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Short Contribution

Membrane-bound pyroglutamyl-arylamidase activity during the first postnatal month in several rat brain areas

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ABSTRACT Membrane-bound pyroglutamyl-aminopeptidase activity cleaves the pyoglutamate amino acid bond of thyroliberin (TRH). Information concerning developmental variations in TRH has been reported. However, little is known about the ontogeny of the membrane-bound enzyme activities capable of hydrolyzing the mentioned tripeptide. In this work we have described decreases in membrane-bound pyroglutamyl-aminopeptidase (arylamidase) activity, from day 9 to day 20 after birth, in the hypothalamus, the striatum, the frontal, occipital and parieto-temporal cortices and the pituitary gland of the male and the female rat. The developmental profile is similar in rats of both sexes. We have not found significative changes between 20 and 25 postnatal days. The observed decreasing activity is developmentally coincident with the increases in thyroliberin and decreases in Hys-Pro diketopiperazine concentration in different brain areas. It is suggested that membrane-bound pyroglutamyl-peptidase activity could play a part in the normal development of thyroliberin physiology.

KEY WORDS: *pyroglutamyl-peptidase, thyroliberin, rat brain, development*

Two pGlu-aminopeptidases have been described in the rat brain, a cysteine protease and a metalloprotease (O'Cuinn *et al.*, 1990). The former enzyme, pGlu-aminopeptidase I (E.C. 3.4.19.3), cleaves all pGlu-amino acid bonds except pGlu-Pro (Browne and O´Cuinn, 1983). Substrates of pGlu-aminopeptidase I, in addition to TRH, include LH-RH, neurotensin and bombesin (Browne and O'Cuinn, 1983). PGlu-aminopeptidase II (E.C. 3.4.19.-) is membrane-bound and has been purified from the brains of different species (O'Connor and O'Cuinn, 1985; Wilk and Wilk, 1989). This metalloenzyme appears to be a specific TRH degrading enzyme (O'Cuinn *et al.*, 1990).

Alteration of TRH physiology seems to occur during the first postnatal month (Lamberton *et al.*, 1984). However, the mechanisms for the change in brain tissue thyroliberin levels during development are not clear. Thus, different developmental profiles for aminopeptidases controlling TRH activity have been described (Faivre-Baumman *et al.*, 1981; Fuse *et al.*, 1990; Gandarias *et al.*, 1992).

Arylamidases represent a subclass of aminopeptidases which are capable of hydrolizing, apart from peptide substrates, aminoacyl-2-naphtylamides. In the present study, we addressed possible developmental changes in membrane-bound pGlu-arylamidase activity in five brain areas and the pituitary gland of the male and female rat. We have also alluded to made sexual differentiation because several studies have described differences in the physiology of TRH during the first postnatal month depending on the sex of the rat (Gayo *et al.*, 1986).

Figure 1 shows the membrane-bound pGlu-arylamidase activity in the hypothalamus and the pituitary gland of male and female rats. We have seen decreases of the enzyme activity from day 9 to day 15 ($p<0.01$), in the pituitary and up to day 20 in the hypothalamus ($p<0.01$), and a maintenance until postnatal day 25 in both areas. There are no sexual differences. The activity of a membrane-bound enzyme in the frontal and parietal cortices is shown in Fig. 2. As in the hypothalamic-pituitary axis, pGlu-arylamidase activity decreases from 9 to 20 postnatal days ($p<0.01$). We have been unable to detect sexual differences. Finally, Figure 3 shows the membrane-bound pGlu-arylamidase activity in the occipital cortex and in the striatum. The enzyme activity decreases in the cortical area from day 9 to day 15 and in the striatum up to day 20. However there are no significant differences between the 20th and the 25th postnatal days. There is no sexual differentiation of enzyme activity in these areas.

Results obtained in this research show, first, that all the brain areas studied and the pituitary gland have membrane-bound pyroglutamate-arylamidase activity during all the developmental stages assayed. There are no sexual differences in the ages studied. This data contrasts with the sexual differences found in

Abbreviations used in this paper: TRH, thyrotropin releasing hormone or thyroliberin; LH-RH, luteinizing hormone releasing hormone; CNS, central nervous system; pGlu, pyroglutamate; DKP, hystidyl-prolyl diketopiperazine.

