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Evidence for hypothalamic ketone bodies sensing: impact on food intake and peripheral metabolic responses in mice

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Running Head: Hypothalamic ketone bodies sensing and energy homeostasis

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ABSTRACT

Monocarboxylates have been implicated in the control of energy homeostasis. Among them, the putative role of ketone bodies produced notably during high fat diet (HFD) has not been thoroughly explored. In this study, we aimed to determine the impact of a specific rise in cerebral ketone bodies on food intake and energy homeostasis regulation. A carotid infusion of ketone bodies was performed on mice to stimulate sensitive brain areas during 6 or 12 hours. At each time point, food intake and different markers of energy homeostasis were analyzed to reveal the consequences of cerebral increase in ketone bodies level detection. First, an increase in food intake appeared over a 12-hour period of brain ketone bodies perfusion. This stimulated food intake was associated with an increased expression of the hypothalamic neuropeptides NPY and AgRP as well as of phosphorylated AMPK and is due to ketone bodies sensed by the brain as blood ketone bodies levels did not change at that time. In parallel, gluconeogenesis and insulin sensitivity were transiently altered. Indeed, a dysregulation of glucose production and insulin secretion was observed after 6 hours of ketone bodies perfusion which reversed to normal at 12 hours of perfusion. Altogether, these results suggest that an increase in brain ketone bodies concentration leads to hyperphagia and a transient perturbation of peripheral metabolic homeostasis.

Keywords: Energy homeostasis, Monocarboxylate transporters, β-hydroxybutyrate, Obesity, Glucose homeostasis

INTRODUCTION

Dysfunction in both cerebral detection of nutrients and integration of circulating signals has been implicated in the pathogenesis of obesity and associated disorders (11). For this reason, numerous studies have explored the possible role of nutrient and endocrine sensing of hypothalamic brain areas and their involvement in energy homeostasis regulation (34). The most studied circulating energy substrate is glucose which represents a critical nutrient monitored by the brain. As the main energy source for brain cells, glucose also plays an important role in brain energy homeostasis (33). However, evidence have been provided showing that the brain can use alternative energy substrates. For instance, fatty acids and ketone bodies significantly contribute to fulfill brain energy needs under specific conditions (6, 13, 36). Despite the fact that it has been known for decades that cerebral ketone bodies utilization increases under particular metabolic conditions (13), central ketone bodies detection has been poorly studied.

Under basal conditions, blood ketone bodies concentrations are low (< 0.3mmol/L) and their cerebral utilization is considered of little significance. However, ketone bodies levels are increased under conditions such as fasting, type I diabetes or obesity (13). The brain can use ketone bodies when their blood concentration reach ≈ 4 mmol/L, a value close to the K_m of the monocarboxylate transporter MCT1 expressed on endothelial cells of cerebral blood vessels for ketone bodies (24, 31, 44). The brain ability to use ketone bodies varies from one brain area to another (12). Interestingly, the hypothalamus, which is a key player in brain sensing of metabolic signals, presents a higher ketone bodies metabolism than other brain areas (12). Thus, ketone bodies could be considered as a metabolic signal putatively sensed in the hypothalamus and participating in energy homeostasis control. Considering their importance for brain energetics in such circumstances, detection of their circulating levels by the brain might be quite important for homeostatic purposes, and putative defects could be involved in some metabolic disorders including obesity.

So far, different approaches including direct ketone bodies infusion into the brain parenchyma (32), subcutaneous injection of ketone bodies (9, 32) or ketogenic diets (5, 16, 39, 45) have been used to try to uncover the role of ketone bodies in the regulation of food intake. However, these strategies bear some caveats as they either bypass the natural supply route, thus possibly affecting primarily other brain areas (intraparenchymal injection) or they act on other key organs for the regulation of whole body metabolism (liver, adipose tissue) and not

exclusively on the brain (subcutaneous injection, ketogenic diets). Indeed, since ketone bodies are mainly produced in the liver, they must cross the blood-brain barrier in order to reach the brain and exert their central effects. ICV administration bypasses this physiological route. In addition, intraperitoneal injections or ketogenic diets affect peripheral organs as well. Finally, the direct central effect of ketone bodies sensing alone could not be investigated through these strategies, as ketone bodies are certainly sensed by other tissues. Altogether, these previous studies suggest an important role of ketone bodies detection (both at central and peripheral levels) in the regulation of energy homeostasis. However, they were not intended to decipher specifically the role of ketone bodies themselves in food intake control as many other parameters were modified in parallel such as fatty acid levels or blood glucose for instance. Consequently, as described in a recent review from Paoli et al., these different studies gave rise to contradictory results regarding orexigenic vs. anorexigenic effects (30). On one hand, a majority of the studies were performed through exposure to a ketogenic diet and seem to indicate that such diets are associated with decreased hunger and appetite reduction (16, 17, 28, 29, 41, 42). Moreover, a recent study described a role of astrocytic ketone bodies and fatty acids as inhibitors of food intake during short-term high energy diet (23). In contrast, ketogenic diets have been shown to induce the activation of AMPK in brain which is associated with an increased food intake suggesting that brain sensing of ketone bodies could have a positive action on food intake behavior (27). Notwithstanding, ketogenic diets cause numerous peripheral effects and alter levels of other nutrients, making it impossible to reveal the unique role of ketone bodies in brain control of food intake.

For these reasons, we investigated in this study the effect of a rise in central ketone bodies on body energy homeostasis. But in contrast to previous studies, we used for this purpose carotid-catheterized animals that were infused with ketone bodies toward the brain to mimic the normal passage via the circulation without significant changes in their concentration in the rest of the circulation and we recorded some of their behavioral and metabolic responses.

MATERIAL and METHODS

Animals

C57BL6 male mice (8-weeks-old; Janvier) were individually housed in a controlled environment (12 h light/dark cycle, light on at 7:00 am, 22°C), with ad libitum access to food (Kliba Nafag standard diet # 3336, Kaiseraugst, Switzerland) and water. Ketone bodies

infusion in unrestrained mice was done as described previously in rat and adapted for mice (26). Briefly, after pentobarbital anesthesia (50 mg/kg), a silicone tubing (internal diameter 0.31 mm, external diameter 0.64 mm) was inserted in the right carotid and pushed 5 mm in the cranial direction. The catheter was led subcutaneously in the middle of the neck between the 2 blades and externalized for further connection to the infusion pump. Mice were then housed 1 week for surgical recovery, before starting the infusion experiments. During the experiments mice were connected to an external infusion pump and NaCl or BHB (adjusted pH at 7.4) was infused at 30 µL/h for either 6 or 12h. All procedures involving mice followed the European Communities Council Directive (86/609/EEC) and were approved by a local committee.

Immunohistochemistry

Anesthetized NPY-GFP mice were intra-cardiacally perfused with 10ml heparin-PBS (50U/ml) and 10ml of 4% paraformaldehyde. Brains were then removed and preserved in 30% sucrose at 4°C, embedded in Tissue freezing medium (Jung, Nussloch, Germany) and frozen in dry ice before sectioning. Three sets of coronal sections (30µm thick) were cut on a cryostat (Leica Biosystems CM 3050S, Dusseldorf, Germany). After three PBS rinses sections were incubated 20 minutes at room temperature (RT) in 2% donkey normal serum (Sigma, Buchs, Switzerland) in PBS 0.3% Triton X-100. Sections were incubated with rabbit anti-MCT1 (1:500)(38) and goat anti-GFP (1:1000, SicGen, Carcavelos, Portugal) two days at 4°C. After three PBS rinses, sections were incubated with secondary antibodies (Jackson ImmunoResaearch, Sulffolk, UK) anti-goat IgG(H+L) Cy3 (1:500) and anti-rabbit IgG Fab Alexa594 (1:500) for 150 minutes at room temperature. Sections were incubated two minutes in 1 µg/ml Hoechst 33342 (Invitrogen, Lucerne, Switzerland)) to label nuclei, mounted onto gelatin-coated glass slides and coverslip with Fluoromount (Sigma). Acquisitions and quantifications were performed on a laser confocal microscope (Zeiss LSM 700, Zeiss, Feldbach, Switzerland) with x40 and x63 objectives and using ZEN software.

Feeding test

Unrestrained mice were infused for either 6 or 12 hours with a solution of 50 mM DL-β-Hydroxybutyric acid (BHB; Sigma, Buchs, Switzerland) at 30µL/h (Figure 1A). Such a concentration and rate allowed the animals to receive a dose of 20µg/h of BHB. This concentration represents the lowest concentration tested showing an impact on food intake but

without inducing mouse sickness (determined by observing breathing difficulties and activity). The infusion started 5 hours after the beginning of the dark period to avoid the increased blood ketone bodies level normally induced during fasting periods and thus that could cause an increased ketone bodies concentration response during feeding periods as it likely occurs in obesity or type 1 diabetes. At 6 hours and 12 hours after the beginning of the perfusion, food intake was measured. At each time point, mice were sacrificed and hypothalamus, cortex, liver and blood were removed for further analysis.

Pyruvate and Insulin tolerance tests

Mice received an intraperitoneal pyruvate (2 mg/g) or insulin (0.5mU/g for Insulin Tolerance Test (ITT) or 2mU/g for Counter regulatory response) injection after 6h and 12h of ketone bodies infusion. Blood was collected from the tail vein at - 30, 0, 15, 30, 45, 60, 90, and 120 min for determination of glucose levels.

RNA extraction, reverse transcription and quantitative real time PCR

Tissues were lysed and homogenized in 300µl of lysis buffer (RLT Buffer, Qiagen, Basel, Switzerland) using the Fast prep 24 lyzer (MPbio, Luzern, Switzerland) according to the manufacturer's instructions. Total RNA was isolated on spin columns with silica-based membranes (RNeasy Mini Kit, Qiagen), following the manufacturer's instructions. RNA was eluted with 30µl of H_2O. A small amount of purified RNA (200ng) was reverse transcribed in a volume of 50µl using the RT High Capacity RNA-to-cDNA Kit (Applied Biosystems, Rotkreuz, Switzerland). Synthesized cDNA was then stored at -20°C. Quantitative real-time PCR analysis was performed with the Applied Biosystems 7900 (Applied Biosystems) Real-Time PCR System. The Taq polymerase master mix employed was the Power SYBR Green (Applied Biosystems). Primer sequences used for mRNA quantification were directed against NPY (Neuropeptide Y), AgRP (Agouti Related Protein), POMC (Pro-Opio MelanoCortin), CART (Cocaine and Amphetamine Related Transcript), BHBDH (β-Hydroxybutyrate Deshydrogenase), HMGcs2 (3-hydroxy-3-methylglutaryl-CoA synthase 2), PEPCK (Phosphoenolpyruvate CarboxyKinase), G6Pase (Glucose-6Phosphatase), as well as β−2-microglobulin mRNA used as an endogenous control (primer sequences in Supplementary Table 1). Data were then analyzed with RQManager 1.2 software (Applied Biosystems) for relative quantitation. Relative quantitation (RQ) of gene expression was based on the comparative Ct method using the $2^{-\Delta\Delta CT}$ method.

