Leu-arylamidase Activity Levels During the Estrous Cycle and Pregnancy in Several Extrahypothalamic Areas of the Rat Brain

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Abstract. A wide variety of behavioral changes in the female rat have been associated with the estrous cycle, pregnancy, and the postpartum period and their accompanying hormonal fluctuations. Since aminopeptidase activity, that presumably controls the activity of several neuroactive peptides, has been implicated in the control of these hormonal changes, the present study examined the tissue levels of Leu-arylamidase activity (Leu-ArA) in the cortices (frontal, parietal and occipital), striatum, hippocampus, amygdala, pineal gland and medulla oblongata during the estrous cycle, different stages of pregnancy (2nd, 7th, 14th and 20th postinsemination day) and the postpartum day. In the estrous cycle, Leu-ArA was significantly increased during the afternoon of the proestrous in the occipital cortex, amygdala and medulla oblongata. In general, higher enzyme levels were found during the middle stages of pregnancy. There is a significant rise after parturition in the occipital cortex, and clear but insignificant increases in the striatum and medulla. It is suggested that several extrahypothalamic areas may mediate several effects on Gn-RH secretion from the hypothalamus, and that Leu-ArA could be implicated.

Key words: Leu-arylamidase — Estrous cycle — Pregnancy — Rat brain

Introduction

The arylamidases represent a subclass of aminopeptidases which are characterized by their ability to hydrolyze amino acid naphthylamides in addition to peptide substrates (Jonhson and Hersh 1990). In the last few years, these enzymes have been implicated in the regulation of the release dynamics of gonadotropins (Gn) from the pituitary. Thus, the first works of Kuhl and Taubert (1975) and Kuhl

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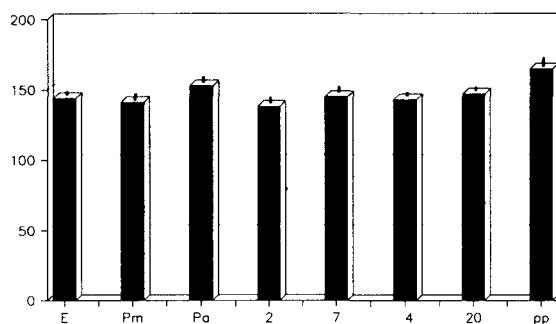
et al. (1978) showed how L-cystine-arylamidase, which is capable of inactivating LH-RH in the hypothalamus and the pituitary, fluctuates its activity during the four stages of the estrous cycle, measuring the minimal activity for the whole cycle between 12.00 h and 16.00 h of the proestrous day. Most recently, it has been shown that, in the hypothalamus, Leu-arylamidase activity (Leu-ArA) does not behave inversely but in a parallel way with respect to the release of Gn during the estrous cycle of the rat, the enzyme levels being higher during the proestrous than in the estrous stage (de Gandarias et al. 1988). It has been suggested that this aminopeptidase activity (and presumably others) (de Gandarias et al. 1989a; 1990) could play a role in the hormonal changes that take place in the hypothalamic-pituitary axis during the estrous cycle, possibly by regulating the levels of several neuroactive peptides. It is necessary to note that it is now clearly established that the secretion of Gn is controlled at the hypothalamic level not only by the classical neurotransmiters but also by numerous peptides present in the brain (Martini et al. 1989). Nevertheless, the Gn surge from the pituitary is not only controlled by the release from the hypothalamus of Gn-releasing hormone (Gn-RH), but also by a complex nervous extrahypothalamic circuitry. Thus, several changes in classical transmitters (Desan et al. 1988) and in the levels of neuropeptides (NP) (Frankfurt et al. 1986; Dyer and Bicknell 1989) and its receptors (Dyer and Bicknell 1989; Weiland and Wise 1990) have been described in the cortex, medulla and limbic system during the cycle. Also, changes have been demonstrated in these brain areas during pregnancy (Wardlaw and Frantz 1983; Desan et al. 1988; Dyer and Bicknell 1989; Glaser et al. 1990); originally, it was suggested that changes in limbic neurotransmitters might possibly contribute to the control of the hormonal changes which occur during pregnancy and the postpartum days. In the present paper, we describe the levels of Leu-ArA measured during the estrous cycle and during different stages of pregnancy in several brain areas of the rat, in order to provide more information on the possible extrahypothalamic control of the hormonal surge from the hypothalamic-pituitary axis.

