Na^+/Cl^-/CREATINE TRANSPORTER ACTIVITY AND EXPRESSION IN RAT BRAIN SYNAPTOSOMES

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Abstract

Creatine is involved in brain ATP homeostasis and it may also act as neurotransmitter. Creatine transport was measured in synaptosomes obtained from the diencephalon and telencephalon of suckling and 2 month-old rats. Synaptosomes accumulate [^{14}C]-creatine and this accumulation was Na^+- and Cl^--dependent and inhibited by high external K^+. The latter suggests that the uptake process is electro- genic. The kinetic study revealed a K_m for creatine of 8.7 μM. A 100-fold excess of either non-labelled creatine or guanidi- nopropionic acid abolished NaCl/creatine uptake, whereas GABA uptake was minimally modified, indicating a high sub- strate specificity of the creatine transporter. The levels of NaCl/creatine transporter (CRT) activity and those of the 4.2 kb CRT transcript (Northern's) were higher in the dienceph- alon than in the telencephalon, whereas the 2.7 kb transcript levels were similar in both brain regions and lower than those of the 4.2 kb. These observations suggest that the 4.2 kb transcript may code for the functional CRT. CRT activity and mRNA levels were similar in suckling and adult rats. To our knowledge the current results constitute the first description of the presence of a functional CRT in the axon terminal mem- brane

that may serve to recapture the creatine released duringthe synapsis.

Key words: creatine transport, CRT, brain.

Introduction

Until recently the only known role for creatine in brain was that played in ATP homeostasis via the creatine/phospho-creatine/creatine kinase system (Brosnan and Brosnan, 2007; Andres et al., 2008). Almeida et al. (2006) reported that neurons release creatine in an action potential-dependent manner, suggesting a role for creatine as a neurotransmitter, particularly as co-transmitter on GABA postsynaptic receptors. It has also been suggested that creatine regulates appetite and weight by acting on specific hypothalamic nuclei(Galbraith et al., 2006).

The physiological relevance of creatine is strengthened by the devastating consequences that result from creatine deficiency. Three disorders that cause either absence or a severe decrease of creatine in the brain have been de- scribed: guanidinoacetate-methyltransferase (GAMT), argi- nine: glycine amidinotransferase (AGAT) and Na^+/Cl^-/creatine transporter (SLC6A8 or CRT) deficiencies (see reviews of Brosnan and Brosnan, 2007; Andres et al., 2008). In AGAT- and GAMT-deficient patients, high doses of oral creatine supplementation produces a partial restoration of thecerebral creatine pool after several months. The liver and kidney synthesize most of the body's creatine, but AGAT and GAMT mRNAs have been detected in the embryonic and adult rat brain, suggesting that the brain may synthesis its own creatine. However, despite the presence in brain of the creatine biosynthetic enzymes, the symptoms due to CRT deficiency cannot be corrected by creatine supplementation (see review of Brosnan and Brosnan, 2007; Andres etal., 2008).

The creatine concentration in the mammalian brain is 4 to 20-fold greater than that in the liver and possibly the major supply route of creatine to the brain is of peripheral origin. CRT mRNA has been identified throughout the rat brain (Schloss et al., 1994; Happe and Murrin, 1995; Saltarelli et al., 1996; Braissant et al., 2001,

2005; Gal-braith et al., 2006) with high levels in the myelinated tracts, cerebellar granule cells, hippocampal pyramidal cells, several brainstem nuclei and the choroid plexus (Saltarelli et al., 1996). Within the brain cells, CRT mRNA was detected in neurons and oligodendrocytes but not in astrocytes (Braissant et al., 2001, 2005; Ohtsuki et al., 2002).

As Na^+/Cl^-/creatine transport (SLC6A8 or CRT) activity has only been measured at the plasma membrane (see reviews of Speer et al., 2004; Brosnan and Brosnan, 2007), the presence of CRT mRNA in neurons suggests the presence of CRT protein in their plasma membrane. The mitochondria also transports creatine (Wazel et al., 2002). However, CRT does not appear to mediate creatine transport in the mitochondria because the mitochondria creatine transporter has different sensitivity to creatine analogs and a 1000 times lower affinity for creatine than the plasma membrane CRT. In addition, mitochodria creatine transport must be independent of the electrochemical Na^+ gradient.

Creatine uptake activity has been observed in rat cortical cultures (Almeida et al., 2006), neuroblastoma cell cultures (Daly, 1985), astroglial-rich cell cultures (Möller and Hamprecht, 1989) and hippocampal slices (Lunardi et al., 2006). These studies, however, do not characterized brain creatine transport in terms of affinity and specificity for creatine, and of ionic and membrane voltage dependence. The present work shows that synaptosomes obtained from rat diencephalon and telencephalon have in their membranes a functional Na (CRT)/Cl/creatine transporter.