Blood analysis

Insulin, (ultra-sensitive Elisa Kit, Millipore, Zug, Switzerland), glucagon (Glucagon RIA, Millipore), plasma lactate (The Edge analyser, Hasselt, Belgique), glucose (Benecheck plus Multi-monitoring system, Hasselt, Belgique) and ketone bodies concentration (D-β-Hydroxybutyrate) (Free Style precision, Abbott, Baar, Switzerland) were measured after the injection at several time points using the indicated kit. Ketone bodies, lactate and glucose were measured at the end of the perfusion from blood taken from the tail vein. Insulin and glucagon were measured in blood taken from cardiac blood withdrawal,

Liver glycogen measurement

Briefly, 100mg of tissue stored at -80°C were homogenized in citrate buffer (NaF 50mM, Citric acid 100mM, pH 4.2) and then centrifuged at 5000g for 10 min at 4°C. Supernatant was removed, and 460µl were incubated with 40µl of a solution of amyloglucosidase 50U/ml (Sigma) diluted in sodium citrate buffer, while 460 µl were incubated with 40µl of sodium citrate buffer only. The tubes were shaken for 30min at 55°C. Then, 10µl of each sample were

deposited in 96-well plates with 200µl of a RTU (Ready to Use) buffer (BioMerieux, Geneve, Switzerland), incubated at room temperature for 20min. The optical density was read at 505nm by spectrophotometry. The difference between conditions with amyloglucosidase and conditions with buffer only represented the glycogen content of the liver sample. Glycogen was expressed as milligrams of glucose resulting from glycogen hydrolysis per gram of tissue.

Western Blot analysis

Proteins were separated with 10% SDS-PAGE. Antibodies against MCT1, MCT2, MCT4, and β-Tubulin were used. After transfer and blocking, membranes were probed in 1% nonfat milk prepared in TBS-T with 1/1,000 rabbit anti-MCT1, anti-MCT2 (38), 1/500 anti-MCT4 (Santa Cruz, Heidelberg, Germany), 1/1000 for AMPK and pAMPK (Cell Signaling, Berverly, MA, USA) and 1/10000 rabbit anti-β-Tubulin (Cell Signaling, Berverly, MA, USA) overnight at 4°C. Specific band for each protein was detected using a goat anti-rabbit (1/10,000 in TBST-1X) peroxidase-conjugated secondary antibody (GE Healthcare, Piscataway, NJ, USA) incubated for 1 hour at room temperature. Bands were revealed with a chemiluminescence kit (BioRad, Reinach, Switzerland) and processed with a ChemiDoc XRS + system (BioRad, Reinach, Switzerland) for densitometry analysis.

Electrophysiology

Brain slices (250µm) were prepared from adult NPY-GFP mice (6-8 weeks old) as previously described (7). Slices were incubated at room temperature (RT), in oxygenated extracellular medium containing (in mM): 118 NaCl, 3 KCl, 1 $MgCl_2$, 25 NaHCO3, 1.2 NaH_2PO_4, 1.5 $CaCl_2$, 5 Hepes, 2.5 D-glucose (osmolarity adjusted to 310mOsM with sucrose, pH 7.3) for a recovery period (at least 60minutes). Once in the recording chamber, slices were perfused at 2-3 ml/min with the same extracellular medium. Slices were viewed with a Nikon microscope (EF600) outfitted for fluorescence (fluorescein filter) and IR-DIC (Infrared-Differemcial interference contrast) videomicroscopy. Viable arcuate NPY neurons were visualized using a X60 water immersion objective (Nikon) with a fluorescence video camera (Nikon). Borosilicate pipettes (4-6MΩ; 1.5mm OD, Sutter Instrument) were filled with filtered extracellular medium. Cell-attached recordings were made using a Multiclamp 700B amplifier, digitized using the Digidata 1440A interface and acquired at 3kHz using pClamp 10.3 software (Axon Instruments). Pipettes and cell capacitances were fully compensated. After a stable baseline was established, BHB (5mM) was perfused for 10minutes. The firing

activity was measured over the last minute of the BHB perfusion and compared with the firing rate measured 1 min before the perfusion.

Statistical analysis

Results are presented as mean ± SEM. Statistical analysis was performed using Prism 6.01. Normality was tested with the Kolmogorov-Smirnov test. Depending of the result of the normality test, an unpaired Student *t*-test or an unpaired *t*-test with Welch's correction (when equal variance was not assumed) was used. Significant differences are indicated as *, **, or *** on graphic representations for p values < 0.05, 0.01, or 0.001, respectively.

RESULTS

Food intake and hypothalamic neuropeptide mRNA expression in mice with intracarotid infusion of β-hydroxybutyrate (BHB).

Food intake measurement shows a significant increase in the amount of pellets ingested after 6 and 12 hours of intracarotid infusion in mice receiving β-hydroxybutyrate compared to those receiving NaCl (1.06 ± 0.07g for the NaCl group vs. 1.32 ± 0.08g for the BHB group at 6h, p=0.016; 1.40 ± 0.09g for the NaCl group vs 1.62 ± 0.10g for the BHB group at 12h, p=0.047) (Figure 1B). Moreover, a closer analysis of the first 6 hours of infusion reveals that food intake began to be significantly stimulated only after 4 hours of infusion in the BHB group compared to NaCl infused mice (0.47 ± 0.04g for the NaCl group vs. 0.70 ± 0.09g for the BHB group at 4h, p=0.025; 0.77 ± 0.05g for the NaCl group vs. 1.04 ± 0.10g for the BHB group at 5h, p=0.020) (Figure 1C). Interestingly, circulating ketone bodies levels remained unchanged at 6 hours (0.33 ± 0.03mM in NaCl group vs 0.42 ± 0.03mM in BHB group, n=15 in each group, p=0.026) while it increased at 12 hours, demonstrating that the effect observed at 6 hours can be due only to ketone bodies acting at the cerebral level (Figure 1E). In accordance with food intake stimulation, the measurement of the pAMPK/AMPK ratio shows an increased activation at 6 hours only (0.11 ± 0.03 a.u. for the NaCl group vs. 0.29 ± 0.05 for the BHB group at 6h, p = 0.009) (Figure 1D. Analysis of the mRNA levels of the hypothalamic neuropeptides involved in food intake regulation revealed an increase for the orexigenic neuropeptide NPY (1.0 ± 0.07 a.u. for the NaCl group vs. 1.46 ± 0.18 a.u. for the BHB group at 6h, p = 0.029; 1.0 ± 0.08 a.u. for the NaCl group vs. 1.71 ± 0.21 a.u. for the BHB group at 12h, p = 0.012) (Figure 1F) and AgRP (1.0 ± 0.09 a.u. for the NaCl group vs 2.12 ± 0.33 a.u. for the BHB group at 6h, p = 0.005; 1.0 ± 0.08 a.u. for the NaCl group vs.

1.63 ± 0.20 a.u. for the BHB group at 12h, p = 0.005) (Figure 1G). In contrast, mRNA expression of the antagonistic (and thus anorexigenic) neuropeptides POMC and CART were not altered during BHB infusion (Figure 1H and 1I, respectively).

MCT1 expression in hypothalamic orexigenic NPY neurons and their acute electrophysiological response to BHB exposure in vitro.

Interestingly, the presence of MCT1 on some hypothalamic neurons had been reported previously (1). However, the precise identity of these neurons (e.g. orexigenic vs. anorexigenic) was not provided in that study. Given our aforementioned results, it was hypothesized that hypothalamic NPY neurons could express MCT1. Immunohistochemistry performed on brain sections of NPY-GFP mice shows that almost 50 % of NPY neurons were MCT1 positive (Figure 2A-E). Surprisingly however, measurements of the electrophysiological activity of arcuate NPY neurons did not reveal any direct modulation in response to short-term BHB exposure (Figure 2F and 2G).

Metabolic characteristics after 6 hours of BHB infusion

First, different blood parameters were measured and a twofold increase in insulin level was evidenced (2.20 ± 1.20 ng/mL for the NaCl group vs 5.02 ± 2.39 ng/mL for the BHB group, p = 0.01, n = 8 NaCl and 6 BHB infused mice) (Figure 3A). Despite such an increase in insulin levels, blood glucose concentration remained unaffected in the BHB group (Figure 3B) while blood lactate level was enhanced (Figure 3C). To test whether this apparent normoglycemia despite an increased insulin level could be due to decreased insulin sensitivity, an insulin tolerance test was performed. However, the results shows that BHB infused mice exhibited instead a higher sensitivity to insulin as shown by the lower glucose concentration induced by insulin injection (Figure 3D) (Area Under Curve (AUC) of 38.76 ± 2.86 for the NaCl group vs 29.19 ± 1.80 for the BHB group, n = 7 for each group, p = 0.02, (Figure 3D, upper panel)). These results rather suggested a putative defect in the counter-regulatory response to hypoglycemia, i.e. of the glucagon release in response to hypoglycemia induced by an insulin load. Indeed, glucagon concentrations were found to be much lower in BHB infused mice compared to the control group at 60 minutes after the insulin induced hypoglycemia (532 ± 88 ng/L for the NaCl group vs 134 ± 50 ng/L for the BHB group, n=6 for each group, p = 0.003) (Figure 3E).

