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Developmental changes of soluble and membrane-bound aspartate aminopeptidase activities in rat brain

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Specific soluble and membrane-bound aspartyl–naphthylamide hydrolyzing activities were assayed in brain subcellular fractions from rat fetuses (19-20 days of gestation), and from 1-week-old and 1-, 5- and 24-month-old rats. Both enzymatic activities showed a heterogeneous distribution, with highest concentrations mainly in the microsomal fraction. Membrane-bound activity was in most cases higher than soluble activity. With the exception of soluble activity in the nuclear and microsomal fractions, significant age-related changes were observed in all fractions for both enzymatic activities. Soluble activity showed a homogeneous developmental profile in most of the fractions, with the lowest levels in 1-month-old rats and the highest in 1-week and 5-month-old animals. However, changes in the microsomal fraction did not follow the pattern displayed by the rest of the fractions. No clear developmental profile in specific membrane-bound activity was observed, each fraction exhibiting a different sequence of changes. Whereas in 24-month-old-rats there was a significant increase in activity in homogenate, nuclear and microsomal fractions, a significant decrease was observed in the synaptosomal fraction. These results may reflect the functional status of the endogenous substrates of the enzymes.

Key words: Aminopeptidase, Subcellular distribution, Brain, Development, Aging.

The brain renin-angiotensin system seems to be complete with the precursor peptides and peptidases necessary for the formation and degradation of biologically active forms of angiotensins. These angiotensins

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serve as ligands at several binding subtypes that are presumed to mediate the well established functions of brain angiotensin that involve regulation of the cardiovascular system, body water balance, cyclic regulation of reproductive hormones and behaviors in concert with angiotensin control over the secretion of pituitary hormones (24).

Peptidases play a major role in the neuropeptides metabolism. These enzymes do not always simply inactivate a peptide, but may change its biological activity (13, 14). Such is the case in the renin-angiotensin system and, although angiotensinases have been described in brain, less is known about their functional importance. Asp-β-naphthylamide hydrolyzing activity (Asp-aminopeptidase activity, AspAP) may be involved in angiotensin metabolism, producing des-Asp^1-angiotensin I and angiotensin III by removing the amino terminal Asp^1 from its substrates angiotensin I and angiotensin II (17). The establishment of the subcellular localization of this activity and its eventual change during development are important in order to understand the regulatory mechanisms controlling the activity of neuropeptides susceptible to be hydrolyzed.

In the present work α-Asp-naphthylamide (AspNNap) has been used, to detect AspAP activity (4, 17). The subcellular distribution of soluble (Sol) and membrane-bound (M-B) AspAP activity from fetal (19-20 days), 1-week-old, and 1-, 5- and 24-month-old rat brains are analyzed.

Materials and Methods

Fetuses (19-20 days), 1-week-old, and 1-, 5- and 24-month-old male Sprague-Dawley rats were used. The rats were housed at a 25 °C constant with lights on from 7.00 to 19.00, and free access to water and food. To avoid diurnal variations (19), all experiments were started at the same time of the day (9.00). To determine fetal age, one male and six female adult rats were kept in a cage overnight, then the male was removed and gestation determined by the presence of sperm in vaginal smears. The fetuses were removed 19-20 days later. Brains were perfused with saline through the left ventriculum under equithensin anesthesia (2 ml/kg b. w.). To obtain enough tissue for each experiment (2.5 - 3 g), the brains of 8-10 fetuses, 5 animals of 1 week and 3 animals of 1, 5 and 24 months of age were pooled and used as starting material. After perfusion, the brains were quickly removed and homogenized in 10 volumes of 0.32 M sucrose (homogenate). Subcellular fractions were obtained according to the method of GRAY and WHITTAKER (9) modified by KRUEGER *et al.* (12).

