EFFECT OF COMBINED TREATMENT WITH IMIPRAMINE AND AMANTADINE ON THE CENTRAL DOPAMINE D₂ AND D₃ RECEPTORS IN RATS

In spite of intensive research, the problem of treating antidepressant-resistant depressive patients has not yet been solved. Our previous studies demonstrated that joint administration of a tricyclic antidepressant drug, imipramine (IMI) with the uncompetitive antagonist of NMDA receptors, amantadine (AMA), produced stronger "antidepressant" effect in the forced swimming test (Porsolt's test) than the treatment with either drug alone given. Since it has been suggested that dopamine receptors, among others, may play a role in anti-immobility effect of IMI, in the present study we examined the effect of AMA (10 mg/kg) and IMI (5 and 10 mg/kg) given separately or jointly, as a single dose or repeatedly (twice daily for 14 days) on the dopamine D₂ and D₃ receptors in the rat brain, using receptor autoradiography.

Following repeated administration of AMA alone or given in combination with IMI (5 mg/kg), the binding of [³H]quinpirole (dopamine D₂/D₃ receptors agonist) was increased, and similar changes were observed at the level of mRNA encoding dopamine D₂ receptors. We used [³H]7-OH-DPAT to selectively label the dopamine D₃ receptors. This experiment has shown that AMA given repeatedly did not induce statistically significant changes in the D₃ receptor binding, while IMI at both used doses, increased the [³H]7-OH-DPAT binding, and this effect was still observed after repeated joint administration of AMA with both doses of IMI. However, using both radioligands, we did not observe any synergistic or even additive effects in the binding studies after joint administration of AMA and IMI. Nevertheless, we can conclude that repeated administration of AMA, given together with IMI, induces the up-regulation of dopamine D₂ and D₃ receptors in the rat brain, and this effect may explain their synergistic action observed in the behavioral studies involving dopaminergic transmission.

Key words: imipramine, amantadine, repeated treatment, dopamine D₂ and D₃ receptors, adaptive changes, rats
INTRODUCTION

All currently used antidepressant drugs (ADs), including both tricyclic and newer agents such as venlafaxine are therapeutically effective in a maximum of 60-70% of depressive patients. The problem of AD-resistant depression has been a subject of extensive studies, yet with no apparent therapeutic success (e.g. 1, 2). Therefore, there is a strong need for alternative antidepressive treatments. Recently, much attention has been focused on the glutamatergic system and on NMDA receptor antagonists in particular. The antidepressive properties of those compounds were suggested over 10 years ago (3) and antidepressive-like actions of competitive NMDA receptor antagonists, such as DL-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 37849) or 2-amino-5-phosphonovaleric acid (AP5), and uncompetitive antagonists such as 1-aminoadamantane (amantadine, AMA) or 1-amino-3,5-dimethyladamantane (memantine) have subsequently been demonstrated in animal models (4-6).

Our previous studies demonstrated that joint administration of tricyclic AD, imipramine (IMI) and the uncompetitive antagonist of NMDA receptors, amantadine (AMA), produced stronger "antidepressant" effect in the forced swimming test (measured as the shortening of immobility time) than the treatment with either of these drugs given separately (7, 8). Positive effects were also observed when the above drugs were used jointly at inactive doses. Moreover, findings of other authors showed that NMDA receptor antagonists, including AMA, induced indirect activation of the dopaminergic system (via blockade of the glutamatergic system) in animals (9). It is also well known that antidepressant drugs administered repeatedly enhance the reactivity of central dopamine D$_2$ and D$_3$ receptors in laboratory animals (10, 11).

