The purpose of the present study was to evaluate the effects of acute and repeated treatment with two antidepressant drugs (ADs) of opposite pharmacological profile, i.e. tianeptine (TIA, serotonin reuptake enhancer) and fluoxetine (FLU, serotonin reuptake inhibitor) on the levels of Met-Enkephalin, (Met-Enk, a member of opioid peptide family, which has been suggested to play a role in the mechanism of action ADs) as well as on mRNA coding for proenkephalin (mRNA PENK) in various regions of the rat brain, pituitary, adrenal glands and plasma. Male Wistar rats were treated acutely or repeatedly (10 mg/kg p.o., twice daily for 14 days) with TIA or FLU. Tissue for biochemical experiments was taken 2 h after last dose of appropriate drug. The levels of Met-Enk were estimated by radioimmunoassay, mRNA PENK was measured using in situ hybridization. From the results obtained in the present study it may be concluded that repeated administration of TIA or FLU induced similar changes in the levels of Met-Enk in the rat hippocampus, striatum, hypothalamus and neurointermediate lobe of pituitary. Such an effect is interesting, especially if one takes into account the differences in pharmacological profile between these two antidepressant drugs. It may be suggested that serotonin level might not be crucial for inducing the alterations in the content of Met-Enk. Since we did not observe any changes in the levels of PENK mRNA in the studied rat brain regions after repeated administration of TIA or FLU, it seems that the observed changes in the levels of Met-Enk do not result from effects of these antidepressants on biosynthesis of PENK, but rather from alterations in the peptide release. Another interesting finding of the present study was that in the anterior lobe of pituitary, adrenal glands and plasma, repeated administration of TIA induced alterations in the contents of Met-Enk, while repeated administration of FLU remained without any effect. It is tempting to speculate that such a differentiation between the effects of these two antidepressants might be linked to the well known feature of TIA (but not FLU) which has been shown to reduce both basal and stress-evoked activity of the hypothalamic-pituitary-adrenal (HPA) axis.

Key words: tianeptine, fluoxetine, Met-Enkephalin, mRNA PENK, rat.
INTRODUCTION

Opioids have been recognized as modulators of emotional processes and mood elevating substances. The euphorogenic and anxiolytic properties of opioids raise the possibility that a dysfunctioning endogenous opioidergic systems may cause the pathogenesis of depression. Indeed, since the work of Kraepelin (1), the opium cure has been recommended for the treatment of depressed patients. Kline et al. (2) were the first to perform clinical trials in different types of psychiatric disorders by use of β-endorphin infusion, and they observed an antidepressant effects. Although results of clinical studies are still controversial, it has been reported that opioid antagonist, naloxone, administered at high doses augments severity of symptoms in depressed patients (3). Furthermore, in the experimental models of depression enhancement of the activity of endogenous opioid systems, by inhibition of enkephalin catabolism, produces antidepressant type effects (4, 5). Other data showed that endogenous opioids and opioid receptors are involved in the mechanism of action of antidepressant drugs (6, 7). Antidepressant drugs inhibit enkephalin binding to synaptosomes of the rat brain (8) and different types of interactions between the μ and δ opioid sites and antidepressant compounds were reported (9). On the other hand, antidepressant treatment induces alterations in both opioid receptors (10-13) and opioid peptide levels in discrete brain regions (14, 15). It has been shown that long-term treatment with tricyclic antidepressants increases the level of enkephalins (14,16), however, also decrease in this parameter was reported (17), but differences in the route of drug administration and dosage should be considered.

Taking into account the monoaminergic theory of depression (18, 19), the efficacy of tricyclic antidepressants is thought to stem from inhibition of the uptake of biogenic amines, such as noradrenaline and serotonin. Opiate compounds are also able to induce this inhibitory effect. Thus, methadone and morphine inhibit noradrenaline reuptake, whereas methadone inhibits both noradrenaline and serotonin reuptake (20).