As blood glucose level could also be influenced by neoglucogenesis, a pyruvate tolerance test was performed and results showed it was reduced in BHB infused mice (Figure 3F) (AUC of 54.50 ± 3.34 for the NaCl group vs 44.27 ± 2.82 for the BHB group, n=5 for each group, p=0.047, (Figure 3F, upper panel)). In accordance, mRNAs of the main enzymes involved in neoglucogenesis, PEPCK and G6Pase, were both decreased in the liver, one of the major sites of neoglucogenesis (1.00 ± 0.27a.u for the NaCl group vs 0.29 ± 0.03 for the BHB group, n=10 for each group, p=0.017 for PEPCK and 1.00 ± 0.19 a.u for the NaCl group vs 0.50 ± 0.08a.u for the BHB group, n=10 for each group, p=0.026 for G6Pase, (Figure 3G and H)). Moreover, the observed increase in lactate level (47.67 ± 3.49 mg/dL for the NaCl group vs 59.50 ± 4.25 mg/dL for the BHB group, n=27 NaCl and 28 BHB mice, p=0.037) could be a consequence of decreased gluconeogenesis (Figure 3C). Another possible contributor to blood glucose level is hepatic glycogen. Indeed, reduced glycogen levels were found in the BHB group compared to the control group (14.60 ± 1.39 µg/mg tissue for the NaCl group vs 9.59 ± 1.11 µg/mg tissue for the BHB group, n=11 for each group, p=0.011), suggesting the putative involvement of hepatic glycogenolysis in the maintenance of glucose homeostasis for BHB infused animals (Figure 3I).

Metabolic characteristics after 12 hours of BHB infusion

The same metabolic evaluation was performed after 12 hours of BHB infusion. Interestingly, it was found that all the parameters measured at 12 hours of BHB infusion were normalized in BHB infused mice to the levels of the control group. Thus, glycemia, insulinemia and lactatemia were not significantly different, and the same was true for the insulin tolerance (Figure 4A-D). As expected, the counter-regulatory response to hypoglycemia shows a normal glucagon release in response to hypoglycemia (Figure 4E). Finally, the pyruvate injection led to a similar neoglucogenic response in BHB and NaCl infused mice (Figure 4F). In accordance, G6Pase and PEPCK mRNA levels were similar, suggesting a normalized endogenous glucose production, which is confirmed by a similar liver glycogen content (Figure 4G-I) and a normalized blood lactate level (Figure 4C).

Hypothalamic and cortical MCT expression as well as hepatic and hypothalamic ketogenesis after BHB infusion

To verify if exposure to BHB alters the capacity of brain cells to take up and use ketone bodies, the expression of the three main cerebral MCTs (MCT1, MCT2 and MCT4) was assessed after either 6 or 12 hours of BHB infusion in both the hypothalamus and cortex.

Expression levels of MCTs remained unchanged in both hypothalamic and cortical areas at 6 and 12 hours of infusion (Figure 5). Then, the impact of cerebral BHB infusion on ketogenesis was evaluated. The expression levels of enzymes involved in ketogenesis (HMG Cs2) or ketone bodies utilization (BHBDH) in both liver and hypothalamus were determined, no significant changes were detected after 6 hours of BHB infusion (Figure 6A-D). At 12 hours of BHB infusion, hypothalamic HMG Cs2 mRNA levels were increased (1.00 ± 0.10 a.u for the NaCl group vs 1.87 ± 0.29a.u for the BHB group, n=9 for each group, p=0.013), without any other significant modification in the liver or for BHBDH mRNA in either the hypothalamus or the liver (Figure 6A-D).

DISCUSSION

Monocarboxylates which include short chain fatty acids and ketone bodies have been shown to undergo some alterations during obesity development (4, 22, 23, 43). However, mechanisms by which they could be implicated in the pathophysiology of obesity have remained elusive. Nonetheless, it was postulated that ketone bodies could be involved in the regulation of food intake and in the control of energy homeostasis. Thus, in this study β-Hydroxybutyrate was infused via the carotid to examine the specific role of this monocarboxylate through its direct and limited action within the central nervous system in the regulation of food intake and energy homeostasis control.

Cerebral ketone bodies perfusion enhances food intake and hypothalamic orexigenic peptide expression

To test this hypothesis, β-Hydroxybutyrate (BHB; 20µg/h) was infused through the carotid for either 6 or 12 hours in order to stimulate brain areas involved in the control of energy homeostasis located primarily in the hypothalamus. The dose chosen (20µg/h) was determined after different concentrations were tested ranging from 2 to 200 µg/h. A dose of 20µg/h most likely represents a high concentration compared to the concentration measured in fasting conditions. However, as the infusion is performed through the right carotid, the final concentration actually permeating the brain will be diluted by the blood coming from the left carotid and the vertebral arteries. In addition, as the rate of infusion is very low (30 µl/h) compared to the carotidic blood flow (0.75 mL/min), the real BHB concentration reaching the targeted brain areas should be much lower than the concentration perfused, although it can not be estimated precisely. As ketogenesis occurs during the fasting period, experiments begun

345 during the dark period 5 hours after light off to prevent the physiological increased in ketone
346 bodies level (10).

347 Our main goal was to study the effect of ketone bodies detection by the brain,
348 independently of possible peripheral effects. Furthermore, ketone bodies concentration
349 increases during high fat diet as well as during fasting. These two metabolic conditions are in
350 opposition in regard to the energy needs, but both are associated with increased food intake.
351 Thus, ketone bodies could be an important signal involved in food intake stimulation. To test
352 this hypothesis a constant cerebral infusion of ketone bodies was administered to determine
353 their direct involvement in food intake control. Mice infused with BHB exhibited a significant
354 increase in food intake after 6 hours. However, the stimulation of food intake only appears
355 between 3 and 6 hours. Thereafter, Control and BHB mice ate the same amount of food
356 without compensation which led to the significant difference in food intake observed at 12
357 hours. (Figure 1B). The enhancement in food intake becomes significant only after 4 hours of
358 BHB infusion, indicating a delayed effect (Figure 1C). A similar delay in the effect of ketone
359 bodies was previously described in an hypothalamic cell line in which AMPK and mTOR
360 phosphorylation increased only after 4 hours of incubation with BHB (20), thus providing a
361 putative molecular mechanism for the BHB effect on food intake. Moreover, these authors
362 reported that activation of these cells by BHB only occurs in high glucose conditions (20).
363 Thus, increased glucose oxidation induces an increase in ATP production that in turn leads
364 first to a decrease in AMPK phosphorylation (AMPKp). This would be consistent with our *in*
365 *vivo* model since the experiments were performed 5 hours after the start of the mouse feeding
366 period which represents the period when mice eat the most (and thus should have the highest
367 circulating blood glucose levels). Indeed, analysis of the phosphorylated/nonphosphorylated
368 AMPK ratio shows such a cyclic activation at 6h which is reversed at 12h (Figure 1D).
369 Subsequently, as BHB is shown to decrease glucose uptake, a resulting decrease of ATP
370 production would ensue and lead to a delayed increase in AMPKp only after the 4 hours
371 infusion (35). Finally, the normalized levels of pAMPK observed at 12h could participate to
372 the normalized food intake during the 6-12h period of measurements confirming a putative
373 biphasic effect.

374 These results contrast with those recently reported by Le Foll et al. (23). Indeed, in this
375 study, the authors described an inhibition of food intake attributed to ketone bodies. However,
376 as they used a different model involving rats on a high fat diet, the increase in circulating fatty
377 acids associated with such a diet (in addition to ketone bodies) could contribute to the

differential observation. In addition, as presented by the authors, the results described seem to be due to central astrocytic ketogenesis induced by fatty acid oxidation. In our case, mice were used as an animal model and we can not exclude a species difference. In addition, as we chose to directly infuse ketone bodies at a high concentration, the concentration reached in the brain could be too elevated to reflect a physiological effect. Moreover, our results mostly suggest that peripheral ketone bodies supply alone could lead to increased food intake but the concomitant presence of elevated circulating fatty acids (as would occur physiologically on a high fat diet) could modulate the response to elevated ketone bodies alone. Another aspect is that the origin of ketone bodies production (central astrocytes vs. peripheral hepatocytes) may make a difference as astrocytes, which can modulate neuronal functions, could have seen such a modulatory role influenced by exposure to high ketone bodies levels. Thus, further investigations are needed to clarify the role of central vs. peripheral ketogenesis. Finally, the differences found in the previous study from Le Foll et al. and ours could also be due to differences in exposure timescales As we investigated short-term (12 hours) infusion of ketone bodies alone to determine the single effect of ketone bodies vs. three days of a high fat diet inducing an increase in both ketone bodies and fatty acids for the study of Le Foll et al., the different results obtained could reflect biphasic effects of ketone bodies on food intake regulation and/or a role of fatty acids overriding the ketone bodies effect. Notably, our results would be consistent with a rapid, short-term effect as food intake stimulation mostly take place within the first six hours of perfusion. A more prolonged cerebral infusion should be performed in the future to clearly determine if such a biphasic effect occurs.

Since the carotid infusion of BHB most likely affects, among others, brain regions such as the hypothalamus, a well-known center for energy homeostasis regulation (2), the mRNA levels of the main neuropeptides involved in food intake control were assessed. An increase in the expression of the orexigenic neuropeptides NPY and AgRP was observed (Figure 1F and 1G), without any significant modification in the anorexigenic neuropeptides POMC and CART (Figure 1H and 1I) after both 6 and 12 hours of BHB infusion. Such an overexpression of orexigenic neuropeptides induced by ketone bodies had been previously described both *in vitro* in the hypothalamic GT1-7 cell line and *in vivo* in diabetic rats (14, 20). However, it is demonstrated here that ketone bodies stimulate orexigenic neuropeptide mRNA expression also under physiological conditions *in vivo*. This result confirms that ketone bodies can stimulate directly the orexigenic neurocircuits *in vivo*.

