The ketone body β -hydroxybutyric acid influences agouti-related peptide expression via AMP-activated protein kinase in hypothalamic GT1-7 cells

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Abstract

β -Hydroxybutyric acid (BHBA) acts in the brain to influence feeding behaviour, but the underlying molecular mechanisms are unclear. GT1-7 hypothalamic cells expressing orexigenic agouti-related peptide (AGRP) were used to study the AMP-activated protein kinase (AMPK) pathway known to integrate dietary and hormonal signals for food intake regulation. In a 25 mM glucose culture medium, BHBA increased intracellular calcium concentrations and the expression of monocarboxylate transporter 1 (MCT1 (SLC16A1)). Phosphorylation of AMPK- α (PRKAA1 and PRKAA2) at Thr¹⁷² was diminished after 2 h but increased after 4 h. Its downstream target, the mammalian target of rapamycin, was increasingly phosphorylated on Ser²⁴⁴⁸ after 2 h but not changed after 4 h of BHBA treatment. After 4 h, BHBA

treatment also increased *Agrp* mRNA expression. This increase was prevented by preincubation with the AMPK inhibitor Compound C. The inhibition of MCT1 activity by *p*-hydroxymercuribenzoate suppressed BHBA-stimulated AMPK phosphorylation but did not prevent BHBA-induced *Agrp* mRNA expression. This finding demonstrates that BHBA triggers the AMPK pathway resulting in orexigenic signalling under 25 mM glucose culture conditions. Under conditions of 5.5 mM glucose, however, BHBA marginally increased intracellular calcium but significantly decreased AMPK phosphorylation and *Agrp* mRNA expression, demonstrating that under physiological conditions BHBA reduces central orexigenic signalling.

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Introduction

As an important intermediate of amino and fatty acid catabolism, D- β -hydroxybutyric acid (BHBA), like glucose, can be used by the brain to provide energy particularly for suckling newborns (Hawkins *et al.* 1971). BHBA may access the brain by crossing the blood–brain barrier as well as entering hypothalamic neurons via the monocarboxylate transporter 1 (MCT1 (SLC16A1); Tildon & Roeder 1988, Ainscow *et al.* 2002, Morris & Felmlee 2008).

The impact of BHBA on satiety has recently been summarised (Laeger *et al.* 2010). In contrast to diabetic hyperketonaemia, which is usually associated with hyperphagia (Goodman 1987, Friedman & Ramirez 1994, Toyonaga *et al.* 2002), i.c.v. application of BHBA rather diminishes food intake (Sakata *et al.* 1982, Arase *et al.* 1988). Based on these findings, it has recently been suggested that BHBA may also be involved in the regulation of hypophagic responses (Laeger *et al.* 2010); however, the underlying molecular mechanisms are still not resolved.

In the hypothalamus, one of the central regulators of food intake and energy homeostasis, the AMP-activated protein

kinase (AMPK (PRKAA2)) pathway is activated in response to an increase in the AMP:ATP ratio and integrates extracellular hormonal and nutrient signals (Hardie *et al.* 1999, Minokoshi *et al.* 2004, Xue & Kahn 2006, Kola 2008). Activation of AMPK (phosphorylation of Thr¹⁷² at the α subunit) leads to an increased expression of orexigenic neuropeptides (NP) such as NPY and agouti-related peptide (AGRP) and a decreased expression of anorexigenic NP such as pro-opiomelanocortin (POMC; Kola 2008). The inhibition of AMPK by Compound C (Cpd C) prevents these expression changes (Iwasaki *et al.* 2007, Shimizu *et al.* 2008).

Incubation of hippocampal neurons with BHBA but without glucose maintains the cellular ATP level (Arakawa *et al.* 1991), suggesting that BHBA may modulate hypothalamic AMPK activity. Upstream kinases of AMPK are the tumour suppressor LKB1 (STK11) kinase and the Ca²⁺/calmodulin-dependent protein kinase I (CAMKI (CAMK1)), the latter dependent on intracellular Ca²⁺ release (Hawley *et al.* 2003, Hurley *et al.* 2005, Witters *et al.* 2006). Once phosphorylated at Thr¹⁷², AMPK may inhibit its downstream target mammalian target of rapamycin (mTOR), which in turn integrates amino acid and insulin signalling and thereby

regulating transcriptional activity (Bolster *et al.* 2002, Kimura *et al.* 2003).

The aim of this study was to investigate the effect of BHBA on hypothalamic orexigenic signalling. GT1-7 cells have been successively used to investigate orexigenic signalling (Yang *et al.* 2005, Li *et al.* 2006, Morrison *et al.* 2007, Hayes *et al.* 2011). Using this model, we could show that BHBA increases intracellular Ca^{2+} release and modulates the phosphorylation of AMPK and mTOR in a time- and glucose-dependent manner. Changes in AMPK phosphorylation were at least in part mediated by MCT1 and accompanied with an altered *Agrp* mRNA expression.