In the present study we evaluated the effects of acute and repeated treatment with two antidepressant drugs of opposite pharmacological profile, i.e. tianeptine (TIA, serotonin reuptake enhancer (21)) and fluoxetine (FLU, serotonin reuptake inhibitor) on the levels of Met-Enkephalin as well as mRNA encoding for proenkephalin in various regions of the rat brain, pituitary, adrenals and plasma.

MATERIALS AND METHODS

Animals and drug administration

Experiments were carried out on male Wistar rats (200-250 g). The animals had free access to food and water before the experiment and were kept at a constant room temperature (22±1°C), under a 12 h light/dark cycle (light on at 7 a.m.). Experimental protocols were approved by the
local Ethics Committee and met guidelines of the responsible Agency of the Institute of Pharmacology.

TIA (Coaxil, Servier, France; 10 mg/kg) and FLU (Farmacom, Kraków, Poland; 10 mg/kg) were dissolved in 0.9% NaCl and administered perorally (p.o.), once or repeatedly (twice daily for 14 days). Drugs were administered at 8-9 o’clock a.m. and 8-9 o’clock p.m. All animals received either saline or test drug, twice daily for 14 days. Those assigned to the group receiving only a single drug treatment were given saline for 13 days and, on day 14, received appropriate drug. This protocol ensured that all animals were handled to the same extent.

**Blood collection, tissue extraction and RIA procedure**

Two hours after the treatment the animals were decapitated with guillotine and the trunk blood was collected into chilled centrifuge tubes containing 100 µl of a 2% solution of ethylenediamine tetraacetic acid (EDTA-Na$_2$)/1.5 ml blood, and immediately centrifuged (3,200 x g; 4°C, 15 min). The plasma was then sampled into polystyrene tubes and stored at –20°C until used for RIA, together with the rat brain structures, pituitary and adrenals, which were quickly removed and frozen on a dry ice. The tissue explants were immersed in 1 ml boiling 2 N acetic acid and maintained at 90°C for 10 min. The tissues were then homogenized using Ultra-Turrax T12 for 0.5-3 min, depending on the mass of tissue. The homogenates were centrifuged (10,000 x g; 4°C) for 30 min.

Following tissue extraction and further purification on Porapak Q (Waters, Milford MA, USA) columns, enkephalins were measured by radioimmunoassay (RIA), as described by Pierzchala et al. (22), using highly specific antibodies (Immunonuclear Corp., Stillwater MN, USA).

**In situ hybridization**

For the in situ hybridization study the brains, pituitary gland and adrenals of rats were rapidly removed and frozen on dry ice. Coronal sections (12 µm thick) were made on cryostat through the appropriate regions, according to Palkovits and Brownstein (23). The sections were thaw-mounted onto chrome-alum pretreated slides, postfixed in 4% paraformaldehyde for 10 min and processed for in situ hybridization by the method described by Young et al. (24). Briefly, a 48-mer synthetic deoxyoligonucleotide (New England Nuclear), complementary to bases 388-435 of the rat proenkephalin (PENK), was labelled using [³⁵S]dATP (1,200 Ci/mM, New England Nuclear) to obtain a specific activity of about 4 x 10⁵ cpm/µl. The sections were hybridized with the labeled oligonucleotide for 20 h at 37°C in a humidified incubator. After washing at 40°C, the sections were dried in a cool-air stream and exposed to a film (Amersham β Max) for 20 days at −70°C. The specificity of in situ hybridization was assessed by pretreatment of some tissue sections with RNAase A (20µg/ml) for 40 min at 30°C, which completely eliminated the hybridization signal with the cDNA probe. Moreover, when the hybridization was carried out in the presence of a 100-fold excess of the unlabeled probe, the signal also disappeared.

Optical density measurements were made from the autoradiograms corresponding to the appropriate brain regions, pituitary and adrenals, using an image analysing system (MCID, Canada). The average optical density values were calculated after subtraction of the film background. The mean optical density values were obtained by averaging out the measurements from autoradiograms of the sections obtained from 5-6 animals per group.

