Reduced metabolism in the hypothalamus of the anorectic anx/anx mouse

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Abstract

The anorectic anx/anx mouse exhibits a mitochondrial complex I dysfunction that is related to aberrant expression of hypothalamic neuropeptides and transmitters regulating food intake. Hypothalamic activity, i.e. neuronal firing and transmitter release, is dependent on glucose utilization and energy metabolism. To better understand the role of hypothalamic activity in anorexia, we assessed carbohydrate and high-energy phosphate metabolism, in vivo and in vitro, in the anx/anx hypothalamus. In the fasted state, hypothalamic glucose uptake in the anx/anx mouse was reduced by ~50% of that seen in wild-type (wt) mice (P < 0.05). Under basal conditions, anx/anx hypothalamus ATP and glucose 6-P contents were similar to those in wt hypothalamus, whereas phosphocreatine was elevated (~2-fold; P < 0.001) and lactate was reduced (~35%; P < 0.001). The anx/anx hypothalamus had elevated total AMPK (~25%; P < 0.05) and GLUT4 (~60%; P < 0.01) protein contents, whereas GLUT1 and GLUT3 were similar to that of wt hypothalamus. Interestingly, the activation state of AMPK (ratio of phosphorylated AMPK/total AMPK) was significantly decreased in hypothalamus of the anx/anx mouse (~60% of that in wt; P < 0.05). Finally, during metabolic stress (ischemia), accumulation of lactate (measure of glycolysis) and IMP and AMP (breakdown products of ATP) were ~50% lower in anx/anx vs wt hypothalamus. These data demonstrate that carbohydrate and high-energy phosphate utilization in the anx/anx hypothalamus are diminished under basal and stress conditions. The decrease in hypothalamic metabolism may contribute to the anorectic behavior of the anx/anx mouse, i.e. its inability to regulate food intake in accordance with energy status.

Key Words
- phosphocreatine
- ATP
- AMPK
- lactate
- glucose uptake
Introduction

Neuronal networks crucial for the regulation of food intake, i.e. satiety and hunger, are located mainly in the hypothalamus. In particular, two populations of neurons in the mediobasal part of the hypothalamus, the arcuate nucleus (Arc), are fundamental in this regulation. These are the agouti-gene-related protein (AGRP) and neuropeptide Y (NPY) expressing neurons that are orexigenic, and the proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) expressing populations that are anorexigenic (Grill & Kaplan 2002, Hillebrand et al. 2002, Morton et al. 2006, Schwartz 2006). Additionally, hypothalamic AMP-dependent protein kinase (AMPK), a sensor of cellular metabolism that responds to changes in energy status (i.e., contents of high energy phosphates), has been implicated in control of feeding, being activated during starvation/food deprivation and inactivated during feeding (Minokoshi et al. 2004, Kahn et al. 2005, Andrews et al. 2008, Ronnett et al. 2009).