A large subpopulation of hypothalamic NPY neurons expresses the transporter MCT1

Ketone bodies are transported in and out of cells by a small group of proteins known as monocarboxylate transporters or MCTs. MCT1 is the first identified member of this group and exhibits a large distribution in the body as well as among brain cells (37). Interestingly, the presence of MCT1 on some hypothalamic neurons had been reported previously (1). However, the precise identity of these neurons (e.g. orexigenic vs. anorexigenic) was not provided in that study. Given our aforementioned results, it was hypothesized that hypothalamic NPY neurons could express MCT1. Immunohistochemistry performed on brain sections of NPY-GFP mice shows that almost 50% of NPY neurons were MCT1 positive (Figure 2A-E). As reported previously(34), brain capillaries (like here in the hypothalamus) strongly express MCT1, a situation which would facilitate the entry of ketone bodies in the brain parenchyma, notably for use as alternative energy fuel by brain cells. In the hypothalamus, NPY neurons expressing MCT1 could be responsible for the enhanced orexigenic activity following BHB infusion by exhibiting a direct sensitivity to circulating ketone bodies. Recently, a mouse model haploinsufficient for MCT1 displaying a resistance to diet-induced obesity and altered food intake has been described, suggesting an important role for monocarboxylates in the regulation of body weight and energy homeostasis regulation (25). Based on the immunocytochemical data presented here, it is postulated that a subpopulation of hypothalamic neurons would be sensitive to circulating ketone bodies and could be responsible for the regulation of food intake which appears altered in haploinsufficient MCT1 mice.

Surprisingly, measurements of the electrophysiological activity of arcuate NPY neurons did not reveal any direct modulation in response to BHB (Figure 2F and 2G). In contrast, a recent study showed using calcium imaging that hypothalamic neurons are activated by an increase in BHB concentration (23). However, in this study the authors did not characterize the specific phenotype of these neurons. In our study, BHB failed to alter the electrophysiological activity of all NPY neurons tested. Such an absence of electrophysiological response from these neurons to ketone bodies could be due to the short duration of exposure and measurement used in our experiment (10 minutes). Indeed, as described above, the stimulatory effect of BHB on food intake only appears after 4 hours of treatment. The same time course was also described for the response *in vitro* (20). Thus, an acute exposure of brain slices to BHB might not be sufficient to observe any long-term

changes in electrophysiological activity. In addition, the denervation of NPY neurons in slices could also explain the lack of response observed upon application of BHB. Removal of inhibition of this population would lead to such a high spontaneous firing rate that it would make BHB-induced changes undetectable. Nevertheless, despite the orexigenic effect of ketone bodies observed here and the lack of effect on POMC mRNA expression, we can not exclude a putative action on POMC neurons that needs to be further explored.

Cerebral ketone bodies perfusion causes a transient rise in insulin level and a decrease in hepatic gluconeogenesis

Circulating insulin levels were significantly elevated after 6 hours of cerebral BHB perfusion (Figure 3A) while blood glucose was not altered in such a condition compared to control mice (Figure 3B). As insulin is known to inhibit food intake when infused in the hypothalamus (40), an eventual brain response to insulin seems counterbalanced by the direct central action of ketone bodies. Ketone bodies, by inducing insulin secretion and stimulating food intake at the same time, appear to have an antagonistic role in central energy homeostasis regulation. A possible explanation could be through a redox signaling mechanism. Indeed, food intake inhibition by insulin was shown to involve an increase of reactive oxygen species production in the hypothalamus (15). In contrast, ketone bodies were shown to reduce reactive oxygen species production in neurons by acting on mitochondrial function (18). Thus, cerebral infusion of ketone bodies could interfere with insulin signaling leading to food intake inhibition by preventing reactive oxygen species production in hypothalamic neurons.

As these observations suggested the possible early development of an insulin resistance, an insulin tolerance test was performed on mice after 6 hours of BHB perfusion. Surprisingly, results rather revealed a more sensitive response to insulin in these animals (Figure 3D). One possible explanation for the enhanced reduction in glucose concentration obtained during the insulin tolerance test is an alteration of counter-regulatory responses to hypoglycemia. Indeed, when the counter-regulation was evaluated following an important insulin injection, lower levels of glucagon were observed after 6 hours of cerebral BHB perfusion compared to control animals (Figure 3E). Interestingly, this reduced glucagon release is observed at 60 minutes after insulin injection, a time when glucose concentration rises to normal values in control animals. Such an observation strongly suggests that insulin sensitivity is not altered but that counter-regulatory response is missing. This response

obtained with BHB is similar to the inhibited counter-regulation observed with central application of lactate (also carried by the MCTs) (3). It is important to notice, as a counter-regulatory response normally occurs in a decreased energy availability condition, that results here seems contradictory with the food intake stimulation observed in parallel. However, it is likely that different neuronal populations are involved in these responses. Indeed, if food intake control is regulated by NPY neurons in the arcuate nucleus, the counter-regulatory response is suggested to be regulated at the level of the ventromedial nucleus, another hypothalamic nucleus (8). However, in view of such contradictory observations, further investigations will be required to determine the precise mechanisms involved.

To further explore the impact of counter-regulation, a pyruvate tolerance test was performed on mice after 6 hours of BHB perfusion. A reduced hepatic gluconeogenesis was revealed in animals after 6 hours of BHB perfusion compared to control animals as evidenced by lower circulating glucose levels obtained following pyruvate injection (Figure 3F). In accordance with reduced hepatic gluconeogenesis, a reduction in the mRNA expression of Glucose-6-Phosphatase (G6Pase; Figure 3H) and Phosphoenolpyruvate carboxykinase (PEPCK; Figure 3G) was evidenced in the liver of animals perfused with BHB compared to controls. In parallel, it was observed that blood lactate level was enhanced while liver glycogen levels were reduced in mice perfused with BHB. As G6Pase is common to gluconeogenesis and glycogenolysis, which classically leads to glucose release, it appears in our case that the observed glycogen breakdown would not lead to glucose release but rather to a glycolytic processing of glucose residues arising from glycogen. In other words, the observed glycogen degradation would lead to a hepatic lactate production, thus explaining the increased lactate level, reinforced by the decreased gluconeogenesis which would prevent hepatic lactate utilization and rather promote circulating lactate accumulation. Such an effect could be the consequence of elevated insulin levels and sensitivity as hyperinsulinemia was previously shown to inhibit hepatic gluconeogenesis (19)

In contrast to the observation made after 6 hours, insulin level was no longer altered after 12 hours of BHB perfusion (Figure 4A) while blood glucose level was still normal (Figure 4B). Similarly, the insulin tolerance test showed that insulin sensitivity was not different between BHB infused animals and control animals (Figure 4D). When the counter-regulatory response was evaluated, again no difference in glucagon levels (Figure 4E) was detected. The pyruvate tolerance test did not reveal any difference in gluconeogenesis (Figure 4F) after 12 hours of BHB perfusion. In the liver, both G6Pase and PEPCK mRNA levels

were the same between control and BHB infused mice (Figure 4G and H). In parallel, both circulating lactate concentration and hepatic glycogen levels were similar for control and BHB infused mice, thus confirming that after 12 hours of BHB perfusion, all parameters returned to normal levels. These data underline the transient nature of the changes induced by central ketone bodies infusion, despite a constant exposure to them, suggesting some forms of central desensitization or habituation. Since it was previously shown that the level of expression of all three cerebral MCT isoforms was modified as a consequence of obesity (36), a possible effect of cerebral BHB perfusion on MCT expression either in the hypothalamus or in the cortex was investigated. The results obtained at the protein level for MCT1, 2 and 4 did not reveal any modification of expression following the infusion of BHB neither in the hypothalamus nor in the cortex (Figure 5). This result is not surprising when considering that it took several weeks of high fat diet to observe a significant enhancement in the cerebral expression of MCTs (25). Nevertheless, our results suggest that hypothalamic (and cortical) ketone bodies transport is not altered during this relatively short cerebral exposure to ketone bodies, although we can not exclude a hypothetic posttranslational modification of the transporters.

As the hypothalamus represents a key brain region for the regulation of body energy homeostasis, several peripheral metabolic markers were analyzed to further determine the impact of cerebral BHB infusion on peripheral metabolism. Hence, the concentration of several circulating metabolites and hormones was measured. First of all, ketone bodies concentration measured after 6 hours of central BHB perfusion was not modified compared to controls, indicating that the observed effects are due to the exogenous cerebral BHB infusion (Figure 1E). At 12 hours of BHB infusion, an increase in ketone bodies concentration was detected (Figure 1E). However, mRNA expression levels for HMG CoA synthase, the rate limiting enzyme involved in ketogenesis, did not show any alteration in the liver (the main ketogenic organ (21)) (Figure 6B). Thus, the elevated circulating ketone bodies level observed after 12 hours of BHB infusion is most likely due to BHB accumulation in the systemic circulation following carotid infusion for such an extended period. More surprisingly, HMG CoA synthase mRNA levels increased in the hypothalamus after 12 hours but not after 6 hours of cerebral BHB perfusion (Figure 6C). In parallel, mRNA expression levels for BHBDH (the essential enzyme for ketone bodies utilization) were analyzed but no modification was observed at any time point neither in the liver nor in the hypothalamus (Figure 6D and 6E). Since the rate-limiting enzyme for ketone bodies synthesis is not affected

at 6 hours, it suggests that the increased food intake (which is significant already at 6 hours) is dependent on the exogenous ketone bodies infusion and not on hypothalamic ketogenesis. In addition, these results do not support a local or global decrease in ketone bodies utilization. Thus, the most likely explanation for the measured increased ketone bodies concentration remains a consequence of the carotid-infused BHB on the global blood concentration. Brain ketogenesis can only produce low concentrations of ketone bodies as measured in a recent study in the VMH in which the authors measured a concentration of ~ 20 µM when the blood concentration can be up to 300 µM(23). It can be concluded that such a small local VMH production is unlikely to contribute significantly to the increased circulating ketone bodies observed in mice after 12 hours of BHB perfusion.

To summarize, this study provides evidence that the simulated elevated cerebral ketone bodies concentration induces specific brain responses that lead to a transient increase in food intake and peripheral energy metabolism alterations. These modifications include decreased hepatic glucose production after glycogen hydrolysis. Thus, ketone bodies sensed by the brain seem to represent an energetic stress signal that leads to energy intake in order to maintain physiological functions. It is accompanied by transient peripheral metabolic responses triggered via the hypothalamus since the observed phenotype is reversed if the BHB infusion lasts up to 12 hours (Figure 7). In conclusion, we demonstrate here that ketone bodies represent an important signal that leads to energy preservation and supply as they stimulate adaptive responses to compensate for a perceived energy deficit.

