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Molecular cloning of rat brain mitochondrial carrier protein-1 cDNA and its up-regulation during postnatal development[1]

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Abstract

Brain mitochondrial carrier protein-1 (BMCP1), a new member of the mitochondrial uncoupling carrier, has been shown to be expressed predominantly in the brain of the mice and humans. We cloned rat BMCP1 cDNA and investigated its mRNA level during postnatal development and under various metabolic conditions. The nucleotide sequence of the cDNA revealed that rat BMCP1 protein was composed of 322 amino acid residues, and was 99 and 96% identical to the mouse and human proteins and 29, 33 and 35% identical to rat uncoupling protein (UCP) 1, UCP2 and UCP3, respectively. The molecular weight was predicted to be 36 017 Da and the protein of this size was detectable when the cDNA was expressed in vitro. Using Northern blot analysis, the corresponding mRNA, approximately 1.8-kb in size, was found expressed predominantly in the cerebrum, cerebellum and hypothalamus. A unique developmental pattern was identified in the brain, where BMCP1 expression was low in their fetal life, but significantly elevated in the first postnatal week. Thereafter BMCP1 mRNA was maintained to be gradually increased. In 48-h fasted or insulin-induced hypoglycemic rats, BMCP1 mRNA expression in the hypothalamus slightly, but significantly, decreased compared with that in their appropriate controls. The present results indicate that BMCP1 may be involved in pathogenesis of mitochondrial dysfunction in neurons induced by aging or neurodegenerative disorders, and perhaps in energy balance in the brain.

Keywords: cDNA cloning; Amino acid sequence; Gene expression; Brain mitochondrial carrier protein-1; Developmental alteration

1. Introduction

Uncoupling proteins (UCPs) in the inner mitochondrial membrane are unique in the ability to dissipate the proton electrochemical gradient generated by respiratory activity [1–6]. The proteins thus function to uncouple mitochondrial respiration from ATP synthesis [1,2]. UCP1 is expressed exclusively in brown adipose tissue (BAT), a crucial site in thermogenesis and energy expenditure in rodents [7,8]. Unlike UCP1, UCP2 is widely expressed in the human and rodent tissues [4,5], and UCP3 is preferentially expressed in skeletal muscle and BAT [9,10].

Our previous report has demonstrated that UCP2 mRNA is also expressed in the rat hypothalamus

Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline

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[1] The nucleotide sequence reported in this paper has been submitted to the GSDB/DDBJ/NCBI GenBank Data Libraries with accession number AB028879.

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[11]. A recent study on in situ hybridization histochemistry detected an intense signal for UCP2 mRNA in the hypothalamus of mouse, particularly in the suprachiasmatic nucleus, the paraventricular hypothalamic nucleus, the arcuate nucleus, the dorsal motor nucleus of the vagus nerve, and the choroid plexus [12]. Taken together, the specifically localized expression of UCP2 mRNA in the hypothalamus suggests that the protein may play some essential roles in those neurons, although the details of its physiological role are still uncertain.

The brain mitochondrial carrier protein-1 (BMCP1), a novel member of the UCP family recently cloned, is predominantly expressed in the central nervous systems in the mice and humans [13]. In situ hybridization analysis has shown it is particularly abundant in the cortex, hippocampus, thalamus, amygdala, and hypothalamus. The predicted amino acid sequence of BMCP1 revealed low level of identity with the other UCPs. When expressed in yeast; however, BMCP1 possessed marked uncoupling activity, resulting in a decrease in mitochondrial membrane potential [13].

The decrease in mitochondrial membrane potential is important evidence explaining impending apoptosis of neuronal cells in response to aging or neurodegenerative disorders [14–16]. From this viewpoint, we cloned rat BMCP1 cDNA and investigated as to whether BMCP1 may be modulated at its mRNA level along developmental periods of fetal and postnatal lives. We also investigated its implication in brain energy expenditure using fasted or insulin-induced hypoglycemic rats, since there have been numerous reports that UCPs expression is regulated by dietary and hormonal manipulations [4,6,17–21].

2. Materials and methods

2.1. Animals

Male Wistar King A (WKA) rats at 12 weeks of age were used in the present study. WKA fetal rats, postnatal rat pups and adult rats were used in the developmental experiment. Animals were housed in a sound-proof room illuminated daily from 07:00 to 19:00 h (a 12:12-h light:dark cycle) and maintained at 21 ± 1°C with humidity at 55 ± 5%. They were allowed free access to tap water and standard pellet rat chow (CE-2, CLEA Japan, Tokyo, Japan), unless otherwise stated.

