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

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

3

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6

7 **Running Head:** Hypothalamic ketone bodies sensing and energy homeostasis

8

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28 **ABSTRACT**

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

45 **Keywords:** Energy homeostasis, Monocarboxylate transporters,  $\beta$ -hydroxybutyrate, Obesity,  
46 Glucose homeostasis

47

48

## 49 INTRODUCTION

50 Dysfunction in both cerebral detection of nutrients and integration of circulating  
51 signals has been implicated in the pathogenesis of obesity and associated disorders (11). For  
52 this reason, numerous studies have explored the possible role of nutrient and endocrine  
53 sensing of hypothalamic brain areas and their involvement in energy homeostasis regulation  
54 (34). The most studied circulating energy substrate is glucose which represents a critical  
55 nutrient monitored by the brain. As the main energy source for brain cells, glucose also plays  
56 an important role in brain energy homeostasis (33). However, evidence have been provided  
57 showing that the brain can use alternative energy substrates. For instance, fatty acids and  
58 ketone bodies significantly contribute to fulfill brain energy needs under specific conditions  
59 (6, 13, 36). Despite the fact that it has been known for decades that cerebral ketone bodies  
60 utilization increases under particular metabolic conditions (13), central ketone bodies  
61 detection has been poorly studied.

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

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

81 exclusively on the brain (subcutaneous injection, ketogenic diets). Indeed, since ketone bodies  
82 are mainly produced in the liver, they must cross the blood-brain barrier in order to reach the  
83 brain and exert their central effects. ICV administration bypasses this physiological route. In  
84 addition, intraperitoneal injections or ketogenic diets affect peripheral organs as well. Finally,  
85 the direct central effect of ketone bodies sensing alone could not be investigated through these  
86 strategies, as ketone bodies are certainly sensed by other tissues. Altogether, these previous  
87 studies suggest an important role of ketone bodies detection (both at central and peripheral  
88 levels) in the regulation of energy homeostasis. However, they were not intended to decipher  
89 specifically the role of ketone bodies themselves in food intake control as many other  
90 parameters were modified in parallel such as fatty acid levels or blood glucose for instance.  
91 Consequently, as described in a recent review from Paoli et al., these different studies gave  
92 rise to contradictory results regarding orexigenic vs. anorexigenic effects (30). On one hand, a  
93 majority of the studies were performed through exposure to a ketogenic diet and seem to  
94 indicate that such diets are associated with decreased hunger and appetite reduction (16, 17,  
95 28, 29, 41, 42). Moreover, a recent study described a role of astrocytic ketone bodies and fatty  
96 acids as inhibitors of food intake during short-term high energy diet (23). In contrast,  
97 ketogenic diets have been shown to induce the activation of AMPK in brain which is  
98 associated with an increased food intake suggesting that brain sensing of ketone bodies could  
99 have a positive action on food intake behavior (27). Notwithstanding, ketogenic diets cause  
100 numerous peripheral effects and alter levels of other nutrients, making it impossible to reveal  
101 the unique role of ketone bodies in brain control of food intake.

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

## 108 **MATERIAL and METHODS**

### 109 *Animals*

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

113 infusion in unrestrained mice was done as described previously in rat and adapted for mice  
114 (26). Briefly, after pentobarbital anesthesia (50 mg/kg), a silicone tubing (internal diameter  
115 0.31 mm, external diameter 0.64 mm) was inserted in the right carotid and pushed 5 mm in  
116 the cranial direction. The catheter was led subcutaneously in the middle of the neck between  
117 the 2 blades and externalized for further connection to the infusion pump. Mice were then  
118 housed 1 week for surgical recovery, before starting the infusion experiments. During the  
119 experiments mice were connected to an external infusion pump and NaCl or BHB (adjusted  
120 pH at 7.4) was infused at 30  $\mu$ L/h for either 6 or 12h. All procedures involving mice followed  
121 the European Communities Council Directive (86/609/EEC) and were approved by a local  
122 committee.

123

#### 124 *Immunohistochemistry*

125 Anesthetized NPY-GFP mice were intra-cardiacally perfused with 10ml heparin-PBS  
126 (50U/ml) and 10ml of 4% paraformaldehyde. Brains were then removed and preserved in  
127 30% sucrose at 4°C, embedded in Tissue freezing medium (Jung, Nussloch, Germany) and  
128 frozen in dry ice before sectioning. Three sets of coronal sections (30 $\mu$ m thick) were cut on a  
129 cryostat (Leica Biosystems CM 3050S, Dusseldorf, Germany). After three PBS rinses  
130 sections were incubated 20 minutes at room temperature (RT) in 2% donkey normal serum  
131 (Sigma, Buchs, Switzerland) in PBS 0.3% Triton X-100. Sections were incubated with rabbit  
132 anti-MCT1 (1:500)(38) and goat anti-GFP (1:1000, SicGen, Carcavelos, Portugal) two days at  
133 4°C. After three PBS rinses, sections were incubated with secondary antibodies (Jackson  
134 ImmunoResearch, Suffolk, UK) anti-goat IgG(H+L) Cy3 (1:500) and anti-rabbit IgG Fab  
135 Alexa594 (1:500) for 150 minutes at room temperature. Sections were incubated two minutes  
136 in 1  $\mu$ g/ml Hoechst 33342 (Invitrogen, Lucerne, Switzerland)) to label nuclei, mounted onto  
137 gelatin-coated glass slides and coverslip with Fluoromount (Sigma). Acquisitions and  
138 quantifications were performed on a laser confocal microscope (Zeiss LSM 700, Zeiss,  
139 Feldbach, Switzerland) with x40 and x63 objectives and using ZEN software.