Materials and Methods

Cell culture of hypothalamic GT1-7 cells

Murine-immortalised GT1-7 hypothalamic cells were kindly provided by Dr Franz Schäfer, University of Heidelberg, Germany. GT1-7 cells were developed by Mellon *et al.* (1990) from a tumour obtained from a transgenic mouse in which the gonadotrophin-releasing hormone (GNRH (GNRH1)) promoter sequence drives the expression of SV40 T antigen (Tg(Lhb-TAg)#Plm) but does not alter the expression of *Agrp*. GT1-7 cells were seeded on 6 cm culture plates and maintained in DMEM (with 4.5 g/l (25 mM) or 1 g/l (5.5 mM) glucose, L-glutamine, sodium pyruvate and 3.7 g/l NaHCO_3) supplemented with 10% (v/v) FCS and 1% (v/v) penicillin–streptomycin solution (PAN Biotech GmbH, Aidenbach, Germany) at 37 °C in 5% CO_2 atmosphere. The culture medium was changed twice a week and cultures were passaged at 80% confluence after trypsinisation (0.05%, w/v). Changes in cell morphology and growing conditions were carefully monitored using an inverted microscope. To reduce mitogenic effects, GT1-7 cells were precultured in 0.1% (v/v) FCS/DMEM for 24 h. BHBA was added to the culture medium either alone or 5 min after preincubation with the MCT1 inhibitor *p*-hydroxymercuribenzoate (pHMB; Sigma–Aldrich; dissolved to 0.5 mM stock solution in H_2O) to block the transfer (Deuticke 1982) or the AMPK inhibitor Cpd C (Zhou *et al.* 2001; Cpd C; Merck; dissolved to 10 mM in dimethyl sulfoxide (DMSO)) in concentrations and times as indicated. Control incubations were performed using H_2O or DMSO as described earlier. The dilution of the cell culture medium by pHMB was not higher than 2:1000 and by Cpd C was not higher than 1:1000.

Western immunoblot analysis

The cell culture medium was withdrawn and GT1-7 cells were solubilised in 80 µl lysis buffer containing 50 mM Tris (pH 7.8), 1 mM EDTA (Pharmacia Biotech), 10 mM NaF (Fisher Scientific, Schwerte, Germany), 1% (v/v) Igepal CA-630 (Sigma–Aldrich), 0.1% (v/v) Triton X-100 (Pharmacia Biotech), 0.5% (v/v) deoxycholic acid (DOC; Sigma), 0.1% (w/v) sodium dodecyl sulfate (SDS; USB

Corporation, Cleveland, OH, USA) and Roche Phospho-Stop tablets (one tablet/10 ml buffer; Roche) on ice. After centrifugation (4 °C, 10 min, 15 700 g), the supernatant was collected and the protein content was quantified using CBQCA Protein Quantitation Kit C-6667 (Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's protocol. For SDS gel electrophoresis, 25 µg sample solutions were diluted to the same amount with SDS sample buffer containing 62.5 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% (v/v) bromophenol blue. The samples were boiled for 5 min and electrophoresed through a 12% (w/w) SDS polyacrylamide gel. Proteins were then transferred to nitrocellulose membranes. Membranes were blocked with 3% (w/v) BSA in TBST buffer (20 mM Tris/HCl, 0.9% (w/v) NaCl, 0.05% (v/v) Tween-20; pH 7.6) and incubated with the primary rabbit antibodies against AMPK- α , phospho-AMPK- α Thr¹⁷², mTOR, phospho-mTOR Ser²⁴⁴⁸ (each from Cell Signaling Technology, Inc., Danvers, MA, USA), chicken-anti-MCT1 (Millipore Corporation, Billerica, MA, USA) or after stripping and re-probing with mouse-anti- β -tubulin antibody (Covance, Inc., Emeryville, CA, USA) at 4 °C for 12 h (each 1:1000 dilution). Membranes were then washed with TBST, incubated with the corresponding HRP-conjugated anti-mouse, anti-rabbit or anti-chicken IgG (each 1:3000, 60 min at room temperature). After washing three times with TBST, the membranes were transferred to ECL solution (Pierce ECL Western Blotting Substrate; Thermo Scientific, Rockford, IL, USA) for 1 min and exposed to Clear Blue X-Ray Film (CL-XPosure Film; Thermo Scientific) for 0.5–2 min. Bands were scanned and quantified using ImageJ 1.42q (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The level of phosphorylation of an assayed enzyme was calculated relative to the total amount of this enzyme. MCT1 levels were assessed relative to β -tubulin levels.

RNA extraction, reverse transcriptase and quantitative real-time PCR analysis

Total RNA of GT1-7 cells was extracted using a NucleoSpin RNA XS kit (Macherey-Nagel GmbH, Düren, Germany), subsequently quantified by measuring the absorbance at 260 and 280 nm (NanoPhotometer; Implen, Munich, Germany) and stored at –80 °C until analysis. The quality of extracted RNA was judged by northern analysis and inspection of the 28S and 18S rRNA bands. The extracted RNA (0.5 µg) was subjected to a RT-PCR using Transcriptor High Fidelity cDNA Synthesis Kit (Roche) with anchored oligo(dT)₁₈ primer (2.5 µM) at 65 °C for 10 min to ensure denaturation of RNA secondary structures. Subsequently, Protector RNase Inhibitor (20 U, 1 mM), deoxynucleotide mix (1 mM), dithiothreitol (5 mM) and Transcriptor High Fidelity Reverse Transcriptase (10 U) were added and RT was performed using MgCl_2 (8 mM) for 30 min at 50 °C. The transcriptase was subsequently inactivated by heating at 85 °C for 5 min.

