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Stress Influences Brain Enkephalinase, Oxytocinase and Angiotensinase Activities: A New Hypothesis

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Key Words
Acute immobilization stress · Aminopeptidases · Prefrontal cortex · Amygdala · Hippocampus

Abstract
Brain enkephalin and oxytocin are anxiolytic agents involved in the response mechanism to stress. Degrading enzymes such as enkephalinase and oxytocinase could also be associated with this response. The effect of acute immobilization stress on enkephalinase and oxytocinase activities was determined in the soluble and membrane fractions of the medial prefrontal cortex, hippocampus and amygdala using alanyl- and leucyl-beta-naphthylamide as substrates, the latter in the presence and absence of 20 mM L-methionine. No change in aminopeptidase activities was observed in the prefrontal cortex of stressed rats. In contrast, enkephalinase activity decreased in the soluble fraction of the hippocampus but increased in the membrane fraction. In the amygdala, soluble oxytocinase and membrane enkephalinase activities decreased in stressed animals. These results show that acute immobilization stress affects differentially enkephalinase and oxytocinase activities depending on the fraction and brain region analyzed. A reduction in the activity of soluble enkephalinase in the hippocampus and soluble oxytocinase as well as membrane enkephalinase in the amygdala may suggest higher availability/longer action of enkephalin and oxytocin at these locations. This may explain the relative importance of these enzymatic activities in the anxiolytic properties proposed for enkephalins and oxytocin in the hippocampus and amygdala during stress conditions. This interpretation is not applicable to membrane enkephalinase activity in the hippocampus. However, alanyl-beta-naphthylamide hydrolyzing activity not only measures enkephalinase activity, it also reflects the angiotensinase-induced metabolism of angiotensin III to angiotensin IV. Therefore, our results may also mirror an increase in the formation of Ang IV in hippocampus and a decrease in the amygdala in acute stress. In conclusion, aminopeptidase activities in the hippocampus and amygdala may affect enkephalin, oxytocin and angiotensin III metabolism during acute immobilization stress and therefore be involved in the anxiolytic response.

Introduction

The analysis of the neurochemical basis underlying stress response is important to appropriately understand and search solutions for stress-related emotional and behavioral problems including anomalous social behaviors

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[1]. Brain endogenous opioids such as beta-endorphin, enkephalin or dynorphin, as well as their receptors expressed in limbic structures responsible for stress responses, have been involved in the central anxiolytic reaction to stressful stimuli [2]. It has been observed that enkephalins are released in the central nervous system, particularly in the amygdala, in response to stress [3]. Furthermore, brain oxytocin is also an important regulator of stress response as an anxiolytic agent [4, 5]. Indeed, oxytocin receptor agonists or activation of the extracellular signal-regulated kinase 1/2 cascade have been proposed as therapeutic mechanisms to activate the anxiolytic properties of the peptide [6, 7]. In addition, other brain neuropeptides such as angiotensin II as well as its receptor AT_1 have been described to be increased in brain under stress conditions, [8] including immobilization stress [9].

Enkephalin and oxytocin are in part regulated by means of aminopeptidases such as enkephalinase and oxytocinase that inactivate them [10–12]. Therefore, these enzymes may be involved in the anxiolytic response to stressful stimuli. In the present work, the effect of acute immobilization stress on enkephalinase and oxytocinase activities was determined in the soluble and membrane-bound fractions obtained from the homogenates of samples of the medial prefrontal cortex, hippocampus and amygdala. These areas are indeed critical brain regions implicated in the response to stress [13].

However, these locations are not only implicated in stress response but are also potential targets of centrally released neuropeptides such as oxytocin. Its release from magnocellular neurons of the hypothalamus and its posterior diffusion through extracellular fluid might allow it to act on distant locations such as the amygdala where it would act as modulator of certain behaviors [14].

The purpose of this work was therefore to analyze the activity of enzymes involved in the degradation of two anxiolytic agents, enkephalin and oxytocin.

