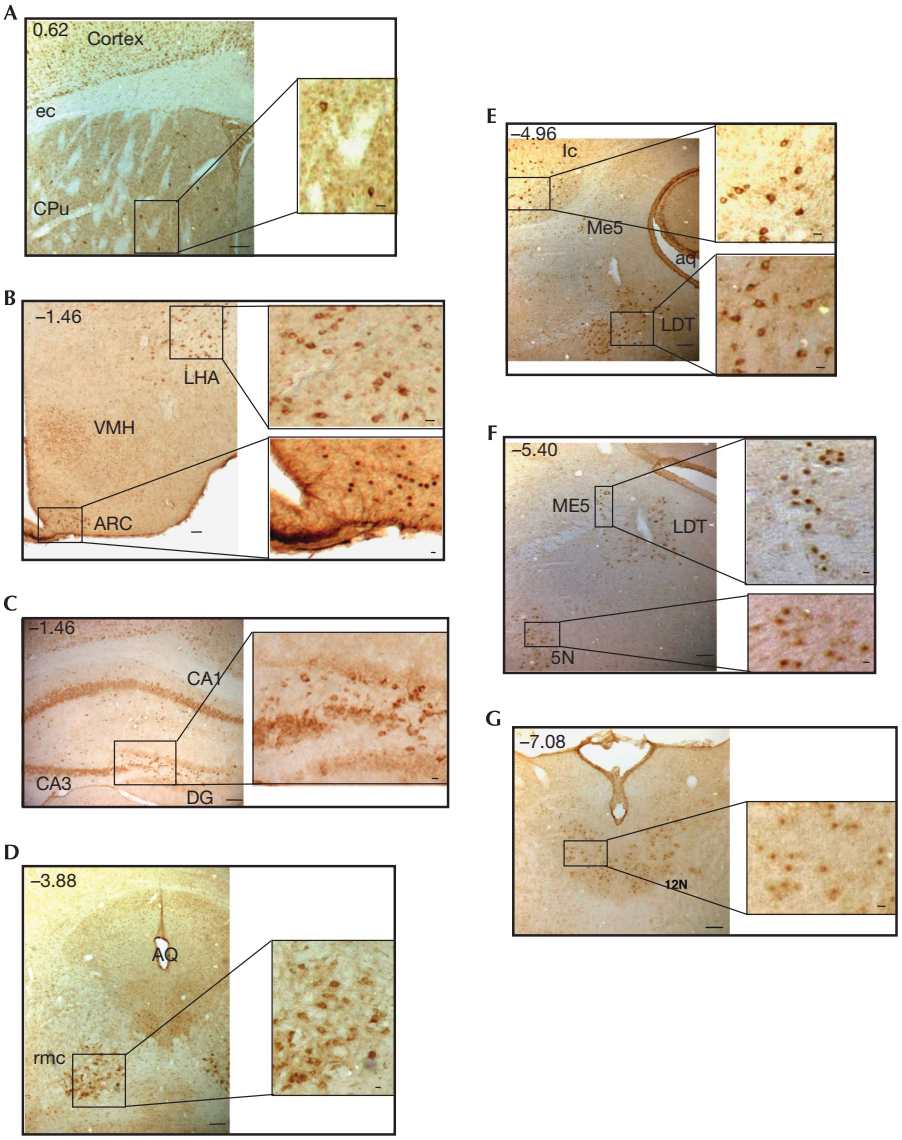

Fig 1 | Expression of CREB-regulated transcription co-activator 2 in the central nervous system. CRTC2 is expressed in several areas of the CNS in differing subcellular localizations. (Representative coronal sections of $n = 4$ brains. Position from Bregma in millimetres at the top left of each image. Scale bar, 0.1 mm.) 5N, motor trigeminal nucleus; 12N, hypoglossal nucleus; aq, aqueduct; ARC, arcuate nucleus; CA, cornu ammonis (Ammon's horn); CPu, caudate putamen; CNS, central nervous system; CRTC2, CREB-regulated transcription co-activator 2; DG, dentate gyrus; ec, external capsule; Ic, inferior colliculus; LDT, laterodorsal tegmental nucleus; LHA, lateral hypothalamic area; ME5, mesencephalic trigeminal nucleus; rmc, red nucleus magnocellular; VMH, ventromedial hypothalamus.