The aim of the present experiment was to examine the effect of IMI (5 and 10 mg/kg) and AMA (10 mg/kg), used separately and jointly, as a single dose or repeatedly (twice daily for 14 days), on the development of adaptive changes in central dopamine D$_2$ and D$_3$ receptors. A tissue for biochemical assays (rat brains) was collected 24 h after a single (acute treatment) or the last dose (repeated treatment) of IMI and AMA. The autoradiography of dopamine D$_2$/D$_3$ receptors was performed in the nucleus accumbens septi, nucleus caudatus and in the islands of Calleja using [$^3$H]quinpirole, an agonist of these receptors. Furthermore, selective binding of [$^3$H]7-OH-DPAT, an agonist of dopamine D$_3$ receptors was examined in the shell region of the nucleus accumbens septi and the islands of Calleja, i.e. the brain regions abundant in these receptors. Additionally, we measured the level of mRNA encoding dopamine D$_2$ receptors using in situ hybridization technique.
MATERIALS AND METHODS

Animals

The experiments were carried out on male Wistar rats, ca. 80 days old, weighing 220-230 g at
the beginning of the study. After 14 days of repeated drug administration, the weight of animals
increased up to 270-300 g. The animals had free access to food and water before the experiment and
were kept at constant room temperature (22 ± 1°C), under a 12/12 h light/dark cycle (light on at 7
a.m.). Experimental protocols were approved by Ethics Committee and met guidelines of the
relevant agency of the Institute of Pharmacology.

Drugs

Amantadine hydrochloride (AMA) was purchased from Sigma (St. Louis, USA), and
imipramine hydrochloride (IMI) was from Polfa (Kraków, Poland).

Drug administration

IMI (5 or 10 mg/kg) and AMA (10 mg/kg) were dissolved in distilled water and were
administered perorally (po) with a stomach tube, once (acute treatment) or repeatedly (twice daily
for 14 days). All animals were handled in the same manner twice daily for 14 days. Control animals
received vehicle for the whole experimental period while the repeatedly treated animals received
the appropriate drug. The animals treated acutely received saline for 13 days, and on the day 14,
they received the appropriate drug. Using this experimental paradigm, we avoided the effect of a
single intragastric intubation which inevitably, as a stressful event for an animal, may mask or
change the actual effect of acute administration of the studied drug. Moreover, all groups of
animals, treated acutely or repeatedly, were taken for experiments at the same time.

The rats used for biochemical experiments were sacrificed at 24 h after a single (acute
treatment) or the last dose (repeated treatment) of IMI or AMA. The tissue was dissected out, frozen
on dry ice and stored until used for autoradiography or in situ hybridization.

Biochemical studies

After administration of IMI and AMA or vehicle, the rat brains were carefully removed and
rapidly frozen in dry ice liquid n-heptane. Consecutive coronal sections (12 µm) were cut at -19°C
using a cryostat Jung CM 3000 (Leica). The effect of the drugs on dopamine D<sub>2</sub>/D<sub>3</sub> receptor
expression was evaluated in the coronal sections at the levels 1.0-1.7 mm from bregma, including
caudate putamen, nucleus accumbens septi, olfactory tubercles and islands of Calleja, according to
the Paxinos and Watson Rat Brain Atlas (12).

Dopamine D<sub>2</sub>/D<sub>3</sub> receptor binding in the rat nucleus accumbens septi, caudate
putamen and islands of Calleja - an autoradiographic procedure

Receptor binding with [³H]quinpirole (NEN Du Pont, specific activity: 44.50 Ci/mmol) was
visualized using the procedure described by Levant and de Souza (13) and Rogoż and Dziedzicka-
Wasylewska (14). Briefly, the sections were preincubated for 10 min at room temperature in 50 mM
Tris-HCl buffer (pH 7.4) containing the following ions: 5 mM KCl, 2 mM MgCl<sub>2</sub> and
2 mM CaCl<sub>2</sub>. The sections were then incubated at room temperature in the same buffer with 10 nM
radioligand for 90 min. Non-specific binding was determined with 1 µM (+)-butaclamol. The
experiment was terminated by dipping the sections in ice-cold buffer and rinsing them twice in
Dopamine D₃ receptor binding in the rat nucleus accumbens septi and islands of Calleja - an autoradiographic procedure