The results were statistically assessed by a one-way analysis of variance (ANOVA), and inter-group differences were analyzed by Dunnett’s multiple-range test.
RESULTS

The effect of an acute and repeated treatment with TIA or FLU on the levels of Met-Enk in the studied tissues and plasma of the rat are summarized in Table 1.

Single administration of TIA resulted in an increase in the level of Met-Enk in pituitary gland (both intermediate and anterior lobes), while in the hypothalamus, adrenals, plasma and striatum, the decrease in the level of Met-Enk was observed. In addition, single administration of FLU induced the decrease in the level of Met-Enk in the hypothalamus, adrenals, plasma and striatum.

On the other hand, both TIA and FLU, administered repeatedly, decreased the level of Met-Enk in striatum and hypothalamus, but in the hippocampus as well as in the neurointermediate lobe of pituitary, the increase in the level of Met-Enk was observed after repeated administration of both TIA or FLU.

In the anterior lobe of pituitary, adrenal glands and plasma, the effects of repeated administration of TIA or FLU were differentiated, i.e. FLU did not induce any significant changes in the level of Met-Enk, while an increase in the level of this peptide in an anterior lobe of pituitary and adrenals, and a decrease in this parameter in plasma, was observed following repeated administration of TIA.

No significant changes were observed in the level of PENK mRNA following acute or repeated administration of TIA or FLU in the rat brain, pituitary or adrenal glands (Table 2).

