Biochemical Characterization of the Hippocampal and Striatal Na,K-ATPase Reveales Striking Differences in Kinetic Properties

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Abstract The activities and basic enzymatic properties of Na K-ATPase were examined in synaptosomal plasma membranes (SPM) prepared from rat hippocampus and striatum A kinetic analysis showed profound differences in apparent affinities for ATP (K) between hippocampal (1 21 mmol/l) and striatal (0 76 mmol/l) enzyme preparations as well as in the corresponding V_{max} values However physiological efficiencies were almost the same The complex pattern of dose response curves to ouabain indicated the presence of two high affinity forms of Na K ATPase in the striatum (very high $K_i = 3\ 73 \times 10^{-8}$ mol/l and high $K_i = 4\ 21 \times 10^{-5}$ mol/l) and one high affinity form in the hippocampus (K = 6 6 × 10^{-7} mol/l) In addition both SPM preparations contained one low affinity form with similar K The very high affinity form had positive cooperativity for ouabain inhibition of Na K ATPase activity in contrast to high and low affinity forms which exhibited negative cooperativity The respective contributions of ouabain sensitive forms to the total activity were estimated as 22% 46% 19% for the striatum and 36% 45% for the hippocampus These data clearly demonstrate striking differences in kinetic properties of the hippocampal and striatal Na K ATPase that may be due to the isoenzyme diversity and adaptation to specific physiological demands of the examined rat brain regions

Key words· Na K ATPase – Synaptic plasma membranes Hippocampus Striatum Rat

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Introduction

Sodium, potassium-adenosine triphosphatase (Na,K-ATPase, EC 3 6 1 37) is a family of ATP-dependent enzymes that mediate active transport of Na^+ and K^+ across the plasma membrane of almost all animal cells In the nervous tissue, this protein plays a key role in maintaining the electrical potential underlying excitability of nerve cells (Stahl 1986 Albers et al 1994) It is known to be composed of at least two subunits catalytic (α) and glycoprotein (β) There may also be an associated subunit γ (Collins and Leszyk 1987) Recently, it has been found that α and β subunits of Na K-ATPase exist in several isoforms in nervous tissue In the rat brain at least three isoforms of the α subunit (α_1 α_2, α_3) (Sweadner 1985, Herrera et al 1987 Jewell et al 1992) each being the product of a separate gene (Shull et al 1986), and three β subunit isoforms (β_1, β_2 β_3) (Schmalzing and Gloor 1994 Malik et al 1996) have been predicted from cDNA cloning experiments Each of the subunit isoforms of the enzyme has a distinctive and markedly variable tissue (Young and Lingrel 1987 Gick et al 1993) and cellular distribution (Watts et al 1991 Cameron et al 1994 Lecuona et al 1996), forming potentially up to six structurally distinct Na,K-ATPase isoenzymes that express different kinetic properties (Blanco et al 1995a b) However, the same sets of subunit isoforms have distinct enzymatic properties in different tissues (Sun and Ball 1992) implying the importance of the milieu in the regulation of pump activity The aim of this work was to clarify regional enzymatic specificities of Na K-ATPases in synaptic plasma membrane preparations from the hippocampus the striatum and for comparative purpose the whole female rat brain The presence of multiple enzyme isoforms of Na K-ATPase in the examined brain regions was determined on the basis of affinity for the specific inhibitor ouabain since it is known that high-affinity ouabain binding sites are associated with α_2 and α_3 while low-affinity ouabain binding is associated with α_1 subunit isoform (Sweadner 1989) To identify the presence of individual α subunit isoform two discriminatory criteria were used apparent affinities to ouabain and differences in cooperativity of ouabain binding

Materials and Methods

Experiments were performed on 2-month-old female Wistar rats weighing 200 250 g The animals were under permanent macroscopic surveillance, and those with behavioral signs of possible brain damage were excluded from experiments After decapitation with a guillotine (Harvard Apparatus), the brains from 13 animals for each experiment were rapidly removed Fresh whole brains (WB) striata (Str) and hippocampi (Hippo) were pooled (3 and 10/pool respectively) for immediate preparation of synaptosomal plasma membranes (SPM) The SPMs were prepared according to the method of Cohen et al (1977) as modified by Towle

and Sze (1983) As described previously (Horvat et al 1995) SPM preparations were obtained after centrifugation in a discontinuous sucrose gradient (0 8 mol/l, 1 0 mol/l and 1 2 mol/l) at 90,000 × *g* for 105 min The band containing SPM was removed from the 1 0 1 2 mol/l sucrose interface, diluted with 5 mmol/l Tris-HCl pH 7 4 and pelleted by centrifugation at 15,000 × *g* for 20 min Resulting pellets were resuspended in 5 mmol/l Tris-HCl pH 7 4 and kept at −70 °C until use The purity of SPM was assessed with the use of 5 mmol/l NaN_3 ohgomycin (2 μg/ml) 1 mmol/l NaF and 1 mmol/l theophilline The results obtained indicated no significant cross-contamination (less than 7%) Protein content was determined by the method of Lowry et al (1951) as modified by Markwell et al (1978)