Materials and Methods

Female Sprague-Dawley rats, bred in our colony and maintained under conditions of controlled illumination (lights on from 07.00 to 19.00) and temperature (24 °C), with unlimited access to water and standard rat chow, were used in this investigation. The rats were three months old. This is important in view of several age-related changes on aminopeptidase activities described previously (de Gandarias et al. 1989b;c;d). Timing of the estrous cycle was determined by examining daily vaginal smears and only rats showing two or more regular cycles were selected for the experiments. The cyclic animals were grouped into estrous (at 10.00 h) and proestrous (at 10.00 h and 15.00 h) groups ($n = 610$ each). The pregnancy stages examined were 2, 7, 14 and 20 postinsemination

days ($n = 10$ each) (the day of insemination was considered day zero of gestation) Also the parturition day ($n = 10$) was included The animals were perfused with saline plus 50 mmol/l phosphate buffer, pH 7 4, from the left cardiac ventricle under Equithensin anesthesia (2 ml/kg body wt) The brains were quickly removed and cooled on dry ice Brain samples, taken by dissection, were the frontal, parietal and occipital cortices, amygdala, striatum, hippocampus, medulla oblongata and pineal gland The brain samples were homogenized (in 10 mmol/l Tris HCl, pH 7 4) and ultracentrifuged ($100,000 \times g$, 35 min) The resulting supernatant was used for the analysis of arylamidase activity and proteins All preparative steps were carried out at $4\,^\circ$C Aminopeptidase (arylamidase) activity was measured fluorimetrically in triplicate using Leu-2-naphthylamide (Leu-NA) as substrate, by the method of Greenberg (1962) with a slight modification (Alba et al 1989) 10 μl aliquots of soluble fraction were incubated with 1 ml of Leu-NA (1 mg/100 ml) in serum albumin (10 mg/100 ml) and dithiothreitol (10 mg/100 ml) in 50 mmol/phosphate buffer, pH 7 4 The reaction was stopped by the addition of 1 ml of 0 1 mol/l acetate buffer solution, pH 4 2 The 2-naphthylamine released was determined by measuring the fluorescence intensity at 412 nm with excitation at 345 nm Relative fluorescence was converted to picomoles of 2-naphthylamine by comparison with a calibration curve Protein
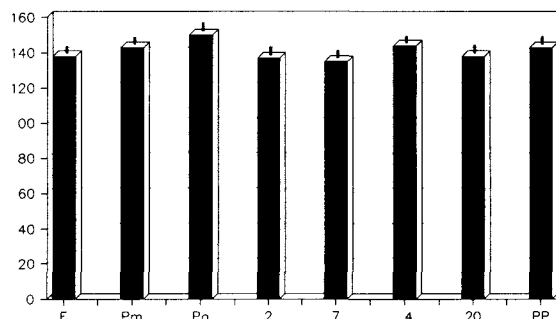
FRONTAL CORTEX