EXPERIMENTAL PROCEDURES

Chemicals

[^{3}H]-GABA and [α^{32}P]-UTP were purchased from GE Healthcare, Europe, GmbH, [^{14}C]-creatine from Moravek Biochemicals Inc., USA and anti-CRT antibody from Alpha Diagnostic International, Inc., USA. All the other reagents used were obtained from Sigma-Aldrich, Spain.

Animals

Eighteen days-old (suckling) and 2 month-old (adults) Wistar rats were used in the current study because in the kidney the maximal differences in CRT activity versus age were found between suck- ling and 2 month-old rat (García-Delgado et al., 2007). They were provided by the animal care facility of the University of Sevilla. Rats were humanely handled and sacrificed in accordance with the European Council legislation 86/609/EEC concerning the protection of experimental animals. Mothers and adult rats were fed with a rat chow diet (Panlab 04) *ad libitum* and had free access to water.

Solutions and synaptosomes preparation

The brains of three to four suckling rats and of two adult rats were pooled to obtain a synaptosomal preparation of either telencephalon or diencephalon. Rats were anesthetized with a lethal i.p. injection of pentobarbital (50 mg/kg) and the brain was rapidly removed and dropped into ice cold PBS solution. The telencephalon and diencephalon were dissected according to the atlas of Paxinos and Watson (1986), cutting off the olfactory bulb, striatum nucleus and hippocampus.

Synaptosomal fractions from telencephalon and diencephalon were prepared using a discontinuous ficoll gradient as de- scribed by Booth and Clark (1978) with some modifications. Briefly, the tissues were homogenized in (in mmol/L) 320 sucrose, 1 EDTA, 1 EGTA, 10 Tris–HCl (pH 7.4). The homogenate was spun at 1000 g

for 10 min and the resultant supernatant was centrifuged at 12,000 g for 20 min. The pellet was re-suspended in 3 ml of (in mmol/L) 320 sucrose and 10 Tris–HCl (pH 7.4). The 3 ml were carefully loaded in a discontinuous ficoll gradient (13% and 7.5% ficoll (w/v) in 320 mmol/L sucrose and 10 mmol/L Tris–HCl, pH 7.4). The gradient was centrifuged at 98,000 g for 30 min in a swing-out rotor centrifuge. Myelin and synaptosomes banded at the first and second interphases respectively, with the mitochondria being pelleted at the bottom. The synaptosomal band was collected and spun at 12,000 g for 20 min. The final synaptosomal pellet was re-suspended in a pH 7.4 buffer contain- ing (in mmol/L) 240 mannitol, 10 glucose, 4.8 Kgluconate, 2.2 Cagluconate, 1.2 $MgSO_4$, 1.2 KH_2PO_4 and 25 HEPES-Tris. All of the steps were carried out at 4 °C.

Protein and enzyme assays

Protein was measured by the method of Bradford (1976), using γ globulin as the standard.

Enzyme assays were carried out in a Hitachi U-2800A recording spectrophotometer. Citrate synthase activity was assayed at 25 °C by the method of Srere (1969), in the presence of 0.1% TritonX-100 to release maximal activity. Ouabain-sensitive, K^+- activated phosphatase activity, a partial reaction of the Na^+, K^+, ATPase, was assayed at 37 °C by the method of Colas and Maroux (1980), in the presence of 20 mM K^+, using p-nitrophenyl phosphate as a substrate. Ouabain-sensitive, K^+-activated phosphatase was defined as the difference in activity measured in the presence and absence of 2 mM ouabain. Creatine and GABA uptake into synaptosomes. Either [^{14}C]-creatine or [^{3}H]-GABA uptake into synaptosomes was measured at 37 °C using a rapid filtration technique as described (García-Delgado et al., 2007). Briefly, 200 –250 µg of synapto- somes were added to tubes containing (in mmol/L) 100 NaCl, 40 mannitol, 10 glucose, 4.8 Kgluconate, 2.2 Cagluconate, 1.2 $MgSO_4$, 1.2 KH_2PO4, 25 HEPES-Tris (pH 7.4) and either 6 µM [^{14}C]-creatine or 10 nM [^{3}H]-GABA. When required NaCl was isosmotically replaced by mannitol. After

designated periods of time, uptake was terminated by the addition of 1 ml of NaCl-free ice cold buffer (stop solution). The samples were immediately filtered, under vacuum, through a 0.45 µm pore size Millipore filter pre-wetted with water. Filters were further washed with 5 ml of ice cold stop solution, dissolved in 5 ml of Ready-Protein (Beckman, Germany) scintillation fluid and the radioactivity determined by liquid scintillation spectrometry. Non-specific isotope binding to the filters was determined separately by adding stop solution to the synaptosomes before addition of uptake buffer and it was subtracted from the total radioactivity of each sample. All experiments were done in triplicate.