Na K ATPase assay

Na,K ATPase activity was determined by measuring the inorganic phosphate (Pi) liberated from the hydrolysis of ATP Typical incubation mixture for the Na K ATPase activity measurement contained 50 mmol/l Tris-HCl pH 7 4 1 mmol/l EDTA 100 mmol/l NaCl 20 mmol/l KCl 5 mmol/l $MgCl_2$ 10 20 μg of SPM proteins and 2 mmol/l ATP (Sigma) in the final volume of 200 μl The activity obtained in the absence of Na^+ and K^+ was attributed to Mg-ATPase and it was subtracted from the total Na K-ATPase activity The mixture was preincubated for 4 min at 37 C and the reaction was started by the addition of ATP After 5 min of incubation the reaction was interrupted by the addition of 3 mol/l perchloracetic acid and immediately cooled in ice water The liberated Pi was determined according to the method of Fiske (1966) with slight modifications using KH_2PO_4 as a reference standard

Ouabain inhibition

Ouabain (Calbiochem) was used as a specific inhibitor of Na K-ATPase in order to analyze enzyme heterogeneity in WB Str and Hippo in terms of ouabain sensitivity This was studied by incubating SPM preparations in the assay medium containing ouabain in a final concentration varying from 10^{-10} to 10^{-3} mol/l In all experiments with ouabain SPM preparations were preincubated for 15 min at 37 °C before substrate addition since it has been demonstrated that inhibition levels induced by a single dose of ouabain remained stable during at least 30 min (Berrebi-Bertrand et al 1990) Inhibition percentages were calculated by comparing the activities in the presence and absence of ouabain after subtracting ouabain-insensitive Mg ATPase activity measured in the presence of 0 1 mmol/l ouabain

Statistical evaluation

The results were analyzed using the Student's *t*-test and the values of $p < 0.05$ were accepted as significant

Results

Na,K-ATPase activity was measured in SPM prepared from Hippo, Str and WB in order to establish regional specificity for maximum enzyme activity Table 1 shows that Na,K-ATPase moiety in the total ATPase activity is the highest in Hippo and the lowest in Str ($p < 0.005$) Similarly, the dependence of enzyme activity on the duration of incubation displayed a remarkable distinction between Hippo and Str Although the hydrolysis of Pi linearly increased during the first 10 min in all investigated brain regions, the maximum activity, attained after 10 min of incubation, was twice as high in Hippo than in Str and WB (results not shown)

Table 1. Specific activities of Na,K-ATPase and Mg ATPase in rat brain SPM and their percent contribution to the total ATPase activity

SPM preparation	ATPase activity (μmol Pi/mg protein/min)		
	Total ATPase	Na,K-ATPase	Mg-ATPase
Whole Brain ($n = 8$)	1 70 ± 0 02	0 95 ± 0 03 (56 2 ± 1 9)%	0 75 ± 0 01 (43 8 ± 1 9)%
Hippocampus ($n = 8$)	1 66 ± 0 03	1 08 ± 0 03* (64 9 ± 1 8)%	0 58 ± 0 02* (35 1 ± 1 8)%
Striatum ($n = 10$)	1 35 ± 0 07*	0 72 ± 0 02* (53 4 ± 1 4)%	0 64 ± 0 02* (46 6 ± 1 4)%

ATPase activities were monitored in standard incubation mixture (see Materials and Methods) containing 2 mmol/l ATP and optimal amount of SPM proteins Values represent means ± S E M from n determinations * $p < 0.005$ (vs whole brain ATPase activity)