The expression level for *Agrp* was determined relative to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) using the following primers: *Agrp*, sense 5'-TGA CTG CAA TGT TGC TGA GTT GTG-3' and anti-sense 5'-TAG GTG CGA CTA CAG AGG TTC GTG-3' (391 bp fragment); *Gapdh*, sense 5'-AAC TTT GGC ATT GTG GAA GG-3' and anti-sense 5'-ACA CAT TGG GGG TAG GAA CA-3' (223 bp). Primer searches were carried out against previously identified genes using the Basic Local Alignment Search Tool (BLAST) program (<http://www.ncbi.nlm.nih.gov/BLAST/>) of the GenBank database (National Center for Biotechnology Information, Washington, DC, USA).

For quantification of *Agrp* and *Gapdh* transcript levels, real-time PCR was performed using LightCycler FastStart DNA Master^{plus} SYBR Green I (Roche) under each optimised condition of annealing at 59 °C, according to the manufacturer's protocol. An aliquot of the PCR product was electrophoresed on a 3% (w/v) agarose gel and visualised by ethidium bromide staining to confirm purity and size. To analyse the relative changes in gene expression, the $2^{-\Delta\Delta C_T}$ method was used (Livak & Schmittgen 2001).

Ca²⁺ imaging and confocal laser scanning microscopy

Hypothalamic GT1-7 cells were washed with PBS (50 mM Na₂HPO₄, 0.85% (w/v) NaCl, 0.25 (w/v) KCl; pH 7.4), incubated with the cell-permeant acetoxymethyl ester Fluo-4 at 3 µM and 0.07% (v/v) Pluronic F-127 (Molecular Probes) in 0.1% (v/v) FCS/DMEM for 45 min at 37 °C in 5% CO₂ atmosphere. After loading the GT1-7 cells were washed with PBS and incubated in indicator-free 0.1% (v/v) FCS/DMEM for further 30 min. Afterwards, GT1-7 cells were incubated in a Microscope Incubator (XL-3-LSM; Pecon, Erbach, Germany) at 37 °C in 5% CO₂ atmosphere and analysed for fluorescence at a confocal laser scanning microscope (LSM 5 Pascal, Axiovert 200M, Zeiss, Jena, Germany) and Zen 2007 Software (Carl Zeiss) before and after BHBA stimulation in concentrations as indicated. For the measurement of green fluorescence, 488 nm line of Argon laser (30 mW) and a 505–530 nm narrowband filter were used. Stacks of images (ten images per second, 256 × 256 pixel) were recorded using a 20× lens and the provided software. Analysis 3.2 (SIS, Münster, Germany) was used to create regions of interest (ROIs) exactly enclosing the GT1-7 cells and to measure the mean intensity in ROIs according to Pöhlend *et al.* (2008).

Statistical analysis

Data were statistically analysed by Systat version 10 using the *F*-test (see figure legends) and a two-way (BHBA concentration and incubation time) ANOVA (main effects: BHBA treatment and incubation time) followed by comparison of means using the Tukey's test. A statistically significant difference was considered when $P \leq 0.05$.

Results

Effect of BHBA on AMPK and mTOR signalling in hypothalamic GT1-7 cells

Based on the previous studies showing that neurons incubated for 2 h with BHBA revealed increased ATP levels (Arakawa *et al.* 1991), we first cultured hypothalamic GT1-7 cells in 25 mM glucose-containing medium as described earlier (Li *et al.* 2006, Coyral-Castel *et al.* 2008, Hayes *et al.* 2011) and investigated whether BHBA modulates AMPK phosphorylation. Incubation with 1.5, 3 and 6 mM BHBA for 2 and 4 h revealed that treatment with 6 mM BHBA resulted in a 21% decrease of AMPK- α phosphorylation after 2 h, whereas after 4 h AMPK phosphorylation significantly increased by 132% when compared with $t=0$ (Fig. 1A; for each time $n=4-8$). After 1, 6 and 8 h, AMPK- α phosphorylation was not changed when compared with $t=0$ (data not shown). In addition, GT1-7 cells treated with only 1.5 and 3 mM BHBA revealed a significant decrease in AMPK phosphorylation after 2 h, but after 4 h AMPK phosphorylation did not differ in comparison with $t=0$ (Fig. 1A). In addition, the loading control with β -tubulin revealed no differences between time points.

As BHBA is primarily used by the brain when glucose is reduced (Lindsay & Setchell 1976, Arakawa *et al.* 1991), we next studied the effect of BHBA on AMPK- α phosphorylation in a lower (5.5 mM) glucose-containing medium, which is more physiological (see also section on BHBA and glucose concentrations). In contrast to the results described in Fig. 1A, incubation with 1.5 and 3 mM BHBA for 2 and 4 h, respectively, revealed that treatment with 3 mM BHBA resulted in a 42% decrease of AMPK- α phosphorylation after 4 h, whereas 1.5 mM BHBA had no effect on AMPK- α phosphorylation (Fig. 1B; for each time $n=5-11$). The use of 6 mM BHBA, however, resulted in a 35% decrease of AMPK- α phosphorylation after 4 h. These results demonstrate that 6 mM BHBA induced a significant ($P < 0.05$) alteration of AMPK- α phosphorylation after 2 and 4 h. Hence, we used 6 mM BHBA in the following experiments.