Based on its role in the regulation of food intake, the hypothalamus has been extensively studied in an animal model of anorexia, the anx/anx mouse (Son et al. 1994, Broberger et al. 1997, 1998, 1999, Johansen et al. 2000, Fetissov et al. 2005, Lachuer et al. 2005, Mercader et al. 2008a, Nilsson et al. 2008, 2011, Lindfors et al. 2011). The anx mutation arose spontaneously at the Jackson Laboratory in the mid-seventies. The anx/anx mouse is indistinguishable from its normal siblings, i.e. homozygote and heterozygote wild-type (wt) mice, at birth. However, during the first postnatal weeks, they gradually decrease their food intake, develop starvation and emaciation. By three weeks of age, they weigh approximately half as much as wt sibling mice and die around or shortly after this early point in life (Maltais et al. 1984). The serum level of the food intake regulating hormone leptin is reduced in the anx/anx mice (Johansen et al. 2000). Reduced leptin should in accordance to the energy homeostatic mechanisms give rise to increased hunger signaling (Hillebrand et al. 2002, Morton et al. 2006, Schwartz 2006), thus indicating an intrinsic hypothalamic problem in the anx/anx mice underlying their paradoxical response to negative energy balance. Abnormal expression and immunohistochemical labeling pattern for several neuropeptide/transmitter systems in the hypothalamus have been documented (Son et al. 1994, Broberger et al. 1997, 1998, 1999, Johansen et al. 2000, 2007, Fetissov et al. 2005, Nilsson et al. 2008). Additionally, signs of hypothalamic inflammation and degeneration have been shown (Lachuer et al. 2005, Mercader et al. 2008b, Nilsson et al. 2008, 2011). Microglia cells of a morphology indicative of an activated state are dramatically increased exclusively in the hypothalamic AGRP system of these mice, starting from postnatal day 12-15. The activated microglia are present solely in close vicinity to, and occasionally even embracing, the AGRP-expressing neuronal cell bodies in the arcuate nucleus, as well as in the projection areas of these same neurons, e.g. in the paraventricular nucleus of the hypothalamus, the lateral hypothalamic area, the medial preoptic region and the bed nuclei of stria terminalis. Interestingly, the occurrence of activated microglia coincides also in time with a reduction of AGRP-positive neuronal fibers in these animals (Nilsson et al. 2008). Microarray studies have revealed an inflammatory profile in the hypothalamus of these mice (Lachuer et al. 2005, Mercader et al. 2008a). Expression of a marker of axonal degeneration, i.e. activated caspase 6, has been documented in the hypothalamic food intake-regulating systems (Nilsson et al. 2011). The phenotypes of the anx/anx mice are also associated with mitochondrial dysfunction, in particular, lower levels and capacity of complex I (CI) in the oxidative phosphorylation system, which is associated with increased levels of reactive oxygen species (ROS) in the hypothalamus (Lindfors et al. 2011).

Firing of neurons in the central nervous system, including the hypothalamus, is dependent on the rate of carbohydrate utilization (Silver & Erecinska 1998, Gilbert et al. 2006, Lutas et al. 2014), which therefore can be used as a marker of neural activity. Based on the described aberrancies in the hypothalamus of the anx/anx mouse, and their paradoxical response to negative energy balance, we examined whether there are alterations in hypothalamic metabolism of these mice.

Materials and methods

Animals

The local ethical committee (Stockholms norra djurförsöksnämnd) approved all experiments involving animals. Heterozygous anx breeding pairs (B6C3Fe–a/a–anx A+/a) originally obtained from the Jackson Laboratory were used to set up an intercross. Genotyping was performed using simple sequence-length polymorphism markers mapped to the sub-chromosomal region where the anx mutation is situated.

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(Maltais et al. 1984, Lindfors et al. 2011). anx/anx mice and their wt siblings were housed at room temperature (22°C) with a 12:12-h light–darkness cycle and had free access to food and water, via milk from the mothers. Female and male mice were killed by decapitation. Anesthesia was not administered as it would interfere with the metabolism (Toyama et al. 2004). Killing occurred at postnatal day (P) 19–21 in the fed state, unless stated otherwise. The average weight (±S.E.) for anx/anx and wt mice was 4.6±0.2 and 7.2±0.3 g (P<0.001), respectively.

Spectrophotometric analysis of respiratory chain enzyme activities in hypothalamic tissue

For analysis of respiratory chain subunits, mice were decapitated and the hypothalamus was rapidly dissected out and kept on ice until analyzed (approximately 30 min). Three animals from each genotype were pooled for the analysis, and five different such pools per genotype were analyzed. The respiratory chain enzyme activities were determined in isolated mitochondria from the tissue as previously described (Wibom et al. 2002), with the exception that a buffer containing 10 mM Tris–HCl, 250 mM sucrose, 3 mM EGTA and 4 mg/mL BSA, pH 7.7 was used for the homogenization procedure.

Spectrophotometric analysis of citrate synthase activity in the hypothalamic tissue

For quantitative analysis of the amount of mitochondria in anx/anx vs wt hypothalamus, we used a mitochondrial marker, citrate synthase (CS) (Alp et al. 1976). anx/anx and wt animals (n=5 per genotype) were decapitated at P19, and the hypothalamus was rapidly dissected out, frozen in isopentane and kept at −70°C until further processed.

After thawing, the tissue was homogenized in a solution containing Triton X-100 (0.05% vol/vol), 50 mmol/L KH$_2$PO$_4$ and 1 mmol/L EDTA, pH 7.5. The homogenate was spun in a microcentrifuge for a minute, and CS activity was determined in the supernatant as previously described (Alp et al. 1976). The assay was performed on an automated photometer (Konelab T20 xti, Thermo Scientific).