Glucose levels regulate hypothalamic CRTC2

After 24 h of fasting, hypothalamic CRTC2 expression seemed to be reduced (particularly in the ARC; Fig 2A) and CRTC2 in the PVH redistributed to the cytoplasm (Fig 2A). By contrast, the subcellular localization of CRTC2 was unaltered in the VMH and LHA (data not shown).

To assess whether the subcellular translocation of CRTC2 was triggered by phosphorylation, we analysed hypothalamic and liver extracts from fed and 24 h fasted mice, as well as fasted mice 15 min after intraperitoneal glucose injection (Fig 2B). Hypothalamic CRTC2 phosphorylation consistently reflected nutrient state, being mostly dephosphorylated in fed mice, more phosphorylated after fasting and predominantly dephosphorylated after glucose injection. It should be noted that the phosphorylation dynamics of hypothalamic CRTC2 are opposite to those in the liver under identical metabolic conditions (Fig 2B). Cortex CRTC2 phosphorylation was unaltered by metabolic state (supplementary Fig S1 online). We further analysed phosphorylation changes specifically at CRTC2 Ser 171 (P171–CRTC2), a site important for subcellular localization in liver cells (Koo *et al*, 2005). Hypothalamic P171–CRTC2 increased in response to fasting, and 2 h re-feeding and intraperitoneal glucose injection reversed this effect (Fig 2C). Interestingly, CRTC2 Ser 171 dephosphorylation was more robust after alteration of glucose than with re-feeding. Cultured