In 25 mM glucose-containing medium, phosphorylation (inhibition) of the downstream target of AMPK, mTOR, at Ser²⁴⁴⁸ significantly increased to 129% after 2 h when incubated with 6 mM BHBA (Fig. 2A; for each time $n=6-8$). After 4 h, mTOR phosphorylation did not differ compared with $t=0$ ($P=0.1$). In contrast, in 5.5 mM glucose-containing medium, phosphorylation of mTOR did not change upon BHBA stimulus (Fig. 2C; for each time $n=5$).

Role of MCT1 for BHBA-induced AMPK phosphorylation

The uptake of BHBA into cells might be mediated by the MCT1 system and its presence has recently been confirmed in hypothalamic neurons (Ainscow *et al.* 2002, Morris & Felmlee 2008). Thus, we examined whether BHBA influences the expression of MCT1. We found that in 25 mM glucose-

Figure 1 Effect of BHBA on AMPK in hypothalamic GT1-7 cells. GT1-7 cells maintained in (A) 25 mM or (B) 5.5 mM glucose-containing medium were stimulated with or without BHBA in concentrations as indicated for 2 and 4 h. Cell lysates were examined by western blot analysis with AMPK- α and phospho-specific AMPK- α Thr¹⁷² antibodies. The densitometric ratio of pAMPK/AMPK was calculated against the ratio of untreated controls and is presented as mean $\pm$ S.D. (for each time $n_{25}=4-8$ and $n_{5.5}=5-11$). The β -tubulin blot served as loading control. Mean values with different lowercase letters differ with $P<0.05$ (Tukey's test; ANOVA: $P<0.05$, $F_A=3.8$, $F_B=2.0$). A representative blot is shown below the graphs.

containing medium, 6 mM BHBA significantly increased MCT1 expression after 2–4 h, suggesting an elevated intracellular uptake of BHBA (Fig. 2B; for each time $n=6-8$). Under conditions of 5.5 mM glucose, however, BHBA did not alter *Mct1* expression (Fig. 2D; for each time $n=5$).

To explore whether AMPK phosphorylation depends on MCT1, GT1-7 cells grown in 25 mM glucose were pre-incubated with the MCT1 inhibitor pHMB (1 μ M) and subsequently stimulated with BHBA (6 mM) for 4 h. Figure 3A shows that inhibition of MCT1 significantly reduced (but not fully suppressed) BHBA-induced phosphorylation of AMPK- α (Fig. 3A; for each time $n=5-6$). Interestingly, pHMB alone also stimulated AMPK- α phosphorylation (Fig. 3A).

The effect of BHBA on AMPK phosphorylation was abolished when cells were preincubated with the specific AMPK inhibitor Cpd C (10 μ M; Fig. 3A).

In low-glucose medium (5.5 mM), diminished AMPK- α phosphorylation elicited by BHBA was prevented by either blockade of MCT1 or AMPK respectively (Fig. 3B; for each time $n=5$). These results suggest that intracellular BHBA uptake mediated by MCT1 may trigger AMPK signalling.

BHBA increases Ca^{2+} efflux in GT1-7 cells

As the Ca^{2+} /CAMK and the Ca^{2+} /CAMK kinase (CAMKK (CAMKK2)) act as upstream signals for the phosphorylation of AMPK- α at Thr¹⁷² (Hawley *et al.* 2005, Hurley *et al.* 2005), we evaluated the effect of BHBA on intracellular $[\text{Ca}^{2+}]_i$. Our results reveal that treatment with 6 mM BHBA in 25 mM glucose led to a rapid $[\text{Ca}^{2+}]_i$ increase, as demonstrated by the increased intracellular Fluo-4 fluorescence (Fig. 4A and C). The onset of $[\text{Ca}^{2+}]_i$ increase with 2 mM BHBA occurred at a later time, to a lower extent, and was sustained for a shorter period of time compared with the 6 mM BHBA stimulus (Fig. 4A; $n=5$). Under conditions of 5.5 mM glucose, however, we only found a marginal $[\text{Ca}^{2+}]_i$ increase after treatment with 6 mM but not with 2 mM BHBA (Fig. 4B; $n=4$).

Regulation of *Agrp* mRNA expression by BHBA under different glucose concentration

AMPK is known to control food intake by regulating the expression of orexigenic and anorexigenic NP in the hypothalamus (Minokoshi *et al.* 2004, Shimizu *et al.* 2008). Therefore, we next investigated the impact of BHBA on orexigenic *Agrp* expression. In 25 mM glucose, we found that transcripts of orexigenic *Agrp* relative to *Gapdh* were significantly increased after 4 and 7 h incubation with 6 mM BHBA (Fig. 5A; for each time $n=5-12$). As AMPK phosphorylation and *Agrp* expression were highest after 4 h, subsequent blocking experiments were performed for this time only. Blockade of AMPK by Cpd C (10 μ M) significantly decreased BHBA-induced *Agrp* expression (Fig. 5A), indicating that BHBA elicits orexigenic signalling specifically via AMPK. Furthermore, treatment with the MCT1 inhibitor pHMB (1 μ M) did not affect BHBA-mediated *Agrp* expression (Fig. 5A), suggesting that BHBA may either enter the cells by bypassing MCT1 or it binds to a BHBA membrane receptor without entering the cells. Regardless, our results demonstrate that BHBA activates the AMPK $\rightarrow$ AGRP pathway under conditions of 25 mM glucose.