Dopamine D₃ receptors were labeled with [³H] 7-OH-DPAT (Amersham, specific activity: 155 Ci/mmol), as described by Lévesque et al. (15) and Maj et al. (11). Briefly, the tissue sections were first preincubated for 10 min at room temperature in 50 mM HEPES /NaOH buffer (pH 7.5), containing 1 mM EDTA and 0.1% bovine serum albumin. The sections were then incubated in the buffer described above with 0.5-1 nM of [³H]7-OH-DPAT. To determine non-specific binding, parallel sections were incubated in the presence of 10 µM dopamine. Following the incubation, the tissue sections were washed four times in ice-cold 50 mM HEPES/NaOH buffer (pH 7.5), containing 100 mM NaCl, rinsed twice in distilled water and then dried in cool air.

The level of mRNA encoding dopamine D₂ receptors in the nucleus accumbens septi and caudate putamen - in situ hybridization

The effect of IMI and AMA on the level of mRNA encoding dopamine D₂ receptors was determined as described previously (16), using a commercially available mixture of 48-mer synthetic deoxyoligonucleotides complementary to bases 4-51, 766-813 and 901-948 of the rat D₂ dopamine receptor (NEN Du Pont), which was labeled using [³⁵S]dATP (1.200 Ci/mmol, ICN Biomedicals, Inc.) with terminal transferase (Roche Molecular Biochemicals). Each experimental group consisted of 6-8 rats.

Data analysis

The autoradiograms were analyzed using a computer imaging system MCID-M1 (Canada) and quantified with the use of computer-generated curves derived from the standards. Film images of sections showing non-specific binding were subtracted from the images of adjacent sections with total binding, thus permitting direct observation of images representing specific binding on screen. The effect of each treatment on regional densities of the labeled receptors was compared with the appropriate control level using ANOVA, followed by Dunnett's test.

RESULTS

Figure 1 shows typical autoradiograms representing the localization of binding of two radioligands used in the present study, i.e. [³H]quinpirole (Figure 1A) and [³H]7-OH-DPAT (Figure 1B) in the brain slices obtained from control rats. The basal level of mRNA encoding dopamine D₂ receptors, measured using in situ hybridization is presented in panel C (Figure 1). Figure 1D shows diagrammatic representation of the areas used in optical density analysis (bregma 1.0-1.7 mm), according to Paxinos and Watson (12).
Dopamine D₂/D₃ receptor binding in the rat nucleus accumbens septi, caudate putamen and islands of Calleja

AMA given acutely increased the binding of [³H]quinpirole to dopamine D₂/D₃ receptors in the shell region of the nucleus accumbens septi but not in the other brain regions studied (Table 1). IMI, neither at a dose of 5 nor 10 mg/kg, given acutely, affected the binding of [³H]quinpirole. AMA given acutely together with the lower dose of IMI (5 mg/kg) increased the binding of [³H]quinpirole in the nucleus accumbens as well as in the islands of Calleja. However, AMA given jointly with the higher dose of IMI (10 mg/kg) significantly increased the binding of [³H]quinpirole in all studied brain regions, i.e. in the nucleus accumbens septi (shell and core), caudate putamen (lateral and medial) as well as in the islands of Calleja (Table 1).

When administered repeatedly, AMA increased the binding of [³H]quinpirole in all studied brain regions (except for the islands of Calleja, where the result did not reach statistical significance), similarly to IMI (10 mg/kg) given repeatedly, while IMI at a dose of 5 mg/kg, given repeatedly, did not induce statistically significant increase in the binding of [³H]quinpirole. Repeated joint administration of AMA with the lower dose of IMI (5 mg/kg) increased the binding of [³H]quinpirole in all brain regions studied, but not any further than