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AUTHOR CONTRIBUTIONS

LC designed experiments; LC, SG, XF, CR and AH conducted experiments; LC, SG, XF performed data analyses; LC, CL and LP contributed to the discussion and writing of the manuscript. LP supervised the project and edited the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Effect of intracarotid BHB infusion on food intake as well as on orexigenic and anorexigenic hypothalamic neuropeptide mRNA expression. (A) Schematic representation of time course procedures for experiments. **(B)** Food intake determined at 6h and 12h of NaCl or BHB infusion by substracting the weight of food pellet remaining at that time from the weight of food provided at the start of the experiment. **(C)** Food intake determined each hour during the 6 first hours of NaCl or BHB infusion by substracting the weight of food pellet remaining at that time from the weight of food provided at the start of the experiment. **(D)**

Hypothalamic AMPKp/AMPK protein expression ratio at 6h and 12h in mice infused with either NaCl or BHB. Upper panels show bands from representative samples chosen from the western blots of all samples run in parallel and rearranged to appear side-by-side. Lower panel provides the quantification of AMPKp/AMPK ratio. **(E)** Blood Ketone bodies concentration at 6h and 12h in mice infused with either NaCl or BHB **(F)** NPY mRNA expression at 6h and 12h in mice infused with either NaCl or BHB **(G)** AgRP mRNA expression at 6h and 12h in mice infused with either NaCl or BHB **(H)** POMC mRNA expression at 6h and 12h in mice infused with either NaCl or BHB **(I)** CART mRNA expression at 6h and 12h in mice infused with either NaCl or BHB. mRNA expression for each hypothalamic peptide was determined by quantitative reverse transcriptase–PCR at 6h, 12h and 24h of NaCl or BHB infusion. n = 8-24 animals per condition and were statistically analyzed with unpaired Student *t*-test (with Welch's correction for NPY 6h and 12h, as well as for AgRP 6h and 12h). a.u., arbitrary units.

Figure 2. Immunocolocalization of the MCT1 transporter on NPY neurons and electrophysiological response of NPY neurons to BHB (A) Immunohistochemistry in hypothalamic arcuate nucleus for MCT1 (red) and NPY neurons (green). **(B) (C) and (D)** Higher magnification of the selection in panel A. NPY neurons are in green (B), MCT1 appears in red (C) and colocalization is indicated by arrows in panel D. **(E)** Representative orthogonal projection of an NPY neuron expressing MCT1. NPY-GFP mice were used for the labeling of the NPY neurons. N=3 mice analyzed (4slices per mice). **(F)** Whole-cell current-clamp recordings of ARC NPY neurons as extracellular BHB concentration is altered. Increased BHB had no effect on electrical activity of NPY neurons. **(G)** Quantification of action potential frequency. n = 9 NPY neurons recorded from 4 male mice and were statistically analyzed with an unpaired Student *t*-test.

Figure 3. Effect of intracarotid BHB infusion on circulating nutrients and key metabolic hormone concentrations at 6h BHB infusion. (A) Insulin at 6h in mice infused with either NaCl or BHB **(B)** Blood Glucose level at 6h in mice infused with either NaCl or BHB **(C)** Blood Lactate level at 6h in mice infused with either NaCl or BHB **(D)** Intraperitoneal insulin tolerance test performed at 6h of BHB infusion, and Area under curve of plasma glucose levels during ITT. **(E)** Glucagon levels 60min following an insulin induced hypoglycemia. **(F)** Intraperitoneal pyruvate tolerance test performed at 6h of BHB infusion, and Area under curve of plasma glucose levels during the pyruvate tolerance test. **(G)** Liver PEPCK mRNA expression at 6h in mice infused with either NaCl or BHB. **(H)** Liver G6Pase mRNA

expression at 6h in mice infused with either NaCl or BHB. **(I)** Liver Glycogen content determined as the glucose produced by glycogen hydrolysis at 6h in mice infused with either NaCl or BHB. n = 8-24 animals per condition and were statistically analyzed with an unpaired Student *t*-test. a.u., arbitrary units.

Figure 4. Effect of intracarotid BHB infusion on circulating nutrients and key metabolic hormone concentrations at 12h BHB infusion. (A) Insulin at 12h in mice infused with either NaCl or BHB **(B)** Blood Glucose level at 12h in mice infused with either NaCl or BHB **(C)** Blood Lactate level at 12h in mice infused with either NaCl or BHB **(D)** Intraperitoneal insulin tolerance test performed at 12h of BHB infusion, and Area under curve (AUC) of plasma glucose levels during ITT. **(E)** Glucagon levels 60min following an insulin induced hypoglycemia. **(F)** Intraperitoneal pyruvate tolerance test performed at 6h of BHB infusion, and Area under curve of plasma glucose levels during the pyruvate tolerance test. **(G)** Liver PEPCK mRNA expression at 12h in mice infused with either NaCl or BHB. **(H)** Liver G6Pase mRNA expression at 12h in mice infused with either NaCl or BHB.**(I)** Liver Glycogen content determined as the glucose produced by glycogen hydrolysis at 12h in mice infused with either NaCl or BHB. n = 8-24 animals per condition and were statistically analyzed with an unpaired Student *t*-test (with Welch's correction for lactatemia). a.u., arbitrary units.

Figure 5. MCT1, MCT2 and MCT4 protein expression in hypothalamus and cortex following intracarotid BHB infusion. (A) Hypothalamic MCT1 protein expression at 6h and 12h in mice infused with either NaCl or BHB. Upper panels are representative Western Blots. Lower panel provides the quantification of MCT1 protein expression. **(B)** Hypothalamic MCT2 protein expression at 6h and 12h in mice infused with either NaCl or BHB. Upper panels are representative Western Blots. Lower panel provides the quantification of MCT2 protein expression. **(C)** Hypothalamic MCT4 protein expression at 6h and 12h in mice infused with either NaCl or BHB. Upper panels are representative Western Blots. Lower panel provides the quantification of MCT4 expression. **(D)** Cortical MCT1 protein expression at 6h and 12h in mice infused with either NaCl or BHB. Upper panels are representative Western Blots. Lower panel provides the quantification of MCT12 protein expression. **(E)** Cortical MCT2 protein expression at 6h and 12h in mice infused with either NaCl or BHB. Upper panels are representative Western Blots. Lower panel provides the quantification of MCT2 protein expression. **(F)** Cortical MCT4 protein expression at 6h and 12h in mice infused with either NaCl or BHB. Upper panels are representative Western Blots. Lower panel provides the

quantification of MCT4 protein expression. MCTs protein levels in BHB groups are expressed as the percentage of the corresponding NaCl treated group (set at 100%). B-tubulin was used as internal reference. Data represent the mean ± SEM with n = 7 animals per condition and were statistically analyzed with unpaired Student *t*-test.

Figure 6. Effect of intracarotid BHB infusion on the mRNA expression of ketogenic enzymes in the hypothalamus and the liver. (A) Hepatic HMG CoA synthase mRNA expression at 6h and 12h in mice infused with either NaCl or BHB **(B)** Hypothalamic HMG CoA synthase mRNA expression at 6h and 12h in mice infused with either NaCL or BHB **(C)** Hepatic BHBDH mRNA expression at 6h and 12h in mice infused with either NaCl or BHB. **(D)** Hypothalamic BHB dehydrogenase (BHBDH) mRNA expression at 6h and 12h in mice infused with either NaCl or BHB Data represent the mean ± SEM with n = 9-12 animals per condition and were statistically analyzed with an unpaired Student *t*-test (with Welch's correction for hypothalamic HMG CoA synthase 6h). a.u., arbitrary units.

Figure 7. Schematic representation of the main transient metabolic and behavioral alterations induced by intracarotid BHB infusion.

Supplementary Table 1

Name	**Forward sequence**	**Reverse sequence**
NPY	ATGCTAGGTAACAAGCGAATGG	TGTCGCAGAGCGGAGTAGTAT
AgRP	ATGCTGACTGCAATGTTGCTG	CAGACTTAGACCTGGGAACTCC
CART	CCCGAGCCCTGGACATCTA	GCTTCGATCTGCAACATAGCG
POMC	ATGCCGAGATTCTGCTACAGT	TCCAGCGAGAGGTCGAGTTT
BHBDH	TGCAACAGTGAAGAGGTGGAGAAG	CAAACGTTGAGATGCCTGCGTTGT
HMGcs2	TGGTTCAAGACAGGGACACAGAAC	AGAGGAATACCAGGGCCCAACAAT
PEPCK	GGCCCCGGGAGTCACCATCA	TGCCGAAGTTGTAGCCGAAGAAGG
G6Pase	AACGTCTGTCTGTCCCGGATCTAC	ACCTCTGGAGGCTGGCATTG
β-2 Microglobulin	CCCCACTGAGACTGATACATACG	CGATCCCAGTAGACGGTCTTG