RNA preparation and Northern blotting

Total RNA was extracted from either telencephalon or diencephalon as described by Chomczynski and Sacchi (1987) and Poly (A^+)RNA was isolated by the method of Badley et al. (1988) as previously reported by us (Murillo-Carretero et al., 1999). [α^{32}P]- UTP-labelled antisense riboprobe of CRT was generated from a rat cDNA fragment as described (Peral et al., 2002). 10 µg of poly(A^+) RNA were loaded into individual lanes of a single gel. Relative quantification of mRNA was determined with a phospho- imager system (Fuji Photo Film Co., Ltd.) using the PCBAS pro- gram (Raytest GmbH). Expression of CRT mRNA was normalized to levels of cyclophilin mRNA. The size of the transcripts was evaluated by ribosomal RNA.

RT-PCR assays

Total RNA was extracted from either telencephalon or diencephalon using RNeasy® kit (Qiagen, USA). cDNA was synthesized from 1 µg of total RNA using QuantiTest® reverse transcription kit (Qiagen) as described by the manufacturer. Real-time PCR was performed with iQ™SYBR® Green Supermix (BioRad, Spain), 0.4 µM primers and 1 µl cDNA, as described (García-Delgado et al., 2007). Controls were carried out without cDNA. Amplification was run in a Min- iOpticon™ System (BioRad) thermal cycler (94 °C/3 min; 35 cy- cles of 94 °C/40 s; 58 °C/40 s, and 72 °C/40 s). Following

amplification, a melting curve analysis was performed by heating the reactions from 65 to 95 °C in 1 °C intervals while monitoring fluorescence. Analysis confirmed a single PCR product at the predicted melting temperature. Primers for CRT were based upon the published sequence of rat CRT (GenBank Database) and were antisense, TTCTATTACCTGGTCAAGTCCT and sense, GCCTCAAGACTTTGTTCTCC. Glyceraldehyde-3-phosphate de- hydrogenase (GAPDH) served as reference gene and was used for samples normalization. The primer sequences for GAPDH were antisense, TGCACCACCAACTGCTTAGC and sense, GGCATGGACTGTGGTCATGAG. The cycle at which each sample crossed a fluorescence threshold, Ct, was determined, and the triplicate values for each cDNA were averaged. Analyses of real- time PCR were done using the comparative Ct method with the Gene Expression Macro software supplied by BioRad.

Western assays

SDS-Page was performed according to Laemmli (1970) on a 10% polyacrylamide gel. Protein samples of synaptosomes obtained from diencephalon and telencephalon of suckling and 2 month-old at were dissolved in Laemmli sample buffer and boiled 5 min before being loaded, electrophoresed and electrotransferred onto a nitrocellulose membrane (Micron Separations Inc., USA) as described (Peral et al., 2002). A total of 70 µg protein was loaded to each lane. The blots were probed with a commercial polyclonal anti-N-terminal peptide (Alpha Diagnostic International, Inc.) of rat CRT (1:500 dilution) for 16 h at 4 °C. After washing with TBST buffer (165 mM NaCl, 100 mM Tris/HCl, pH 7.5, and 0.1% Tween 20), the blot was incubated with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (SIGMA, Spain) (1:3000 dilution) for 1 h at room temperature. The blots were subsequently incubated with an anti-β-actin antibody to normalize CRT density values. The immunoreactive bands were viewed using the enhanced chemiluminescence procedure (GE Healthcare, Europe). The relative abundance of the bands was quantified using PCBAS version 2.0 (Fuji).

Statistical analysis

Data are presented as mean±SEM for n separated animals. In the Figures, the vertical bars that represent the SEM are absent when they are less than symbol height. Comparison between different experimental groups was evaluated by the two-tailed Student's *t*-test. One-way ANOVA followed by Dunnett's test was used for multiple comparisons (GraphPad Prism Program) in Fig. 3. Differences were set to be significant only for $P<0.05$.

RESULTS

Characterization of the synaptosomes preparation

The activity of enzymes characteristic of plasma mem- branes (ouabain-sensitive, K^+-activated phosphatase, a partial reaction of the Na^+, K^+, ATPase) and mitochondria (citrate synthase) were measured in homogenate, synaptosomes and mitochondria fractions.