2.2. Preparation of fasted, insulin-induced hypoglycemic and STZ-DM rats

Fasted rats were prepared by deprivation of food for 48-h with free access to tap water. Hypoglycemic rats were made by treatment with daily subcutaneous injections of 3 U insulin (Lente Iszilin; Shimizu, Shizuoka, Japan) for 3 days. Streptozotocin-induced diabetic (STZ-DM) rats were made by injecting STZ (Sigma, St. Louis, MO, USA) into the tail vein at a dose of 60 mg/kg under light ether anesthesia and were sacrificed 12 days after the injection. Blood samples were collected from each rat through a chronically indwelling silicone catheter implanted in the right external jugular vein [22]. Serum samples were immediately frozen at −20°C until serum metabolites were measured. Serum insulin concentration was quantified using an insulin radioimmunoassay kit (Rat insulin [^{125}I] assay system; Amersham, Buckinghamshire, UK). Concentration of Serum glucose and free fatty acids (FFA) was measured with commercially available kits (Merckauto Glucose; Kanto, Tokyo, Japan, NEFA-SS'Eiken'; Eiken, Tokyo, Japan).

2.3. Tissue preparation and total RNA extraction

After rats were sacrificed, various tissues including the cerebrum, cerebellum, hypothalamus, heart, lung, kidney, liver, spleen, stomach, large intestine, small intestine, testis, skeletal muscle, BAT, and epididymal white adipose tissue (WAT) were surgically dissected to investigate the tissue-specific expression of BMCP1. In the developmental experiment, whole brain was surgically removed from fetal rats at the 18th day of pregnancy, postnatal rat pups consisting of the 1st and 4th day, and the 1st, 2nd and 4th week of age, and adult rats consisting of 10 and 28 weeks of age (four for each group). The hypothalamus taken from fasted, STZ-DM and insulin-induced hypoglycemic rats and their appropriate controls (five for each group) was also used in the experiments under various metabolic conditions. The tissue samples were immediately frozen in liquid nitrogen to be

stored at −80°C until use. Total RNAs were extracted as previously described [23].

2.4. Cloning of rat BMCP1 cDNA using RT-PCR and transcription and translation in vitro

Total RNA (1 μg) from WKA rat hypothalamus was reverse-transcribed by oligo(dT)$_{12-18}$ priming using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, USA). The single-strand cDNAs were subjected to PCR for cloning of rat BMCP1 cDNA. For amplification of rat BMCP1 cDNA, we prepared a set of primers, P1 and P2 (sense primer P1, 5′-CCGAGGAACTGGCAAGATC-3′ and antisense primer P2, 5′-TGGGCTGATGGGTTTCCAG-3) which were specific for the sequence of the mouse BMCP1 cDNA [13]. PCR was carried out with Taq DNA polymerase (Amersham, Buckinghamshire, UK) and 20 pmol each of the sense and antisense primers. The reaction profile was as follows: denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, for 30 cycles. The PCR products of approximately 1000-bp were subcloned into pCR II vector (TA cloning kit; Invitrogen, San Diego, USA) for sequencing. The nucleotide sequences were determined by the dideoxynucleotide chain termination method, using synthetic oligonucleotide primers which were complementary to the vector sequence and ABI373A automated DNA Sequencing System (Perkin-Elmer, Norwalk, USA). All DNA sequences were confirmed by reading both DNA strands.

BMCP1 full-length cDNAs inserted into pCR II vector (pCR-rBMCP1) were linearized by digestion with restriction enzymes *Bam*HI or *Eco*RV for the transcription of sense or antisense RNA, respectively. Sense and antisense RNAs were transcribed and capped in vitro with T7 and Sp6 RNA polymerase, respectively, using a Message Machine kit (Ambion, Austin, USA). The resulting RNA was translated in a rabbit reticulocyte lysate system (Promega, Madison, USA) in the presence of [^{35}S]methionine. The translation products were electrophoresed in a 12.5% polyacrylamide gel and detected by fluorography.