#### 140 *Feeding test*

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

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

152 *Pyruvate and Insulin tolerance tests*

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

157 *RNA extraction, reverse transcription and quantitative real time PCR*



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

#### 176 *Blood analysis*

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

#### 184 *Liver glycogen measurement*

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

190 deposited in 96-well plates with 200 $\mu$ l of a RTU (Ready to Use) buffer (BioMerieux, Geneve,  
191 Switzerland), incubated at room temperature for 20min. The optical density was read at  
192 505nm by spectrophotometry. The difference between conditions with amyloglucosidase and  
193 conditions with buffer only represented the glycogen content of the liver sample. Glycogen  
194 was expressed as milligrams of glucose resulting from glycogen hydrolysis per gram of tissue.

#### 195 *Western Blot analysis*

196 Proteins were separated with 10% SDS-PAGE. Antibodies against MCT1, MCT2, MCT4,  
197 and  $\beta$ -Tubulin were used. After transfer and blocking, membranes were probed in 1% nonfat  
198 milk prepared in TBS-T with 1/1,000 rabbit anti-MCT1, anti-MCT2 (38), 1/500 anti-MCT4  
199 (Santa Cruz, Heidelberg, Germany), 1/1000 for AMPK and pAMPK (Cell Signaling,  
200 Beverly, MA, USA) and 1/10000 rabbit anti- $\beta$ -Tubulin (Cell Signaling, Beverly, MA, USA)  
201 overnight at 4°C. Specific band for each protein was detected using a goat anti-rabbit  
202 (1/10,000 in TBST-1X) peroxidase-conjugated secondary antibody (GE Healthcare,  
203 Piscataway, NJ, USA) incubated for 1 hour at room temperature. Bands were revealed with a  
204 chemiluminescence kit (BioRad, Reinach, Switzerland) and processed with a ChemiDoc XRS  
205 + system (BioRad, Reinach, Switzerland) for densitometry analysis.

#### 206 *Electrophysiology*

207 Brain slices (250 $\mu$ m) were prepared from adult NPY-GFP mice (6-8 weeks old) as previously  
208 described (7). Slices were incubated at room temperature (RT), in oxygenated extracellular  
209 medium containing (in mM): 118 NaCl, 3 KCl, 1 MgCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 1.5  
210 CaCl<sub>2</sub>, 5 HEPES, 2.5 D-glucose (osmolarity adjusted to 310mOsM with sucrose, pH 7.3) for a  
211 recovery period (at least 60minutes). Once in the recording chamber, slices were perfused at  
212 2-3 ml/min with the same extracellular medium. Slices were viewed with a Nikon microscope  
213 (EF600) outfitted for fluorescence (fluorescein filter) and IR-DIC (Infrared-Differential  
214 interference contrast) videomicroscopy. Viable arcuate NPY neurons were visualized using a  
215 X60 water immersion objective (Nikon) with a fluorescence video camera (Nikon).  
216 Borosilicate pipettes (4-6M $\Omega$ ; 1.5mm OD, Sutter Instrument) were filled with filtered  
217 extracellular medium. Cell-attached recordings were made using a Multiclamp 700B  
218 amplifier, digitized using the Digidata 1440A interface and acquired at 3kHz using pClamp  
219 10.3 software (Axon Instruments). Pipettes and cell capacitances were fully compensated.  
220 After a stable baseline was established, BHB (5mM) was perfused for 10minutes. The firing

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

### 223 *Statistical analysis*

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

## 229 **RESULTS**

### 230 *Food intake and hypothalamic neuropeptide mRNA expression in mice with intracarotid* 231 *infusion of $\beta$ -hydroxybutyrate (BHB).*

232 Food intake measurement shows a significant increase in the amount of pellets ingested after  
233 6 and 12 hours of intracarotid infusion in mice receiving  $\beta$ -hydroxybutyrate compared to  
234 those receiving NaCl (1.06  $\pm$  0.07g for the NaCl group vs. 1.32  $\pm$  0.08g for the BHB group at  
235 6h, *p*=0.016; 1.40  $\pm$  0.09g for the NaCl group vs 1.62  $\pm$  0.10g for the BHB group at 12h,  
236 *p*=0.047) (Figure 1B). Moreover, a closer analysis of the first 6 hours of infusion reveals that  
237 food intake began to be significantly stimulated only after 4 hours of infusion in the BHB  
238 group compared to NaCl infused mice (0.47  $\pm$  0.04g for the NaCl group vs. 0.70  $\pm$  0.09g for  
239 the BHB group at 4h, *p*=0.025; 0.77  $\pm$  0.05g for the NaCl group vs. 1.04  $\pm$  0.10g for the BHB  
240 group at 5h, *p*=0.020) (Figure 1C). Interestingly, circulating ketone bodies levels remained  
241 unchanged at 6 hours (0.33  $\pm$  0.03mM in NaCl group vs 0.42  $\pm$  0.03mM in BHB group, *n*=15  
242 in each group, *p*=0.026) while it increased at 12 hours, demonstrating that the effect observed  
243 at 6 hours can be due only to ketone bodies acting at the cerebral level (Figure 1E). In  
244 accordance with food intake stimulation, the measurement of the pAMPK/AMPK ratio shows  
245 an increased activation at 6 hours only (0.11  $\pm$  0.03 a.u. for the NaCl group vs. 0.29  $\pm$  0.05  
246 for the BHB group at 6h, *p* = 0.009) (Figure 1D. Analysis of the mRNA levels of the  
247 hypothalamic neuropeptides involved in food intake regulation revealed an increase for the  
248 orexigenic neuropeptide NPY (1.0  $\pm$  0.07 a.u. for the NaCl group vs. 1.46  $\pm$  0.18 a.u. for the  
249 BHB group at 6h, *p* = 0.029; 1.0  $\pm$  0.08 a.u. for the NaCl group vs. 1.71  $\pm$  0.21 a.u. for the  
250 BHB group at 12h, *p* = 0.012) (Figure 1F) and AgRP (1.0  $\pm$  0.09 a.u. for the NaCl group vs  
251 2.12  $\pm$  0.33 a.u. for the BHB group at 6h, *p* = 0.005; 1.0  $\pm$  0.08 a.u. for the NaCl group vs.