PARIETAL CORTEX

Figure 1. Leu-arylamidase activity levels during the estrous cycle (E= Estrus, Pm= Proestrus morning, Pa= Pro estrus afternoon) and during pregnancy (2, 7, 14, 20= days, PP= postpartum day) in the frontal and parietal cortices Values represent mean $\pm$ S E M (units of arylamidase/mg protein)
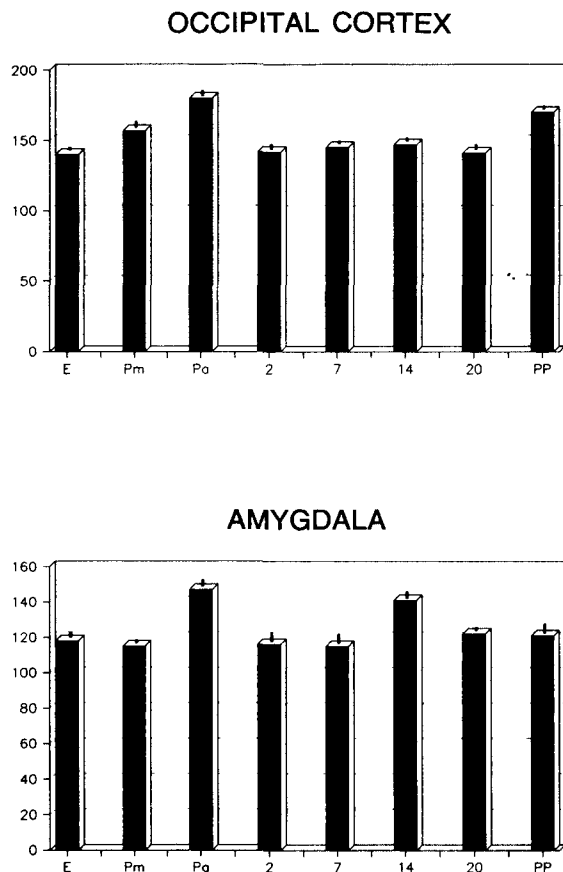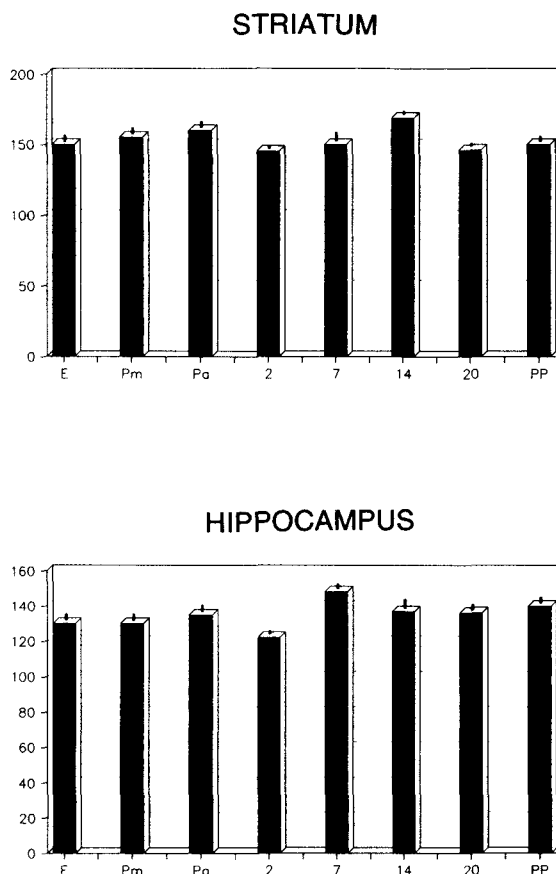
OCCIPITAL CORTEX

Figure 2. Leu-arylamidase activity levels during the estrous cycle (E= Estrus; Pm= Proestrus morning; Pa= Proestrus afternoon) and during pregnancy (2, 7, 14, 20= days; PP= postpartum day) in the occipital cortex and in the amygdala. Values represent mean ± S.E.M (units of arylamidase/mg protein).

AMYGDALA

concentration was measured in triplicate by the method of Bradford (1976). The results were recorded as units of Leu-ArA per milligram of protein. One unit of arylamidase activity is the amount of the enzyme that hydrolyzes one picomol of Leu-NA per minute. Differences between means were analyzed by the PLSD-Fisher's test, and ANOVA test was used for inter-groups comparisons.