Aminopeptidases exhibit broad substrate specificity. A single enzyme can have several substrates and the same substrate may be hydrolyzed by several enzymes. In addition, the metabolism of neuropeptides may be accomplished by soluble or membrane-bound enzymes (table 1). To evaluate enkephalinase activities, we assayed alanyl aminopeptidase (AlaAP) activity in the membrane-bound fraction (aminopeptidase M; EC 3.4.11.2) [11] and in the soluble fraction (puromycin-sensitive aminopeptidase; EC 3.4.11.14) [12]. Membrane-bound alanyl-beta-naphthylamide hydrolyzing activity, in addition to enkephalinase, also reflects the angiotensinase activity responsible for the metabolism of angiotensin III to angiotensin IV [15]. For oxytocinase activity, placental leucyl aminopeptidase (P-LeuAP; EC 3.4.11.3) was assayed using leucyl-beta-naphthylamide in the presence of 20 mM of L-methionine [16]. Leucyl-beta-naphthylamide-hydrolyzing activity (LeuAP; EC 3.4.11.1), in the absence of L-methionine in the medium of incubation, was also assayed for comparative purposes. This enzymatic activity was also reported as an enkephalinase [17] and also hydrolyzes dynorphins [18], substance P [19] and angiotensin II [20]. In addition, using this substrate, a novel neuron-specific aminopeptidase that metabolizes enkephalins could be detected [21].

Table 1. Brain enzymes in the soluble and membrane-bound fraction from brain sample homogenates detected by means of aminoacyl-β-naphthylamides used in the present work

Main names/EC number/ artificial substrate/fraction	Endogenous substrates [ref.]
AlaAP; AP M/EC 3.4.11.2/ Ala-β-NNap/membrane	angiotensin III [42], enkephalins [42], bradykinins [42], endorphins [42]
AlaAP; PSAP/EC 3.4.11.14/ Ala-β-NNap/soluble	enkephalins [12]
LeuAP/EC 3.4.11.1/Leu-β-NNap/soluble, membrane	enkephalins [17], angiotensin II [20], substance P [19], dynorphins [18]
NSAP/EC 3.4.11.-/Leu-β-NNap/soluble	enkephalins [12]
P-LeuAP; oxytocinase/ EC 3.4.11.3/Leu-β-NNap + L-methionine 20 mM/ membrane, soluble?	oxytocin [34, 38], vasopressin [34, 38], enkephalins [38], dynorphins [38], angiotensin III [34]

The susceptible endogenous substrates hydrolyzed by these enzymes are also indicated. AP M = Aminopeptidase M; NSAP = neuron-specific aminopeptidase; PSAP = puromycin-sensitive aminopeptidase.

Material and Methods

Ten adult male Wistar rats weighing 200–250 g were housed under controlled temperature (25°C), with a regular 12-hour light/12-hour dark cycle (light on from 7.00 h) and received food and water ad libitum. For the stress study, 5 animals were maintained in their original home cages as control whereas 5 animals were randomly selected and placed individually inside plastic cyl-

inders (21 cm in length and 6 cm in diameter) during 1 h. Both ends of the cylinders were closed with ventilation sliding doors. Animals were sacrificed immediately after the end of the immobilization period [22].

Aminopeptidase activity levels are affected by daytime and light-dark conditions [23]. Since some of their substrates may exhibit seasonal differences in their levels [24], the response to stress conditions may also be under seasonal influence [25]. In consequence, differences in peptidase activities depending on the season in which we perform the experiments are plausible. Therefore, to avoid these possible differences, rat brains were perfused with saline in light conditions (between 9.00 a.m. and 12.00 noon) through the left cardiac ventricle under equithensin anesthesia (2 ml/kg body weight) (equithensin contained: 42.5 g/l chloralhydrate dissolved in 19.76 ml ethanol, 9.72 g/l Nembutal®, 0.396 L/L propylenglycol and 21.3 g/l magnesium sulfate in distilled water), quickly removed (less than 60 s) and cooled in dry ice. The experiments were done in summer (northern hemisphere). To collect the selected tissue samples, brains were individually defrosted and after they reached the appropriate consistence, sliced by hand with a blade. The selected areas were identified in the slices and their coordinates checked in the stereotaxic atlas of Paxinos and Watson [26]. The medial prefrontal cortex was dissected between 12.70 and 11.20 mm anterior to the interaural line (AIL), the hippocampus was dissected between 7.12 and 5.40 mm AIL and the amygdala was dissected between 7.12 and 5.40 mm AIL. Tissue samples were homogenized in 10 vol of 10 mM HCl-Tris buffer (pH 7.4) and ultracentrifuged at 100,000 *g* for 30 min (4°C) to obtain the soluble fraction. Soluble enzymatic activity and protein content were assayed in triplicate in the resulting supernatants. The pellets were rehomogenized in HCl-Tris buffer (pH 7.4) plus 1% Triton X-100 to solubilize membrane proteins. After centrifugation (100,000 *g*, 30 min, 4°C) membrane-bound activity and proteins were measured in triplicate in the supernatants. To ensure complete recovery of activity, the detergent was removed from the medium by adding adsorbent polymeric Biobeads SM-2 (100 mg/ml) and shaking the samples for 2 h at 4°C.