By contrast, cells incubated in 5.5 mM glucose responded with decreased (42–46%) *Agrp* mRNA expression after 4 and 7 h of BHBA treatment (Fig. 5B; for each time $n=7-13$), indicating that the ratio between BHBA and glucose determines feed intake-related signalling. This reduction could be prevented by pre-incubation with Cpd C, indicating that BHBA diminishes orexigenic signalling via AMPK under

Figure 2 Effect of BHBA on mTOR and MCT1 in hypothalamic GT1-7 cells. GT1-7 cells cultured in 25 mM (A and B) or 5.5 mM (C and D) glucose were stimulated with or without BHBA (6 mM). Cell lysates were probed with (A and C) antibodies against Ser²⁴⁴⁸ mTOR and total mTOR or with (B and D) antibodies against MCT1 and β-tubulin. The densitometric ratio of pmTOR/mTOR and MCT1/β-tubulin, respectively, was calculated. The ratio between BHBA-treated and -untreated controls is presented as mean ± s.d. (for each time $n_{25} = 6-8$ and $n_{5.5} = 5$). Mean values with different lowercase letters differ with $P < 0.05$ (Tukey's test; ANOVA: $P < 0.05$, $F_A = 3.4$, $F_B = 6.5$, $F_C = 0.4$, $F_D = 0.5$). A representative blot is shown below the graphs.

low-glucose conditions. In contrast to the result described for Fig. 5A, pHMB also prevented the reduction of BHBA-induced *Agrp* mRNA expression (Fig. 5B). Again, these results demonstrate that BHBA is a trigger for the AMPK → AGRP pathway but whether it increases or decreases orexigenic signalling depends on glucose concentration.

Discussion

BHBA and glucose concentrations

Under normoglycaemic conditions, ketone bodies may occur in substantial concentrations (~ 0.5 mM) in humans (Laffel 1999). In ruminants, BHBA plasma concentration is even higher during and after food intake due to oxidation of ruminal butyrate in ruminal epithelial cells (van Soest 1994, Duske *et al.* 2009). During times of high energy demands, such as pregnancy, lactation, exercise and fasting, glucose becomes less available and energy stores are mobilised from body fat, and ketone bodies become an important respiratory fuel (Hawkins & Biebuyck 1979). Thus, fasting may increase blood BHBA levels by 5–8 mM (Lindsay & Setchell 1976, Cahill & Veech 2003). In adult humans with diabetic ketoacidosis, circulating BHBA may even reach 3–14 mM while glucose is about 26 mM (Friedman & Ramirez 1994, Sheikh-Ali *et al.* 2008). In humans as well as in ruminants, circulating BHBA is also elevated in response to chronic consumption of a ketogenic diet (Brehm *et al.* 2003, Boden *et al.* 2005, Duske *et al.* 2009). Because of the relatively high fat and low carbohydrate content in rodent milk (mouse: 17–30% fat, 1–2% carbohydrates), BHBA is touted as one of the most important energy sources of suckling rodents (Hawkins *et al.* 1971, Görs *et al.* 2009). Thus, it seems that among species (gastric, hind-gut or reticulorumen fermenters) and their developmental stages, a different hypothalamic sensitivity to BHBA exists. The similarity between all species, however, is that the mammalian brain can use BHBA instead of glucose primarily under hypoglycaemic conditions and that the extent of usage depends on the physiological state (Owen *et al.* 1967, Lindsay & Setchell 1976, Laeger *et al.* 2010).

The extracellular glucose concentration in the brain is about 25% of the circulating glucose and varies between different brain areas and physiological states (Silver & Erecinska 1994, de Vries *et al.* 2003). Under hyperglycaemic conditions, the extracellular glucose level in the brain may reach > 10.5 mM (Silver & Erecinska 1994), while it is about 1.4 mM in fed rats and 0.7 mM in overnight-fasted rats (de Vries *et al.* 2003). Thus, the 25 mM glucose concentration used in our *in vitro* study seems to correspond to about 100 mM plasma glucose, which appears to be pathophysiological. This raises the question about the physiological relevance of our cell culture approach. However, because of the different metabolic rate and the diffusion-controlled availability of nutrients, for example, the glucose concentration determined *in vivo* could

Figure 3 Effect of BHBA on AMPK in hypothalamic GT1-7 cells. GT1-7 cells kept in (A) 25 mM or (B) 5.5 mM glucose were preincubated with either 1 μ M of the MCT1 inhibitor pHMB or with 10 μ M of the AMPK inhibitor Cpd C, each for 5 min followed by the incubation with or without 6 mM BHBA for 4 h. Cell lysates were examined by western blot analysis with phospho-specific AMPK- α Thr¹⁷² and normalised against total AMPK- α . The densitometric ratio of pAMPK/AMPK was determined. Data are calculated as fold change of untreated controls and are presented as mean $\pm$ s.d. (for each time $n_{25} = 5-6$ and $n_{5.5} = 5$). Mean values with different lowercase letters differ with $P < 0.05$ (Tukey's test; ANOVA: $P < 0.05$, $F_A = 4.5$, $F_B = 3.2$). A representative blot is shown below the graphs.

not be simply applied to the cell culture. GT1-7 cells were generated via tumourigenesis (Mellon *et al.* 1990) and possess a approximately ten times higher glucose uptake and glycolysis rate compared with non-oncogenic cells (Dang & Semenza 1999). Considering this fact, 25 and 5.5 mM glucose used in cell culture would correspond to 2.5 and 0.55 mM of extracellular glucose available for non-oncogenic cells, which in turn reflects the glucose concentration as measured in the brain (Silver & Erecinska 1994). Moreover, a number of recent investigations (Lee *et al.* 2005, Li *et al.* 2006, Coyral-Castel *et al.* 2008, Wen *et al.* 2010, Hayes *et al.* 2011) also used glucose concentrations between 5.5 and 25 mM for

GT1-7 cells, making our results comparable with these studies, while the use of < 5.5 mM glucose led to cell death (Honegger *et al.* 2002).