Fig. 1. Example autoradiograms of [³H] quinpirole binding (A), [³H] 7-OH-DPAT binding (B) and in situ hybridisation for D₂ mRNA (C). D shows diagrammatic representation of the areas used in optical density analysis: Shell - nucleus accumbens shell, Core - nucleus accumbens core, CP-l – caudate putamen lateral, CP-m – caudate putamen medial, ICj - islands of Calleja.
Table 1. Effect of imipramine (IMI) given alone or in combination with amantadine (AMA) on the binding of \( ^3H \) quinpirole to dopamine D\(_2\)/D\(_3\) receptors in the rat brain. IMI (5 or 10 mg/kg) and AMA (10 mg/kg) were given at a single dose or repeatedly (twice daily, 14 days). Rats brains were taken for an autoradiographic analysis 24 h after the the last administration of IMI or AMA. Data represent means ± SEM (fmol/mg protein), n = 6-8. Statistical significance was evaluated by ANOVA, followed by Dunnett's test. *p<0.005, **<0.001 vs. vehicle-treated group.

| Treatment | Nucleus accumbens septi | | | | | Island of Calleja |
|-----------|-------------------------|-----------------|-----------------|-----------------|-------------------|
|           | shell core               | medial lateral  |                               |                 |
| Vehicle   | 34.59 ± 3.59             | 40.08 ± 3.68    | 41.80 ± 5.43     | 51.51 ± 4.77    | 74.16 ± 4.44      |
| IMI 5, single | 44.55 ± 2.77    | 45.54 ± 3.81    | 47.39 ± 5.01     | 54.59 ± 5.80    | 87.68 ± 5.35      |
| IMI 10, single | 38.77 ± 3.69    | 39.10 ± 4.40    | 41.53 ± 3.66     | 49.95 ± 6.91    | 77.93 ± 5.59      |
| AMA 10, single | 59.64 ± 4.16** | 47.21 ± 5.15    | 46.44 ± 4.86     | 49.23 ± 7.56    | 83.78 ± 5.53      |
| IMI 5+AMA 10, single | 59.74 ± 5.35** | 59.91 ± 4.46*   | 57.25 ± 5.71     | 58.25 ± 6.13    | 100.0 ± 6.32*     |
| IMI 10+AMA 10, single | 56.98 ± 3.76** | 63.51 ± 5.14** | 62.32 ± 3.45*    | 71.88 ± 7.04    | 106.2±8.10**      |
| IMI 5, repeated | 42.26 ± 4.57    | 52.82 ± 4.54    | 48.24 ± 4.90     | 55.01 ± 5.34    | 75.78 ± 6.28      |
| IMI 10, repeated | 46.79 ± 4.56    | 57.22 ± 4.70*   | 59.73 ± 5.64     | 68.92 ± 5.57    | 98.92 ± 3.75*     |
| AMA 10, repeated | 52.43 ± 3.08*  | 57.47 ± 4.55*   | 61.24 ± 3.08*    | 71.77 ± 4.56    | 85.77 ± 4.71      |
| IMI 5+AMA 10, repeated | 53.92 ± 3.58* | 58.45 ± 3.58*   | 62.43 ± 2.63*    | 70.06 ± 4.23    | 95.74 ± 4.76*     |
| IMI 10+AMA 10, repeated | 50.27 ± 2.85    | 47.52 ± 4.11    | 54.59 ± 2.78     | 57.07 ± 3.22    | 82.39 ± 7.29      |

either of the drugs given alone. Such an effect was not observed when AMA was administered jointly with the higher dose of IMI (Table 1).

Dopamine D\(_3\) receptor binding in the rat nucleus accumbens septi and islands of Calleja

The binding of \( ^3H \)7-OH-DPAT was studied in the shell region of the nucleus accumbens septi and the islands of Calleja, i.e. in the brain regions where this radioligand binds selectively to the dopamine D\(_3\) receptors.

AMA given acutely increased the binding of \( ^3H \)7-OH-DPAT in both of these brain regions. Similar effect was observed after acute administration of IMI, given acutely at both studied doses, i.e. 5 and 10 mg/kg. Joint acute administration of AMA and IMI resulted in the increase in the \( ^3H \)7-OH-DPAT binding, although the effect was statistically significant in the rat brains obtained from animals receiving higher dose of IMI (10 mg/kg) (Table 2).