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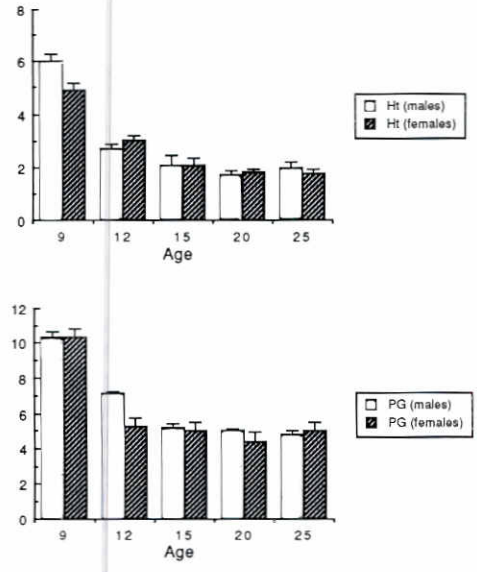

Fig. 1. Membrane-bound pyroglutamyl-arylamidase activity levels in the hypothalamus (Ht, upper) and the pituitary gland (PG, lower) *of male and female rats during different stages of development (9,12,15,20,25= postnatal days). Values represent mean±SEM (units of arylamidase/mg protein).*

soluble pGlu-aminopeptidase (Gandarias *et al.*, unpublished results). However, it is remarkable that the LHRH is a substrate for the soluble form, while membrane-bound activity has a high degree of specificity for TRH.

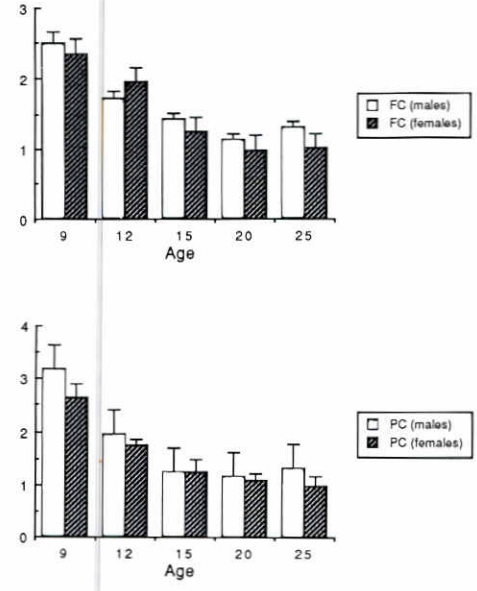

Fig. 2. Membrane-bound pyroglutamyl-arylamidase activity levels in the frontal (FC, upper) and the parietal (PC, lower) cortices *of male and female rats during different stages of development (9,12,15,20,25= postnatal days). Values represent mean±SEM (units of arylamidase/mg protein).*

We also reported here on the changes of membrane-bound pGlu-arylamidase during the rat brain development. The age-related alterations produced in male and female rats are very similar to those described by Vargas *et al.* (1992) utilizing tritiated TRH as substrate. Thus, brain enzyme activity decreases from the second to the third week after birth. The decreasing activity of membrane-bound pGlu-aminopeptidase (arylamidase) in the brain regions investigated is in accord with increasing levels of TRH. In this sense, several authors described decreases in hypothalamic and extrahypothalamic TRH content up to the third postnatal week (Aratan-Spire *et al.*, 1983; Nemeskeri *et al.*, 1985). Moreover, hypothalamic TRH mRNA content remained unchanged in this period (Taylor *et al.*, 1990). These data might reinforce the hypothesis that this enzyme plays an active role in controlling biological activity of TRH. However, the maintenance or slight changes of the enzymatic activity observed between day 20 and 25 cannot explain the dramatic two fold increase in the peptide content at this age