252 1.63 ± 0.20 a.u. for the BHB group at 12h, p = 0.005) (Figure 1G). In contrast, mRNA  
253 expression of the antagonistic (and thus anorexigenic) neuropeptides POMC and CART were  
254 not altered during BHB infusion (Figure 1H and 1I, respectively).

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

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

265 *Metabolic characteristics after 6 hours of BHB infusion*

266 First, different blood parameters were measured and a twofold increase in insulin level was  
267 evidenced (2.20 ± 1.20 ng/mL for the NaCl group vs 5.02 ± 2.39 ng/mL for the BHB group, p  
268 = 0.01, n = 8 NaCl and 6 BHB infused mice) (Figure 3A). Despite such an increase in insulin  
269 levels, blood glucose concentration remained unaffected in the BHB group (Figure 3B) while  
270 blood lactate level was enhanced (Figure 3C). To test whether this apparent normoglycemia  
271 despite an increased insulin level could be due to decreased insulin sensitivity, an insulin  
272 tolerance test was performed. However, the results shows that BHB infused mice exhibited  
273 instead a higher sensitivity to insulin as shown by the lower glucose concentration induced by  
274 insulin injection (Figure 3D) (Area Under Curve (AUC) of 38.76 ± 2.86 for the NaCl group  
275 vs 29.19 ± 1.80 for the BHB group, n = 7 for each group, p = 0.02, (Figure 3D, upper panel)).  
276 These results rather suggested a putative defect in the counter-regulatory response to  
277 hypoglycemia, i.e. of the glucagon release in response to hypoglycemia induced by an insulin  
278 load. Indeed, glucagon concentrations were found to be much lower in BHB infused mice  
279 compared to the control group at 60 minutes after the insulin induced hypoglycemia (532 ± 88  
280 ng/L for the NaCl group vs 134 ± 50 ng/L for the BHB group, n=6 for each group, p = 0.003)  
281 (Figure 3E).

282 As blood glucose level could also be influenced by neoglucogenesis, a pyruvate tolerance test  
283 was performed and results showed it was reduced in BHB infused mice (Figure 3F) (AUC of  
284  $54.50 \pm 3.34$  for the NaCl group vs  $44.27 \pm 2.82$  for the BHB group,  $n=5$  for each group,  
285  $p=0.047$ , (Figure 3F, upper panel)). In accordance, mRNAs of the main enzymes involved in  
286 neoglucogenesis, PEPCK and G6Pase, were both decreased in the liver, one of the major sites  
287 of neoglucogenesis ( $1.00 \pm 0.27$  a.u for the NaCl group vs  $0.29 \pm 0.03$  for the BHB group,  
288  $n=10$  for each group,  $p=0.017$  for PEPCK and  $1.00 \pm 0.19$  a.u for the NaCl group vs  $0.50 \pm$   
289  $0.08$  a.u for the BHB group,  $n=10$  for each group,  $p=0.026$  for G6Pase, (Figure 3G and H)).  
290 Moreover, the observed increase in lactate level ( $47.67 \pm 3.49$  mg/dL for the NaCl group vs  
291  $59.50 \pm 4.25$  mg/dL for the BHB group,  $n=27$  NaCl and 28 BHB mice,  $p=0.037$ ) could be a  
292 consequence of decreased gluconeogenesis (Figure 3C). Another possible contributor to blood  
293 glucose level is hepatic glycogen. Indeed, reduced glycogen levels were found in the BHB  
294 group compared to the control group ( $14.60 \pm 1.39$   $\mu$ g/mg tissue for the NaCl group vs  $9.59 \pm$   
295  $1.11$   $\mu$ g/mg tissue for the BHB group,  $n=11$  for each group,  $p=0.011$ ), suggesting the putative  
296 involvement of hepatic glycogenolysis in the maintenance of glucose homeostasis for BHB  
297 infused animals (Figure 3I).

#### 298 *Metabolic characteristics after 12 hours of BHB infusion*

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

#### 309 *Hypothalamic and cortical MCT expression as well as hepatic and hypothalamic ketogenesis* 310 *after BHB infusion*

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

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

## 323 **DISCUSSION**

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

### 332 **Cerebral ketone bodies perfusion enhances food intake and hypothalamic orexigenic** 333 **peptide expression**

334 To test this hypothesis,  $\beta$ -Hydroxybutyrate (BHB; 20 $\mu$ g/h) was infused through the  
335 carotid for either 6 or 12 hours in order to stimulate brain areas involved in the control of  
336 energy homeostasis located primarily in the hypothalamus. The dose chosen (20 $\mu$ g/h) was  
337 determined after different concentrations were tested ranging from 2 to 200  $\mu$ g/h. A dose of  
338 20 $\mu$ g/h most likely represents a high concentration compared to the concentration measured in  
339 fasting conditions. However, as the infusion is performed through the right carotid, the final  
340 concentration actually permeating the brain will be diluted by the blood coming from the left  
341 carotid and the vertebral arteries. In addition, as the rate of infusion is very low (30  $\mu$ l/h)  
342 compared to the carotidic blood flow (0.75 mL/min), the real BHB concentration reaching the  
343 targeted brain areas should be much lower than the concentration perfused, although it can not  
344 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

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

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



410 **A large subpopulation of hypothalamic NPY neurons expresses the transporter**  
411 **MCT1**

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

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

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

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

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

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

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

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

501 In contrast to the observation made after 6 hours, insulin level was no longer altered  
502 after 12 hours of BHB perfusion (Figure 4A) while blood glucose level was still normal  
503 (Figure 4B). Similarly, the insulin tolerance test showed that insulin sensitivity was not  
504 different between BHB infused animals and control animals (Figure 4D). When the counter-  
505 regulatory response was evaluated, again no difference in glucagon levels (Figure 4E) was  
506 detected. The pyruvate tolerance test did not reveal any difference in gluconeogenesis (Figure  
507 4F) after 12 hours of BHB perfusion. In the liver, both G6Pase and PEPCK mRNA levels