Figure 1

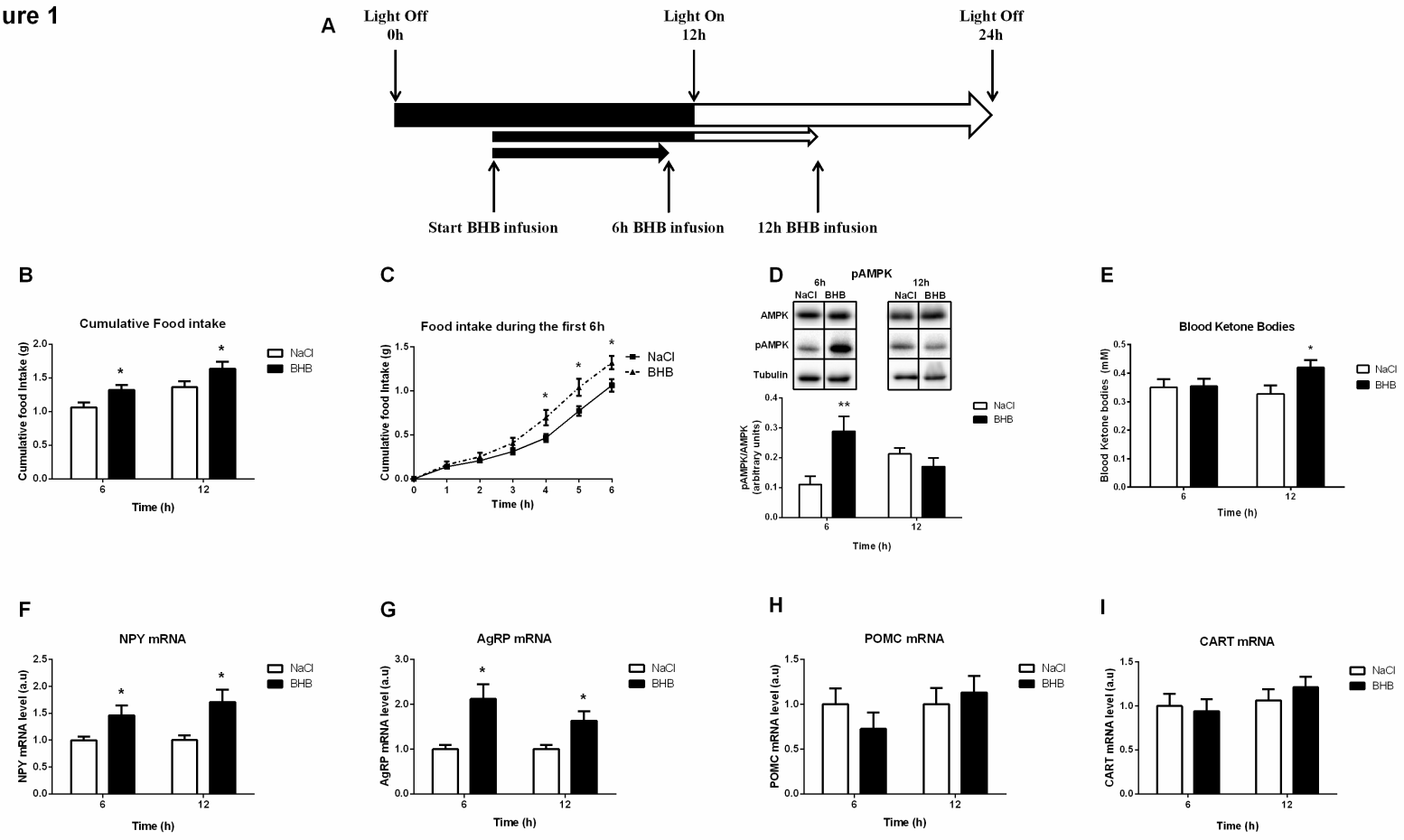
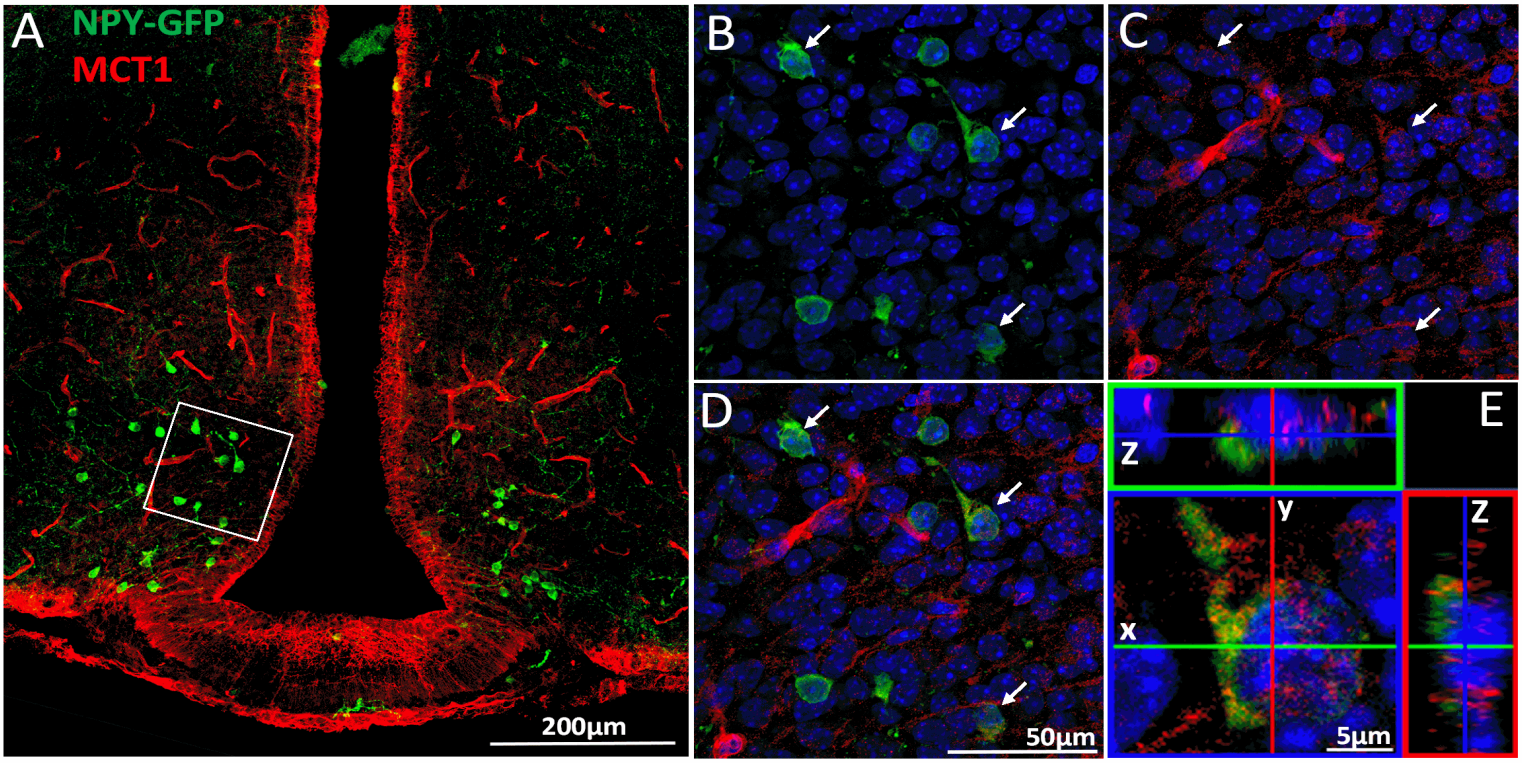
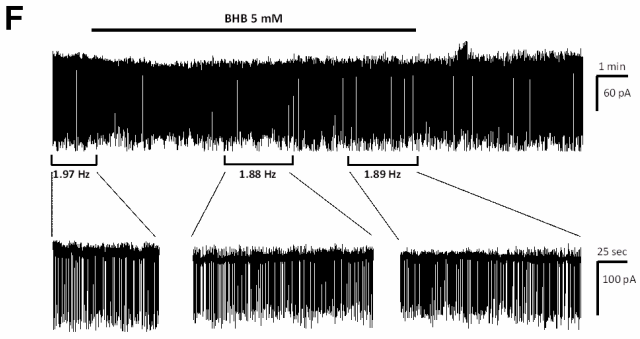

A
Light Off
0h
Light On
12h
Light Off
24h
Start BHB infusion
6h BHB infusion
12h BHB infusion
B
Cumulative Food intake
Cumulative food Intake (g)
Time (h)
NaCl
BHB
C
Food intake during the first 6h
Cumulative food Intake (g)
Time (h)
NaCl
BHB
D
pAMPK
6h
12h
NaCl
BHB
AMPK
pAMPK
Tubulin
pAMPK/AMPK (arbitrary units)
Time (h)
E
Blood Ketone Bodies
Blood Ketone bodies (mM)
Time (h)
F
NPY mRNA
NPY mRNA level (a.u)
Time (h)
G
AgRP mRNA
AgRP mRNA level (a.u)
Time (h)
H
POMC mRNA
POMC mRNA level (a.u)
Time (h)
I
CART mRNA
CART mRNA level (a.u)
Time (h)