One might assume that differences also exist between circulating and extracellular BHBA concentrations in the brain, but currently there are no data about it. However, further studies in whole animals are required to determine extracellular BHBA brain concentrations under various physiological conditions. Those data would help to examine, in a physiologically relevant range, which concentrations in the brain are to be adjusted in *in vivo* experiments to provoke alterations in feed intake signalling and in feed intake. When, however, the glucose/BHBA ratio (see below) instead of the absolute metabolite concentration is considered, our cell culture model mimics the *in vivo* situation and allows the investigation on principle signalling pathways induced by BHBA.

BHBA uptake via MCT1

Investigations on dissociated brain cells of rats revealed two forms of neuronal BHBA uptake: diffusion and carrier-mediated transport systems (Tildon & Roeder 1988). Members of the H⁺-coupled MCT (SLC16A1) family (MCT1, MCT2 (SLC16A7) and MCT4; Morris & Felmlee 2008) and members of the solute carrier (SLC) group, the sodium-coupled MCT1 (SMCT1; SLC5A8), have been identified as neuron-specific transporters for BHBA (Martin *et al.* 2006). MCT1 is abundantly distributed throughout the brain with high levels in the hypothalamic region and adjusted to the local glucose transporter GLUT1 (SLC2A1; Maurer *et al.* 2004). In our study, we demonstrated significantly increased MCT1 expression by 4 h of BHBA incubation in 25 mM glucose-containing medium. However, to date, detailed mechanisms of transcriptional regulation of MCT1 expression by BHBA are not known. In addition, inhibition of MCT1 via 1 μ M pHMB suppresses (but not fully prevents) BHBA-induced AMPK phosphorylation in 25 mM glucose and AMPK dephosphorylation in 5.5 mM glucose respectively. Higher concentrations of pHMB (1 mM), however, did not lead to a further MCT1 blocking effect but rather to cell death (data not provided). Furthermore, we have shown that in 25 mM glucose-containing medium, pHMB stimulates AMPK phosphorylation in the absence of BHBA, because the entrance of other metabolites (fuels) transported by MCT1, such as lactate, pyruvate, acetate and acetoacetate, is also blocked, which would thus lead to an increase in AMPK phosphorylation. Moreover, although pHMB suppresses AMPK phosphorylation in the presence of BHBA, it did not prevent increases in *Agrp* expression in 25 mM glucose. These results suggest that BHBA triggers *Agrp* expression not exclusively via MCT1. For example, BHBA could affect intracellular signalling without entering the cell by binding to the membrane G protein-coupled receptor 109A (GPR109A (NIACR1)), which has been shown to be expressed in several portions of the brain in multiple species (Tigtemeyer *et al.* 2011).

Figure 4 BHBA increases $[Ca^{2+}]_i$ in GT1-7 cells cultured in (A) 25 and (B) 5.5 mM glucose-containing DMEM. After stimulation with BHBA (2 and 6 mM), changes of the intracellular Fluo-4 fluorescence were recorded by confocal laser-scanning microscopy (C, e.g. in 25 mM glucose). Control incubations were performed with water (D). The fluorescence intensity after application of BHBA or water (indicated by an arrow), respectively, was recorded in five (25 mM glucose) and four (5.5 mM glucose) independent experiments each with seven to ten cells. Data are presented as mean $\pm$ s.d. Significant differences ($P < 0.05$; ANOVA) in 25 mM glucose between the control and the 6 mM BHBA group occur > 50 s after application and between the control and the 2 mM BHBA group > 64 and < 96 s. Significant differences in 5.5 mM glucose between the control and the 6 mM but not the 2 mM BHBA treatment occurred > 50 and < 100 s after application.

The latter assumption is supported by the finding that under conditions of 5.5 mM glucose, BHBA indeed reduced AMPK phosphorylation but did not alter MCT1 expression. Thus, under low-glucose conditions, cells seem to dispense for the regulation of MCT1 expression, enabling BHBA to enter the cells particularly via a non-MCT1 mediated pathway. In this case, supraphysiological BHBA is not required for an increase in MCT1 expression.