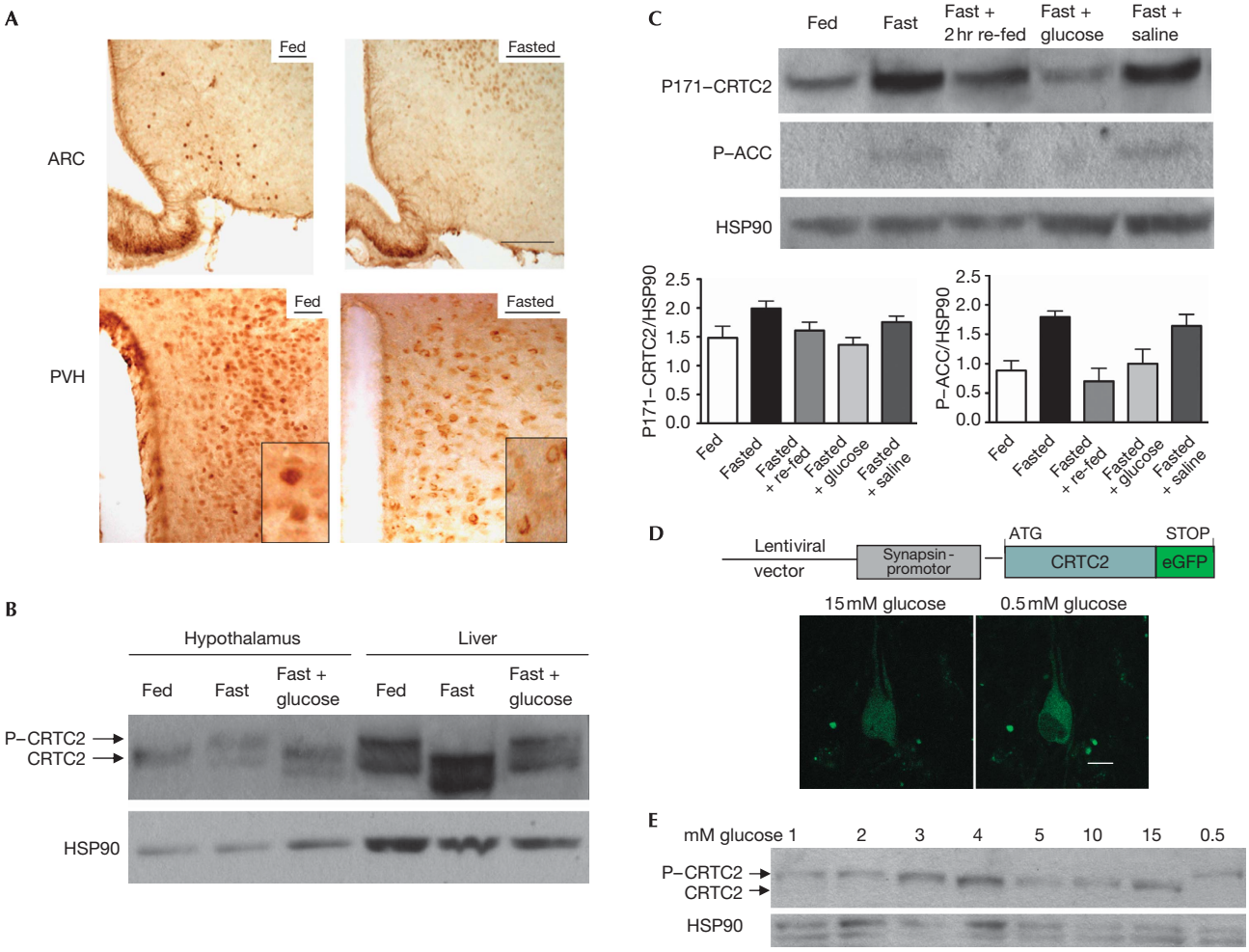

Fig 2 | Hypothalamic CREB-regulated transcription co-activator 2 subcellular localization and phosphorylation is regulated by glucose state. (**A**) The subcellular localization of CRTC2 in the ARC and PVH is regulated by feeding state. CRTC2 is nuclear in the ARC and PVH in *ad libitum* fed mice (left panels). After 24 h fasting CRTC2 diminishes and moves to the cytoplasm (right panels). Scale bar, 0.25 mm. (**B**) Hypothalamic CRTC2 phosphorylation is regulated by nutrient state. In contrast to liver, hypothalamic CRCT2 is dephosphorylated in the fed state, 24 h fasting increases its phosphorylation, whereas intraperitoneal glucose injection (0.1 mg/kg, 15 min) reverses this effect (representative of $n = 4$). (**C**) Hypothalamic CRTC2 phosphorylation at Ser 171 is sensitive to nutrient state. A 24 h fast increases hypothalamic P171–CRTC2, whereas 2 h re-feeding and intraperitoneal glucose injection reverses this effect. CRTC2 phosphorylation closely resembles phosphorylation of the AMPK target gene ACC in the same tissue. Bottom: quantification of $n = 3$. (**D**) Hypothalamic CRTC2 subcellular localization is regulated by glucose. Primary hypothalamic cultures expressing a neuron-specific CRTC2–eGFP chimeric protein showed nuclear CRTC2–eGFP exclusion after 15 min of incubation at low glucose (representative neuron of $n = 3$ experiments). Scale bar, 10 μm. (**E**) CRTC2 phosphorylation is regulated within physiological hypothalamic glucose concentration ranges. In hypothalami, incubated with the indicated glucose concentrations, CRTC2 phosphorylation decreases above 3–4 mM glucose (representative of $n = 4$). ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ARC, arcuate; CRTC2, CREB-regulated transcription co-activator 2; eGFP, enhanced green fluorescent protein; HSP90, heat-shock protein 90; PVH, paraventricular hypothalamic.

hypothalamic neurons transduced with a lentivirus driving neuron-specific expression of CRTC2–eGFP (enhanced green fluorescent protein) fusion protein (Fig 2D; Bittinger *et al*, 2004) showed nuclear CRTC2–eGFP exclusion within 15 min of incubation at low glucose levels. Consistent with *in vivo* experiments (Fig 2B,C), glucose is thus one of the stimuli that regulates hypothalamic CRTC2 subcellular distribution. Furthermore, in hypothalamic explants, CRTC2 phosphorylation decreased above 3–4 mM glucose, showing that CRTC2 activity is regulated by physiological hypothalamic glucose ranges (Fig 2E; Silver & Erecinska, 1994).

AMPK activity regulates hypothalamic CRTC2

In 0.5 mM glucose, only 14% of cultured hypothalamic neurons showed nuclear CRTC2–eGFP, whereas at high glucose this increased to 60%. This glucose-induced redistribution was mimicked by AMPK inhibition with compoundC at 0.5 mM glucose (Fig 3A). Conversely, nuclear CRTC2–eGFP in neurons cultured in 15 mM glucose redistributed significantly to the cytoplasm after addition of the AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR; Fig 3A). Neurons in which CRTC2 did not translocate in response to glucose and/or AMPK activity changes presumably reflect hypothalamic CRTC2