Results

Figure 1 shows the frontal and parietal cortex Leu-arylamidase activity during the estrous cycle and during pregnancy. No significant changes were observed in these brain regions throughout the cycle and gestation. The activities of Leu-arylamidase in the occipital cortex and the amygdala are given in Fig. 2. ANOVA test detected significant differences in both areas throughout the cycle ($p < 0.05$). Thus, the levels in the occipital cortex during the proestrus (morning $p < 0.05$, afternoon $p <$

Figure 3. Leu-arylamidase activity levels during the estrous cycle (E= Estrus; Pm= Proestrus morning; Pa= Proestrus afternoon) and during pregnancy (2, 7, 14, 20= days; PP= postpartum day) in the striatum and hippocampus. Values represent mean ± S.E.M. (units of arylamidase/mg protein).

STRIATUM

HIPPOCAMPUS

0.01) were significantly higher than those measured during the estrus phase. In the amygdala, there were no differences in the morning values measured between estrus and proestrus, but the afternoon levels were higher for proestrus than for estrus ($p < 0.05$). ANOVA did not show any significant changes throughout pregnancy but, in the amygdala, the enzyme levels were higher on day 14 than during the rest of the gestation phases ($p < 0.05$); in the occipital cortex, there was a significant increase after parturition ($p < 0.05$). Figure 3 shows Leu-ArA values measured in the striatum and the hippocampus during the cycle and during pregnancy. As with the frontal and parietal cortices, ANOVA did not show any significant differences throughout both states. However, higher levels were observed on 14 day of gestation in the striatum (comparing with days 2 or 20) ($p < 0.05$), and in the hippocampus, there was a rise in the enzyme activity between postinsemination days 2 and 7 ($p < 0.05$). Finally, Figure 4 shows the activities of Leu-arylamidase during the
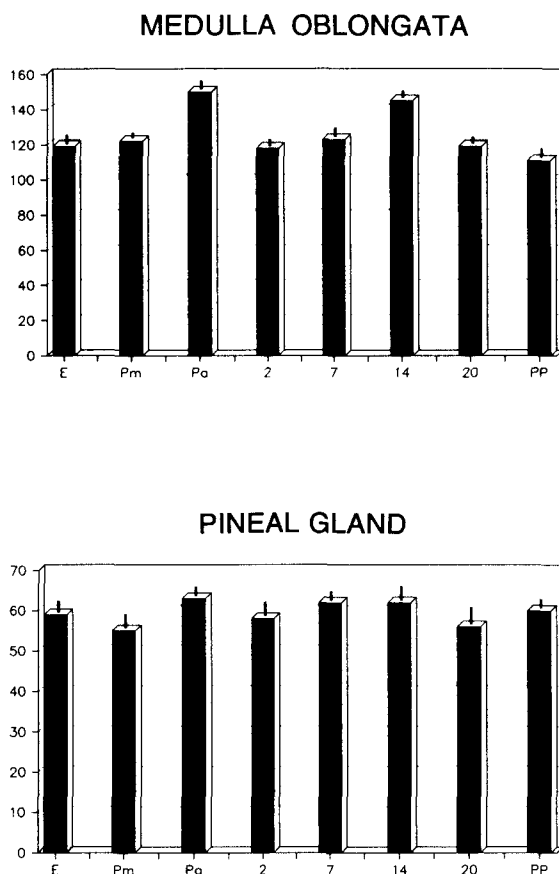
MEDULLA OBLONGATA

Figure 4. Leu-arylamidase activity levels during the estrous cycle (E= Estrus, Pm= Proestrus morning, Pa= Proestrus afternoon) and during pregnancy (2, 7, 14, 20= days, PP= postpartum day) in the medulla oblongata and in the pineal gland Values represent mean ± S E M (units of arylamidase/mg protein)