Ex vivo autoradiography analysis of hypothalamic glucose uptake

For analysis of glucose uptake, mice (n=5 per genotype) were fasted for 5–6 h prior to intraperitoneal injection of [3H]-2-deoxy-glucose (0.3 mCi/mL of sterile saline, 10 mL/kg body weight). Forty-five min after injection (sufficient time for adequate glucose uptake to occur (Wong et al. 2011)), mice were decapitated, and brain and liver were rapidly dissected out and frozen in ice-cold isopentane. The tissues were stored at −80°C. Blood glucose concentration was measured at the time of killing using an Accu-Chek blood glucose analyzer (Roche Diagnostics). Serum glucose values were calculated by multiplying the blood values by 1.15 (Frank et al. 2012) to estimate glucose-specific activity in serum (see below). Brain tissue was cryosectioned (14 μm) and exposed to phosphor imaging plates (Fujiﬁlm Plate BAS-TR2025, Fujiﬁlm, Tokyo, Japan). Quantitative analysis of plates was performed using Multi Gauge 3.2 phosphorimager software (Fujiﬁlm) as described elsewhere (Miyamoto et al. 2000, Waldron et al. 2015).

The radioactivity in serum was measured with a scintillation counter by adding 5 µL serum to 90 µL ddH$_2$O, and then, 10 mL scintillation fluid (Emulsifier safe, Perkin Elmer). The glucose uptake into hypothalamus, motor cortex and thalamus and liver was calculated by the following formula: (µmol glucose per mL serum/dpm per mL serum) × dpm/g tissue = µmol glucose/45 min/g tissue) as described elsewhere (Beglopoulos et al. 2016).

Western blot analysis of AMPK and GLUTs in hypothalamic tissue

For Western blot, animals were decapitated and the hypothalamus was rapidly dissected out, frozen in isopentane and stored at −70°C until further processed. 7–10 animals per genotype were used for this analysis. The hypothalamic tissue was homogenized in lysis buffer (20 mM HEPES, pH 7.4, 40 mM KCl, 1 mM dithiothreitol, 0.3% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), 1 mM phenyl-methylsulfonyl fluoride, 1 mg/mL leupeptin, 1 mg/mL pepstatin, and 1 mM EDTA). The lysates were centrifuged at 700×g for 15 min, 4°C, and proteins were measured in supernatant by the Bradford assay (Bio-Rad). 30 µg total protein were loaded onto NuPAGE 4–12% gels (Invitrogen). Proteins were transferred onto Immobilon-FL membranes (Millipore). Membranes were blocked in 3% BSA and incubated overnight at 4°C with the following primary antibodies: total AMP-activated protein kinase (AMPK-T, 1:1000, Cell Signaling Technology), phosphorylated AMPK (AMPK-P, 1:1000, Cell Signaling Technology), glucose transporter (GLUT) 4 (1:5000, Chemicon/Millipore) GLUT1 (1:5000, Chemicon/Millipore) and GLUT3 (combination of...
two antisera directed against different epitopes of GLUT3, 1:250, The Human Protein Atlas, Stockholm, Sweden). The membranes were then incubated for 1 h in the darkness with secondary antibody (donkey anti-rabbit at 1:20,000 dilution) and were scanned with an Odyssey infra-red imager (LI-COR Biosciences, Lincoln, NE, USA) after washing. Band intensities were quantified by ImageJ and normalized with the intensities of bands after incubation with anti-beta-actin (1:2500, Abcam).