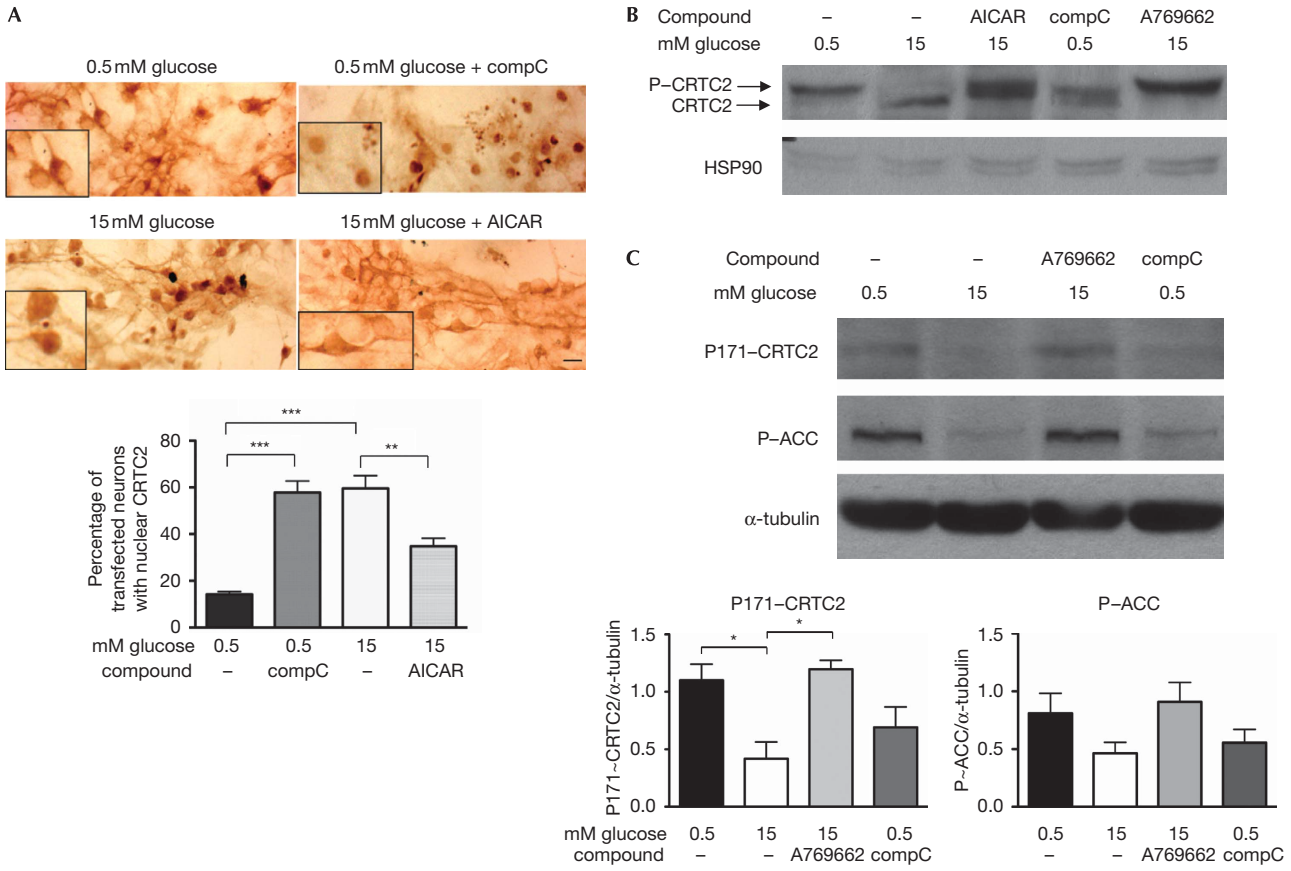

Fig 3 | Hypothalamic CREB-regulated transcription co-activator 2 is regulated by AMPK-mediated phosphorylation. (**A**) CRTC2 subcellular localization is regulated by AMPK activity. In primary hypothalamic cultures, neuron-specific CRTC2–eGFP was redistributed from the cytoplasm to the nucleus in response to inhibition of AMPK (compoundC, 10 μM, top panels); conversely, activation of AMPK (AICAR, 0.5 mM) causes CRTC2 nuclear export (bottom panels; quantification of percentage nuclear CRTC2–eGFP neurons is mean of five random fields per incubation). Scale bar, 10 μm. (**B**) CRTC2 phosphorylation is regulated by AMPK activity. AMPK activators (AICAR, 0.5 mM; A769662, 10 μM) or inhibitors (compoundC, 10 μm) override glucose effects on CRTC2 phosphorylation state and show that CRTC2 is a downstream target of AMPK (representative of $n = 4$). (**C**) Hypothalamic CRTC2 phosphorylation at Ser 171 is sensitive to AMPK activity. Increase in glucose levels significantly decreases P171–CRTC2, whereas A769662 reverses this effect. AMPK inhibition reduces P171–CRTC2 at low glucose. Phosphorylation state of the AMPK target ACC indicates AMPK activity. CRTC2 Ser 171 phosphorylation is thus sensitive to glucose and AMPK activity. Bottom: quantification of $n = 4$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; CRTC2, CREB-regulated transcription co-activator 2; eGFP, enhanced green fluorescent protein; HSP90, heat-shock protein 90.

subpopulations that are differentially regulated, which is consistent with our previous observations of heterogeneous CRTC2 regulation during fasting.