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

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

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

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

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## 568 **AUTHOR CONTRIBUTIONS**

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

## 572 CONFLICT OF INTEREST

573 The authors declare no conflict of interest.

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698

## 699 **FIGURE LEGENDS**

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



707 Hypothalamic AMPKp/AMPK protein expression ratio at 6h and 12h in mice infused with  
708 either NaCl or BHB. Upper panels show bands from representative samples chosen from the  
709 western blots of all samples run in parallel and rearranged to appear side-by-side. Lower panel  
710 provides the quantification of AMPKp/AMPK ratio. **(E)** Blood Ketone bodies concentration  
711 at 6h and 12h in mice infused with either NaCl or BHB **(F)** NPY mRNA expression at 6h and  
712 12h in mice infused with either NaCl or BHB **(G)** AgRP mRNA expression at 6h and 12h in  
713 mice infused with either NaCl or BHB **(H)** POMC mRNA expression at 6h and 12h in mice  
714 infused with either NaCl or BHB **(I)** CART mRNA expression at 6h and 12h in mice infused  
715 with either NaCl or BHB. mRNA expression for each hypothalamic peptide was determined  
716 by quantitative reverse transcriptase–PCR at 6h, 12h and 24h of NaCl or BHB infusion. n = 8-  
717 24 animals per condition and were statistically analyzed with unpaired Student *t*-test (with  
718 Welch’s correction for NPY 6h and 12h, as well as for AgRP 6h and 12h). a.u., arbitrary  
719 units.

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

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

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

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

758 **Figure 5. MCT1, MCT2 and MCT4 protein expression in hypothalamus and cortex**  
759 **following intracarotid BHB infusion. (A)** Hypothalamic MCT1 protein expression at 6h and  
760 12h in mice infused with either NaCl or BHB. Upper panels are representative Western Blots.  
761 Lower panel provides the quantification of MCT1 protein expression. **(B)** Hypothalamic  
762 MCT2 protein expression at 6h and 12h in mice infused with either NaCl or BHB. Upper  
763 panels are representative Western Blots. Lower panel provides the quantification of MCT2  
764 protein expression. **(C)** Hypothalamic MCT4 protein expression at 6h and 12h in mice infused  
765 with either NaCl or BHB. Upper panels are representative Western Blots. Lower panel  
766 provides the quantification of MCT4 expression. **(D)** Cortical MCT1 protein expression at 6h  
767 and 12h in mice infused with either NaCl or BHB. Upper panels are representative Western  
768 Blots. Lower panel provides the quantification of MCT12 protein expression. **(E)** Cortical  
769 MCT2 protein expression at 6h and 12h in mice infused with either NaCl or BHB. Upper  
770 panels are representative Western Blots. Lower panel provides the quantification of MCT2  
771 protein expression. **(F)** Cortical MCT4 protein expression at 6h and 12h in mice infused with  
772 either NaCl or BHB. Upper panels are representative Western Blots. Lower panel provides the

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

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

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

788

789

790 **Supplementary Table 1**

Name	Forward sequence	Reverse sequence
NPY	ATGCTAGGTAACAAGCGAATGG	TGTCGCAGAGCGGAGTAGTAT
AgRP	ATGCTGACTGCAATGTTGCTG	CAGACTTAGACCTGGGA ACTCC
CART	CCCGAGCCCTGGACATCTA	GCTTCGATCTGCAACATAGCG
POMC	ATGCCGAGATTCTGCTACAGT	TCCAGCGAGAGGTTCGAGTTT
BHBDH	TGCAACAGTGAAGAGGTGGAGAAG	CAAACGTTGAGATGCCTGCGTTGT
HMGcs2	TGGTTCAAGACAGGGACACAGAAC	AGAGGAATACCAGGGCCCAACAAT
PEPCK	GGCCCCGGGAGTCACCATCA	TGCCGAAGTTGTAGCCGAAGAAGG
G6Pase	AACGTCTGTCTGTCCCGGATCTAC	ACCTCTGGAGGCTGGCATTG
$\beta$ -2 Microglobulin	CCCCACTGAGACTGATACATACG	CGATCCCAGTAGACGGTCTTG