2.5. Northern blot analysis

Total RNAs (20 μg/lane) were electrophoresed on 1.2% formaldehyde-agarose gel, and the RNAs that were separated were transferred onto a Biodyne B membrane (Pall Canada, Mississauga, Ont., Canada) in 20×SSC by capillary blotting [24], and immobilized by exposure to ultraviolet light (0.8 J). Prehybridization and hybridization were carried out as described [25], using the ^{32}P-labeled 1043-bp DNA fragment excised after digesting pCR-rBMCP1 with *Eco*RI restriction enzyme, as a probe. Membranes were washed under high stringency conditions [24]. After washing the membranes, the hybridization signals were analyzed with a BIO-image analyzer BAS 2000 (Fuji Film Institution, Tokyo, Japan). The band intensities obtained by Northern blots were normalized to those of 18S ribosomal RNAs. The results were expressed as % changes ± S.E.M. of the mean appropriate control values taken as 100%.

2.6. Statistical analysis

Data were expressed as the mean ± S.E.M. The statistical significance between two groups was assessed by Student's unpaired *t*-test and among more than three groups by one-way analysis of variance followed by Scheffe's multiple comparison test.

3. Results

3.1. Cloning of rat BMCP1 cDNA and transcription and translation in vitro

To obtain the nucleotide sequence of rat BMCP1 cDNA, RT-PCR was carried out using total RNA from WKA rat hypothalamus as a template. Sequences of both strands were determined for more than 95% of the cDNA fragment. Analysis of the deduced amino acid sequence revealed that rat BMCP1 was a protein composed of 322 amino acid residues and was 99 and 96% identical to the mouse and human counterparts, and 29, 33 and 35% identical to rat UCP1, 2 and 3, respectively, as shown in Fig. 1. Transcription and translation in vitro was carried out using the cloned cDNA and a protein product of approximately 36 kDa that is of expected size was synthesized by SDS-PAGE.