**Analysis of metabolites and ATP turnover in hypothalamic tissue**

Animals were divided into two groups and killed by decapitation. For one group of animals, representing the basal condition, the whole head was immediately frozen in liquid N$_2$, freeze-dried and the hypothalamus was dissected out. In another group of animals, representing the ischemic/stress condition, the hypothalamus was dissected out immediately after decapitation and then rapidly frozen in liquid N$_2$ (a process taking approximately 1 min) and subsequently freeze-dried. Ice-cold 0.5 M perchloric acid was added to the freeze-dried hypothalamic, kept in an ice bath for 20 min and then centrifuged at 10,000 $g$ at 4°C. The supernatant was neutralized with 2.2 M KHCO$_3$ and centrifuged as described previously. The latter supernatant was assayed for phosphocreatine (PCr), creatine (Cr), ATP, glucose 6-P and lactate with enzymatic techniques (changes in NAD(P)H) adapted for fluorometry (Lowry 1972), as well as assayed for ATP, ADP, AMP and IMP (changes in NAD(P)H) adapted for fluorometry (Lowry et al. 1964, Sahlin & Katz 1989, Katz 1988). To adjust for admixture of non-hypothalamic tissue (e.g. blood), metabolite values were divided by the sum of PCr + Cr (total Cr) and then multiplied by the mean total Cr content for the whole material. Total Cr did not differ significantly between groups under any condition studied (data not shown) and averaged 41.9 ± 2.0 µmol/g dry weight for the whole material. Anaerobic ATP turnover was calculated from the following equation: –ΔATP – ΔCr + (1.5Δ lactate) (Lowry et al. 1964, Katz 1988), where $\Delta$ refers to the mean difference between ischemia and basal values.

**Statistical analysis**

Statistically significant ($P<0.05$) differences between groups were determined with the unpaired two-tailed $t$ test. All values are presented as means ± S.E.M.

**Results**

**Respiratory chain enzyme activities in the anx/anx hypothalamus**

To assess the mitochondrial capacity of the anx/anx hypothalamus, we measured the enzymatic activity of the different complexes (C) in the respiratory chain in isolated mitochondria and found small but significant decreases in activities of CI (NADH:coenzyme Q reductase) (0.33 ± 0.01 vs 0.38 ± 0.01 units/unit CS, $P=0.01$) and of CI+III (NADH:cytochrome c reductase) (1.99 ± 0.02 vs 2.27 ± 0.06 units/unit CS, $P=0.003$) in anx/anx vs wt hypothalamus. No differences were seen in CII (succinate dehydrogenase) (0.21 ± 0.01 vs 0.22 ± 0.00 units/unit CS, $P=0.23$), CII+III, (succinate:cytochrome c reductase) (0.55 ± 0.01 vs 0.57 ± 0.01 units/unit CS, $P=0.11$) or CIV (cytochrome c oxidase) (2.77 ± 0.08 vs 2.85 ± 0.09 units/unit CS, $P=0.54$). No difference in total tissue CS activity was seen when comparing anx/anx and wt hypothalamus (7.6 ± 0.6 vs 7.4 ± 0.4 µmol/min/g, respectively, $P=0.8$), indicating similar levels of mitochondria in the two groups. Thus, we confirm the previously documented mitochondrial CI dysfunction in the anx/anx mouse, which cannot be explained by a difference in mitochondrial content (Lindfors et al. 2011).

**Metabolites in the anx/anx hypothalamus**

In the basal state, anx/anx and wt hypothalamus exhibited similar contents of ATP and glucose 6-P, whereas PCr was significantly higher and lactate was significantly lower in anx/anx vs wt hypothalamus (Table 1). One minute of ischemia (as explained in the Materials and methods section) resulted in marked depletion of ATP and PCr, and accumulation of lactate, in both anx/anx and wt hypothalamus (Table 1). However, ATP and PCr were significantly higher, whereas lactate was significantly lower, in the tissue from anx/anx vs wt after ischemia (Table 1). Particularly evident were the markedly smaller accumulations of AMP and IMP (breakdown products of ATP) in the ischemic hypothalamus of the anx/anx mouse (Fig. 1). IMP is a particularly sensitive indicator of net ATP degradation (Sahlin & Katz 1989, Spencer & Katz 1991). The ATP/AMP ratio was significantly increased in anx/anx vs wt hypothalamus in the ischemic condition (Fig. 1E). It could be argued that the dissection of the hypothalamus after decapitation prior to freezing (ischemic condition) significantly influenced the metabolite values during ischemia. This is, however, unlikely as the absolute
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values for ATP, PCr, lactate and AMP after ischemia in the present study were very similar to values reported earlier for mouse whole brain and cerebrum one min after decapitation (assuming a wet/dry ratio of 4.3) (Lowry et al. 1964, Lin et al. 1993). Further, metabolite levels in the basal state were also similar in the present study to those in whole brain and cerebrum (Lowry et al. 1964, Lin et al. 1993).