Repeated administration of AMA did not change the binding of \( ^3H \)7-OH-DPAT, but this binding was increased following repeated administration of IMI (10 mg/kg). When these two drugs were administered repeatedly together, the binding of \( ^3H \)7-OH-DPAT was also increased, but not any further than in a group of animals receiving IMI (10 mg/kg) alone. However, in a group of animals administered AMA together with the lower dose of IMI (5 mg/kg), we observed statistically significant increase in the binding of \( ^3H \)7-OH-DPAT, although...
Table 2. Effect of imipramine (IMI) given alone or in combination with amantadine (AMA) on the binding of [3H] 7-OH-DPAT to dopamine D_{3} receptors in the rat brain. IMI (5 or 10 mg/kg) and AMA (10 mg/kg) were given at a single dose or repeatedly (twice daily, 14 days). Rats brains were taken for an autoradiographic analysis 24 h after the the last administration of IMI or AMA. Data represent means ± SEM (fmol/mg protein), n = 6-8. Statistical significance was evaluated by ANOVA, followed by Dunnett's test. *p<0.005, **p<0.001 vs. vehicle-treated group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nucleus accumbens septi (shell)</th>
<th>Islands of Calleja</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>17.24 ± 0.83</td>
<td>31.54 ± 1.17</td>
</tr>
<tr>
<td>IMI 5, single</td>
<td>23.03 ± 1.52**</td>
<td>40.90 ± 1.87**</td>
</tr>
<tr>
<td>IMI 10, single</td>
<td>21.85 ± 0.71*</td>
<td>39.48 ± 2.18*</td>
</tr>
<tr>
<td>AMA 10, single</td>
<td>23.10 ± 0.97**</td>
<td>39.84 ± 2.63*</td>
</tr>
<tr>
<td>IMI 5 + AMA 10, single</td>
<td>20.59 ± 1.87</td>
<td>37.41 ± 1.53</td>
</tr>
<tr>
<td>IMI 10 + AMA 10, single</td>
<td>22.12 ± 2.01*</td>
<td>39.47 ± 2.00*</td>
</tr>
<tr>
<td>IMI 5, repeated</td>
<td>20.43 ± 0.83</td>
<td>35.40 ± 2.12</td>
</tr>
<tr>
<td>IMI 10, repeated</td>
<td>22.58 ± 0.68**</td>
<td>38.96 ± 1.10*</td>
</tr>
<tr>
<td>AMA 10, repeated</td>
<td>20.00 ± 0.99</td>
<td>32.87 ± 1.81</td>
</tr>
<tr>
<td>IMI 5 + AMA 10, repeated</td>
<td>22.89 ± 1.00**</td>
<td>40.02 ± 1.57**</td>
</tr>
<tr>
<td>IMI 10 + AMA 10, repeated</td>
<td>22.96 ± 0.65**</td>
<td>40.36 ± 1.61**</td>
</tr>
</tbody>
</table>

Repeated administration of IMI alone at 5 mg/kg did not induce statistically significant increase in dopamine D_{3} receptor binding (Table 2).

The level of mRNA encoding dopamine D_{2} receptors in the nucleus accumbens septi and caudate putamen

Slight although statistically significant increase in the level of mRNA encoding dopamine D_{2} receptor (D_{2} mRNA) was observed in the core region of the nucleus accumbens septi at 24 h after acute administration of IMI (10 mg/kg) or AMA, however joint administration of these two drugs did not change this parameter (Table 3).

When IMI (10 mg/kg) or AMA were administered repeatedly (separately or jointly), the significant increase in the level of D_{2} mRNA was observed in all studied brain regions, i.e. in the nucleus accumbens septi and in the caudate putamen. The statistically significant increase in the D_{2} mRNA was also observed in the group of animals receiving repeatedly lower dose of IMI (5 mg/kg) together with AMA, although this dose of IMI, given repeatedly but alone, did not induce any change in the level of D_{2} mRNA (Table 3).