To determine if Na,K-ATPase activity differs between brain regions because of different quantities of the enzyme required for the maximum activity, we measured the enzyme activity as a function of total SPM proteins (ranging from 5-40 μg) Fig 1*A* shows that Na,K-ATPases from all 3 sources of SPM exhibit similar patterns of activation by increasing amounts of protein In Str and WB the enzyme reached almost equal maximum specific activity (0 832 and 0 865 μmol Pi/mg protein/min, respectively), but the amount of SPM protein needed to obtain these activities was about 2 5-fold higher in WB (17–20 μg) than in Str (8 μg) (Fig 1*B*) Considerably higher specific enzyme activity was detected in Hippo (1 060 μmol Pi/mg protein/min), at optimal SPM concentration found to be 12 μg These protein concentrations were used in further experiments as optimal

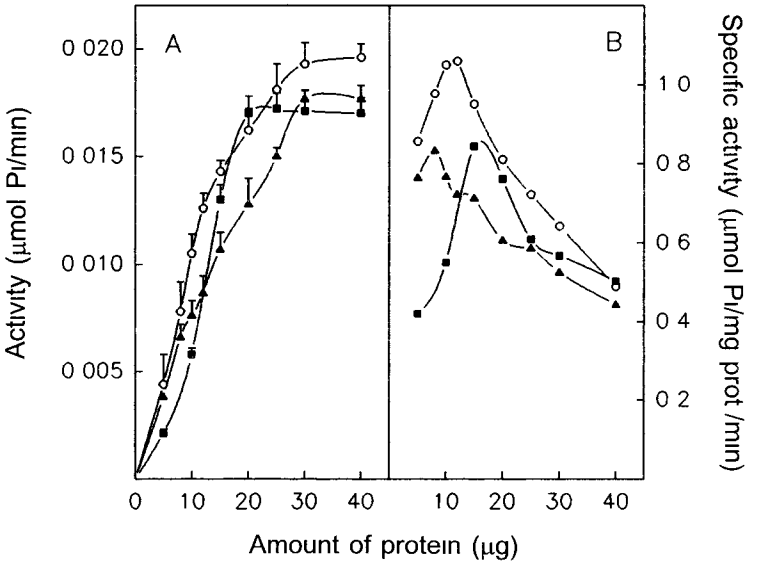

Figure 1. Effect of varying membrane protein concentrations on the Na K-ATPase activity expressed as µmol Pi/min (*A*) and µmol Pi/mg protein/min (*B*). Enzyme activities were measured at 37°C as a function of different amounts of the whole brain (■) hippocampal (○) and striatal (▲) SPM proteins (from 5–40 µg) in the presence of 2 mmol/l ATP. The enzymatic assays were carried out as described in Materials and Methods. Symbols represent the means ± S.E.M. of two experiments on three separate SPM preparations.

The results concerning the substrate dependence of Na K-ATPase activity are presented in Fig. 2. Similar patterns of activation by increasing ATP concentrations were observed in all brain regions, but markedly lower activity at plateau was detected in Str. The kinetic parameters K_m and V_{max} were calculated from the Eadie–Hofstee semi-reciprocal plot of V vs. $V/[S]$ using software package ENZEDUC 1.0 for computation of hyperbolic enzyme kinetics (Marino and Fediuni 1996). V_{max}, K_m and V_{max}/K_m derived from these plots are shown in Table 2. It can be noticed that regional variations are marked for K_m values. The Na K-ATPase in Str exhibits almost 2-fold higher apparent affinity for ATP than in Hippo and WB. On the other hand, Hippo and WB Na K-ATPases have a significantly higher V_{max} values (52%, $p < 0.005$) than Str enzyme. Table 2 also displays V_{max}/K_m ratios which represent enzymatic physiologic efficiency (phys. eff.) (Grissar et al. 1979, 1980) or 'kinetic power' of an enzyme reaction that is related to the evolution of catalytic efficiency, conformational flexibility and enzyme organization (Keleti 1988). This ratio also minimizes the standard error of the estimated kinetic constants (Duggleby and Clarke 1991). The analyses of data indicate that, despite the