AlaAP, P-LeuAP and LeuAP were measured fluorometrically using the aminoacyl-β-naphthylamides (aaNNap) AlaNNap and LeuNNap as substrates according to the modified method of Greenberg [27] described elsewhere [28]: 10 μl of each supernatant was incubated for 30 min at 25°C with 1 ml of the substrate solution: 2.14 mg/100 ml AlaNNap or 2.92 mg/ 100 ml LeuNNap, 10 mg/100 ml bovine serum albumin, and 10 mg/100 ml dithiothreitol in 50 mM of phosphate buffer, pH 7.4. For P-LeuAP determination 20 mM of L-methionine was added to the corresponding substrate solution [16]. Adding 1 ml of 0.1 mol/l of acetate buffer, pH 4.2, stopped all the reactions. The amount of β-naphthylamine, product of the enzymatic activity, was measured fluorometrically at 412 nm emission wavelengths with an excitation wavelength of 345 nm. The sensitivity of the method allows measurements of aminopeptidases in the picomolar range. Proteins were quantified in triplicate by the method of Bradford [29], using bovine serum albumin as a standard. Specific soluble and membrane-bound aminopeptidase activities were expressed as picomoles of AlaNNap, or LeuNNap hydrolyzed per minute per milligram of protein. Fluorogenic assays were linear with respect to time of hydrolysis and protein content. The experimental procedures for animal use and care were in accordance with the European Communities Council Directive 86/609/EEC.

For the statistical analysis, the difference between groups was evaluated with one-way analysis of variance (ANOVA). Post hoc comparisons were made using Tukey's test; p values below 0.05 were considered significant.

Results

The distribution and the effect of acute immobilization stress on aminopeptidase activities in the medial prefrontal cortex, hippocampus and amygdala are represented in figure 1. The highest levels for all the activities determined were observed in the amygdala, especially that corresponding to the soluble fraction. AlaAP (enkaphalinase, angiotensinase) and LeuAP (enkephalinase, angiotensinase) and P-LeuAP (oxytocinase) activities did not change significantly in the prefrontal cortex in stress conditions. However, we observed significant changes in the hippocampus and amygdala. While AlaAP activity (enkephalinase, angiotensinase) decreased significantly ($p = 0.018$) in the soluble fraction of hippocampus, it increased significantly ($p = 0.012$) in the membrane-bound fraction of stressed rats. In the amygdala, although the three soluble activities analyzed demonstrated a tendency to decrease in stressed animals, only P-LeuAP (oxytocinase) activity reached statistical significance ($p = 0.005$). In addition, membrane-bound AlaAP activity (enkephalinase and angiotensinase) also decreased significantly ($p = 0.012$) in stressed animals.

Discussion

Because of their catalytic activity, neuropeptidases not only inactivate neuropeptides but also change their biological activities by causing the formation of new peptides with properties different from their precursors. For example, decreased oxytocinase or enkephalinase activities produce an increase in oxytocin and enkephalin. The existence of an intracellular renin-angiotensin system in neurons was hypothesized. This implies a major role for soluble aminopeptidase activities involved in the control of the active angiotensin peptides [30]. In the present report, discrepancies are observed between soluble and membrane-bound activities in their response to acute immobilization stress. Although soluble and membrane-bound activities might hydrolyze the same substrates, the regulatory mechanisms of both enzymatic pools may be independent and local changes due to this condition may determine this differential response. Metabolism of neu-