The activity of the ouabain-sensitive, K^+-activated phosphatase (in nmol substrate. min^{-1}. mg of $protein^{-1}$) was 31.8±0.1, 70±0.1 and 7.3±0.1 (n=5) for homogenate, synaptosomes, and mitochondria fractions, respectively and that of citrate synthase (in µmol substrate. min^{-1}. mg of $protein^{-1}$) was 0.37±0.01, 0.40±0.01, 1.9±0.05 (n=5), respectively. Enrichment factors were calculated by reference to the homogenate activity. The mitochondria fraction showed a 5-fold enrichment of citrate synthase activity and 0.23% enrichment of Na^+, K^+, ATPase activity. Synaptosomes are not enriched in citrate synthase activity (1-fold enrichment) and they show a 2.2-fold enrichment of Na^+, K^+, ATPase activity.

Since synaptosomes contain plasma membrane, mitochondria and cytosolic vesicles, the observed enrichment in Na^+, K^+, ATPase activity and the lack of enrichment in citrate synthase activity may indicate that the synatosomes fraction mainly contain synaptosomes.

Effect of osmolarity on creatine uptake insynaptosomes

Thirty minutes creatine uptake versus external osmolarity was measured in the presence of NaCl to determine whether creatine is transported into an intra synaptosomal space (Fig. 1). An increase in medium osmolarity from 300mosmol/L to 1400 mosmol/L reduced creatine uptake into the synaptosomes, indicating that creatine is transported into an osmotically active space.

Creatine uptake into brain synaptosomes

Synaptosomes isolated from either telencephalon or diencephalon of 2 month-old rat take up [^{14}C]-creatine in a NaCl- dependent manner (Fig. 2). In nominally NaCl-free conditions creatine transport into telencephalon synaptosomes was similar to that observed in diencephalon synaptosomes. NaCl-dependent creatine transport was significantly higher inthe diencephalon than in the telencephalon.

The volume of the synaptosomes was evaluated by assuming that NaCl-independent creatine uptake reached passive equilibrium at 30 min (Fig. 2 dashed line). The 30 in uptake value of 9 pmol creatine/mg protein corresponds to a volume value of 1.5 µl/mg protein and the inside: outside creatine ratio was of approximately 5:1 for the diencephalon and 3:1 for the telencephalon synaptosomes. These observations indicate that synaptosomes have concentrated creatine by a factor of five and three, respectively.

Effect of Na^+, Cl^- or electrical membrane potential on creatine uptake into synaptosomes

Fig. 3 summarizes the effects of either electrical mem- brane potential, Na^+, Cl^- or NaCl chemical gradients on creatine uptake in synaptosomes isolated from the telencephalon of 2 month-old rat. The voltage across the synaptosomal membrane was decreased with high external K^+ concentration plus 20 µM valinomycin. An inside directed electrochemical chemical gradient either of NaCl, Na^+ or Cl^- were created by the addition to the incubation buffer of either 80 mmol/L NaCl, N-methyl-

glucamine Cl or Nagluconate, respectively. NaCl gradients were abolished by isosmotic replacement of NaCl with mannitol.

The results revealed that membrane depolarization inhibited creatine uptake (second column). Either Na^+-or Cl^--free conditions (third and fourth column, respectively) reduced creatine uptake to that measured under NaCl-free conditions (last column).

The observations discussed so far indicate that creatine crosses the synaptosomal membrane via a concentrative, electrogenic and Na^+-and Cl^--dependent transport system.

Specificity of creatine uptake into synaptosomes

Either creatine or GABA uptake into synaptosomes obtained from the telencephalon of 2 month-old rat was measured in the absence and presence of the compounds listed in Table

1. The concentration of cold creatine, guanidinopropionic acid (GPA) and guanidinoacetic acid (GAA) were 100 times higher than that of the radiolabeled substrate.

The data revealed that cold creatine and GPA, a high- affinity substrate of CRT, inhibited creatine uptake to the values reached in nominally NaCl-free condition. GAA also significantly inhibited creatine uptake, though to a lesser extent. On the other hand, GPA has a small significant inhibitory effect on GABA uptake and neither creatine nor GAA significantly modified GABA uptake.

Kinetic study of creatine uptake

The relationship between initial rate of creatine uptake (1 min incubation) into synaptosomes and creatine concentration is given in Fig. 4. The concentration of creatine ranged from 5 to 150 µM. Uptake was measured in the presence and absence of NaCl in the incubation buffer. Synaptosomes were obtained from the telencephalon of 2 month-old rat.

Creatine uptake measured in the nominal absence of NaCl showed a linear relationship with its concentration in the incubation buffer (data not shown). The difference between total creatine uptake and that observed in the absence of NaCl follows first-order kinetics (Fig. 4). The Eadie–Hofstee plot of the saturable component (Fig. 4, insert) yielded a linear relationship, consistent with the participation of a single transport system in the creatine uptake process.

The calculated apparent K_m and V_{max} values for creatine uptake were 8.7±1.2 μM and 7.5±0.8 pmol min^{-1} mg $protein^{-1}$, respectively.