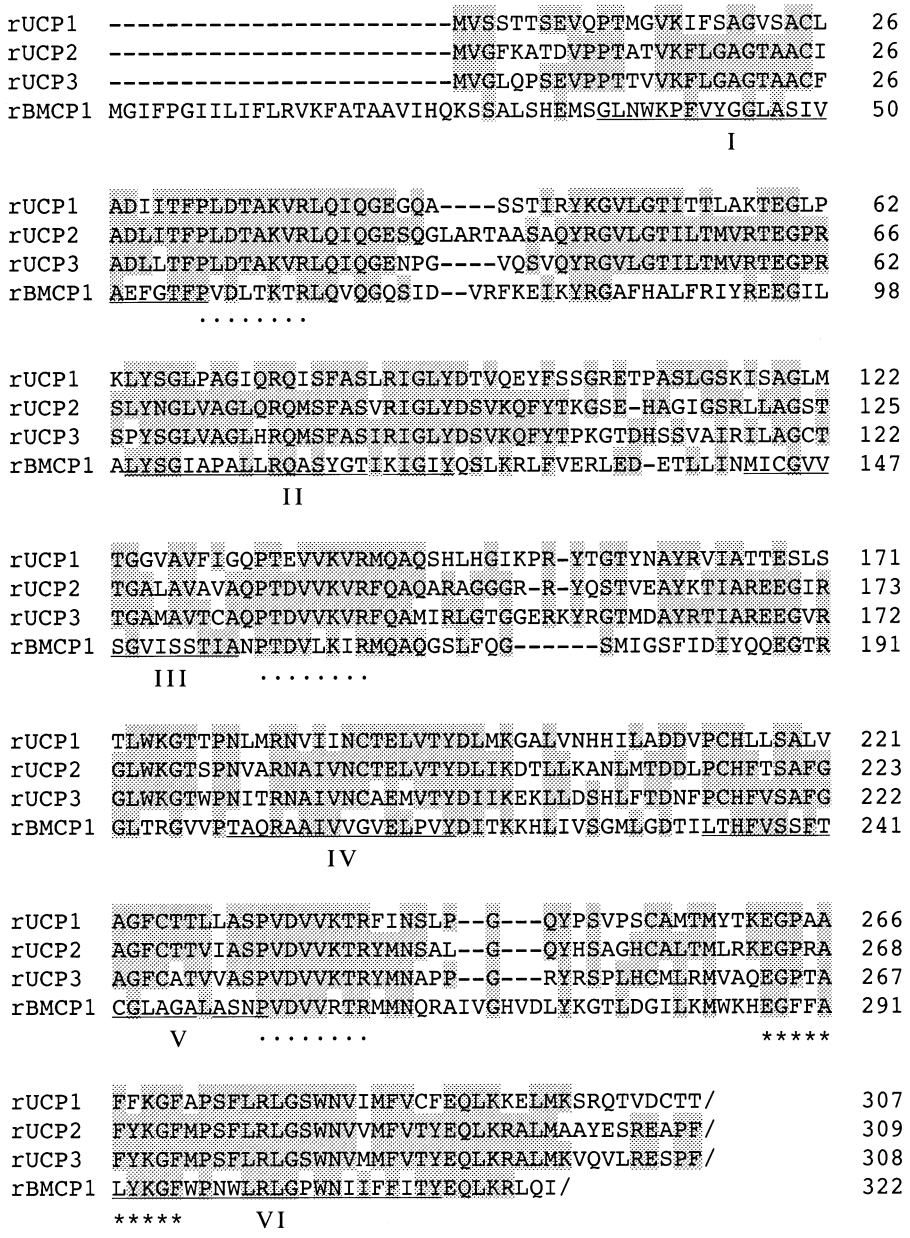


Fig. 1. Alignment of the predicted amino acid sequences of rat BMCP1 and the other UCPs. The amino acids residues conserved among these four uncoupling carriers are shaded. Six putative transmembrane domains are underlined and labeled I–VI. Three mitochondrial carrier motifs are indicated by dots and the putative purine nucleotide binding site is indicated by asterisks. Amino acid numbers are to the right. Gaps are inserted to maximize homology.

3.2. *Expression levels of BMCP1 mRNA in various tissues of rats*

The tissue-specific pattern of rat BMCP1 mRNA expression was characterized by Northern blot analysis. As shown in Fig. 2, BMCP1 mRNA was highly expressed in rat brain, including the cerebrum, cerebellum and hypothalamus as a 1.8-kb mRNA. In addition to the primary expression in the brain, lesser levels of BMCP1 mRNA were present, in the heart, lung, kidney and some other tissues.

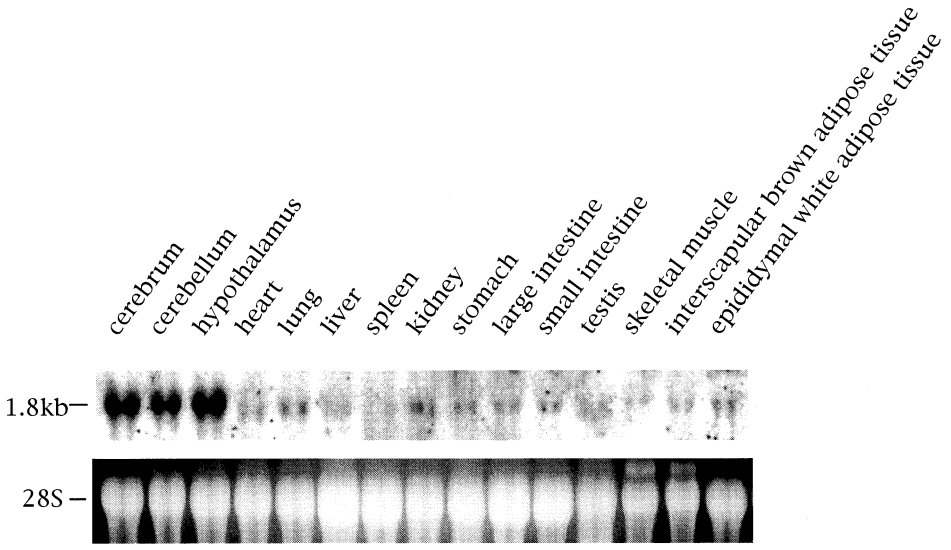
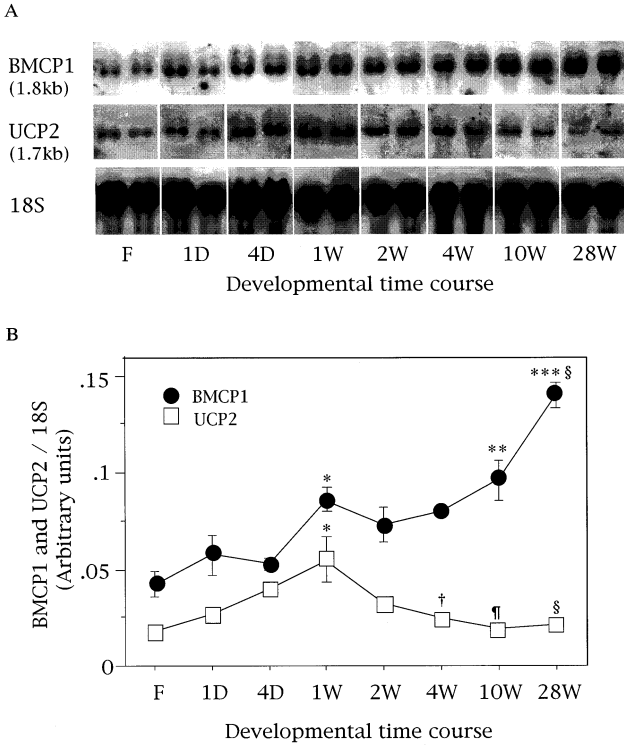