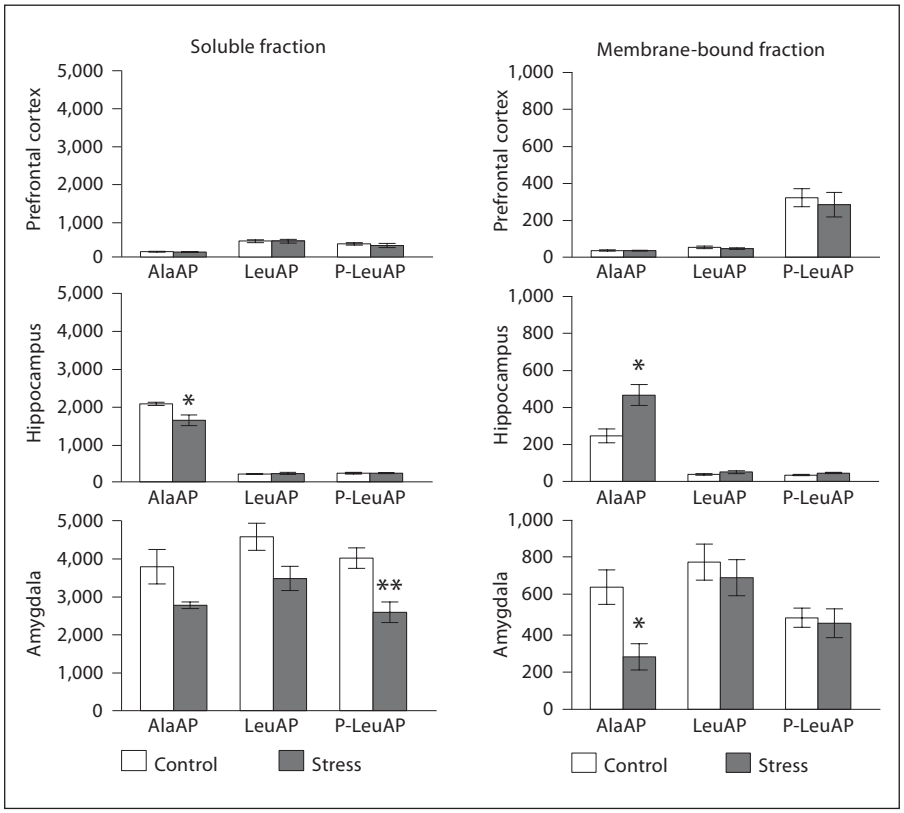

Fig. 1. AlaAP, P-LeuAP and LeuAP activities in the medial prefrontal cortex, hippocampus and amygdala of control (white bars, n = 5) and stressed animals (gray bars, n = 5). Values represent mean ± SEM of specific AlaAP, LeuAP, and P-LeuAP activities expressed as picomoles of alanyl- or leucyl-β-naphthylamide hydrolyzed per minute per milligram of proteins. Asterisks represent differences between control versus stressed animals: * $p < 0.05$, ** $p < 0.01$.

ropeptides by membrane-bound peptidases is a major mechanism for the regulation of their function. There is, however, also evidence for uptake systems such as for cholecystokinin octapeptide [31] or for Met-enkephalin [32] in rat brain synaptosomes. Therefore, these systems could participate, in conjunction with the extra- and intracellular metabolism of neuropeptides by peptidases, in the control of neuropeptide levels and function.

Several forms of oxytocinase have been described. Initially, the enzyme was named cystinyl aminopeptidase (CysAP) because CysNNap was used as a substrate for its measurement [33]. Subsequently, Tsujimoto et al. [34] purified a human placental LeuAP (EC 3.4.11.3) that hydrolyzed oxytocin, vasopressin and angiotensin III, and concluded that P-LeuAP and CysAP activities were shared by the same molecule. However, Itoh and Nagamatsu [35] purified an aminopeptidase that hydrolyzed oxytocin and vasopressin, with properties different from LeuAP (EC 3.4.11.1), AlaAP (EC 3.4.11.2) and P-LeuAP (EC 3.4.11.3). This enzyme hydrolyzed L-Cys-*p*-nitroanilide with the highest specificity. In addition, two different forms of oxytocinase were later purified [36]. These results suggest the existence of several forms of oxytocinase. P-LeuAP exists in both membrane-bound and soluble forms [37] and has also been identified in the brain where it was localized only in the membrane fraction. No soluble form of the enzyme was detected [38]. The present data, however, suggest that a soluble form of the enzyme could also be present in the brain.