Creatine transport in suckling and 2 month-old rats

In order to test for different brain regions we prepared synaptosomes from the telencephalon and diencephalon of suckling and 2 month-old rats and measured creatine uptake in the presence and absence of NaCl. NaCl-dependent creatine uptake was evaluated as the difference between total uptake and that measured in NaCl-free conditions. NaCl- dependent and NaCl-independent creatine uptake were similar in suckling and 2 month-old rats (Fig. 5).

Northern assay

CRT mRNA expression in telencephalon and diencephalon of 2 month-old rat was examined by Northern hybridization. Fig. 6 shows that the antisense riboprobe hybridized to two transcripts of 4.2- and 2.7 kb. The abundance of the 4.2 kb transcript in diencephalon was significantly higher than in telencephalon. The abundance of the 2.7 kb transcript was similar in the two brain regions examined and 9 –13-times lower than that of the 4.2 kb transcript, respectively.

CRT mRNA levels in suckling and 2 month-old rats

The expression of CRT mRNAs in the telencephalon and diencephalon of 18 day-old and 2 month-old rats was examined by the real-time PCR technique. Fig. 7 shows

that CRT mRNA levels were higher in the diencephalon as compared with the telencephalon and they were of the same magnitude in suckling and 2 month-old rats.

CRT protein expression

CRT protein expression was examined by Western blot- ting. 70 µg protein of synaptosomes obtained from either telencephalon or diencephalon of suckling and 2 month- old rats were loaded per line. The polyclonal anti-N-terminal-CRT antibody detected polypeptides of 55-, 70-, 80-, 97- and 116 kDa in the synaptosome protein (Fig. 8). The 55 kDa band was absent in the synaptosomes obtained from the telencephalon of 18 day-old rat and it was very faint in those from 2 month-old rat. The 97 kDa band was not observed in the synaptosomes obtained from the di-

encephalon. Once corrected by the β-actin, the intensities of the 70- and 80 kDa polypeptides bands were similar in the four synaptosomes preparations tested.

DISCUSSION

The brain pools of ATP are small and do not significantly change during brain activation because they are efficiently replenished via the creatine/phosphocreatine/creatine kinase system. Creatine is a very polar molecule and it may cross with difficulty lipidic membranes. The presence of CRT mRNA in brain cells suggests that CRT protein is at their plasma membrane and functional experiments (Almeida et al., 2006; Daly, 1985; Möller and Hamprecht, 1989; Lunardi et al., 2006) have suggested that CRT is present in the neurons plasma membrane.

The present study reveals that the membrane of synaptosomes has a functional Na^+/Cl^-/creatine transporter (CRT) because the characteristics of creatine transport in brain synaptosomes resemble those found in other tissuesfor CRT (Dai et al., 1999; García-Delgado et al., 2001; Peral et al., 2002; Ohtsuki et al., 2002). Thus it was found that: (1) synaptosomes accumulated creatine in the presence of an inwardly directed

electrochemical NaCl gradient; (2) concentrative creatine uptake was Na^+- and Cl^--dependent, because it required the simultaneous presence of both ions in the incubation buffer; (3) concentrative creatine uptake was electrogenic as it was inhibited by a decrease in the voltage across the synaptosome mem- brane; (4) there was a single NaCl-dependent creatine transport system with high affinity (8.7 μM) and specificity for creatine. The stoichiometry of the transporter has not been evaluated in the current study, but its electrogenicity suggests that at least two Na^+ and one Cl^- are required totransport one creatine molecule.

The obtained apparent K_m value is similar to the K_m value found in COS-7 cells transfected with human brain CRT (Sora et al., 1994) and is lower than that measured in astroglial cell cultures (Möller and Hamprecht, 1989) and COS-7 cells (Guimbal and Kilimann, 1993) that express rabbit brain/spinal cord CRT.

The human CRT is strongly related to a subfamily of protein sequences that includes the GABA transporters (Chen et al., 2004). The terminal nerves present Na^+/Cl^-/GABA transporters that mediate the rapid reuptake ofGABA and contribute to the termination of GABAnergic synapsis. The current results reveal that a 100-fold excess of either unlabeled creatine or GPA abolished NaCl-de- pendent creatine uptake, whereas that of GABA was minimally modified. These findings indicate that synapto- somes have a specific creatine-preferring transport system different from GABA transporters. Creatine transport was significantly inhibited by GAA, which agrees with reports showing that CRT transports GAA across the blood-cerebrospinal fluid barrier (Tachikawa et al., 2008).

Northern hybridization analysis revealed the presence in rat brain of two CRT mRNA transcripts of 4.2 and 2.7 kb. The presence of different transcripts is believed to arise from alternative polyadenylation (Nash et al., 1994).