AMPK and GLUTs in anx/anx hypothalamus

AMPK-T and GLUT4 proteins were significantly elevated (~25% and ~60%, respectively) in anx/anx compared to those in wt hypothalamus, whereas AMPK-P, GLUT1 and GLUT3 were similar between groups (Fig. 2A). The ratio AMPK-P/AMPK-T was decreased by ~40% in anx/anx hypothalamus (Fig. 2B).

Carbohydrate utilization in the anx/anx mouse

The higher content of PCr and lower degree of AMPK activation (AMPK-P/AMPK-T) suggested a lower degree of metabolism in the anx/anx hypothalamus in the basal condition. To directly examine this possibility, we assessed the rate of glucose uptake in hypothalamus of conscious anx/anx mice. Blood glucose concentration in the fasted state averaged 5.6 ± 0.6 mM in anx/anx and 7.9 ± 0.6 mM in wt mice (P=0.04), which agrees with our recent findings for both groups under comparable conditions (Lindfors et al. 2015). In the fasted state, serum insulin does not differ significantly between groups (Lindfors et al. 2015). Glucose uptake was significantly reduced in the hypothalamus of anx/anx compared to that in wt mice (Fig. 3). The lower glucose uptake was observed in other areas of the brain, as well as in the liver (Fig. 3). To assess whether hypometabolism also occurred under stress conditions, carbohydrate and energy metabolism were quantified in ischemic hypothalamus (~1 min of ischemia). Under this condition, glycolysis (lactate accumulation) was reduced by almost 40%, whereas anaerobic ATP turnover was ~10% lower in the anx/anx group (Table 2). Thus, carbohydrate utilization was diminished under both resting and stress conditions in the anx/anx hypothalamus. It should be noted that the term carbohydrate utilization refers to glycolysis from utilization of intracellular glucose and glycogen that are trapped in the cell during ischemia (no significant

Table 1 Metabolites in the anx/anx hypothalamus in basal state and one minute of ischemia.

<table>
<thead>
<tr>
<th></th>
<th>Basal state</th>
<th>Ischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCr</td>
<td>Cr</td>
</tr>
<tr>
<td>wt</td>
<td>6.5 ± 0.4</td>
<td>35.0 ± 0.4</td>
</tr>
<tr>
<td>anx/anx</td>
<td>12.2 ± 1.0***</td>
<td>29.2 ± 1.0***</td>
</tr>
<tr>
<td>wt</td>
<td>0.6 ± 0.1</td>
<td>41.9 ± 0.1</td>
</tr>
<tr>
<td>anx/anx</td>
<td>1.6 ± 0.4*</td>
<td>40.9 ± 0.4*</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. and were obtained by enzymatic analyses; n=5–9 hypothalami per genotype. Values are given in µmol/g dry weight. *P<0.05; ***P<0.001 (unpaired t-test).

G6P, glucose 6-P; PCr, phosphocreatine.

Figure 1 Metabolites in the anx/anx hypothalamus. Metabolites in the anx/anx and wt mouse hypothalamus under basal conditions and after 1 min of ischemia analyzed by HPLC. (A) IMP; (B) ADP; (C) ATP; (D) AMP and (E) ATP/AMP. Values are means ± S.E.M., n=3–7 hypothalami per genotype. ***P<0.001 between groups (unpaired t-test).

Figure 2A-2B AMPK and GLUTs in anx/anx hypothalamus.
glucose transport under this condition), as demonstrated elsewhere (Lowry et al. 1964), and glucose uptake under basal conditions, which is assumed to reflect glycolysis as well.

**Discussion**

The major finding of the present study is that the anorexia of the anx/anx mouse is associated with a lower rate of hypothalamic glucose utilization.

Diseases associated with mitochondrial dysfunction (in brain and skeletal muscle) are generally associated with a stressed metabolic profile, as judged by lower levels of phosphocreatine and elevated levels of lactate as well as increased rates of glucose uptake (Wredenberg et al. 2006, Jeppesen et al. 2007, Milone & Wong 2013). These metabolic responses occur, presumably, to ensure set point levels of ATP. In some cases, however, mitochondrial dysfunction has been associated with reduced glucose uptake and hypometabolism, e.g. in epilepsy and Alzheimer’s disease (Chandrasekaran et al. 1996, Tenney et al. 2014). This is similar to what we see in the anx/anx hypothalamus in the present study. The hypothalamic metabolism is evidenced by a lower glucose uptake rate and decreased lactate content, as well as elevated PCr content in the basal state. Furthermore, the ratio of AMPK-P/AMPK-T is decreased in the basal state, which is consistent with lower metabolic stress in the anx/anx hypothalamus. This is somewhat reminiscent of the hypometabolic state associated with hibernation (Healy et al. 2011) and likely reflects lower neural activity, as seen, for example, in Alzheimer’s disease (Cunnane et al. 2011).