As exogenous CRTC2–eGFP is expressed in neurons that might not normally contain CRTC2, we investigated endogenous CRTC2 modulation in intact hypothalami. Mimicking *in vitro* results, incubation of hypothalami with AICAR increased CRTC2 phosphorylation despite high glucose, whereas compoundC reduced it; AMPK activity thus overrides glucose effects on CRTC2 phosphorylation (Fig 3B). In addition, a new, selective AMPK activator A769662, which activates AMPK by a mechanism distinct from AMP (Cool *et al*, 2006; Sanders *et al*, 2007), also increased CRTC2 phosphorylation (Fig 3B). We further analysed phosphorylation changes specifically at CRTC2 Ser 171, a site crucial for AMPK-dependent CRTC2 regulation in the liver (Koo *et al*, 2005). Indeed, decreases in hypothalamic P171–CRTC2 in response to high glucose were reversed by A769662, and compoundC reduced P171–CRTC2 despite low glucose (Fig 3C). Acetyl-CoA carboxylase (ACC) is the only previously reported hypothalamic AMPK target, and its phosphorylation corresponds with active P–AMPK (Fig 2C; supplementary Fig S2 online). Indeed, P171–CRTC2 changes consistently paralleled ACC phosphorylation under the same conditions, both *in vivo* (Fig 2C) and *in vitro* (Fig 3C). These data show that hypothalamic CRTC2 is a new downstream target of neuronal AMPK, and that glucose-regulated CRTC2 Ser 171 phosphorylation and subcellular distribution is mediated by AMPK activity. Interestingly, although neurons and pancreatic β-cells share similar glucose-sensing mechanisms, this is in contrast to CRTC2 in β-cells in which Ser 171 phosphorylation is not regulated by AMPK (Jansson *et al*, 2008).

CRTC2 regulates hypothalamic CRE genes

Insulin recepter substrate 2 (*Irs2*) is a transcriptional target of CRTC2 in the liver (Canettieri *et al*, 2005) and hypothalamic IRS2