The pattern of CRT mRNA expression, that of the 4.2 kb transcript and that of CRT activity were similar. Thus thehigher levels of transport activity in the diencephalon as compared with telencephalon were associated with higher levels of CRT mRNA and of the 4.2 kb transcript. The abundance of the 2.7 kb transcript was similar in

the two brain regions under study and 9 –13-times lower than that of the 4.2 kb transcript, respectively. All these observations suggest that the 4.2 kb transcript may code for the functional CRT protein.

Our observations do not give conclusive results on the molecular weight of brain CRT. Of the five polypeptides detected by the antibody (55-, 70-, 80-, 97- and 116 kDa) only the 70- and 80 kDa polypeptides were present in the diencephalon and telencephalon of suckling and 2 month-old rats, suggesting that the size of the brain CRT is either 70- or 80 kDa. However, the intensity of the 70- and 80-kDa bands was independent of either the age of the animal or the brain region examined. The antibody used in the current study detected in the apical membrane of rat enterocytes polypeptides of 57-, 65-, 80- and 116 kDa and the results suggested that the 65 kDa polypeptide represents the plasma membrane CRT (García-Miranda et al., in press). Previous studies on the size of CRT protein have also yielded conflicting results. A CRT cDNA clone from human brain with an open reading frame of 1905 base pairs predicted a protein of 635 amino acids (≈70.5 kDa) (Sora et al., 1994). Estimates of CRT molecular weight in various tissues vary from 40 to 210 kDa and in brain from 55 to 84 kDa (see Speer et al., 2004; Sartorius et al., 2008). Immunoreactive bands of >100 kDa may represent aggregates of CRT (see Speer et al., 2004). The discrepancy on the identification of the CRT protein in tissues has been attributed to the specificity of the available antibodies, anomalous behaviour of hydrophobic proteins in gels, the cross-reaction of the anti-CRT antibodies with non-CRT polypeptides and tissue-specific glycosylation (Speer et al., 2004). As these authors concluded, the anti-CRT antibodies available should only be used with those pre- cautions in mind.

CONCLUSION

In conclusion, this is the first report showing the presence in the membrane of synaptosomes of a functional CRT transporter. CRT activity and CRT mRNA levels show brain regional differences. The presence of CRT in the presynaptic membranes

will allow the local transport of creatine to replenish the phosphocreatine pool and thus maintaining essentially constant the ATP levels. If creatine acts as a central neurotransmitter (Almeida et al., 2006), the transporter would allow the rapid reuptake of creatine and termination of the synapsis.

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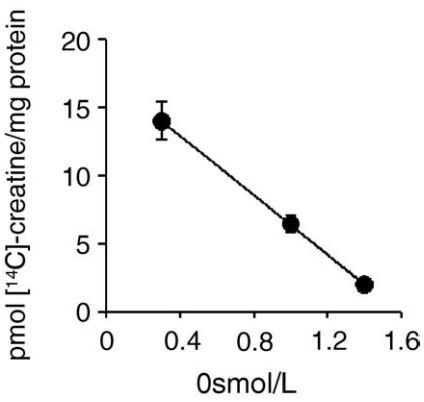

Fig. 1. External osmolarity and creatine uptake into synaptosomes obtained from the telencephalon of 2 month-old rat. Medium osmolar- ity was increased by the addition of mannitol. 6 μM [^{14}C]-creatine uptake was measured in the presence of NaCl during 30 min. Each point represents the mean±SEM of three separated synaptosomes preparations.

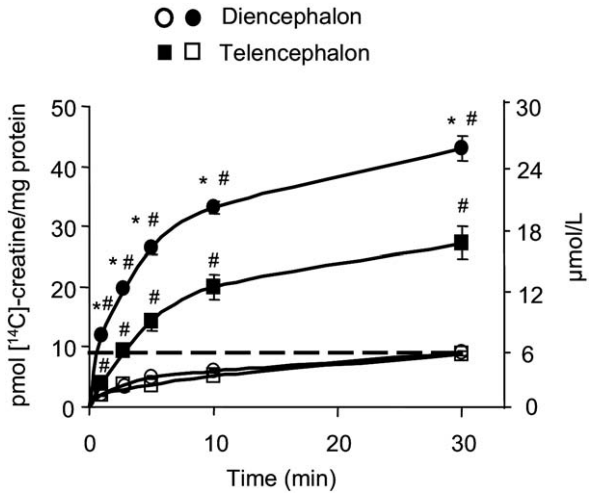

Fig. 2. Time course of creatine uptake. Synaptosomes were isolated from the telencephalon and diencephalon of 2 month-old rats. Six micromolar [^{14}C]-creatine uptake was measured in the presence (■●) and absence (eŒ) of NaCl in the incubation buffer. NaCl was isos- motically substituted by mannitol. The dashed line represents the passive uptake value expected at equilibrium. Means±SEM, n=4. # P<0.001 as compared with NaCl-free conditions; * P<0.001, as compared with telencephalon.