Oxytocinase/vasopressinase has been identified as an insulin-regulated membrane aminopeptidase associated with the GLUT4-containing vesicle [39]. In the brain, oxytocinase hydrolyzes oxytocin, vasopressin and opioid peptides such as enkephalins or dynorphin [38]. It was suggested that this enzyme was identical to the AT_4 receptor for angiotensin IV involved in learning and memory [40]. However, this assumption was recently challenged and it was proposed that the AT_4 receptor was not the insulin-regulated membrane aminopeptidase molecule but the type I tyrosine kinase receptor, c-Met [41].

Soluble AlaAP (puromycin-sensitive aminopeptidase; EC 3.4.11.14) is especially abundant in the brain where it was identified as the major enkephalin-degrading activity [12]. Membrane-bound AlaAP (Aminopeptidase M; EC 3.4.11.2) hydrolyzes enkephalins, bradykinins, endorphins and is also the enzyme responsible for the metabo-

lism of angiotensin III to angiotensin IV [42]. The hippocampus, amygdala, and prefrontal cortex are important brain regions implicated in the mediation of stress effects on learning and memory [43].

The present results show that acute immobilization stress affects differentially enkephalinase and oxytocinase activities depending on the fraction and the brain region analyzed. Clearly, enkephalinase activity is reduced in the soluble fraction of the hippocampus and in the membrane fraction of the amygdala whereas oxytocinase is reduced in the soluble fraction of the amygdala. This may suggest higher availability/longer action of enkephalin and oxytocin at these locations. Such observation reflects the relative importance of these enzymatic activities in the anxiolytic properties proposed for enkephalins and oxytocin in the hippocampus and amygdala during stress conditions.

However, as membrane alanyl-beta-naphthylamide hydrolyzing activity also reflects the angiotensinase responsible for the metabolism of angiotensin III to angiotensin IV, our results may also suggest a role for the angiotensin metabolites. Our data suggest indeed a decrease in the formation of angiotensin IV in the amygdala. In contrast, there is an increased formation of angiotensin IV in the hippocampus. Therefore, acute immobilization stress affects angiotensin IV availability in the hippocampus and amygdala in an opposite manner. It was reported that angiotensin IV administered into the hippocampus significantly enhances long-term potentiation in a dose- and time-dependent manner [44]. Although the involvement of the amygdala in anxiety-related processes is well established [3], the role of the hippocampus is not conclusive [45]. The present data, however, clearly indicate that aminopeptidase activities in the hippocampus and amygdala are affected during acute immobilization stress and may therefore change enkephalin, oxytocin and angiotensin III metabolism, which supports their role in the anxiolytic response to acute stress. Moreover, an increase in substance P release in the medial nucleus, but not in the central nucleus of the amygdala, was observed in response to immobilization stress [46]. Furthermore, the analysis of maternal aggression and the role of brain oxytocin in lactating Wistar rats support the hypothesis. Indeed, oxytocin release was higher within the central nucleus of the amygdala for high anxiety-related behavior compared with low anxiety-related behavior during a maternal defense test [47]. In the present results, reduced levels of oxytocinase activity suggest higher levels of oxytocin in immobilization stress conditions. Similar to the above-mentioned reports [46, 47], further studies analyzing the local release and/or local concentrations of the neuropeptide in the extracellular fluid using in vivo micropush-pull superfusion and microdialysis techniques could confirm the present hypothesis. In addition, the blockade of the enzymatic activity by means of specific aminopeptidase inhibitors, microinjected locally in limbic structures, could clarify the functional importance of the present results [46]. In conclusion, the analysis of the participation of aminopeptidase activities during stress response may open new therapeutic perspectives that contemplate the use of inhibitors of the involved enzymes to prolong the half-life of anxiolytic agents. This hypothesis is worth investigating further.

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