Different neuronal populations respond differently to metabolic stress (Schreiber & Baudry 1995), and this has been attributed to the subtype of ATP-sensitive potassium channel (K-ATP) expressed. A subtype of K-ATP channel consisting of Kir6.2 and SUR1 subunits, expressed, e.g., by dopaminergic neurons in substantia nigra, becomes activated by mitochondrial CI dysfunction, leading to ceased electrical activity, hyperpolarization and reduced firing (Liss et al. 1999). Kir6.2/SUR1 K-ATP channels are expressed by hypothalamic neurons i.e. the POMC/CART and AGRP/NPY neurons, and by a few other cell populations including the pancreatic beta-cells and brain stem neurons (Miki et al. 2001, Ibrahim et al. 2003, van den Top et al. 2007). High amounts of energy are required for firing of action potentials and neurotransmitter release, and mechanisms that inhibit such activities would conserve energy during conditions of negative energy balance (Attwell & Laughlin 2001, Sengupta et al. 2010).
Table 2  ATP turnover in the anx/anx hypothalamus.

<table>
<thead>
<tr>
<th></th>
<th>-ΔATP (µmol/g dry weight)</th>
<th>-ΔPCr (µmol/g dry weight)</th>
<th>+1.5ΔLactate (µmol/g dry weight)</th>
<th>ATP turnover (µmol/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>13.4</td>
<td>5.8</td>
<td>31.4</td>
<td>50.6</td>
</tr>
<tr>
<td>anx/anx</td>
<td>13.7</td>
<td>10.6</td>
<td>20.5</td>
<td>44.8</td>
</tr>
</tbody>
</table>

Values are calculated from the mean values shown in Table 1. ΔATP, ΔPCr, and ΔLactate values are given as µmol/g dry weight. ATP turnover = -2ΔATP - ΔPCr + (1.5ΔLactate).

Uncontrolled generation of ROS in NPY/AGRP neurons can also cause diminished firing of these neurons, thus resulting in a reduced orexigenic drive (Andrews et al. 2008, Horvath et al. 2009).

The marked difference in glucose uptake between anx/anx and wt mice can be attributed to several factors. First, the higher concentration of blood glucose in wt mice can result in a higher mass action effect and therefore a higher glucose uptake. The difference in blood glucose between groups in the present study is ~2 mM. Noteworthy is that large increases in blood glucose can be associated with significant reductions in mouse brain glucose utilization (Vannucci et al. 1997, Toyama et al. 2004) and, conversely, chronic hypoglycemia can result in increased brain glucose uptake (McCall et al. 1986). Indeed, in general, chronic hyperglycemia decreases and chronic hypoglycemia increases brain glucose uptake (McCall 1992). Further, mass action should be most significant under conditions where extracellular glucose concentration is below the $K_m$ for the glucose transporter. In brain, the major glucose transport proteins are considered to be GLUT1 and GLUT3 (Simpson et al. 2007, Zhang et al. 2014). The $K_m$ of these transporters for glucose is generally in the 1–3 mM range (Augustin 2010, Thorens & Mueckler 2010), i.e., lower than the blood glucose concentration in anx/anx and wt mice. Therefore, it is unlikely that the lower blood glucose concentration is primarily responsible for the marked decrease in glucose uptake in anx/anx mice.

An alternative possibility is that there is a decreased availability of GLUT proteins in anx/anx mice. However, neither GLUT1 nor GLUT3 protein levels are decreased in the anx/anx hypothalamus. Consistent with our results is the finding that the decreased brain glucose uptake in hyperglycemic db/db mice is also not associated with decreases in brain GLUT1 or GLUT3 protein levels (Vannucci et al. 1997). Indeed, under normoglycemic conditions in adult normal animals, the levels of glucose transporter proteins are not considered to limit brain glucose utilization (Vannucci et al. 1997).