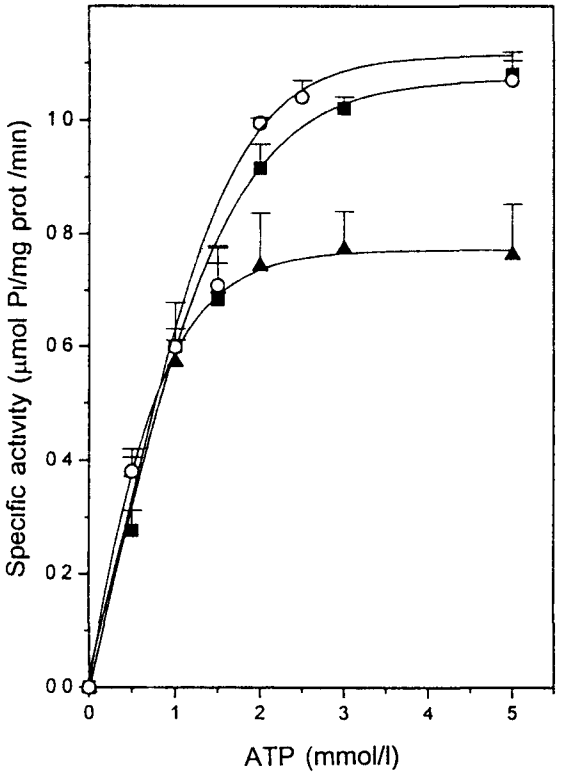

Figure 2. Substrate concentration dependence of the whole brain (■) hippocampal (○) and striatal (▲) Na,K ATPase activity. The enzyme substrate affinity was determined at different ATP concentrations (0.5–5.0 mmol/l) as described in Materials and Methods. Symbols represent means ± S.E.M. of four determinations (run in duplicate) on four separate SPM isolations. The lines represent the best fits obtained by fitting data according to the Michaelis-Menten hyperbolic enzyme kinetics in Microcal Origin 3.5 scientific graphic software package.

differences in K_m and V_{max}, the physiological efficiency of Na,K-ATPase is almost equal in WB, Hippo and Str ($p > 0.05$).

The presence of multiple enzyme isoforms of Na,K-ATPase was determined on the basis of affinity for the inhibitor ouabain. The fraction of total Na,K ATPase activity (i.e. residual Na,K-ATPase activity) remaining at each ouabain concentration after subtracting ouabain-insensitive Mg ATPase activity measured in the presence of 0.1 mmol/l ouabain is shown in Fig. 3. As can be seen, in all SPM preparations analyzed, up to 70% of the activity was inhibited with 10 µmol/l ouabain while complete inhibition was obtained with 1 mmol/l drug. The obtained curves

Table 2 Kinetic parameters as determined from the data presented in Fig 2

SPM preparation	K (mmol/l)	V_{max} (μmol Pi mg protein^{-1} min^{-1})	Phys eff (V_{max}/K_m)
Whole Brain	1 29 ± 0 02	1 28 ± 0 02	0 99 ± 0 01
Hippocampus	1 21 ± 0 18	1 28 ± 0 04	1 06 ± 0 03
Striatum	0 76 ± 0 06*	0 84 ± 0 05*	1 10 ± 0 03

Kinetic parameters were estimated using Eadie Hofstee plot Values represent means ± S E M from at least four determinations (run in duplicate) on four separate SPM isolations * $p < 0$ 005 (vs kinetic parameters obtained for whole brain Na K ATPase)

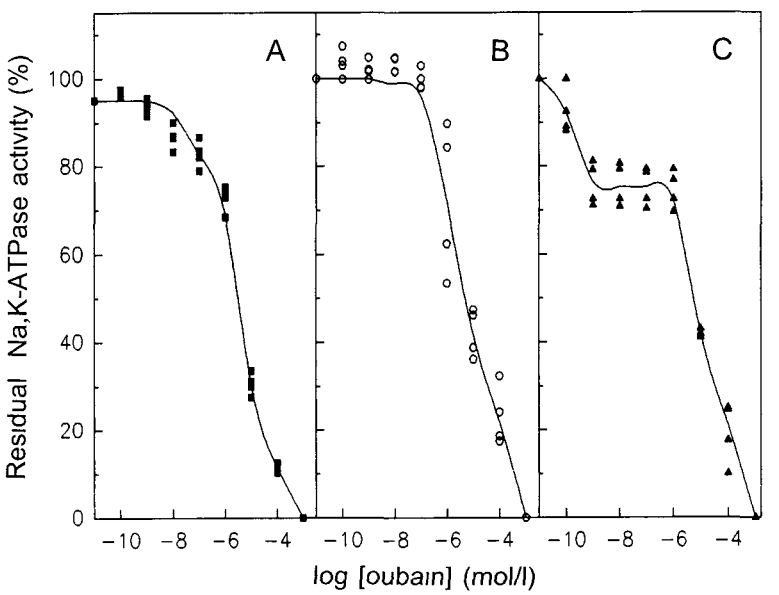

Figure 3 Dose response curves to ouabain of the whole brain (*A*) hippocampal (*B*) and striatal (*C*) Na K ATPase to ouabain Na K-ATPase was measured in the presence of various concentrations of ouabain (10^{-10} 10 ^{3} mol/l) Activities were expressed as percentages of the respective activity detected in the absence of ouabain after subtraction of ouabain resistant (Mg ATPase) activity obtained in the presence of 0 1 mmol/l drug SPM preparations were preincubated with ouabain for 15 min at 37°C before ATP addition Symbols represent the results of four experiments on two different SPM preparations

were fitted for one two and three independent inhibitory states for ouabain The best fits were achieved for the presence of one inhibitory state of high affinity in