Figure 2


A
NPY-GFP
MCT1
200µm
B
C
D
50µm
E
z
y
x
z
5µm


F
BHB 5 mM
1 min
60 pA
1.97 Hz
1.88 Hz
1.89 Hz
25 sec
100 pA

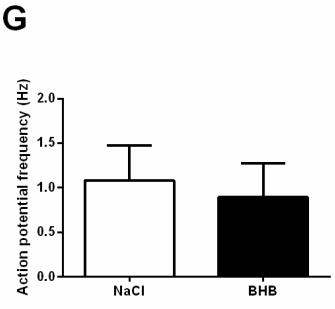

G
Action potential frequency (Hz)
2.0
1.5
1.0
0.5
0.0
NaCl
BHB

Figure 3

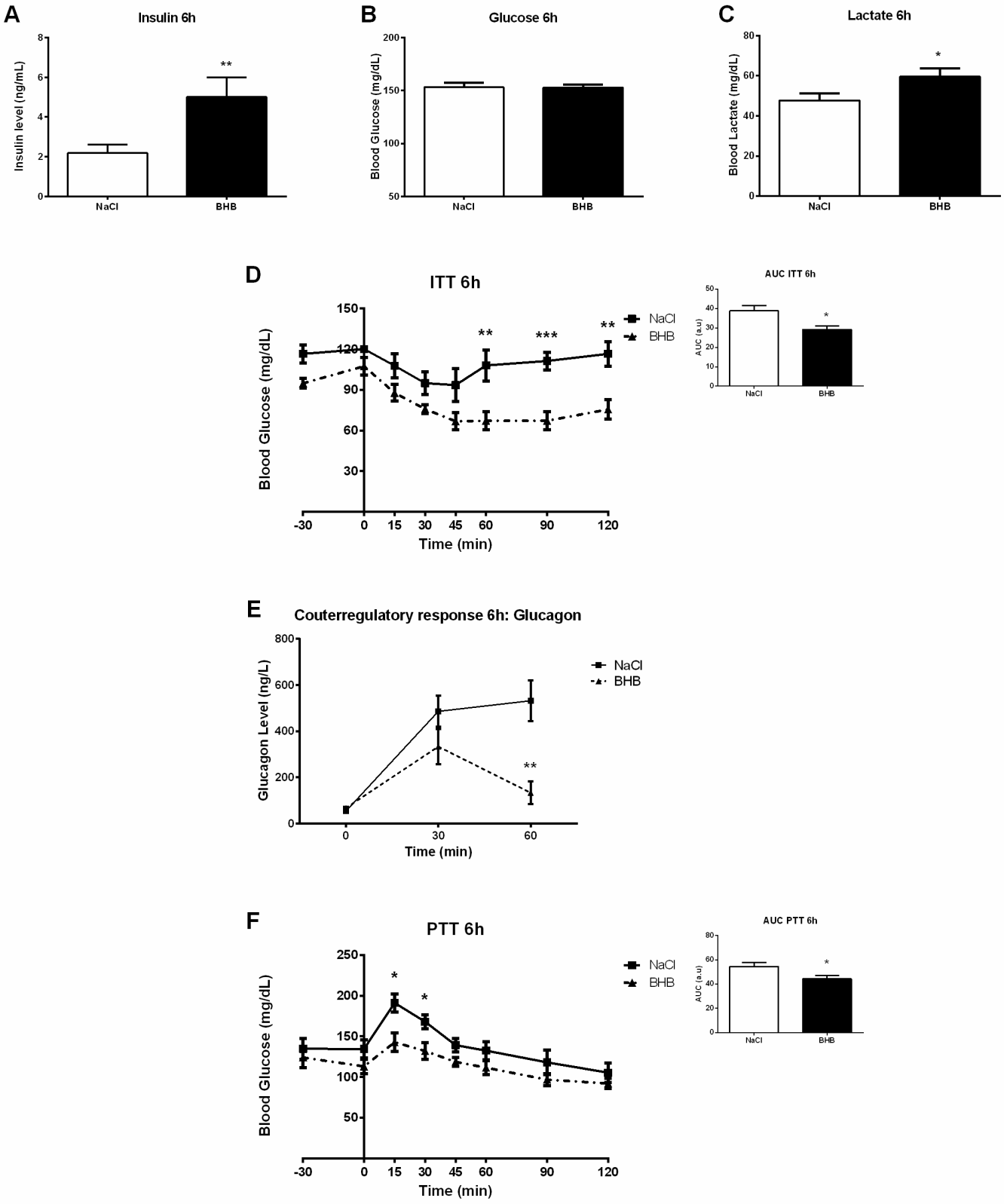

A
Insulin 6h
Insulin level (ng/mL)
NaCl
BHB
B
Glucose 6h
Blood Glucose (mg/dL)
C
Lactate 6h
Blood Lactate (mg/dL)
D
ITT 6h
Blood Glucose (mg/dL)
Time (min)
AUC ITT 6h
AUC (a.u)
E
Couterregulatory response 6h: Glucagon
Glucagon Level (ng/L)
F
PTT 6h
AUC PTT 6h
G
Liver PEPCK mRNA 6h
mRNA level (a.u)
H
Liver G6Pase mRNA 6h
I
Liver glycogen 6h
Glycogen content (µg/mg of liver)

Figure 4

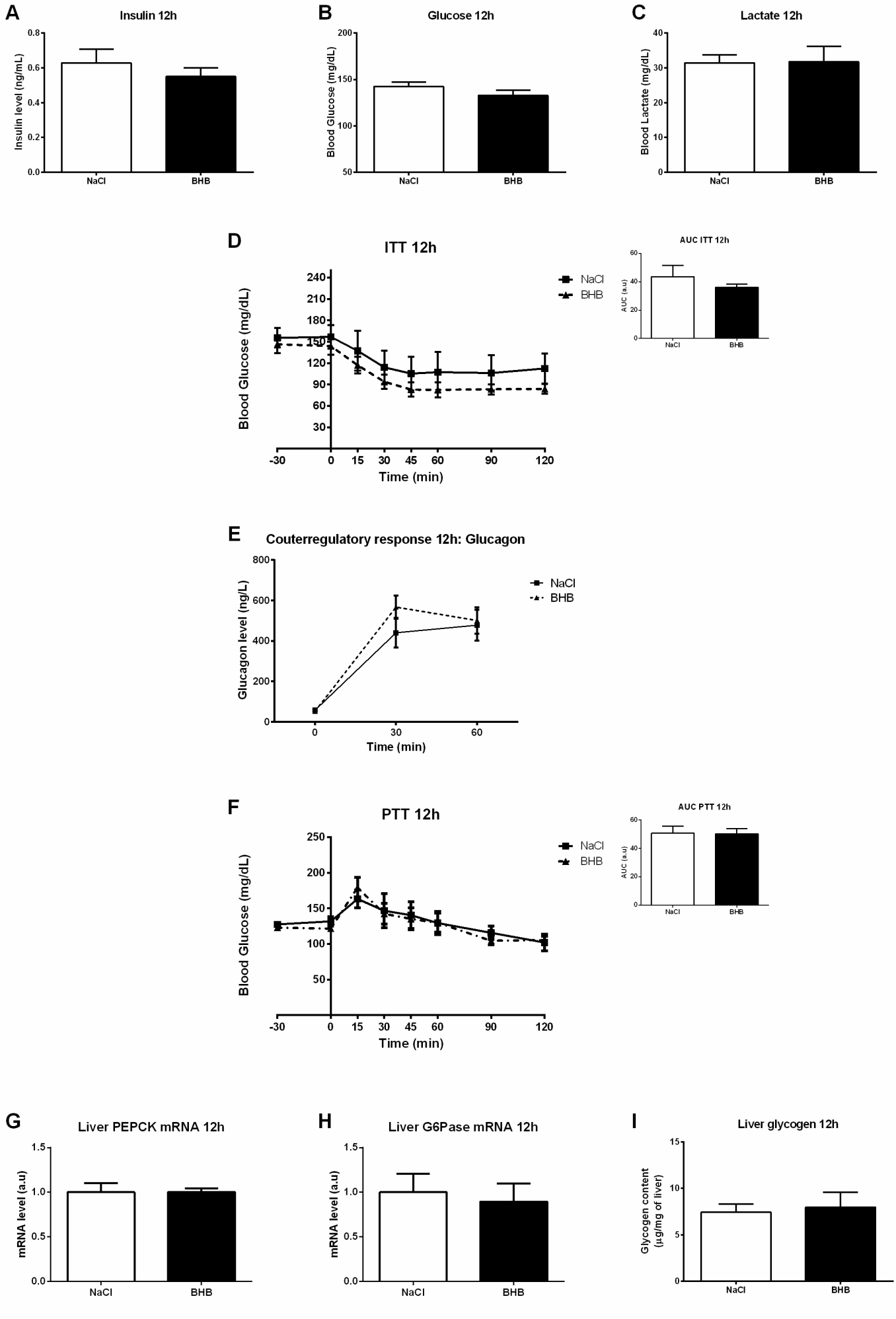

A
Insulin 12h
Insulin level (ng/mL)
0.0
0.2
0.4
0.6
0.8
NaCl
BHB
B
Glucose 12h
Blood Glucose (mg/dL)
50
100
150
200
NaCl
BHB
C
Lactate 12h
Blood Lactate (mg/dL)
0
10
20
30
40
NaCl
BHB
D
ITT 12h
Blood Glucose (mg/dL)
30
60
90
120
150
180
210
240
-30
0
15
30
45
60
90
120
Time (min)
NaCl
BHB
AUC ITT 12h
AUC (a.u)
0
20
40
60
NaCl
BHB
E
Couterregulatory response 12h: Glucagon
Glucagon level (ng/L)
0
200
400
600
800
0
30
60
Time (min)
NaCl
BHB
F
PTT 12h
Blood Glucose (mg/dL)
50
100
150
200
250
-30
0
15
30
45
60
90
120
Time (min)
NaCl
BHB
AUC PTT 12h
AUC (a.u)
0
20
40
60
NaCl
BHB
G
Liver PEPCK mRNA 12h
mRNA level (a.u)
0.0
0.5
1.0
1.5
NaCl
BHB
H
Liver G6Pase mRNA 12h
mRNA level (a.u)
0.0
0.5
1.0
1.5
NaCl
BHB
I
Liver glycogen 12h
Glycogen content (µg/mg of liver)
0
5
10
15
NaCl
BHB