Briefly, the procedure separated the primary fractions into P_1 (1,000 *g*, nuclear), P_2 (12,500 *g*, crude mitochondrial), P_3 (100,000 *g*, microsomal) and high-speed supernatant S_3 (cytosolic). Further fractionation of the crude mitochondrial pellet was obtained by centrifugation (100,000 *g*) of fraction P_2 on a discontinuous sucrose gradient (0, 7.5 and 14 %), which yielded fractions A (myelin), B (synaptosomal) and C (mitochondrial). Samples from P_1, B, C, P_3 and total homogenate were individually homogenized in 10 mM Tris-HCl buffer (pH 7.4) and centrifuged (100,000 *g*, 30 min, 4 °C). These supernatants and those of fraction S_3 (cytosolic) were used to test soluble AspAP activity and proteins, assayed in triplicate. The resultant pellets and fraction A (myelin) were homogenized in Tris-HCl 10 mM buffer (pH 7.4) containing 1 % Triton X-100. After centrifugation (100,000 *g*, 30 min, 4 °C), the supernatants were used to test membrane-

bound AspAP activities and proteins, also in triplicate.

AspAP activity was determined fluorometrically with AspNNap as the substrate, according to the method of CHEUNG and CUSHMAN (4) modified as follows: 10 ml of each supernatant was incubated for 120 min at 37 °C with 1 ml of the substrate solution (1 mg/100 ml AspNNap, 10 mg/100 ml BSA and 10 mg/100 ml $MnCl_2$ in 50 mM HCl-Tris buffer, pH 7.4). Activity was previously shown to be linear with time and protein content after 120 min of incubation. The reaction was stopped by adding 1 ml 0.1 M acetate buffer (pH 4.2).

The β-naphthylamine released as a result of enzymatic activity was measured fluorometrically at an emission wavelength of 412 nm with an excitation wavelength of 345 nm. Proteins were measured in triplicate by the method of BRADFORD (2). Specific soluble and membrane-bound activities were expressed as *p*mol of AspNNap hydrolyzed per min per mg of protein.