Hippo (Fig 3*B*) and two inhibitory states of high affinity in WB (Fig 3*A*) and Str (Fig 3*C*). In addition all SPM preparations exhibited one inhibitory state of low affinity for ouabain. The three states were denoted as 'very high-' 'high' and 'low-affinity' enzyme forms as adopted from Berrebi-Bertrand et al (1990). The apparent affinities for ouabain (K_i) and the respective contributions of each inhibitory form to the total enzyme activity as calculated from these curves, are presented in Table 3. As can be seen the apparent K_i for the 'very high-" and 'high-affinity' forms markedly differed ($p < 0.05$) while K_i for the 'low affinity' form were almost the same ($p \gg 0.05$) in all SPM preparations. Table 3 also shows Hill coefficients (n) as calculated according to Hill equation for inhibitors that display sigmoidal dose-response curves (Segel 1968). Since the 'very high-affinity' forms of WB and Str have $n_H \geq 1$ they appear to exhibit positive cooperativity the same holds for the high affinity form in Hippo with $n_H > 1$ classifying it in the category of 'very high affinity' form (Sweadner 1985, Urayama and Sweadner 1988). On the other hand the 'high' and 'low affinity' forms have $n_H < 1$ indicating negative cooperativity.

Discussion

The existence of multiple forms of Na,K-ATPase has been known for many years (Sweadner 1989). However the presence of multiple forms of each subunit greatly complicates the analysis of the enzyme spatial distribution and regulation. Each cell type probably has its unique combination of regulatory controls including regulation of isoform selection, levels of gene expression, sensitivities to various modulators and spatial distribution (Fambrough 1988). In the past few years a great progress has been done in mapping the cellular distribution of each isoform of the sodium pump in the rat brain using *in situ* hybridization histochemistry (Schneider et al. 1988, Brines et al. 1991) and immunocytochemistry (Cameron et al. 1994). Additionally the enzymatic properties of individual α and β isoforms have been determined (Jewell et al. 1992, Blanco et al. 1995a,b). Despite intense studies, the correlation of enzymatic properties of the individual isoforms to their physiological role in differential neural cell type and different brain regions is still not fully understood. The aim of the present study was to distinguish some of the differences in basic enzymatic properties that exist between Na,K-ATPases in distinct brain regions of adult female rats.

A comparison of specific activities of Na,K-ATPases from the hippocampal, striatal and whole rat brain SPM revealed that both the highest activity and per cent contribution to the total ATPase activity (65%) are associated with hippocampal SPM preparations. This is in agreement with findings from autoradiographic (Spyropoulos and Rainbow 1984, Caspers et al. 1987) and hybridization (Schneider et al. 1988, Brines et al. 1991) studies that the number of functional sodium pump

Table 3. Apparent affinities for ouabain (K_i) Hill coefficients (n_H) and respective contributions (%) of very high- , high- and low -affinity enzyme forms (derived from Fig 3)

SPM preparation	Enzyme form								
	Very high-			High-			Low-		
	K_i (nmol/l)	n_H	%	K_i (μmol/l)	n_H	%	K_i (mmol/l)	n_H	%
Whole brain	24 3 ± 0 2	1 01 ± 0 02	17	12 7 ± 0 8	0 83 ± 0 02	50	0 21 ± 0 01	0 72 ± 0 01	21
Hippocampus	660 ± 12*	1 11 ± 0 07	36				0 31 ± 0 09	0 72 ± 0 02	45
Striatum	37 3 ± 1 2*	0 97 ± 0 03	22	42 1 ± 1 6*	0 86 ± 0 03	46	0 27 ± 0 07	0 74 ± 0 01	19

Means ± S E M from four determinations on two different SPM preparations * $p < 0\ 005$ (vs whole brain K_i values)

molecules is the highest in the hippocampus the olfactory bulbs the cortex and the cerebellum