PINEAL GLAND

cycle and during pregnancy in the medulla oblongata and in the pineal gland In the latter brain area, no significant changes in any of the stages under study were appreciated However, in the medulla ANOVA showed significant differences throughout the estrous cycle ($p < 0.05$) but not during pregnancy There were no differences in morning values measured between estrus and proestrus, but proestrus levels at 15 00 h were higher than those measured for the estrus period ($p < 0.005$) and proestrus morning ($p < 0.05$) During gestation, the levels on day 14 were higher than those on days 2 and 20 ($p < 0.05$)

Discussion

The ovarian steroids oestradiol and progesterone convey information on the status of the female reproductive system to the brain, which is able to integrate these

signals and respond by altering its release of Gn-RH from the hypothalamus. However, since hypothalamic Gn-RH-secreting neurons do not concentrate oestradiol or progesterone (Dyer and Bicknell 1989), it is likely that some of these steroidal actions are achieved through intermediate central pathways. Thus, it has been established that the secretion of the gonadotropins is controlled at the hypothalamic level not only by the classical transmitter (e.g. norepinephrine, dopamine, serotonin, etc.), but also by numerous peptides present in the brain, such as the opioid peptides (Dyer and Bicknell 1989), neuropeptide Y (Sutton et al. 1988), and substance P (Battmann et al. 1991), to name a few. Numerous proteases capable of inactivating peptide transmitters are present in the brain, but it is likely that there are relatively few physiologically inactive peptide transmitters (Lynch and Snyder 1986). Precisely, aminopeptidase activity has been suggested as a mechanism for the regulation of several kinds of these neurosecretory materials, including those mentioned above (Benuck and Marks 1975; Hersh and McKelvy 1981; Johnson and Hersh 1990). In recent years, several changes in arylamidase activities have been described during the estrous cycle in the rat hypothalamus (de Gandarias et al. 1988; 1989a, 1990), and it has been suggested that they could play a role in the regulation of Gn secretion, possibly by regulating the levels of several neuroactive peptides. Nevertheless, previous experimental reports have demonstrated that also several extrahypothalamic regions of the central nervous system could be implicated in the regulation of the hormonal surge from the hypothalamic-pituitary axis. Those included changes in several neurotransmitters and neuropeptides in the rat cortex, medulla and the limbic system during the estrous cycle (Frankfurt et al. 1986; Desan et al. 1988; Dyer and Bicknell 1989) and during pregnancy (Wardlaw and Frantz 1983; Glaser et al. 1990). The results obtained in the present research show how, Leu-arylamidase activity had significantly higher proestrus afternoon values in the occipital cortex, the amygdala and medulla oblongata, while there were no significant changes in the frontal and parietal cortices, the striatum, the hippocampus and the pineal gland. These findings support the view that the occipital cortex, the amygdala and the medulla could possibly be a part of a complex nervous circuitry involved in the neuroendocrine control of Gn secretion, as also suggested previously (Dyer and Bicknell 1989). On the other hand, slight and insignificant increases in the morning enzyme activity were shown in the above brain areas during the proestrus phase, which coincides with previous reports (de Gandarias et al. 1992). It should be noted that in the rat LH response, standard LH-RH stimulus increases steadily during the estrous cycle, reaching maximal values during the early afternoon of proestrus (Cooper et al. 1974). Also aminopeptidase activity was observed to vary throughout pregnancy. There is a significant rise after parturition in the occipital cortex, and a clear but insignificant increase in the striatum and the medulla. In general, higher (in several cases insignificant) levels of Leu-ArA were found during the middle stages of pregnancy. Changes in several

neuropeptide levels have recently been demonstrated during these phases (Wardlaw and Frantz 1983; Leng et al. 1985; Sander et al. 1989). In summary, along with previous findings, our results suggest that several extrahypothalamic areas may mediate, directly or indirectly, several effects on GnRH secretion from the hypothalamus, and that the aminopeptidases are implicated, possibly by regulating the levels of several neuroactive peptides.

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