Fig. 2. Tissue distribution of rat BMCP1 mRNA. A representative Northern blot analysis is shown. Total RNAs (20 μg/lane) from various tissues in male Wistar King A rats were analyzed. The 28S ribosomal RNA bands visualized with ethidium bromide are shown in the bottom panel.

3.3. Developmental alteration of BMCP1 mRNA expression in the rat brain

As shown in Fig. 3, BMCP1 mRNA levels in the whole brain rapidly increased by 2.0-fold to reach a first peak in the first postnatal week ($P < 0.05$), and increased again with the advance of aging, and were greater by 2.3-fold in the 10th postnatal week ($P < 0.005$) and by 3.2-fold in the 28th postnatal week ($P < 0.0001$) compared to fetal levels. UCP2 mRNA levels in the brain remained low in the fetal life, but gradually increased along with postnatal development peaking in the first postnatal week by 2.2-fold compared with the fetal level ($P < 0.05$). After the fourth postnatal week, UCP2 mRNA began to decrease and returned to the fetal level.



←

Fig. 3. Developmental alterations of BMCP1 and UCP2 mRNA expression in the brain of fetal rats, postnatal rat pups and adult rats. (A) Representative Northern blots of BMCP1 and UCP2. Total RNAs (20 μg/lane) in various stages of life were used. (B) Time course changes in developmental mRNA expression of BMCP1 and UCP2. Closed circles and open squares indicate the mRNA levels in BMCP1 and UCP2, respectively. Values, the mean ± S.E.M. ($n = 4$ for each). F, the fetal life time on the 18th day of pregnancy; 1D and 4D, the 1st and 4th postnatal days; 1W, 2W, 4W, 10W and 28W, the 1st, 2nd, 4th, 10th and 28th postnatal weeks. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0001$ vs. the corresponding fetal value. $^{\dagger}P < 0.05$, $^{\S}P < 0.01$, $^{\P}P < 0.005$ vs. the corresponding value at the 1st postnatal week.

3.4. Regulatory expression of BMCP1 mRNA levels under various metabolic conditions

Fasted rats decreased their body weight (ad libitum-fed group vs. fasted group, 320 ± 4 vs. 282 ± 2 g, $P < 0.0001$), concentration of serum glucose (119 ± 3 vs. 78 ± 6 mg/dl, $P < 0.0001$) and serum insulin (6.9 ± 0.4 vs. 3.1 ± 0.5 ng/ml, $P < 0.0001$), but increased serum FFA (0.38 ± 0.03 vs. 0.88 ± 0.02 mEq/l, $P < 0.0001$). The level of BMCP1 mRNA in the hypothalamus was decreased by 20% in 48-h fasted rats compared with that in the ad libitum-fed controls ($P < 0.05$).