DISCUSSION

In the recently published paper by Rogóź et al. (8), we have shown that combination of ADs (including IMI) with the uncompetitive NMDA receptor
antagonists (including AMA), induced synergistic (hyper-additive) antidepressive-like effects in the forced swim test in rats. Since our previous studies have shown that i.c.v. administration of D₂ antisense oligonucleotides reversed the effect of IMI in the forced swim test, indicating that the dopamine D₂ receptors play a significant role in the behavioral anti-immobility effects of imipramine (17), we designed the present experiments in order to study the effect of AMA and IMI, administered jointly, on the binding of dopamine D₂ and D₃ receptors. We used the agonists, [³H]quinpirole and [³H]7-OH-DPAT, to label these receptors since it has been shown that the binding of an agonist is a better tool for elucidating changes at the level of dopamine D₂/D₃ receptors, at least following treatment with antidepressant drugs (14).

Single administration of AMA increased the binding of both radioligands in the shell region of the nucleus accumbens septi (and in the islands of Calleja in case of the binding of [³H]7-OH-DPAT), and similar increase was observed in group of rats receiving joint administration of AMA with IMI. Our previous results have shown similar increases in the binding of dopaminergic radioligands following single administration of antidepressant drugs, with no changes observed in the functional, behavioral studies (11, 18). Therefore, we interpreted these changes as resulting from the alterations in the membrane fluidity, which has been shown to occur after single administration of IMI (19). It has been suggested that various antidepressant drugs are capable of nonspecific interaction

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nucleus accumbens septi</th>
<th>Nucleus caudatus</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>shell</td>
<td>core</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.028 ± 0.001</td>
<td>0.025 ± 0.001</td>
</tr>
<tr>
<td>IMI 5, single</td>
<td>0.031 ± 0.001</td>
<td>0.030 ± 0.001</td>
</tr>
<tr>
<td>IMI 10, single</td>
<td>0.035 ± 0.002</td>
<td>0.034 ± 0.001*</td>
</tr>
<tr>
<td>AMA 10, single</td>
<td>0.034 ± 0.002</td>
<td>0.033 ± 0.001*</td>
</tr>
<tr>
<td>IMI 5 + AMA 10, single</td>
<td>0.030 ± 0.001</td>
<td>0.030 ± 0.002</td>
</tr>
<tr>
<td>IMI 10 + AMA 10, single</td>
<td>0.028 ± 0.001</td>
<td>0.027 ± 0.001</td>
</tr>
<tr>
<td>IMI 5, repeated</td>
<td>0.031 ± 0.002</td>
<td>0.031 ± 0.002</td>
</tr>
<tr>
<td>IMI 10, repeated</td>
<td>0.031 ± 0.001</td>
<td>0.034 ± 0.001*</td>
</tr>
<tr>
<td>AMA 10, repeated</td>
<td>0.036 ± 0.001*</td>
<td>0.033 ± 0.001*</td>
</tr>
<tr>
<td>IMI 5 + AMA 10, repeated</td>
<td>0.037 ± 0.003*</td>
<td>0.037 ± 0.002**</td>
</tr>
<tr>
<td>IMI 10 + AMA 10, repeated</td>
<td>0.032 ± 0.001</td>
<td>0.033 ± 0.002*</td>
</tr>
</tbody>
</table>

Table 3. Effect of imipramine (IMI) given alone or in combination with amantadine (AMA) on the level of mRNA coding dopamine D₂ receptors in the rat brain. IMI (5 or 10 mg/kg) and AMA (10 mg/kg) were given at a single dose or repeatedly (twice daily, 14 days) Rats brains were taken for an autoradiographic analysis 24 h after the last administration of IMI or AMA. Data represent means ± SEM of optical density values obtained by averaging measurements of 4-5 sections obtained from 6-8 animals per group. Statistical significance was evaluated by ANOVA, followed by Dunnett's test. *p<0.005, **p<0.001 vs. vehicle-treated group.
with the neuronal membranes, which increases membrane fluidity (20, 21). The changes in membrane fluidity may, in turn, influence not only receptor interaction with G proteins and effector systems, but may also change the radioligand binding parameters, what has been directly shown for opioid (22), muscarinic (23) and dopamine D\textsubscript{2} (24) receptors. However, the question remains whether AMA may act in a similar manner.