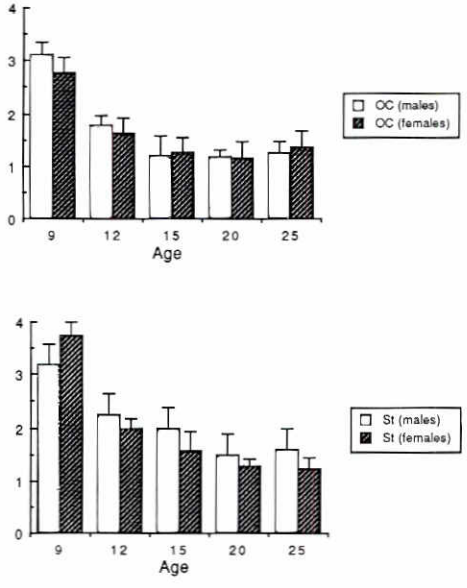

Fig. 3. Membrane-bound pyroglutamyl-arylamidase activity levels in the occipital cortex (OC, upper) and in the striatum (St, lower) *of male and female rats during different stages of development (9,12,15,20,25= postnatal days). Values represent mean±SEM (units of arylamidase/mg protein).*

reported by Aratan-Spire *et al.* (1983). Thus, it could be suggest that an increase in gene transcription, translation or altered posttranslational events are likely to contribute to elevate TRH levels during this period.

The products of the action of pGlu-aminopeptidase on TRH are pGlu-and Hys-ProNH_2. In the absence of further enzyme activity the latter metabolite will nonenzymatically and spontaneously cyclize to His-Pro diketopiperazine (DKP), which is itself reported to possess endocrine activity (Brabant *et al.*, 1981) as well as numerous central nervous activities (Prassad *et al.*, 1982). The high pGlu-aminopeptidase (arylamidase) activity found in 9 day-old rats is in accord with the high concentration of the mentioned metabolite in the brain (Prassad *et al.*, 1983). These data could

reinforce the hypothesis that pGlu-aminopeptidase activity plays an active role in determining the levels of endogenous DKP concentration in the brain.

In summary, this study demonstrated developmental decreases in brain and pituitary gland pGlu-arylamidase activity in both sexes from the 9th to the 15th or 20th postnatal days. Since, these changes are coincident with increases in hypothalamic and extrahypothalamic TRH levels, It could be suggested that this enzyme activity might play a part in the normal development of TRH in rat brain.

Experimental Procedures

Male and female Sprague-Dawley rats, bred in our colony and maintained under conditions of controlled light (12 h) and temperature (24°C), with food and water *ad libitum* were used in this investigation. The ages of the animals were 9 days (n= 9), 12 days (n= 9), 15 days (n= 9), 20 days (n= 9) and 25 days (n= 9) postnatal. In order to avoid proteolytic contamination from blood, animals from five groups were perfused with saline plus 50 mM phosphate buffer, pH 7.4, through the left cardiac ventricle under Equithensin anesthesia. The brains were quickly removed and cooled in dry ice. Brain samples, taken by dissection, were as follows: frontal, parietal and occipital cortex, hypothalamus, striatum and pituitary gland. The brain samples were homogenized (in Tris HCl 10 mM, pH 7.4) and ultracentrifuged (100,000xg, 35 min). The resulting pellets were washed 3 times by suspension in Tris buffer in order to avoid contamination of the soluble form. After, the pellets were homogenized in Tris HCl 10 mM (pH 7.4) plus 1% of Triton X-100 to obtain, after ultracentrifugation (100,000xg, 35 min), supernatant solutions used to determine the membrane-bound activity and protein quantification. All preparative steps were carried out at 4°C. pGlu-arylamidase activity was fluorimetrically measured in triplicate using pGlu-2-naphtylamide as sustrate, by the method of Greenberg (1962), but with the recent modifications described by Alba *et al.* (1989): 10 µl aliquots of membrane-bound fractions were incubated with 1 ml of pGlu-2-naphtylamide (2 mg/100 ml) and serum albumin (10 mg/100 ml) in 50 mM phosphate buffer, pH 7.4. The reaction was halted by the addition of 1 ml of 0.1 M acetate buffer solution, pH 4.2. The 2-naphtylamine released was determined by measuring the fluorescent intensity at 412 nm with excitation at 345 nm. Relative fluorescence was converted to picomoles of 2-naphtylamine by comparison with a standard curve. Protein concentration was measured in triplicate by the method of Bradford (1976).

The results were recorded as units of pGlu-arylamidase activity per mg of protein (mean±SEM). One unit of arylamidase activity is the amount of enzyme that hydrolyzes 1 picomol of pGlu-2-naphtylamide per minute.

Global comparison of the results was done by two way ANOVA test. To compare developmental stages, results were analyzed using one-way ANOVA and significance of the sexual differences was confirmed with Student's *t* test.

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