791

**Figure 1**

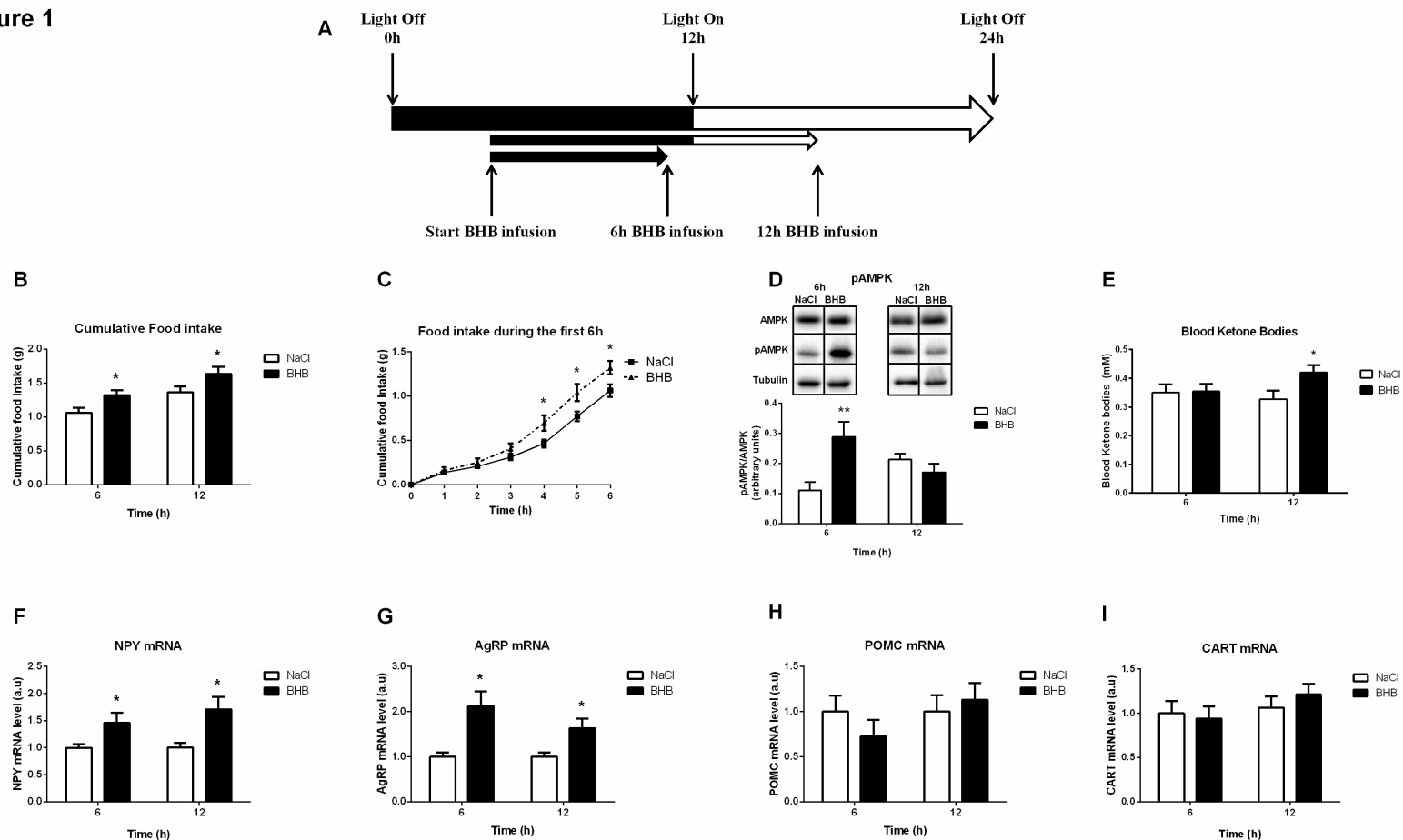
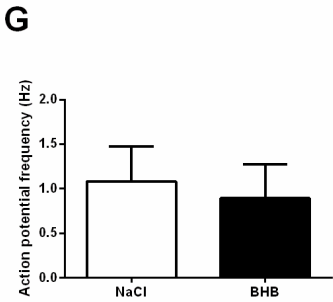