Following repeated administration of AMA alone or in combination with the lower dose of IMI (5mg/kg), the binding of \[^{3}H\]quinpirole was increased, similarly as the level of mRNA encoding dopamine D\textsubscript{2} receptors, indicating that the enhancement in the binding may result from an increase in the translation process, occurring upon repeated administration of the studied drugs.

This observation fits well with the results showing the importance of dopamine D\textsubscript{2} receptor for anti-immobility effects in the forced swim test, and may contribute to further understanding of the mechanisms by which AMA increases the dopaminergic neurotransmission. Many different approaches have been proposed to address this issue. The studies with \textit{in vivo} microdialysis have been shown that local application (1mM, 40 min) of AMA increases the extracellular dopamine levels in the striatum by inhibiting the reuptake of DA and/or by blocking the channel in the NMDA receptor (25). In the more recent study, Peeters et al. (26) have shown that dopamine-induced stimulation of \[^{35}S\]GTP\gamma S binding was significantly enhanced (40\%) in the striatum homogenates obtained from rats treated for 4 days with AMA (40 mg/kg i.p.) compared to vehicle-treated animals, however, that enhancement of the functional response to dopamine stimulation was abolished after 7 days of treatment. These authors proposed that a progressive adaptation of striatal dopamine receptors developed after prolonged exposure to elevated dopamine concentration. Similar adaptation seems to appear in our experimental paradigm, since after repeated joint treatment of AMA with the higher dose of IMI (10 mg/kg) we did not observe any statistically significant increases in the binding of \[^{3}H\]quinpirole in the rat brain, although each of these drugs administered separately induced an increase in the binding of this radioligand. On the other hand, different situation was observed when the lower dose of IMI was used, i.e. 5 mg/kg. This dose of IMI, administered repeatedly, did not induce any statistically significant alterations in the binding of \[^{3}H\]quinpirole, and given together with AMA, it did not change the effect induced by the latter drug. It can be postulated that the results obtained with the use of \[^{3}H\]quinpirole might be interpreted as concerning dopamine D\textsubscript{2} receptors, since no statistically significant increase was observed in the islands of Calleja, where no expression of this subtype of dopamine receptors has been described. Despite numerous studies pointing to the dopaminergic mechanism that accounts for most of antiparkinsonian properties of AMA (reviewed by Huber et al. (27) and implication of other than dopaminergic actions in these effects (e.g. NMDA antagonism, reviewed by Danysz et al. (9)), proofs of the effect of AMA, given at the therapeutically relevant doses on the binding of
dopaminergic receptors seem scarce. Long-term administration of AMA to mice increased a number of postsynaptic dopamine receptors in the striatum, what has been shown with the use of \([\text{H}]\)spiroperidol (28). It has also been proposed that AMA might influence striatal D2 receptors, driving them towards a high-affinity state (29), which would explain why small amounts of dopamine are required for AMA to have a significant effect. Possibly this subtle regulation of dopamine receptors by AMA might account for the different effects of AMA administered with IMI, depending on the dose of this latter drug.