Figure 5

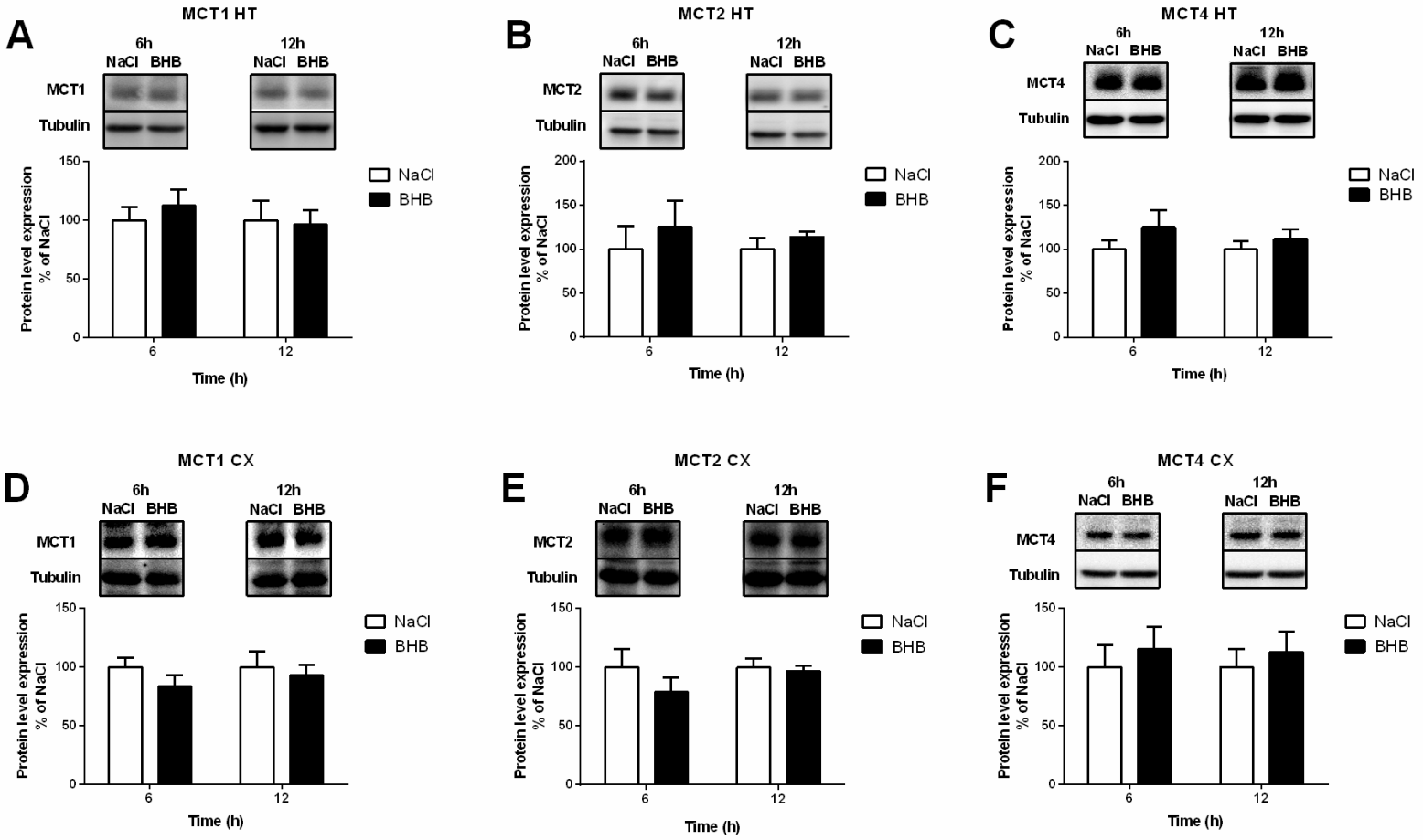

A
MCT1 HT
6h
12h
NaCl BHB
NaCl BHB
MCT1
Tubulin
Protein level expression
% of NaCl
150
100
50
0
6
12
Time (h)
NaCl
BHB
B
MCT2 HT
MCT2
Tubulin
200
150
100
50
0
C
MCT4 HT
MCT4
Tubulin
D
MCT1 CX
E
MCT2 CX
F
MCT4 CX

Figure 6

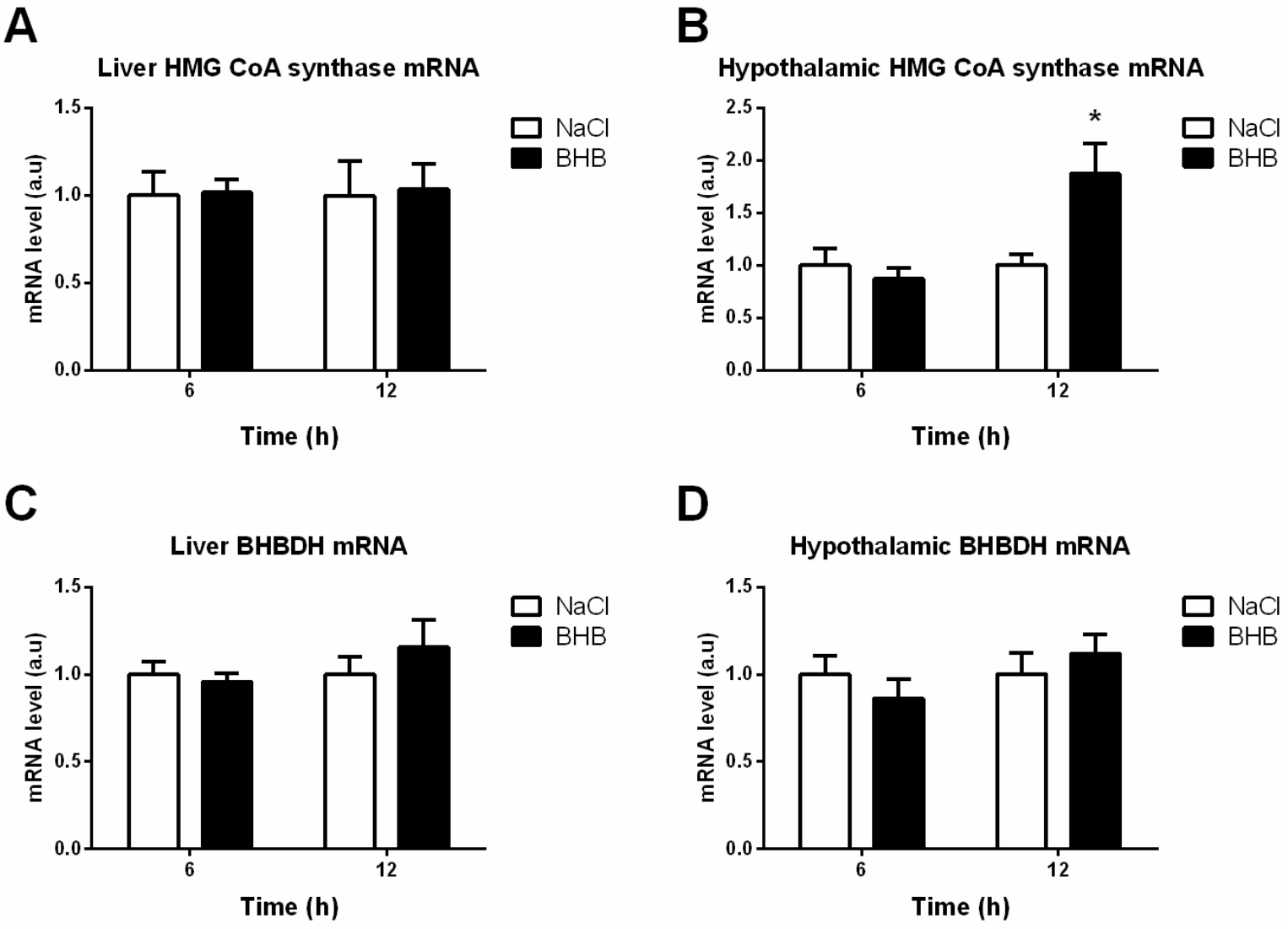
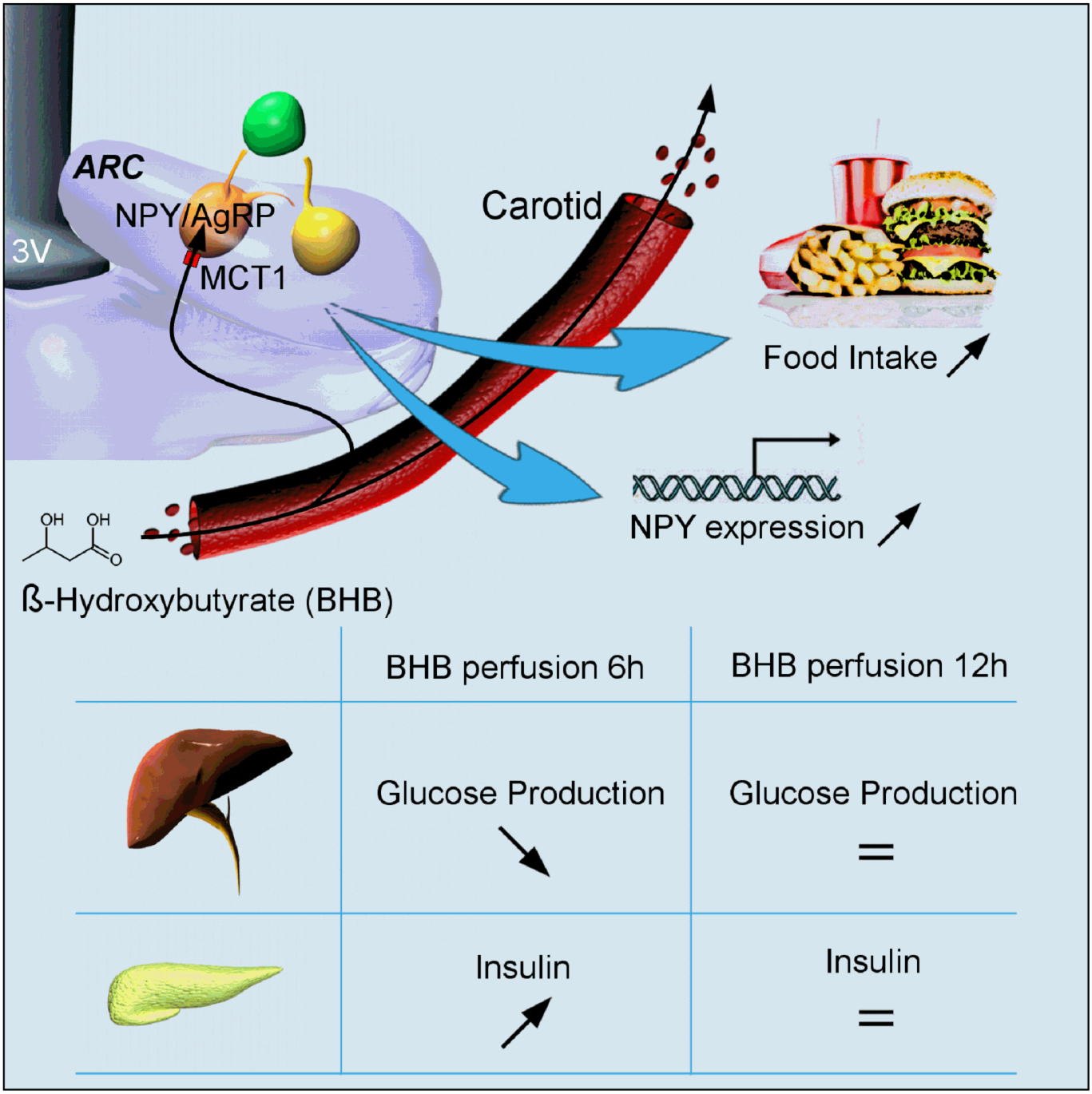

A
Liver HMG CoA synthase mRNA
mRNA level (a.u)
1.5
1.0
0.5
0.0
6
12
Time (h)
NaCl
BHB
B
Hypothalamic HMG CoA synthase mRNA
mRNA level (a.u)
2.5
2.0
1.5
1.0
0.5
0.0
*
6
12
Time (h)
NaCl
BHB
C
Liver BHBDH mRNA
mRNA level (a.u)
1.5
1.0
0.5
0.0
6
12
Time (h)
NaCl
BHB
D
Hypothalamic BHBDH mRNA
mRNA level (a.u)
1.5
1.0
0.5
0.0
6
12
Time (h)
NaCl
BHB

Figure 7


ARC
NPY/AgRP
MCT1
3V
Carotid
Food Intake
NPY expression
OH
OH
O
ß-Hydroxybutyrate (BHB)
BHB perfusion 6h
BHB perfusion 12h
Glucose Production
Glucose Production
=
Insulin
Insulin
=