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Effect of neonatal handling on brain enkephalin-degrading peptidase activities

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Abstract

Neonatal handling decreases neutral endopeptidase 24.11 activity in the amygdala. However, this procedure does not affect aminopeptidase activities in any of the brain areas studied. Neonatal handling has been one of the most commonly used strategies to study the plasticity of the nervous system. The crucial role of the opioids in the control of different aspects of behaviour and development has been well documented. Regarding this subject, the endogenous opioid system might mediate some of the effects induced by neonatal handling. In this work, we have studied the effects of neonatal handling on several enkephalin-degrading peptidases, including soluble and membrane-bound aminopeptidases (puromycin-sensitive and -insensitive) and neutral endopeptidase 24.11 in different rat brain areas. Tyrosine aminopeptidase activities were measured fluorimetrically using tyrosine-β-naphthylamide as substrate and puromycin as selective inhibitor of one of the membrane-enzymes. Dansyl-D-Ala-Gly-Phe(pNO2)-Gly was the fluorogenic substrate for neutral endopeptidase. The reduced neutral endopeptidase 24.11 activity in the amygdala of neonatal handled rats could reduce enkephalin catabolism in this area and it could be responsible for some of the effects induced by neonatal handling.

1. Introduction

The rat brain presents very high plasticity immediately after birth and is very sensitive to exterior stimuli. Neonatal handling has been one commonly used strategy in the study of brain plasticity. Nowadays, it is known that neonatally handled animals have different sensitivity to noxious stimulus when they are adults as compared with non-handled animals. Thus, neonatal handling decreases the sensitivity to painful stimulus (D'Amore et al., 1993, 1995). The emotivity level and the susceptibility to becoming helpless after stressful situations are also reduced in handled rats as compared to non-handled ones (Costela et al., 1995; Tejedor-Real et al., 1998a).

Since the early 1980s, we know that opioid peptides are relevant for several types of social behaviour (Panksepp et al., 1980). Nowadays, it is established that opioid peptides are involved in the regulation of several physiological and pathological processes, some of which are affected by neonatal handling. Concerning this subject, activation of the opioid system by the administration of inhibitors of enkephalin degrading peptidases (aminopeptidases and neutral endopeptidase 24.11) has antidepressant type effects (Baamonde et al., 1992) and produces lower sensitivity to painful stimuli (Valverde et al., 1996; Noble et al., 1997). Moreover, the administration of opioid agonists or inhibitors of the enkephalin metabolism reverts the tendency of the rats to become helpless after receiving stressful stimuli (Tejedor-Real et al., 1993, 1995, 1998a). Therefore, the endogenous opioid peptides and their hydrolyzing peptidases could be physiological substrates of the neonatal handling model.

The aim of the present work was to study the effect of neonatal handling on enkephalin-degrading

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enzymes. Since previous works have reported that neutral endopeptidase 24.11 (enkephalinase) and aminopeptidases are the most important enzymes involved in enkephalin degradation (Hersh, 1982), we have included both in this research.

2. Experimental procedures

2.1. Animals

For all the experiments, subjects ($n = 20$ handled, $n = 20$ control) were the offspring of male and female Wistar albino rats purchased from the Central Animal Service of Cadiz and mated (one male × three females) in our laboratory. Animals were maintained on a 12:12 light-dark schedule with free access to food and water and housed in a temperature and humidity-controlled room with noise kept to a minimum. On the day of birth (day 0), all the litters were cross-fostered and culled to 10 male pups with a mother (Lane-Petter et al., 1968). Females were rejected to avoid hormonal effects during adulthood, the period of biochemical testing. Neonates remained with the dams at all times except during neonatal handling, and were weaned at 21 days of age. Control rats permanently remained in their home cages. Biochemical analysis were performed in adulthood at 60 days of age.

The experiments were conducted according the guidelines laid down in the *Guide for Care and Use of Laboratory Animals* of the Institute of Laboratory Animal Resources National Research Council.

2.2. Handling

Mothers of the handled litters were removed daily from their cages and then the pups were removed, and placed in a plastic container with a paper towel for about 15 min, at room temperature. After handling, the pups, followed by their mothers, were returned to their cage. This procedure was repeated daily from day 1 to day 21 at 12:00 h. Offspring were weaned at 21 days of age. After weaning, the pups were then housed in groups of five belonging to the same initial treatment group and left undisturbed until testing on day 60.

2.3. Chemicals

All the products were purchased from Sigma-Chemical Co.