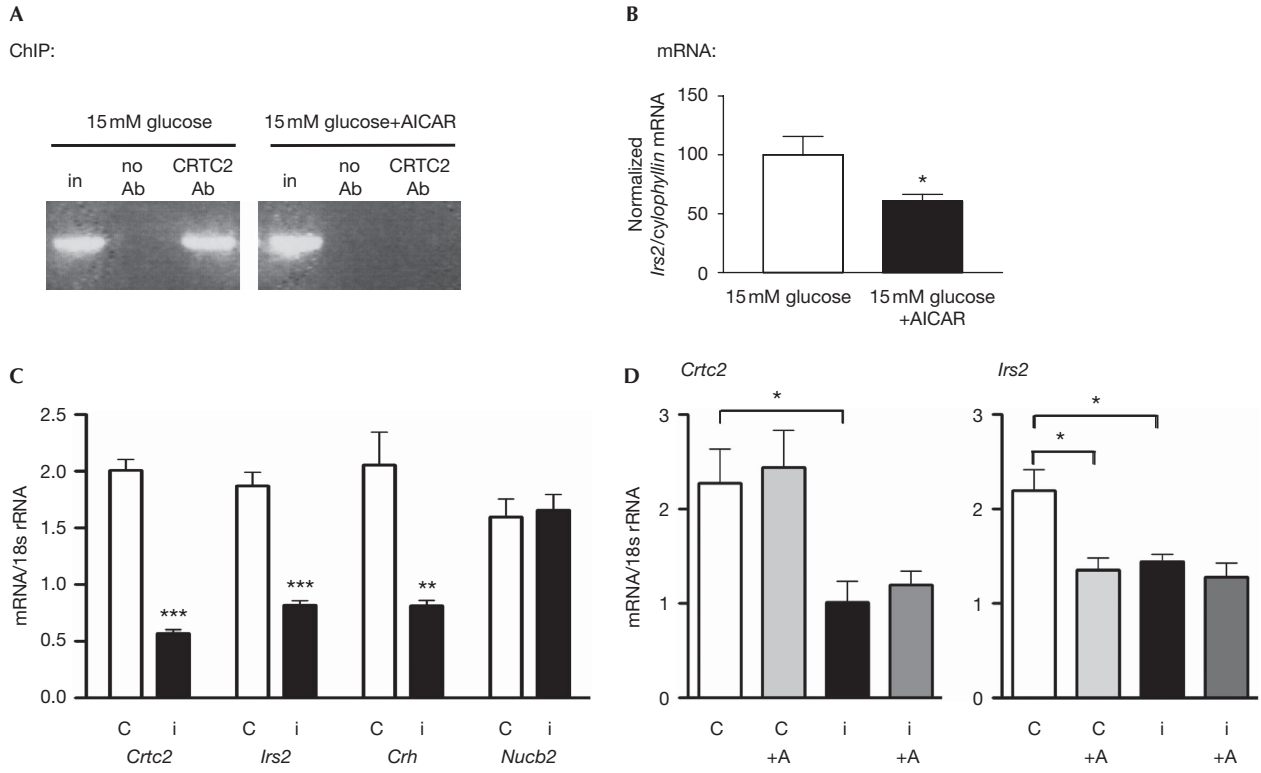

Fig 4 | CREB-regulated transcription co-activator 2 regulates hypothalamic CRE-gene expression. (**A**) AMPK activity regulates hypothalamic CRTC2 occupancy of the *Irs2* promoter. Chromatin immunoprecipitation on mouse hypothalami incubated in the indicated media. Active AMPK removes CRTC2 from the *Irs2* CRE at high glucose. CRTC2 Ab, CRTC2 pull down; in, input DNA; no Ab, no antibody control. Representative of $n = 3$. (**B**) AMPK activation downregulates *Irs2* gene expression at high glucose in primary hypothalamic cultures ($n = 8$). (**C**) CRTC2 is required for appropriate *Irs2* and *Crh* expression. Primary hypothalamic cultures were incubated with control (C) or *Crtc2* RNAi (i) adenovirus. *Crtc2* knock down significantly reduces *Irs2* and *Crh* mRNA expression, whereas *Nucb2* mRNA is unaltered ($n = 4$). (**D**) CRTC2 is required to mediate the transcriptional regulation of *Irs2* mRNA by AMPK. Primary hypothalamic neuronal cultures were incubated with control (C) or *Crtc2* RNAi (i) adenovirus and treated with (+A) or without A769662 (5 μM). *Crtc2* knock down abolishes AMPK's regulation of *Irs2* mRNA expression ($n = 4$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; ChIP, chromatin immunoprecipitation; CRE, cAMP-response element; *Crh*, corticotropin-releasing hormone; CRTC2, CREB-regulated transcription co-activator 2; *Irs2*, insulin receptor substrate 2; *Nucb2*, nucleobindin 2 precursor; RNAi, RNA interference; rRNA, ribosomal RNA.

has an important function in metabolic homeostasis (Kubota *et al*, 2004; Lin *et al*, 2004). As CRTC2 and IRS2 co-localize in the ARC and PVH (data not shown), we analysed whether CRTC2 might also be involved in the transcriptional regulation of *Irs2* in the hypothalamus by using chromatin immunoprecipitation. CRTC2 occupies the CRE site of the *Irs2* promoter at high glucose levels and AICAR treatment prevented this association (Fig 4A), further confirming that CRTC2 activity is regulated by AMPK. Consequently, the addition of AICAR to neurons cultured in high glucose reduced *Irs2* mRNA expression levels (Fig 4B), presumably by removing CRTC2 from the *Irs2* promoter. Furthermore, we found that *Irs2* mRNA expression was increased in neurons cultured in concentrations more than 5 mM glucose (supplementary Fig S3 online), showing that hypothalamic *Irs2* mRNA expression is glucose dependent, as well as AMPK activity dependent.