Table 1. Effect of acute and repeated administration of TIA or FLU on the levels of Met-Enkephalin. The animals were sacrificed 2 h after the acute or repeated treatment with TIA or FLU. The values (mean ± SEM) from 8 animals are given in pg/mg tissue, pg/gland (in case of pituitary) or pg/ml (in case of plasma). The statistical significance was assessed using ANOVA followed, when appropriate, by Dunnett’s test. *p<0.05; **p<0.01 vs control level.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>TIA, 1x</th>
<th>TIA, 14 days</th>
<th>FLU, 1x</th>
<th>FLU, 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus [pg/mg tissue]</td>
<td>1.91 ± 0.12</td>
<td>2.17 ± 0.17</td>
<td>3.25 ± 0.30**</td>
<td>2.87 ± 0.39</td>
<td>4.25 ± 0.36**</td>
</tr>
<tr>
<td>Striatum [pg/mg tissue]</td>
<td>4.97 ± 0.43</td>
<td>2.57 ± 0.41**</td>
<td>2.59 ± 0.19**</td>
<td>2.49 ± 0.29**</td>
<td>3.43 ± 0.31*</td>
</tr>
<tr>
<td>Hypothalamus [pg/mg tissue]</td>
<td>26.94 ± 2.95</td>
<td>8.04 ± 1.44**</td>
<td>9.80 ± 0.89**</td>
<td>9.18 ± 0.98**</td>
<td>12.19 ± 1.50**</td>
</tr>
<tr>
<td>Pituitary (pars anterior) [pg/gland]</td>
<td>624.9 ± 57.3</td>
<td>1470 ± 65.14**</td>
<td>999.3 ± 78.19**</td>
<td>643.6 ± 83.51</td>
<td>732.1 ± 44.92</td>
</tr>
<tr>
<td>Pituitary (pars intermediate) [pg/gland]</td>
<td>312.1 ± 57.6</td>
<td>650.0 ± 75.89*</td>
<td>1334 ± 108.6**</td>
<td>195.6 ± 33.06</td>
<td>1527 ± 106.1**</td>
</tr>
<tr>
<td>Adrenals [pg/mg tissue]</td>
<td>7.43 ± 0.87</td>
<td>4.98 ± 0.96</td>
<td>12.06 ± 1.91**</td>
<td>3.67 ± 0.64**</td>
<td>5.50 ± 0.90**</td>
</tr>
<tr>
<td>Plasma [pg/ml]</td>
<td>73.73 ± 7.36</td>
<td>56.50 ± 3.83</td>
<td>43.80 ± 7.49**</td>
<td>43.64 ± 3.23**</td>
<td>68.59 ± 7.74</td>
</tr>
</tbody>
</table>
Table 2. Effect of acute and repeated administration of TIA or FLU on the level of mRNA encoding for proenkephalin [optical density arbitrary units]. The animals were sacrificed 2 h after the acute or repeated treatment with TIA or FLU. The mean optical density values were obtained by averaging the measurements from autoradiograms of the 4 – 5 sections covering each region of interest, obtained from 6 – 8 animals per group.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>TIA, 1 x</th>
<th>TIA, 14 days</th>
<th>FLU, 1 x</th>
<th>FLU, 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus (dentate gyr.)</td>
<td>0.066 ± 0.003</td>
<td>0.077 ± 0.008</td>
<td>0.083 ± 0.007</td>
<td>0.093 ± 0.014</td>
<td>0.086 ± 0.007</td>
</tr>
<tr>
<td>Striatum (dorsal)</td>
<td>0.388 ± 0.011</td>
<td>0.425 ± 0.024</td>
<td>0.383 ± 0.013</td>
<td>0.365 ± 0.025</td>
<td>0.398 ± 0.017</td>
</tr>
<tr>
<td>Striatum (ventral)</td>
<td>0.422 ± 0.017</td>
<td>0.449 ± 0.027</td>
<td>0.420 ± 0.016</td>
<td>0.408 ± 0.020</td>
<td>0.458 ± 0.016</td>
</tr>
<tr>
<td>Hypothalamus (ventromed.n.)</td>
<td>0.417 ± 0.022</td>
<td>0.442 ± 0.034</td>
<td>0.355 ± 0.033</td>
<td>0.488 ± 0.025</td>
<td>0.457 ± 0.032</td>
</tr>
<tr>
<td>Pituitary (p. anterior)</td>
<td>0.167 ± 0.011</td>
<td>0.158 ± 0.014</td>
<td>0.183 ± 0.016</td>
<td>0.188 ± 0.028</td>
<td>0.210 ± 0.021</td>
</tr>
<tr>
<td>Pituitary (p. intermed.)</td>
<td>0.231 ± 0.021</td>
<td>0.230 ± 0.013</td>
<td>0.206 ± 0.010</td>
<td>0.201 ± 0.013</td>
<td>0.219 ± 0.024</td>
</tr>
<tr>
<td>Adrenals (core)</td>
<td>0.094 ± 0.004</td>
<td>0.099 ± 0.011</td>
<td>0.119 ± 0.003</td>
<td>0.075 ± 0.005</td>
<td>0.086 ± 0.005</td>
</tr>
</tbody>
</table>

DISCUSSION

The present studies were designed in order to find out whether TIA and FLU, two antidepressant drugs of more or less similar clinical efficacy (25), evoke comparable changes at the level of Met-Enk and PENK mRNA in the rat. Whereas classic tricyclic antidepressants and selective serotonin reuptake inhibitors (SSRIs), including FLU, block serotonin reuptake, TIA has been shown to decrease the extracellular serotonin level in the rat brain (26). It also facilitates serotonin uptake in rat (27) and human platelets (28).

Antidepressant treatment induces alterations in opioid peptide levels in discrete brain structures (14-16), however TIA and FLU have not been studied so far in a comparable experimental paradigm. Recently, Uzbay et al. (29) have shown that TIA has a prominent thermal antinociceptive activity in mice, what also may suggest the involvement of endogenous opioid peptide systems. On the other hand, Rosby et al. (30) have shown that chronic FLU treatment increased the levels of PENK mRNA in the rat amygdala to ca. 200% of saline controls. By using DL-p-chlorophenylalanine methyl ester (PCPA) to deplete brain serotonin levels these authors have indicated that the mechanism of PENK mRNA regulation by FLU is serotonin-dependent. However, it has not been answered whether the PENK mRNA is actually translated – leading to increased enkephalin synthesis and release.
From the results obtained in the present study it may be concluded that repeated administration of TIA or FLU induced similar changes in the levels of Met-Enk in the rat hippocampus, striatum, hypothalamus and neurointermediate lobe of pituitary. Such an effect is by itself interesting, especially if one takes into account the differences in pharmacological profile between these two antidepressant drugs. It may be suggested that serotonin level might not be crucial for inducing the alterations in the content of Met-Enk. Since we did not observe any changes in the levels of PENK mRNA in the studied rat brain regions after repeated administration of TIA or FLU, it seems that the observed changes in the levels of Met-Enk do not result from effects of these antidepressants on biosynthesis of PENK, but rather from alterations in the peptide release.