2.4. Sample preparation

To remove plasma peptidase activities, animals were perfused (from the left cardiac ventricle) with saline plus 50 mM of phosphate buffer solution (pH 7.4), under Equithensin (42.5 g/l of cloralhydrate, 9.72 g/l of Nembutal; 22.74 g/l of magnesium sulphate and 396 ml of propylene glycol) anaesthesia (2 ml/kg). After perfusion with phosphate buffer saline (pH = 7.4), the brains were quickly removed and cooled on dry ice. Brain samples, taken by manual brain slicing according to the atlas of Paxinos and Watson (1981), were the frontal, occipital and parietal cortices, the striatum, the hypothalamus, the cerebellum, the thalamus, the brainstem, the hippocampus and the amygdala. The samples (0.1 g tissue/ml) were homogenized (in Tris HCl 10 mM, pH 7.4) and ultracentrifuged (100,000 *g*, 35 min). Samples from the supernatant were used to detect the soluble aminopeptidase (3.4.11.14) activity and proteins. The resulting pellets were washed three times by suspension in Tris buffer to avoid contamination of the soluble form. The pellets were later homogenized in Tris HCl 10 mM (pH 7.4) plus 1% of Triton X-100 to obtain, after ultracentrifugation (100,000 *g*, 35 min) supernatant solutions which were employed to determine the membrane-bound activities (aminopeptidases and neutral endopeptidase) and proteins. All preparatory steps were carried out at 4°C.

2.5. Enzyme assays

Aminopeptidase activities were fluorimetrically measured in triplicate using Tyr-β-naphthylamide as substrate (it has been described that only Tyr-aminopeptidase hydrolyses Tyr-β-naphthylamide; Wagner et al., 1981) by the method of Greenberg (1962) but with recent modifications described by Alba et al. (1989). Briefly, 10 μl of sample (1 mg protein/ml) were incubated with 1 ml substrate solution containing DTT, Albumine, Tyr-β-naphthylamyde in 50 mM phosphate buffer. The reaction was stopped by the addition of 1 ml of buffer acetate solution. The membrane-bound activity was detected entirely, and after treatment with 20 μM puromycin. This concentration was chosen to obtain selective inhibition of the puromycin-sensitive aminopeptidase (3.4.11.-) activity from the membranes, without affecting aminopeptidase M activity, puromycin-insensitive (3.4.11.2) (Giros et al., 1986)

Neutral endopeptidase 24.11 activity was also fluorimetrically measured in triplicate by the method described by Florentin et al. (1984) using dansyl-D-Ala-Gly-Phe(pNO2)-Gly (DAGNPG) as substrate and captopril as inhibitor of the angiotensin converting enzyme. Since neutral endopeptidase is a membrane-bound enzyme, we have measured its activity only in the membranes.

Protein concentration was measured in triplicate by the method described by Bradford (1976). The results were recorded as units of aminopeptidase or neutral

Table 1
Activity levels of neutral endopeptidase 24.11 in 10 rat brain areas of handled ($n = 20$) and non-handled ($n = 20$) animals (values represent the mean ± SEM of units of endopeptidase activity (nmol of hydrolyzed substrate/min) per mg of protein; * t-test $P < 0.05$)

	Control	Handled
Hypothalamus	0.686 ± 0.06	0.658 ± 0.061
Hippocampus	0.753 ± 0.07	0.599 ± 0.065
Amygdala	1.155 ± 0.067	0.752 ± 0.064*
Striatum	0.701 ± 0.078	0.735 ± 0.067
Thalamus	0.791 ± 0.087	0.778 ± 0.062
Brainstem	0.746 ± 0.078	0.585 ± 0.067
Cerebellum	0.450 ± 0.088	0.521 ± 0.062
Frontal	0.603 ± 0.09	0.606 ± 0.044
Occipital	0.633 ± 0.067	0.700 ± 0.055
Parietal	0.503 ± 0.078	0.482 ± 0.045

endopeptidase 24.11 activity per milligram of protein. One unit of neutral endopeptidase 24.11 is the amount of enzyme that hydrolyzes one nanomol of DAGNPG and one unit of aminopeptidase activity is the amount of enzyme that hydrolizes one nanomol of tyrosine-β-naphthylamide per minute.

2.6. Statistical analysis

Differences between groups were calculated by the Student t-test. Statistically significant differences were considered at $P < 0.05$

3. Results

Table 1 shows the effects of neonatal handling on neutral endopeptidase 24.11 activity in certain brain areas of the adult rat. These areas are: the frontal, parietal and occipital cortices, the hypothalamus, the hippocampus, the amygdala, the brainstem, the cerebellum, the thalamus and the striatum. The neutral endopeptidase 24.11 activity is significantly decreased ($P < 0.05$) in the amygdala of handled rats, as compared to control animals. We have also found a decrease in the neutral endopeptidase 24.11 activity in the hippocampus and the brainstem, although the differences did not reach statistically significant values.

Aminopeptidase activity levels in handled and control rats are shown in Table 2. Soluble and membrane-bound puromycin-sensitive aminopeptidase activities are higher than neutral endopeptidase 24.11 activity in all the brain areas under study. The activity levels of the puromycin-insensitive aminopeptidase are in the same range as those obtained in neutral endopeptidase 24.11. When aminopeptidase activities of handled and control rats are compared, significant differences are not obtained, neither in soluble nor in membrane-bound forms.