To investigate whether CRTC2 is required for appropriate CRE gene expression, we used a *Crtc2* RNA interference (RNAi) adenovirus to reduce *Crtc2* mRNA expression by 72% in primary hypothalamic cultures (Fig 4C). This loss of *Crtc2* led to a 56% reduction in *Irs2* and a 60% reduction in corticotropin-releasing hormone (*Crh*) mRNA expression (*Crh* is also a CRE gene regulated by metabolic state in the hypothalamus (Brady *et al*, 1990)). By contrast, the CRE gene nucleobindin 2 precursor (*NucB2*, encoding Nesfatin 1), which is also suppressed by fasting (Oh *et al*, 2006), was unaffected (Fig 4C). Activation of AMPK by A769662 had no effect on *Crtc2* expression, but *Irs2* mRNA was significantly reduced (Fig 4D; in line with previous data using AICAR, Fig 4B). However, following *Crtc2* knockdown, AMPK activation no longer decreased *Irs2* expression (Fig 4D), indicating that CRTC2 is essential for mediating the effect of AMPK on *Irs2* mRNA expression. These data show that co-activation by hypothalamic CRTC2 is crucial in the regulation of a specific set of CRE genes involved in hypothalamic energy-sensing pathways.

DISCUSSION

Given the current obesity epidemic, our need to understand the neuronal processes regulating metabolic balance could not be

more pressing. We show for the first time, to our knowledge, that CRTC2 is expressed in neurons of several distinct CNS regions, and that its phosphorylation and subcellular localization in the hypothalamus are altered by glucose through the alteration of AMPK activity. Our data, therefore, reveal a new role for CRTC2 in the downstream molecular mechanisms of neuronal glucose sensing, and add significantly to our understanding of the link between nutrient sensing and gene regulation. Future experiments will need to investigate whether CRTC2-mediated changes in gene expression coincide with electrophysiological changes in response to alterations of extracellular glucose levels, or whether these are distinct processes occurring in different neurons and/or neuronal cell types.

Given the important role of hypothalamic AMPK in the regulation of energy homeostasis (Minokoshi *et al*, 2004), downstream neuronal AMPK targets are of crucial interest; hypothalamic CRTC2 is only the second such protein to be identified. Other kinases can regulate CRTC2 activity in the liver and pancreas (Koo *et al*, 2005; Dentin *et al*, 2007; Jansson *et al*, 2008); whether SIK1/2 or microtubule affinity-regulating kinase 2 (MARK2) regulate hypothalamic CRTC2 activity, in addition to AMPK, will need further investigation. However, in showing that CRTC2 mediates the transcriptional regulation of *Irs2* by AMPK, we have uncovered a new mechanism through which neuronal AMPK regulates transcription. A reduction of *Irs2* in low fuel states is consistent with AMPK-mediated suppression of hypothalamic catabolic pathways. In addition, CRTC2 might act to shut down the anorectic CRH by reducing *Crh* mRNA during fasting. Indeed, CRH has previously been shown to be suppressed by fasting specifically in the PVH (Brady *et al*, 1990), and CREB has a major role in the transcriptional regulation of *Crh* (Itoi *et al*, 1996). We add that the regulation of *Crh* mRNA requires CREB co-activation by CRTC2, presumably through AMPK-mediated phosphorylation of CRTC2. Taken together, these data show that CRTC2 has an important role in linking nutrient cues with hypothalamic CRE gene expression.

As changes in CRTC2 phosphorylation and subcellular distribution in response to fasting are not uniform across the hypothalamus, neuronal subpopulation-specific regulation of CRTC2 might be well positioned to activate distinct transcriptional programmes in different cell types during different metabolic states. Further specificity might be imparted by the control of a specific set of CREB target genes by hypothalamic CRTC2; we show that not all hypothalamic CRE genes regulated by feeding and fasting are also CRTC2 co-activation dependent. More generally, CNS CRTC2 co-activation might mediate the differential effects of a range of neuronal signals on specific CREB target gene expression.