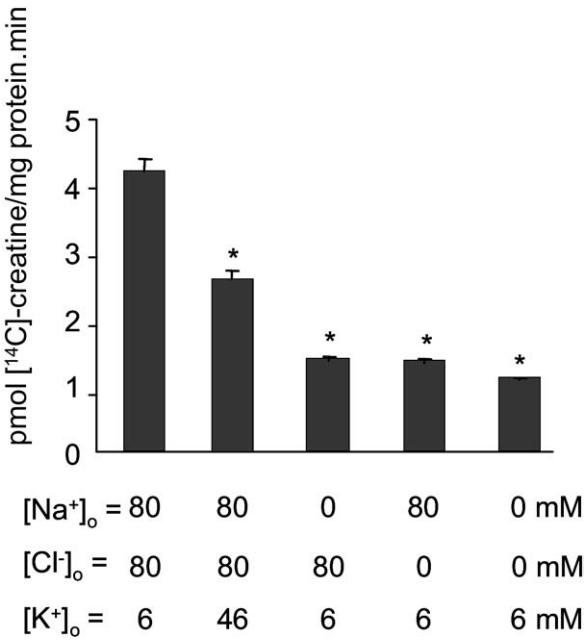

Fig. 3. Effect of electrical membrane potential and ion chemical gra- dients on [^{14}C]-creatine uptake into synaptosomes. Synaptosomes were obtained from the telencephalon of 2 month-old rat. In these experiments the concentration of NaCl was 80 mM and that of man- nitol 80 mM. NaCl was isosmotically replaced either with 80 mmol/L N-methyl-glucamineCl (only electrochemical Cl^- gradient), 80 mmol/L Nagluconate (only electrochemical Na^+ gradient) or 160 mmol/L man- nitol (without NaCl gradients). When 40 mmol/L KGluconate was used, the concentration of mannitol was zero. The concentration of [^{14}C]-creatine in the uptake buffer was 6 µM and the time of uptake 1 min. Means±SEM, n=5. One-way ANOVA showed an effect of Na^+, Cl^- and K^+ on creatine uptake (P<0.0001). Dunnett's test: * P<0.001, as compared with electrochemical NaCl gradient condition, first column.

Table 1. Specificity of either creatine or GABA uptake in synapto- somes isolated from the telencephalum of 2 month-old rat

Modifiers	% uptake rate	
	Creatine	GABA
None	100	100
Creatine	41±2*	92±4
GPA	33±1*	82±3**
GAA	83±4**	93±4
NaCl-free	31±1*	3±0.1*

The uptake of 5 µM [^{14}C]-creatine and that of 5 nM [^{3}H]-GABA weremeasured during 1 min in the absence and presence of variousunlabeled modifiers.

Labeled substrate uptake obtained in the absence of modifiers wasset at 100%.

The concentration of cold creatine, GPA (guanidinopropionic acid) and GAA (guanidinoacetic acid) was 500 µM for creatine uptake and 05 µM for GABA uptake.

Data are Mean±SEM of three separated synaptosome prepara- tions. * $P<0.001$, ** $P<0.05$ as compared with uptake in the absence of modifiers, first row.

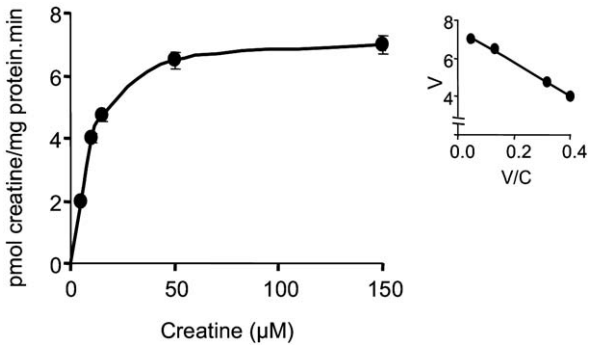

Fig. 4. Creatine uptake into synaptosomes vs. its concentration in the incubation buffer. Synaptosomes were obtained from the telencepha- lon of 2 month-old rat. The creatine concentration in the incubation buffer ranged from 1 to 150 μM and the time of uptake was 1 min. Data represent NaCl-dependent creatine uptake: total uptake minus that measured in NaCl-free buffers. Insert: Eadie–Hofstee plot of data. Means±SEM, $n=4$.