The insulin-induced hypoglycemic rats increased their body weight (PBS control group vs. insulin-induced hypoglycemic group, 314 ± 3 vs. 331 ± 5 g, $P < 0.05$), but decreased serum glucose (109 ± 6 vs. 33 ± 8 mg/dl, $P < 0.001$) and serum FFA (0.40 ± 0.03 vs. 0.20 ± 0.01 mEq/l, $P < 0.0005$) 3 days after daily insulin treatment. The STZ-DM rats decreased their body weight (citrate-buffered control group vs. STZ-DM group, 340 ± 4 vs. 256 ± 5 g, $P < 0.0001$) and serum insulin (8.1 ± 1.3 vs. 2.2 ± 0.6 ng/ml, $P < 0.001$), but increased serum glucose (102 ± 2 vs. 519 ± 16 mg/dl, $P < 0.0001$) and serum FFA (0.41 ± 0.05 vs. 0.79 ± 0.04 mEq/l, $P < 0.001$) 12 days after STZ treatment. BMCP1 mRNA level in the hypoglycemic rats was decreased by 22% compared with that in the PBS controls ($P < 0.01$), leaving the BMCP1 mRNA level of the STZ-DM rats unaffected.

4. Discussion

In the present study, we cloned rat BMCP1 cDNA. The protein was comprised of 323 amino acid residues, and the molecular weight was calculated to be 36 017 Da, the value close to that estimated with SDS-PAGE. The predicted amino acid sequence of rat BMCP1 was less homologous to those of other UCP family members, but conserved six putative transmembrane domains, three mitochondrial transporter protein signature motifs and a putative nucleotide binding site as with other UCPs [4,5,9,10,26]. Together with these findings, the results indicate potently that rat BMCP1 is a member of the mitochondrial proton-transporter proteins.

To confirm tissue-specific characteristics of the rat BMCP1 gene, we compared expression levels of the mRNA in various tissues of rat. BMCP1 mRNA was predominantly expressed in various parts of the rat brain, such as the cerebrum, cerebellum and hypothalamus. These findings were consistent with the tissue distribution of mouse and human BMCP1 [13].

To assess the putative role of brain UCPs during perinatal development and aging, we analyzed BMCP1 and UCP2 expression in the whole brain of the fetus, postnatal rat pups and adult rats. In the present study, both BMCP1 and UCP2 mRNA levels in the brain remained low in the fetus, but they gradually increased along toward postnatal development peaking in the first postnatal week. Then BMCP1 expression continued to increase with aging, and was greater in the 28th week than in fetal life, while UCP2 expression decreased and returned to the fetal level after the fourth postnatal week. This expression pattern of the BMCP1 gene has been found to be markedly different from that of UCP2 in the present study and the other UCPs in the BAT and heart of rodents [27,28]. Therefore, the specific rise of BMCP1 expression in old rats indicates that this protein has a certain role in aging of neuronal cells. There is mounting evidence for mitochondrial involvement not only in neurodegenerative disorders, but also in normal aging [14–16]. Electron transport chain (ETC) functioning was found to be impaired in these disorders and aging [14–16], leading to reduction in ATP production, formation of free-radicals, altered calcium handling and opening of the mitochondrial permeability transition pore, an event linked to cell death in neurons [14,15]. Mitochondrial uncoupling carriers decrease the electron potential of the mitochondrial inner membrane [1–6,13] resulting from proton leakage and participate in energy dissipation by uncoupling respiratory chain function from ATP synthesis. There is no direct evidence linking up-regulation of BMCP1 expression to aging. One possible explanation is that it may be associated with mitochondrial ETC dysfunction which leads to apoptosis of the neuronal cells.

Fasting is well known to modulate UCPs expression [6,17,18]. The level of BMCP1 mRNA in hypo-

thalamus slightly, but significantly, decreased in the 48-h fasted rats compared with the ad libitum-fed controls. To further investigate key factors in the regulation of BMCP1, we assessed concentrations of hypothalamic BMCP1 in STZ-DM and insulin-induced hypoglycemic rats. Akin to fasting, STZ-DM produced hypoinsulinemia and hyper-free fatty acidemia, but did hyperglycemia in contrast to fasting. The expression of BMCP1 mRNA in STZ-DM rats was left unaffected. Conversely, BMCP1 mRNA level in the hypoglycemic group decreased compared with that in the PBS controls. The following idea is still greatly in need of experimental verification; however, these results assume that a decrease in glucose concentration may be one of the regulators for BMCP1 expression. Indeed, glucose is a principal energy source for the mammalian brain. The down-regulation of BMCP1 in response to hypoglycemia may play a homeostatic role under energy deficiency in the brain.

In summary, we isolated a cDNA encoding rat BMCP1 and determined the sequence. BMCP1 expression in the whole brain was increased with the advance of aging. BMCP1 may thus be involved in the pathogenesis of mitochondrial dysfunction in neurons along with aging or neurodegenerative diseases.

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