The importance of the CRTC family of CREB co-activators in metabolic regulation was recently demonstrated using *Crtc1*-deficient mice showing that CRTC1 is involved in the maintenance of energy homeostasis and fertility (Altarejos *et al*, 2008). We hypothesize that hypothalamic CRTC2 and CRTC1 have divergent physiological functions via different transcriptional pathways, as CRTC2 and CRTC1 have overlapping but distinct patterns of expression (Fig 1; Altarejos *et al*, 2008). In addition, co-activation of CREB through CRTC2 was not able to compensate for the loss of CRTC1 in *Crtc1*-deficient mice, suggesting separate transcriptional and physiological roles for the two CREB co-activators.

Our data firmly place CRTC2 as a new component on the ever increasing map of neuronal signalling pathways involved in sensing and regulating nutrient state. Indeed, we have shown that CRTC2 is crucial at the interface of glucose sensing and CRE gene transcription, and future experiments will investigate the physiological roles of CRTC2 in specific areas and subpopulations of the hypothalamus.

METHODS

Mice. Mice were maintained on a 12-h light/dark cycle with free access to water and mouse chow (EURodent Diet 22%, LabDiet, Richmond, IN, USA). Studies were carried out in accordance with the UK Animals (Scientific Procedures) Act and with the approval of the local ethical committee.

Immunohistochemistry and western blot. Immunohistochemistry was carried out as described previously (Liu *et al*, 2003) using the rabbit-anti-TORC2-1cKSCN (1:5,000, specificity confirmed by Bittinger *et al*, 2004 using RNAi approaches).

For western blots, hypothalamus and liver from fed, 24 h fasted and 24 h fasted mice 15 min after intraperitoneal glucose (0.1 mg/kg) or saline injection were homogenized in standard RIPA buffer with protease and phosphatase inhibitors (Roche Diagnostics GmBH, Mannheim, Germany). For *ex vivo* incubations, hypothalami were incubated in HEPES-buffered saline (NaCl 130 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM and HEPES 10 mM, at pH 7.4), supplemented with indicated glucose, AICAR (0.5 mM; Toronto Research Chemicals, North York, ON, Canada), A769662 (10 μM; University of Dundee, UK) or compoundC (10 μM; Merck Chemicals Ltd, Nottingham, UK) for 45 min at 37 °C with 5% CO_2. Standard SDS–PAGE on 6% Tris–HCl gels was carried out and membranes were incubated at 1:5,000 in rabbit-anti-CRTC2 (EMD Biosciences, Gibbstown, NJ, USA; specificity shown using RNAi approaches; Screaton *et al*, 2004), 1:1,200 rabbit anti-phospho-acetyl-CoA carboxylase (Ser 79), 1:200 anti-phospho-Ser 171-CRTC2, 1:1,000 anti-heat-shock protein 90 and 1:2,000 anti-phospho-AMPK-α (Thr 172; all obtained from Cell Signaling Technologies, Danvers, MA, USA).

Chromatin immunoprecipitation assays. Mouse hypothalamic blocks were incubated in HBS containing 15 mM glucose, with and without AICAR (0.5 mM) for 90 min at 37 °C with 5% CO_2 and processed as described by Da Silva Xavier *et al* (2004). See supplementary information online.

Lentiviral generation. A lenti-synapsin–eGFP construct (Liu *et al*, 2006) was digested with *Nhe*I, and *Not*I, and CRTC2–eGFP chimeric cDNA (Bittinger *et al*, 2004) was inserted. Lentiviral preparation and concentration were as described previously (Coleman *et al*, 2003).

Primary hypothalamic rat culture. Isolation of primary rat hypothalamic cultures was carried out as described previously (Mountjoy *et al*, 2007). Synapsin-CRTC2–eGFP lentivirus was added for 24 h, 24 h after plating of primary hypothalamic cultures. See supplementary information online.

Quantitative RT–PCR and RNAi. Five-day-old primary hypothalamic rat cultures were incubated in HBS containing 15 mM glucose with AICAR (0.5 mM for 4 h) and prepared for TaqMan PCR. See supplementary information online.

Three-day-old primary hypothalamic cultures were incubated with control *Gfp*–RNAi or *Crtc2*–RNAi adenovirus (1×10^6 pfu/ml; for details see supplementary information online) for 24 h. On day 5, cultures were incubated in Neurobasal with or without

A769662 (5 μM for 4 h) and prepared for TaqMan PCR. See supplementary information online.

Statistical analysis. Data were analysed in GraphPad Prism using one-way anova with Tukey's *post hoc* test in Figs 3A,C and 4D, and Student's *t*-test in Fig 4B,C.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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