Finally, intracellular variables related to metabolic rate may explain the differences in glucose uptake. It is well documented that the metabolic rate/energy turnover is a key factor in determining glucose transport and utilization in cells. This is particularly clear in skeletal muscle where glucose uptake can increase up to 50-fold during maximal exercise (Katz et al. 1986), and the same principle holds for neurons (see introduction). Thus, the lower rate of glucose uptake in anx/anx mice may primarily be derived from a lower metabolic rate. The metabolic profile of the anx/anx hypothalamus is consistent with this explanation (increased PCr, decreased lactate content and lower AMPK-P/AMPK-T). The question is how a lower metabolic rate would directly affect glucose transport. One possibility is via diminished activation of AMPK, resulting in less translocation of GLUT4 to the plasma membrane, which has been shown in skeletal muscle (Kurth-Kraczek et al. 1999). Although hypothalamic AMPK has been implicated in food intake (Minokoshi et al. 2004, Kahn et al. 2005) to our knowledge, evidence for a role for AMPK in control of glucose transport in hypothalamus has not been reported. Alternatively, a block in glycolysis (for example at phosphofructokinase) could result in the inhibition of glucose phosphorylation (Randle et al. 1964, Katz et al. 1986, Hue & Taegtmeyer 2009), resulting in an accumulation of glucose in the brain. This would decrease the concentration gradient for glucose and thereby decrease glucose uptake. Indeed, measurements in db/db mouse brain show that the decreased glucose utilization is associated with a marked increase in brain glucose levels, resulting in a decrease of about 30% in the plasma/brain glucose ratio (Vannucci et al. 1997). Conversely, the increased brain glucose uptake observed during chronic hypoglycemia is associated with a marked decrease in the brain glucose levels (McCall et al. 1986). Thus, the decreased glucose uptake in anx/anx hypothalamus likely stems from a hypometabolic state, possibly owing to an inhibition of glycolysis. However, this awaits experimental confirmation.

Interestingly, AMPK-T and GLUT4 protein expressions are both elevated in the anx/anx hypothalamus. Such adaptations have been seen in skeletal muscle in response to increases in metabolic stress, such as chronic exercise (Richter & Ruderman 2009, Richter & Hargreaves 2013). Thus, the elevated expression of the two proteins in the presence of hypometabolism is unexpected, and the mechanism for this is not clear at present.

The lower AMPK-P/AMPK-T ratio observed in the anx/anx hypothalamus compared to wt is indicative of...
lower AMPK activation. AMPK is not only of importance in regulating cellular energy status (as discussed previously) but is also implicated in whole body energy homeostasis and hypothalamic systems regulating food intake (Claret et al. 2007). Noteworthy is that the activity of AMPK (as judged by increase in the AMPK-P/AMPK-T ratio) has been shown to increase during fasting to enhance feeding (Kahn et al. 2005) and decrease after refeeding (Ronnert et al. 2009), and lack of AMPK expression in the hypothalamus results in reduced food intake and body weight (Minokoshi et al. 2004). Although AMPK-T was elevated in the anx/anx hypothalamus, the activation level of the enzyme was decreased in the basal state, and this could contribute to the anorexia of the anx/anx mouse.

We conclude that hypothalamic metabolism is reduced in the anorectic anx/anx mouse and that this may contribute to their inability to regulate food intake according to energy status and the premature death of these animals. A potential mechanism underlying this phenomena involving mitochondrial CI dysfunction and a specific subtype of K-ATP channel is discussed previously, but further studies are needed to identify the exact mechanism for the hypometabolism. Further, it is not known whether, or to what extent, activation of microglia, neuroinflammation, neurodegeneration and peripheral hormones (e.g., leptin) contribute to the hypometabolism. Additional research is also required to examine the basis for the reduced glucose uptake in other brain regions, i.e. motor cortex and thalamus. It also remains to be determined if hypometabolism pertains to the whole hypothalamus or to specific hypothalamic cells, i.e. neurons or glia, or even specific neuronal populations such as the orexigenic NPY/AGRP neurons and other populations expressing the specific K-ATP channel. Nevertheless, in view of the findings of the present study, we feel that imaging investigations of hypothalamic activity in humans with anorectic conditions, e.g. anorexia nervosa, are warranted.

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

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