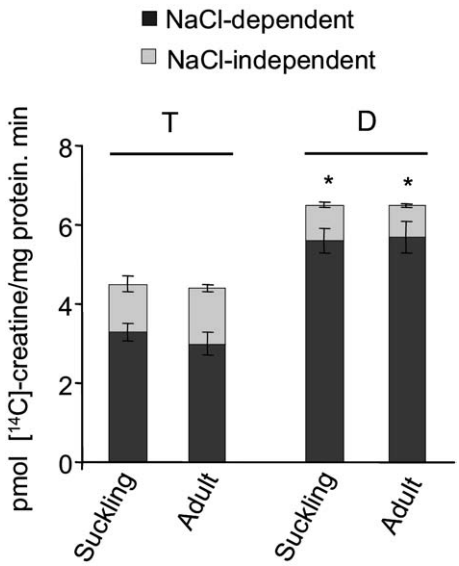

Fig. 5. Creatine uptake into synaptosomes isolated from the telen- cephalon (T) and diencephalon (D) of suckling and 2 month-old rats. The concentration of [^{14}C]-creatine in the uptake buffer was 6 µM and the time of uptake 1 min. Means±SEM. The number of animals per age was four. * $P<0.001$, comparisons between the two brain regions

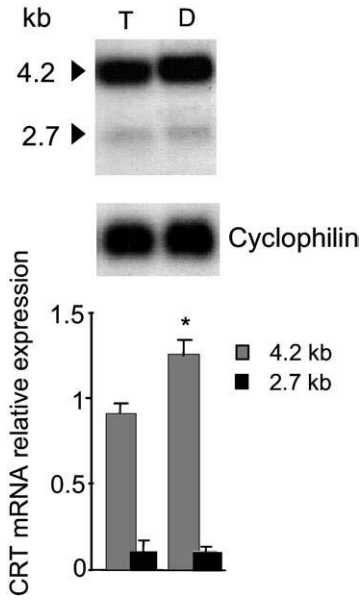

Fig. 6. Northern analysis of CRT mRNA. 10 μg of poly(A^+) RNA obtained from either telencephalon (T) or diencephalon (D) of 2 month-old rats were loaded onto the gel per lane. The sizes of the CRT mRNAs were calculated by ribosomal RNA as molecular weight lad- der. Data were normalized relative to cyclophilin. Histograms represent means±SEM of arbitrary units of CRT mRNA. $n=3$. * $P<0.05$, as compared with telencephalon.

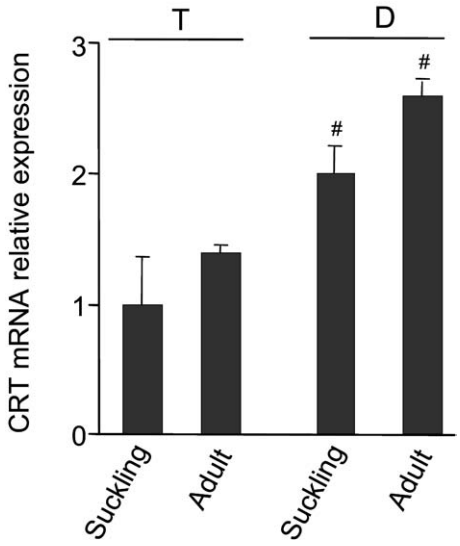

Fig. 7. Brain expression of rat CRT mRNA versus age. Real-time PCR was performed using total RNA isolated from the telencephalon (T) and diencephalon (D) of suckling and 2 month-old rats. Data were normalized to GAPDH. The CRT mRNA levels measured in the telen- cephalon of suckling rats were set at 1. Histograms represent means±SEM of arbitrary units of CRT mRNA abundance. The number of animals per age was five. $^{\#}$ $P<0.05$, comparisons between the two brain regions.

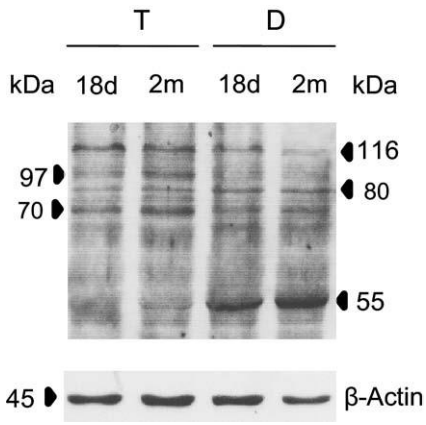

Fig. 8. Western blots of CRT protein. Each blot was loaded with proteins extracted from synaptosomes of telencephalon (T) and diencephalons (D) of 18 day and 2 month-old rat. A total of 70 µg protein was loaded to each lane. The blots were probed with a commercial polyclonal anti-N-terminal-CRT antibody as described in Methods section. The CRT density values were normalized relative to β-actin. The blot is representative of three Western's blots.