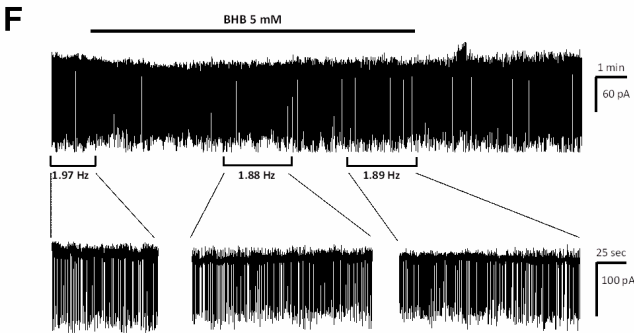
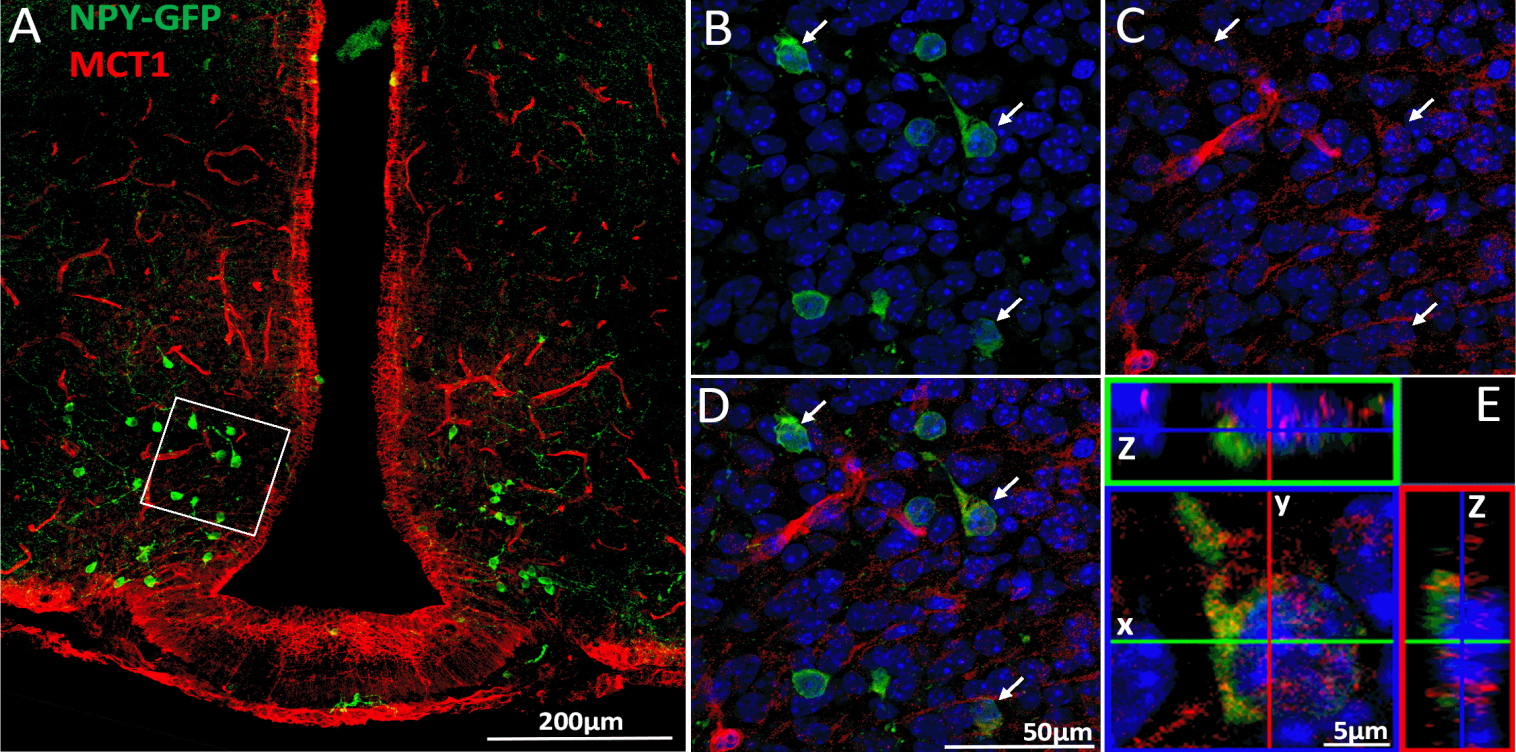
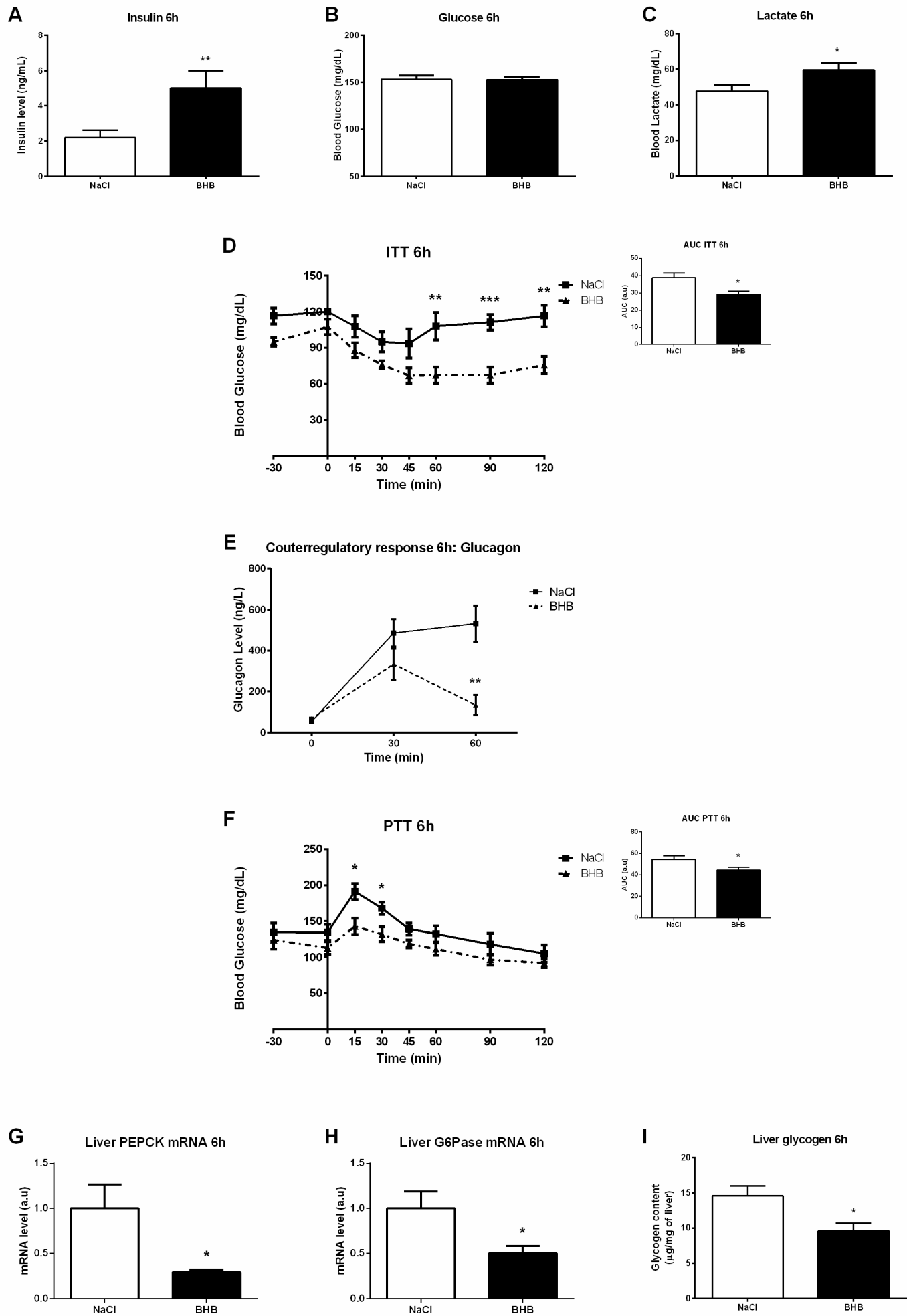