Effect of BHBA on AMPK signalling

We have demonstrated that in the presence of 25 mM glucose, BHBA increases MCT1 expression, which should support the uptake of BHBA into cells for altering AMPK activity. Our results demonstrate that treatment with 1.5, 3 and 6 mM BHBA led to a decline of AMPK- α phosphorylation after 2 h. This means that hypothalamic cells are capable of responding to BHBA by decreasing its AMPK phosphorylation, which is in accordance with elevated ATP levels found in primary neurons incubated for 2 h with 20 mM BHBA (in 10 mM glucose-containing medium; Arakawa *et al.* 1991). Unfortunately, the authors did not show how the ATP level changed after 4 h of BHBA incubation. However, this temporally raised ATP level might be the reason for the diminished AMPK- α phosphorylation as observed in our study 2 h after BHBA treatment. In contrast, after 4 h of BHBA incubation, AMPK phosphorylation was increased. The stimulation of the AMPK pathway might be caused by a reduced glucose uptake as prolonged exposure to BHBA leads to a reduced glucose uptake, as demonstrated for cardiomyocytes (Pelletier & Coderre 2007). Such a reduced glucose uptake (see also section on The glucose/BHBA ratio and food intake) potentially associated with reduced glucose oxidation would decrease the ATP level and thereby increase AMPK phosphorylation.

Studies performed in 5.5 mM glucose-containing medium, however, reveal that BHBA-induced AMPK- α phosphorylation is contrarily regulated compared with culture conditions in 25 mM glucose. This might be due to lowered activity of Ca^{2+} -dependent kinases (see below) located upstream of AMPK or due to the fact that BHBA under low-glucose conditions serves as a metabolite for ATP production and thus contributes to decreased AMPK phosphorylation.

Based on the fact that the loading control β -tubulin did not change between times, changes in the phosphorylation of AMPK- α is not the result of changes in total AMPK- α . This is strengthened by another study with GT1-7 cells, which demonstrated that high glucose supplementation (25 mM) suppresses AMPK- α phosphorylation without changes in the total AMPK- α levels (Lee *et al.* 2005).

Effect of BHBA on mTOR signalling

One of the downstream targets of AMPK, mTOR, is a highly conserved serine/threonine kinase, integrating nutrient and hormonal signals to control growth and development (Bolster *et al.* 2002, Kimura *et al.* 2003). In the arcuate nucleus of the hypothalamus, mTOR is localised in POMC and AGRP/NPY neurons that are involved in food intake regulation (Cota *et al.* 2006). Fasting induces a decrease in mTOR phosphorylation at Ser²⁴⁴⁸ (Cota *et al.* 2006). Our results demonstrate that BHBA increases phosphorylation of mTOR at Ser²⁴⁴⁸ after 2 h, which inversely corresponds to a decline of p-AMPK. Conversely, reduced mTOR phosphorylation

Figure 5 BHBA increases AMPK-mediated orexigenic *Agrp* expression in hypothalamic GT1-7 cells cultured in 25 mM glucose-containing medium (A). In contrast, BHBA decreases *Agrp* expression in hypothalamic GT1-7 cells cultured in 5.5 mM glucose-containing medium (B). GT1-7 cells were either stimulated with BHBA (6 mM) alone or preincubated with 1 μ M of the MCT1 inhibitor pHMB or with 10 μ M of the AMPK inhibitor Cpd C, respectively, followed by BHBA (6 mM) application. At times as indicated, transcript levels of *Agrp* and *Gapdh* were quantified by real-time PCR. 2^{-ΔΔC_T} method was used to analyse the relative gene expression. The graph represents means $\pm$ s.d. originating from three independent experiments (for each time $n_{25} = 5-12$ and $n_{5.5} = 7-13$). Mean values with different lowercase letters differ with $P < 0.05$ (Tukey's test; ANOVA: $P < 0.05$, $F_A = 6.3$, $F_B = 6.3$).

after 4 h BHBA treatment is associated with significantly raised AMPK phosphorylation in a culture medium containing 25 mM glucose. This inverse phosphorylation is in accordance with earlier findings in skeletal muscle cells and in C2C12 myoblast cells, showing that AMPK negatively controls mTOR signalling (Bolster *et al.* 2002, Du *et al.* 2007). Why under culture conditions of 5.5 mM glucose changes in mTOR phosphorylation (despite of observed p-AMPK changes) were not as pronounced as in 25 mM glucose is currently unknown.

Effect of BHBA on $[Ca^{2+}]_i$

Besides the activation of AMPK by LKB1-AMP, Ca^{2+} /CAMKK may activate its downstream target CAMKI that in turn also phosphorylates AMPK- α at

Thr¹⁷² (Witters *et al.* 2006). Therefore, we investigated the effect of BHBA on intracellular $[Ca^{2+}]_i$. Our results show that BHBA elicits a faster increase of $[Ca^{2+}]_i$ compared with AMPK phosphorylation, suggesting that BHBA triggers the AMPK pathway via Ca^{2+} -dependent signalling under conditions of 25 mM glucose. In the presence of 5.5 mM glucose, BHBA induces a only marginal $[Ca^{2+}]_i$ influx, indicating that Ca^{2+} -dependent kinases should not significantly phosphorylate AMPK under these conditions. On the other hand, a previous study revealed that $[Ca^{2+}]_i$ raised via AMPK activation in hypothalamic neurons (Kohno *et al.* 2008). However, further studies, e.g. using calcium-binding agents, are required to determine the association between $[Ca^{2+}]_i$ increase and AMPK signalling triggered by BHBA.