Another interesting finding of the present study is that in the anterior lobe of pituitary, adrenal glands and plasma, repeated administration of TIA induces alterations in the contents of Met-Enk, while repeated administration of FLU remains without any effect. It is tempting to speculate that such a differentiation between the effects of these two antidepressants might be linked to the well known feature of TIA (but not FLU) which has been shown to reduce both basal and stress-evoked activity of the hypothalamic-pituitary-adrenal (HPA) axis. Stressful life events can trigger the onset of depression in predisposed individuals (31), and the response to stress is characterized by behavioural and neuroendocrine changes, primarily those controlled by the HPA axis (32, 33). Furthermore, major depression per se is associated with hyperactivity of the HPA axis (33, 34). Thus, the HPA axis and maladaptation to the effects of stress are thought to be involved in the triggering of stress-induced depression (31). TIA antagonizes stress-induced behavioural deficits in animals models of depression (35, 36) and reduces the HPA axis response to stress. It has been shown that TIA prevents stress-induced or corticosterone-induced morphological changes (dendritic atrophy) in the rat hippocampus (37, 38). Delbende et al. (34) have shown that, in unstressed animals, repeated administration of TIA caused a significant reduction of corticotrophin releasing factor (CRF) content in the hypothalamus (without affecting CRF levels in extrahypothalamic regions such as the cerebral cortex and hippocampus (39).

Numerous studies support an important contribution of endogenous opioid peptide systems in the mediation, modulation, and regulation of stress responses, including endocrine (HPA axis) and behavioural responses. The widespread distribution of enkephalin throughout the limbic system, pituitary and adrenals is consistent with a direct role in the modulation of the stress responses. Met-Enk appears to play an important role in reducing the impact of a wide range of stressors (40). Met-Enk and other opioids have been shown to modify the synthesis and release of hypothalamic releasing agents, such as CRF (41, 42).

Therefore, the results obtained in the present study, which showed that in pituitary gland and adrenals repeated administration of TIA increases the content
of Met-Enk, while decreasing it in the plasma – together with the lack of changes in the level of PENK mRNA – may suggest the inhibition of the peptide release, the effect which remains in agreement with the data indicating the inhibition of HPA axis by TIA (34).

Similar conclusion might be drawn from the results observed after the acute treatment with TIA and FLU – changes in the content of Met-Enk observed 2 h after acute drug administration, without any significant alterations at the level of PENK mRNA suggest that the release of the peptide is affected. However, the elucidation of a role of enkephalinergic peptides play in the effects of TIA on stress-related responses, as well as in the mechanism of antidepressant action of this drug, needs further investigation.

Acknowledgements: The author are grateful to Farmacom, Kraków (Poland) for the generous gift of fluoxetine.

REFERENCES

1. Kraepelin E. Die Psychiatrische Klinik. 1905; Barth, Leipzig
12. Isenberg KF, Cicero TJ. Possible involvement of opiate receptors in the pharmacological profiles


Received: December 5, 2001
Accepted: January 20, 2002

Author’s address: M. Dziedzicka-Wasylewska, Department of Pharmacology, Institute of Pharmacology, Polish Academy of Sciences, 12 Smętna Street, 31-343 Kraków, Poland, e-mail address: wasyl@if-pan.krakow.pl