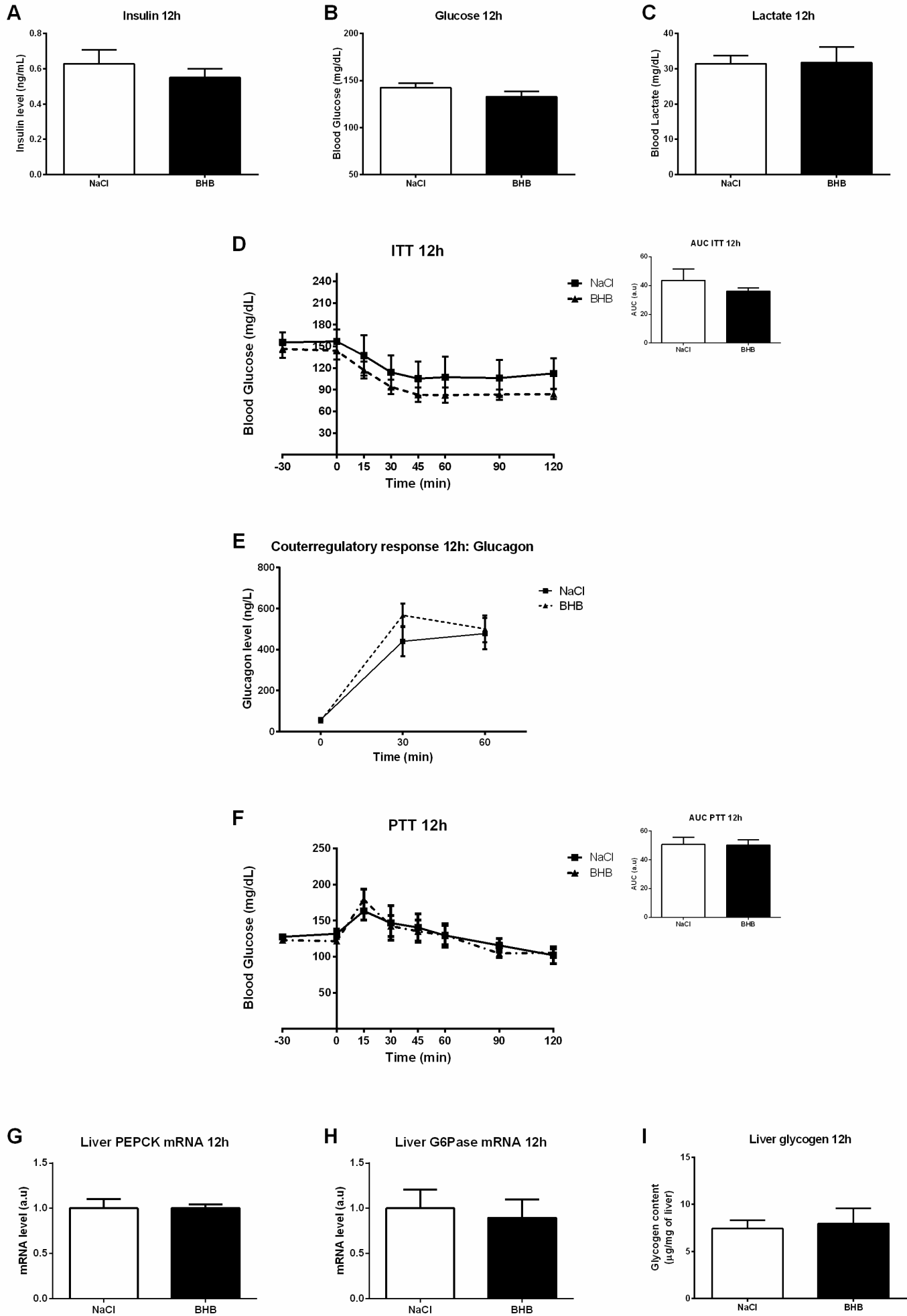
Figure 2



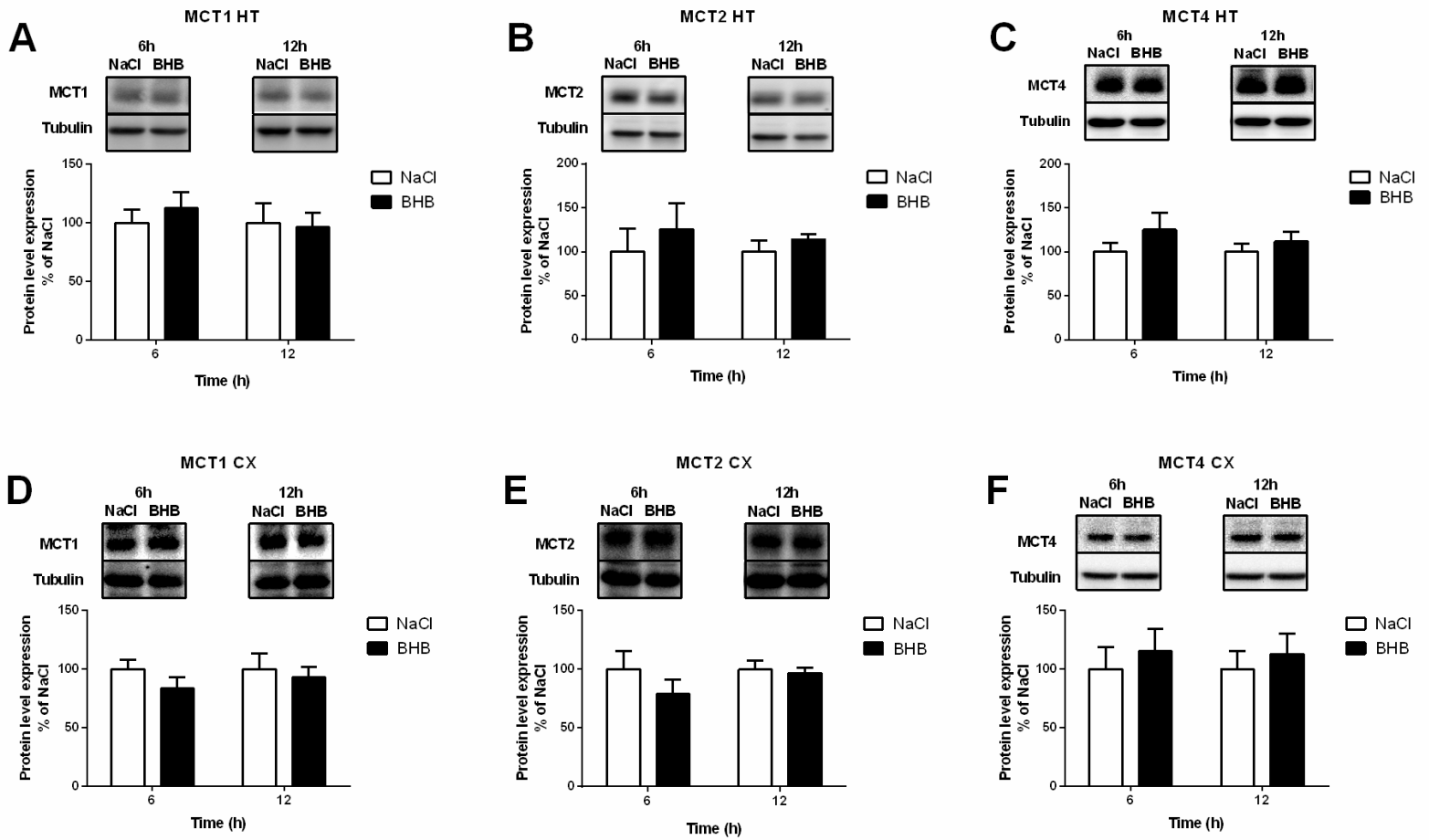
**Figure 3**



**Figure 4**



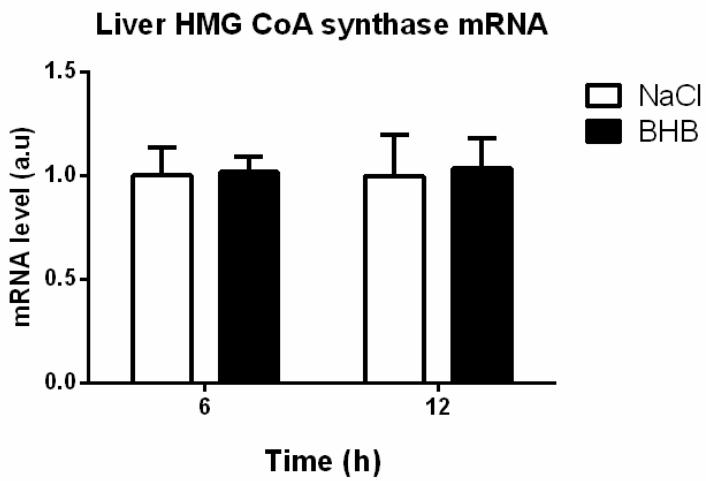