On the other hand, \([\text{H}]\)7-OH-DPAT, which selectively labels dopamine D3 receptors, shows slightly different effect following treatment with AMA and IMI, administered repeatedly separately or in combination. AMA given repeatedly alone did not induce statistically significant changes in the binding of \([\text{H}]\)7-OH-DPAT in the shell region of the nucleus accumbens septi or in the islands of Calleja, while IMI, at both doses, increased the binding of dopamine D3 receptor ligand, and this effect was still sustained after repeated joint treatment of AMA with IMI, used at both doses. The mechanism of such different regulation of these two subtypes of dopamine receptors might result from their different response to the increased dopamine neurotransmission. Namely, the density of dopamine D2 receptors usually decreases following repeated administration of dopaminergic agonists, and increases after treatment with neuroleptic drugs or after chemical lesion of ascending dopaminergic pathway (30, 31), while in case of dopamine D3 receptors the situation is different. Studies using rats with unilateral lesions of the nigrostriatal dopaminergic pathway with 6-hydroxydopamine have shown a progressive appearance of D3 receptor mRNA and binding sites upon repeated intermittent administration of L-DOPA, that occurred in the denervated caudate putamen, a brain area in which this receptor subtype is normally absent (32-34).

Therefore, the differences in the effect of joint repeated administration of AMA with IMI, depending on the dose of IMI (5 or 10 mg/kg) as well as on the dopamine receptor subtype under consideration, might result from the subtle interplay between the amount of presynaptic dopamine released upon repeated treatment with AMA and the influence of repeated treatment of IMI and/or AMA on the biosynthesis of post-synaptic dopamine receptors. Careful consideration of the doses of each drug used in this kind of studies becomes also important if one takes into account the results obtained by Rogóź et al. (7, 8), who have shown that synergistic interaction in the forced swim test was observed after administration of such doses of AMA and IMI which, when given alone, did not exert any significant effect.

Pharmacokinetic interactions can be excluded, since after coadministration of the compounds no increase, or even a decrease in general locomotion, examined in the open field test, was observed (7, 8). Also coadministration of AMA (20 mg/kg) with IMI (5 and 10 mg/kg) did not change the level of IMI and its metabolite, desipramine, in the rat plasma and brain, measured 1 h after forced swimming test exposure (own data, in preparation).
It should be also pointed out that in our biochemical studies measuring the effects of AMA and IMI administered jointly, we did not observe any synergistic or even additive effect of these two drugs on the binding to dopamine D_{2}/D_{3} receptors. However, one should take into account the role the neurotransmitter receptors play in the whole cascade of the signal transduction through the neuronal membrane. The information received by receptor (upon binding of specific agonist) is strongly enhanced via its interaction with appropriate G protein, the effector system and finally - the second messenger. Therefore, small, ca. 30% changes in the radioligand binding may have significant consequences for the given neuron at the postreceptor level, and strong homeostatic mechanisms must operate in order to prohibit excessive up- (or down-) regulation of receptors. This is the most probable explanation of apparently small (although statistically significant) changes observed usually at the level of receptors in the binding studies, as well as the lack of additivity of effects induced by the drugs used in the present study.

It should be mentioned that AMA, the NMDA receptor antagonist used in the study, is the not selective. For example, it has been shown that AMA enhances the hind limb flexor reflex in spinal rats in a manner similar to that of agents affecting noradrenergic transmission (35, 36) and attenuates reserpine-induced hypothermia which is also indicative of noradrenergic properties (5). Our preliminary data showed that AMA (10 mg/kg), given repeatedly, increased the clonidine-induced aggression in mice, and enhanced the action of antidepressant drugs such as IMI. The results suggest that AMA given repeatedly evokes hyperresponsiveness of α_{1}-adrenoreceptors. Such an activity was observed earlier following repeated administration of tricyclic antidepressants (37-39). Additionally, amantadine at therapeutic concentrations, binds to the sigma1 site (9). Some findings indicate that the selective sigma1 ligands, such as SA4503, are active in the forced swimming test and may thus be potential antidepressants (40, 41).

In conclusion, our study shows that repeated administration of AMA alone or together with IMI induces the up-regulation of dopaminergic D_{2}/D_{3} receptors in the rat brain, and this effect may underlie their synergistic action observed in the behavioral studies, which involve dopaminergic transmission.

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