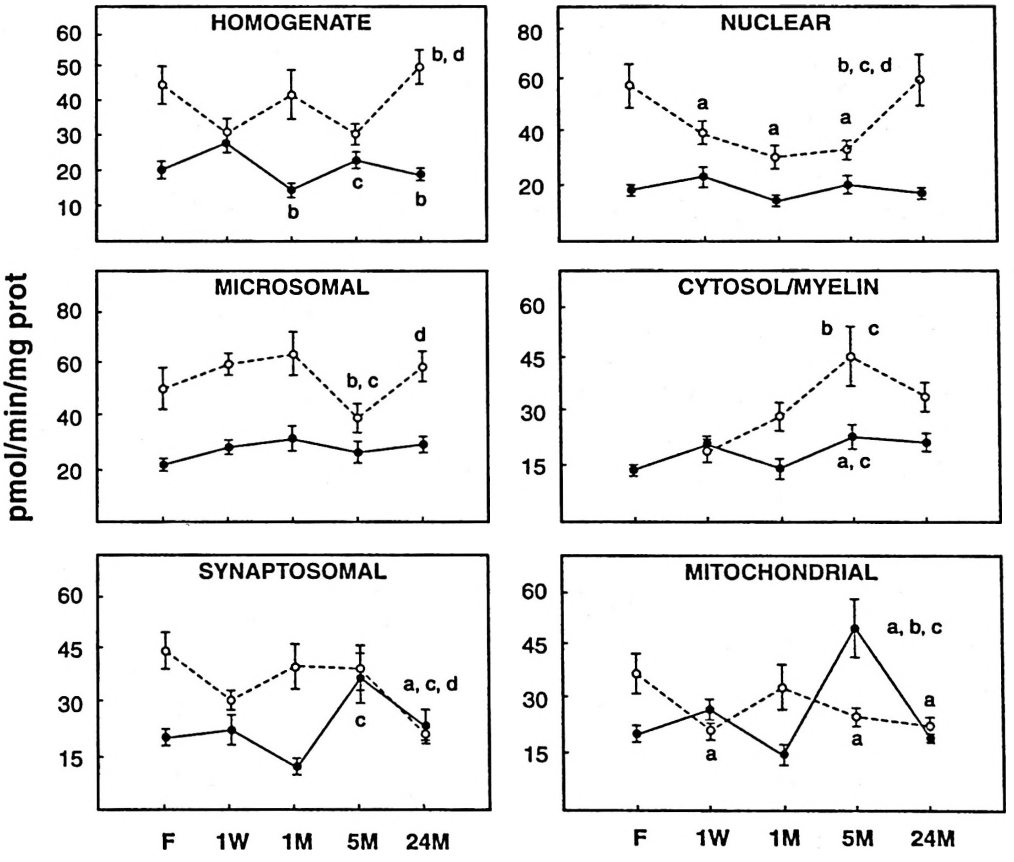

Fig. 1. *Soluble (—) and membrane-bound (---) AspAP activities in homogenate, nuclear, microsomal, cytosol, myelin, synaptosomal and mitochondrial fractions of rat brain tissue.*
Tissues: from fetuses (F; n = 6-8), 1-week-old (1W; n = 9-12), 1-month-old (1M; n = 5-8), 5-month-old (5M; n = 7-12) and 24-month-old rats (24M; n = 5-9). Specific activities are expressed as pmol of AspNNap hydrolyzed per min per mg of protein. In synaptosomal and mitochondrial fractions, data of fetuses correspond to P_2 fraction. Statistical significance ($p < 0.05$): a, vs F; b, vs 1 W; c, vs 1 M; d, vs 5M.

One-way analysis of variance (ANOVA) was used to compare the results between ages; if significant, comparisons between the means were made with the Duncan's test. Values of $p < 0.05$ were considered significant.

Results

The age-related changes of Sol and M-B AspAP activities, between fetal, 1-week, 1-, 5- and 24-month old rat brains, are represented in figure 1. Sol and M-B activities were distributed heterogeneously, membrane-bound activity being in most cases higher than that in the soluble fraction.

The soluble microsomal fraction was the best source of enzymatic activity in fetal, 1-week-old, 1- and 24-month-old rats. However, in 5-month-old rats, soluble activity was predominant in the synaptosomal and mitochondrial fractions. Microsomal and nuclear fractions were also good sources of M-B AspAP activity.

With respect to changes in enzyme activity during development, Sol and M-B activities showed significant age-related changes in all subcellular fractions, with the exception of Sol activity in the nuclear and microsomal fractions. Specific Sol AspAP activity seemed to show a homogeneous pattern during development, with minimum levels in fetal, 1-month-old and 24-month old rat brains, and peaks in 1-week- and 5-month-old rats. The most significant age-related changes were observed in Sol activity in mitochondrial fraction where they increased significantly in 5-month old rats ($p < 0.001$) and decreased in 24-month old ones ($p < 0.001$). However, this pattern differed in the microsomal fraction, which did not exhibit changes during development. On the other hand, there was no clear developmental pattern in specific M-B AspAP activity, which differed significantly from Sol activity, showing in some fractions an opposite profile of development.

Membrane-bound activity increased significantly between 5 and 24 months in the homogenate ($p < 0.01$), nuclear ($p < 0.01$) and microsomal ($p<0.05$) fractions, and decreased significantly between fetuses and 1-week old animals in nuclear ($p < 0.01$) and mitochondrial ($p < 0.05$) fractions. However, the significant decrease in M-B activity in the synaptosomal fraction in 24-month-old rats is noteworthy, given the absence of changes in the younger specimens.

Discussion

Although the subcellular distribution in brain tissue and age-related changes in several aminopeptidase activities have been described previously (5, 11, 15, 16, 18) no studies have investigated the subcellular distribution and developmental profile of AspAP activity in its soluble and membrane-bound forms. Studies of the pattern of subcellular distribution during development and aging may help us to understand the functional role of AspAP activity and its substrates in the central nervous system. In this case, the opposite pattern of development which exhibit both soluble and membrane-bound activities in some fractions, could suggest a reciprocal relationship in their regulatory functions hydrolyzing susceptible substrates.