The most striking difference between Na K-ATPases of investigated brain regions was in their apparent affinities for ATP which was almost twice as high in the striatum than in the hippocampus This difference however was compensated for by significantly higher V_{max} of hippocampal Na K-ATPase resulting in equal physiological efficiencies of the enzymes This suggests that both Na K-ATPases have the potential of possessing distinct capabilities to accommodate to specific cellular conditions that are a reflection of fundamental differences in the milieu Thus different principal neurotransmitters (Akagawa and Tsukada 1979 Segal et al 1980 Bertorello et al 1990 Horiuchi et al 1992) endogenous modulator molecules (Rodriguez de Lores Arnaiz 1993 Kawane et al 1994) and since the present work was done on female rats specific influence of ovarian steroids (Teyler et al 1980 Bettini et al 1992) may potentiate the differences between the hippocampal and striatal Na K-ATPase

The presence of multiple enzyme isoforms of Na K-ATPase in the investigated brain regions was determined on the basis of affinity for the inhibitor ouabain It is known that high affinity ouabain binding sites are associated with α_2 and α_3 subunits while the low-affinity ouabain binding site is associated with the α_1 subunit The low-affinity form has K_i value repeatedly reported to be in the range of 10^{-4} mol/l which makes it easy to identify Similarly, in our study the apparent K_i values for low-affinity form were in the same range In contrast K_i values reported for high-affinity ouabain binding for rat brain Na K-ATPase varied from 0 02 to 1 0 μmol/l (Urayama and Sweadner 1988 Hsu and Guidotti 1989 Berrebi-Bertrand et al 1990) Thus it was difficult to determine which form (α_3 or α_2) corresponds to the very high or the high affinity component of ouabain-sensitive ATPase activity described here However Hill coefficient of $n_H \geq 1$ indicating positive cooperativity and reflecting the amplification of sensitivity to ouabain was reported for α_3 isozyme (Sweadner 1985 Urayama and Sweadner 1988 Shyjan et al 1990) On the other hand $n_H < 1$ implying negative cooperativity and low affinity for ouabain is typical for rat kidney Na K-ATPase which is considered as a model of α_1 containing tissue The results presented here (Table 3) showed that irrespective of the brain region the very high-affinity form has positive cooperativity suggesting that it is predominantly α_3 like whereas negative cooperativity of the low-affinity form indicates its α_1 origin Interestingly, the high-affinity form also demonstrates negative cooperativity This type of reactivity has so far been described only by Berrebi-Bertrand et al (1990) If not the inherent property of α_2 the negative cooperativity might be a consequence of alterations of the transmembrane Na K ATPase segment due to the membrane isolation procedure (Berrebi-Bertrand et al 1990) or modifications in the phospholipid composition (Harris 1985 Yoda and Yoda 1987)

The analysis of the apparent affinity for ouabain showed that hippocampal and striatal Na,K-ATPase had dissimilar sensitivities to ouabain. Thus when compared to the striatal the hippocampal "very high-affinity" form is approximately 18 times less sensitive to ouabain. Additionally, the sum contribution of the high affinity form to ouabain is markedly smaller in the hippocampus, representing 36% of the total enzyme activity in comparison to 68% in the striatum. In contrast, the "low affinity" form is more abundant in the hippocampus (45%) than in the striatum (19%). Considering these results, we proposed that hippocampal Na,K-ATPase might be less sensitive to an endogenous ouabain-like factor, and therefore more sensitive to stimulatory effect of noradrenaline and serotonin (Rodriguez de Lores Arnaiz 1993). Such stimulated Na,K-ATPase activity, followed by hyperpolarization of neuronal membrane, could be responsible for the depression of neuronal spontaneous firing (Segal 1981, Phillis 1992). Conversely, the higher affinity of striatal Na,K-ATPase to ouabain makes it more sensitive to the action of endogenous ouabain, leading to a reduction of its activity. Consequently, cells containing such enzyme activity will be vulnerable to neurotoxic effects of glutamate in the milieu. Since it is known that α_2 and α_3 isoforms are specifically involved in the homeostatic response to excitatory amino acids (EAAs) stimulation (Brines and Robbins 1992), greater contents of both high-affinity forms in the striatum may have a role in the protection of striatal neurons from EAA toxicity.

Taken together, our results suggest that the hippocampal and striatal Na,K-ATPases may have enzymatic properties selected in response to the milieu required for the specific physiological roles of the brain regions investigated. Therefore examination of the effects of different endogenous neuromodulators, neurotransmitters and hormones that will give a better insight into their role in the regulation of the hippocampal and the striatal Na,K-ATPase will be the subject of our further interest.

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