4. Discussion

In this work, it has been shown that there is a statistically significant decrease of neutral endopeptidase 24.11 activity in the amygdala of neonatally handled adult rats. This enzyme is one of the major enzymes which degrades endogenous enkephalin (Roques et al., 1993) and a good correspondence between the brain distribution of the enzyme and the opioid receptor has been found (Waksman et al., 1987). Neutral endopeptidase 24.11 is a membrane-bound enzyme whose active site is directed to the extracellular side of the cell and its more likely function could be to hydrolize peptides after being released (Roques et al., 1993). The results obtained in this research suggest to us that enkephalin extracellular catabolism could be reduced in the amygdala of handled rats.

Table 2
Activity levels of soluble and membrane-bound (puromycin-sensitive and insensitive) aminopeptidases in 10 rat brain areas of handled ($n = 20$) and non-handled ($n = 20$) animals (values represent the mean ± SEM of units of aminopeptidase activity (nmol of hydrolyzed substrate/min) per mg of protein)

	Soluble		Puromycin sensitive		Puromycin insensitive	
	Control	Handled	Control	Handled	Control	Handled
Hypothalamus	19.2 ± 1.21	19.0 ± 1.1	2.3 ± 0.2	2.5 ± 0.1	0.40 ± 0.02	0.45 ± 0.03
Hippocampus	20.5 ± 1.1	20.8 ± 1.2	2.5 ± 0.2	2.6 ± 0.2	0.43 ± 0.04	0.46 ± 0.04
Amygdala	19.2 ± 0.9	19.6 ± 1.1	2.0 ± 0.2	2.2 ± 0.2	0.40 ± 0.04	0.39 ± 0.03
Striatum	19.0 ± 1.3	19.8 ± 1.1	2.2 ± 0.1	2.4 ± 0.2	0.40 ± 0.04	0.39 ± 0.04
Thalamus	19.2 ± 1.2	19.2 ± 1.3	2.5 ± 0.1	2.3 ± 0.1	0.45 ± 0.05	0.45 ± 0.04
Brainstem	16.1 ± 1.5	16.4 ± 0.9	2.9 ± 0.2	3.0 ± 0.2	0.54 ± 0.05	0.56 ± 0.04
Cerebellum	15.9 ± 0.9	15.6 ± 0.9	2.6 ± 0.2	2.5 ± 0.2	0.50 ± 0.04	0.52 ± 0.03
Frontal	17.5 ± 1.1	17.0 ± 1.0	2.1 ± 0.1	2.1 ± 0.2	0.42 ± 0.03	0.43 ± 0.04
Occipital	18.2 ± 1.2	18.4 ± 1.2	2.1 ± 0.2	2.0 ± 0.2	0.43 ± 0.04	0.43 ± 0.04
Parietal	17.9 ± 0.9	17.2 ± 0.9	2.2 ± 0.2	2.1 ± 0.1	0.41 ± 0.04	0.44 ± 0.03

Since neonatal handling causes a great variety of changes in the behaviour of the adult rat, it is not easy to ascertain the behavioural effect that corresponds to this biochemical change. However, some of the recent discoveries about the role of the opioid system in the brain, and specifically in the amygdala, could throw some light on this subject.

Administration of enkephalin-degrading enzyme inhibitors (Tejedor-Real et al., 1993, 1995; 1998b) or enkephalin agonists to the rat reverses the helpless behaviour caused by inescapable shocks and causes hypoalgesia (Valverde et al., 1996; Noble et al., 1997). Amygdala is a brain area involved in painful and stressful stimuli processing (Werka, 1997). The decreased neutral endopeptidase 24.11 in the amygdala of neonatally handled animals observed in this research could have a connection with the hypoalgesia (D'Amore et al., 1993, 1995) and stress resistence (Costela et al., 1995) observed in neonatal handled animals. The reduction of the endopeptidase activity in the brainstem (an area involved in pain transmission and analgesic response), although not significant, agrees with this hypothesis.

Neonatal handling also decreases neutral endopeptidase activity in the hippocampus. In this area, many biochemical modifications in neonatally handled adult rats have been reported (Cain and Routtemberg, 1983; Mohamed et al., 1993; Smythe et al., 1994).

Aminopeptidases are also involved in enkephalin catabolism (Hersh, 1982; Giros et al., 1986). However, in our work, we have not found differences in aminopeptidase activity between handled and non-handled adult rats. This could mean that aminopeptidase activities reached in adulthood, at least in the studied brain areas, are not important in determining the effects of neonatal handling. However, we cannot conclude that these enzymes are not at all affected by neonatal handling, as aminopeptidases could be affected in previous stages of development. With regard to this subject, it is necessary to note that the activity levels of several aminopeptidases are higher in the developing rat brain than in the adult animal (De Gandarias et al., 1997).

In summary, in this work we have shown that neonatal handling reduces the neutral endopeptidase 24.11 activity level in the amygdala. This reduction, observed in a enkephalin-degrading enzyme, would increase the enkephalin activity which could be responsible, at least in part, for some of the behavioural effects of neonatal handling in adult rats. However, since the changes are restricted to this enzyme in only one brain area, it does not seem that this change can explain all the actions of neonatal handling on endogenous opioid system related behaviour or pain perception. Further studies focusing on opioid peptide levels or receptor number and affinity must be carried out to establish more clearly the effects of neonatal handling on the endogenous opioid system.

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