The only aminopeptidase previously reported to be specific for dicarboxylic amino acids is glutamate aminopeptidase (GluAP; Aminopeptidase A; EC 3.4.11.7), which also readily removes the N-terminal Asp residue from α-L-Asp1-angiotensin II (17). GluAP does not hydrolyze β-naphthylamide derivatives of

amino acids other than aspartic and glutamic acids. This specificity also applies to the hydrolysis of true peptide bonds such as those in angiotensin analogs (4). It is also possible that an aspartate aminopeptidase, previously reported to be present in brain (22), but with low activity on β-naphthylamide derivatives, is partially responsible for the hydrolysis of N-terminal Asp-residues of peptides. Therefore, with AspNNap as the substrate, the release of β-naphthylamine may be due to the action of at least two different enzyme activities. In the absence of a purification process, the participation of more than one enzyme cannot be excluded. Because of its specificity and its rapid action on N-terminal aspartic acid residues of angiotensin analogs, Asp-β-naphthylamide hydrolyzing activity in its soluble and membrane-bound forms, may play an important physiological role as an angiotensinase (4). In addition, AspAP can also acts on N-terminal Asp residues other than angiotensin II, such as the cholecystokinin (26-33) octapeptide or cholecystokinin (26-29) tetrapeptide.

A heterogeneous distribution and significant age-related changes for Sol and M-B AspAP activities, both of which have different patterns of distribution and change during development has been documented, these findings imply a different functional role for each activity. Other authors have described a progressive increase in brain proteins until one month of age, with no further changes in adult (16) or aged rat brains (8). Therefore, the heterogeneous developmental changes in AspAP activity in subcellular fractions described in the present work were not due to modifications in protein content.

The high levels of Sol and M-B AspAP activities in the microsomal fraction in most ages tested here may be related to the synthesis or processing of target proteins during axoplasmic transportation. Moreover, the marked changes in Sol AspAP activity in synaptosomes, mitochondria and cytosol support a role for this activity in these locations, suggesting changes in the functional status of their substrates during development and aging. In these fractions, activity peaks in 5-month-old rats, when maturation and development are complete. However, both Sol and M-B activities decrease significantly in the synaptosomal fraction in aged animals, a change that may reflect a loss of the putative functional role of their substrates in nerve-ending events.

Early studies of enkephalinase activity (20) suggested the synaptic neuropeptidase model as one of the major inactivation mechanisms of neuropeptides. This model involves postsynaptic membrane-bound peptidases acting in close concert with a neuropeptide receptor. However, because soluble and membrane-bound forms of aminopeptidase activity, with a heterogeneous subcellular distribution and significant changes during development and ageing, have been described, there may be other mechanisms of regulation. These include the participation of peptidases from glial cells (10), reuptake processes (3, 7), degradation during transport, control of the activity of the degrading enzyme by feedback mechanisms (1), and unidentified mechanisms regulating the concentration, and perhaps the action, of coexisting neuropeptides by the simultaneous presence of different enzyme activities in the same neuron (21). Therefore, the changes that occur in subcellular locations other than the synaptosome ie, the microsome, cytosol or mitochondria, may also reflect regulatory enzymatic control over susceptible endogenous substrates. Also, the soluble and membrane-bound forms of peptidases should be take into consideration in a neuropeptidase model of regulation of neuropeptides.

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Se determina la actividad soluble y unida a membrana responsable de la hidrólisis de la aspártico–β-naftilamida en fracciones subcelulares de cerebro de feto de rata (19-20 días de gestación), ratas de 1 semana, 1 mes, 5 meses y 24 meses de edad. Ambas actividades muestran una distribución heterogénea, con la mayor concentración en la fracción microsomal. La actividad unida a membrana es, en la mayoría de los casos, mayor que la soluble. Excepto la actividad soluble en las fracciones nuclear y microsomal, en el resto de fracciones se observan cambios significativos con la edad, para ambas actividades enzimáticas. La actividad soluble muestra un perfil de desarrollo homogéneo en la mayoría de las fracciones, con los menores niveles en ratas de 1 mes y los mayores en animales de 1 semana y 5 meses de edad. Sin embargo, los cambios observados en la fracción microsomal, no siguen el modelo del resto de fracciones. En la actividad unida a membrana, no se observa un claro perfil de desarrollo, mostrando cada fracción una secuencia diferente de cambios. Mientras que en ratas de 24 meses se observa un aumento significativo en homogenado, fracción nuclear y microsomal, la fracción sinaptosomal muestra un descenso. Estos resultados pueden reflejar el estado funcional de los substratos endógenos de estos enzimas

Palabras clave: Aminopeptidasa, Distribución subcelular, Cerebro, Desarrollo, Envejecimiento.

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