# Figure 5



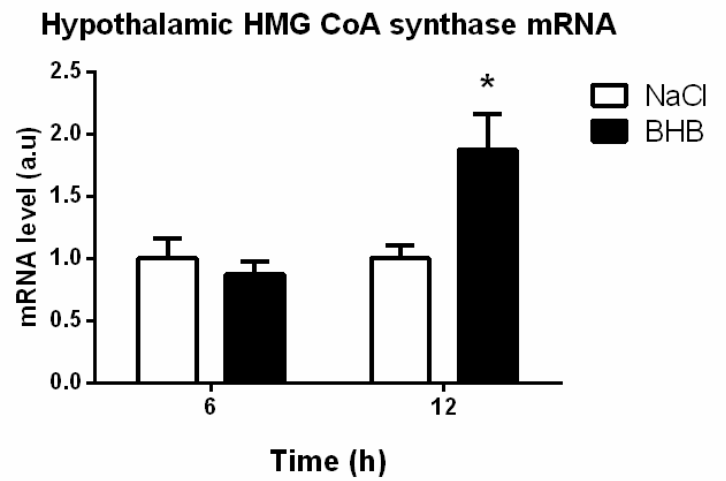


# Figure 6

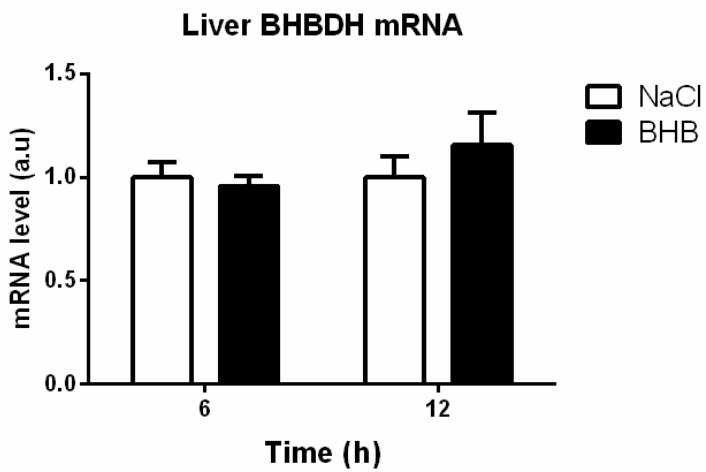
**A**



**B**



**C**



**D**

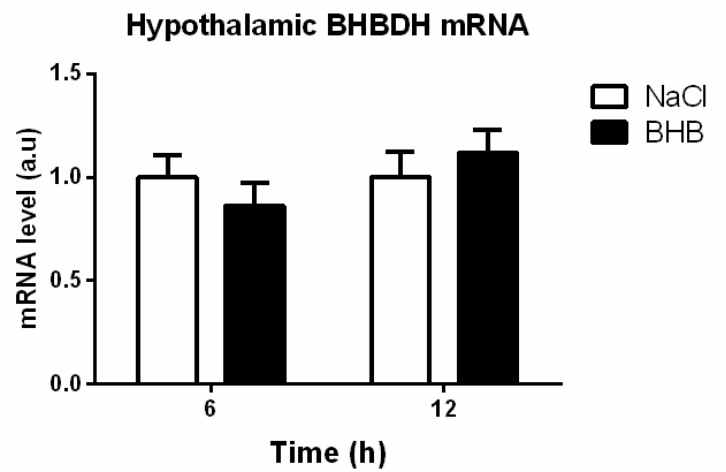


Figure 7