Effect of BHBA on *Agrp* mRNA expression

Hypothalamic AMPK inhibition reduces *Agrp* expression (Shimizu *et al.* 2008) and induces a reduction of food intake (Minokoshi *et al.* 2004, Xue & Kahn 2006). In contrast, increased activation of AMPK in the arcuate nucleus of the hypothalamus leads to an increased expression of *Agrp* (Shimizu *et al.* 2008). Consistent with these findings, we demonstrate that increased AMPK phosphorylation is accompanied with increased *Agrp* expression (25 mM glucose) and decreased AMPK phosphorylation is associated with decreased *Agrp* abundance (5.5 mM glucose). The alteration of *Agrp* expression is mediated via AMPK because Cpd C prevents BHBA-induced aberrant *Agrp* expression under both glucose conditions. Hence, our results demonstrate the existence of the BHBA $\rightarrow$ AMPK $\rightarrow$ AGRP pathway.

The glucose/BHBA ratio and food intake

Whether this pathway is activated or inhibited seems to depend on glucose concentration. In this study using tumorous cells, we were able to demonstrate that in a medium containing 25 mM glucose, 6 mM BHBA elicits increased expression of *Agrp*, whereas under culture conditions of 5.5 mM glucose, *Agrp* expression was significantly reduced. Thus, we investigated glucose/BHBA ratio of $\sim 4:1$ and $\sim 1:1$ respectively. The high glucose/BHBA ratio used in our study might be comparable with high plasma glucose concentrations (glucose/BHBA ratio of $\sim 4:1$) observed in patients with diabetic ketosis (Sheikh-Ali *et al.* 2008), a metabolic situation associated with hyperphagia (Toyonaga *et al.* 2002). Similarly, streptozotocin-treated rats are hyperglycaemic, ketoacidotic and hyperphagic (Goodman 1987, Friedman & Ramirez 1994), although this might also be due to insulin deficiency. Moreover, consumption of high-fat diets, which increase circulating glucose and BHBA concentrations, leads to increased meal size and greater energy intake in rodent and humans (Warwick 1996, Hu *et al.* 2004). Also, pregnant cows possessing higher plasma BHBA and glucose concentrations (glucose/BHBA ratio of $\sim 3:1$) due to feeding a ketogenic diet show the higher feed intake

compared with cows with lower plasma BHBA and glucose concentrations (Duske *et al.* 2009). Whether the increased feed intake in the situation of high glucose is due to or despite high BHBA remains to be investigated. However, the putative orexigenic characteristics of BHBA in the presence of high glucose could be explained by the fact that BHBA acts similar to 2-deoxyglucose as a glucose anti-metabolite, thereby inhibits glucose uptake and ATP production and may stimulate feed intake even at satiation (Minami *et al.* 1995).

BHBA treatment in the lower (5.5 mM) glucose (glucose/BHBA ratio of ~1:1)-containing medium, on the other hand, reflects the metabolic situation present in normo- or hypoglycaemic animals receiving BHBA infusions. Thus, for example, i.c.v. application of BHBA suppresses food intake in the long term in Wistar King A (Sakata *et al.* 1982) and Osborne–Mendel rats (Arase *et al.* 1988). However, in the study of Davis *et al.* (1981), only a numeric reduction of food intake upon i.c.v. BHBA infusion could be observed when compared with saline-treated controls. Women eating a very low-carbohydrate diet reveal significantly increased BHBA and normal glucose levels whereby food intake spontaneously decreased (Brehm *et al.* 2003). Additionally, early lactating cows with reduced glucose but elevated BHBA levels (glucose/BHBA ratio of 1:1) ate less than non-ketotic cows with a 4:1 ratio (Hammon *et al.* 2009). Thus, the majority of these studies indicate that BHBA exerts an anorexigenic role in the brain (in normo- or hypoglycaemia), and our results obtained from the studies in 5.5 mM glucose (glucose/BHBA ratio of 1:1) demonstrates that this may be due to reduced AMPK phosphorylation and reduced *Agrp* expression. The anorexic BHBA trait might be caused by the observation that BHBA serves under normal or lowered glucose condition as metabolite for ATP production and thus trigger for diminished *Agrp* expression. This mechanism would explain earlier findings, showing that the metabolic conversion of BHBA by the brain occurs primarily under hypoglycaemic conditions (Ruderman *et al.* 1974, Lindsay & Setchell 1976).

However, although the cell culture model used requires glucose and BHBA concentrations above those existing *in vivo*, the glucose/BHBA ratio investigated herein is highly comparable with the *in vivo* situation. Considering this fact, we propose a cellular pathway, which integrates BHBA in the control of food intake. Nevertheless, further cell and *in vivo* studies are necessary to confirm our current results by which BHBA regulates *Agrp* expression in dependency of the glucose level.

Conclusions

To summarise, we investigated a cellular pathway triggered in response to BHBA and provide new knowledge about BHBA's possible impact on food intake regulation. We have demonstrated that BHBA is capable of entering the cell not only via MCT1 but also via other routes with subsequent mobilisation of calcium. Furthermore, BHBA modulates

AMPK- α phosphorylation. Associated with an increased AMPK phosphorylation, *Agrp* mRNA expression increases after 4 h of BHBA treatment in a 25 mM glucose-containing medium. However, although *in vitro* conditions for GT1-7 cells require higher glucose concentrations, it is uncertain whether 25 mM glucose reflects any physiological relevance for *in vivo* comparisons. In the presence of 5.5 mM glucose, however, BHBA mobilises intracellular Ca^{2+} to a much lower extent and reduces AMPK phosphorylation and *Agrp* expression after 4 h. The latter cellular signal transduction mechanism may contribute to the understanding of the feed intake depressive effect of BHBA observed after *in vivo* infusions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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