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# The effect of prolonged treatment with imipramine on the biosynthesis and functional characteristics of $D_2$ dopamine receptors in the rat caudate putamen

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**1** The present study shows the effects of imipramine in a single dose (10 mg $kg^{-1}$, p.o.) or following repeated (14 days, twice a day) treatment on the level of mRNA coding for $D_2$ dopamine receptors in the rat caudate putamen (CP). Repeated administration of imipramine resulted in the increase of the level of mRNA coding for $D_2$ dopamine receptors.

**2** Radioligand binding studies with the $D_2$ receptor agonist, [$^3$H]-N-0437, indicated, that following imipramine administration, the affinity of the agonist for the $D_2$ dopamine receptor significantly increased, though without any alterations in the $B_{max}$.

**3** Pharmacological manipulations (by use of forskolin, GppNHp and quinpirole) of the cyclic AMP generating system, *ex vivo* following administration of imipramine indicated that an up-regulation of factors inhibiting cyclic GMP formation takes place.

**4** Most probably it is the $D_2$ dopamine receptor which undergoes functional up-regulation, resulting from the enhancement of its biosynthesis.

**Keywords:** Imipramine; $D_2$ dopamine receptor mRNA; [$^3$H]-N-0437; cyclic AMP; rat caudate putamen

## Introduction

Previous behavioural and neurochemical studies have shown that antidepressant treatments affect brain dopaminergic systems. Chronic administration of antidepressant drugs increases psychostimulant- or apomorphine-induced locomotor activity, possibly as a result of postsynaptic dopaminergic supersensitivity (Maj, 1986; 1990; Maj *et al*., 1984; 1987; 1989; 1991). Antidepressant treatment has also been shown to decrease presynaptic dopamine autoreceptor function. Chronic administration of antidepressant drugs, electroconvulsive shock or deprivation of rapid eye movement sleep, decreased the ability of low doses of apomorphine to produce sedation, an effect mediated by dopamine autoreceptors (Serra *et al*., 1979; 1981a,b; 1990). Although some studies have suggested that chronic antidepressant treatment decreases the function of dopamine autoreceptors, others are not in agreement with this conclusion (Spyraki & Fibiger, 1981; Holcomb *et al*., 1982; Diggory & Buckett, 1984). Alternatively, the hypothesis of 'supersensitivity' of postsynaptic dopamine receptors has been proposed. However, the 'supersensitivity' hypothesis, although well documented in behavioural studies (Spyraki & Fibiger, 1981; Martin-Iverson *et al*., 1983; Arnt *et al*., 1984; Maj *et al*., 1984; Maj & Wedzony 1985; 1988; Płaźnik & Kostowski, 1987), contrasts with the results of dopamine receptor binding which show that the number of $D_2$ receptors, as measured by [$^3$H]-spiperone binding, is unchanged, whereas that of $D_1$ receptors, measured by [$^3$H]-SCH 23390 binding, is even decreased after long-term treatment with antidepressants (De Montis *et al*., 1990; Martin-Iverson *et al*., 1983; Klimek & Nielsen, 1987). However, in further studies by Klimek & Maj (1989) is has been shown that antidepressants administered repeatedly increase the affinity of $D_2$ dopamine receptors for the agonist quinpirole (LY-171555) in the limbic system. This finding is in line with the observation that repeated administration of antidepressants potentiates the hyperactivity induced by dopamine agonists (Spyraki & Fibiger, 1981; Maj, 1990).

Since the clinical effect of antidepressant drugs is generally observed only after prolonged treatment, the biochemical changes requiring such prolonged administration of a drug may suggest alterations at the genomic level. However, until recently little has been known about transcriptional and post-transcriptional factors regulated by chronic drug treatment, although long-term changes in neuronal synaptic function are known to be dependent upon selective regulation of gene expression. Therefore, the present study was intended to obtain information on whether repeated administration of imipramine, one of the most commonly used antidepressant drugs, modifies the biosynthesis of postsynaptic $D_2$ dopamine receptors. This aim was achieved by measuring the level of mRNA coding for $D_2$ dopamine receptors in the rat caudate putamen (CP) by use of *in situ* hybridization. To characterize further the possible changes in the affinity of $D_2$ dopamine receptors following repeated administration of imipramine, we used the potent and selective dopamine $D_2$ receptor agonist, [$^3$H]-N-0437 (VanOene *et al*., 1984; Van der Weide *et al*., 1986). Additionally, some aspects of functional changes in $D_2$ dopamine receptor were analysed by measuring the reactivity of adenylate cyclase in slices of the CP, *ex vivo*, following repeated administration of imipramine.

## Methods

### *Animal treatment*

Male Wistar rats (180–220 g) were treated with either a single dose of imipramine (10 mg $kg^{-1}$ or repeated doses (p.o. twice daily, at 08 h 00 min and 17 h 00 min) for 14 days. Control groups received the equivalent volume of saline. Two or

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seventy two hours after the last dose the animals were decapitated, the CP were dissected and used immediately for radioimmunoassay of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) or frozen for the evaluation of [$^3$H]-N-0437 binding. For the *in situ* hybridization studies the brains of rats were rapidly removed and frozen on dry ice.

### In situ *hybridization*

Coronal sections (12 $\mu$m thick) through the CP were cut on a cryostat. The sections were thaw-mounted onto chrome alum pretreated slides, postfixed in 4% paraformaldehyde for 10 min and processed for *in situ* hybridization as described previously (Dziedzicka-Wasylewska & Rogoż, 1995). Briefly, a mixture of 48-mer synthetic deoxyoligonucleotides complementary to bases 4–51, 766–813 and 901–948 of the rat $D_2$ dopamine receptors (Bunzow *et al*., 1988), was labelled with [$^{35}$S]-dATP (1,200 Ci mmol$^{-1}$, NEN DuPont, U.K.) to obtain a specific activity of about $4 \times 10^5$ c.p.m. $\mu$l$^{-1}$. The sections were hybridized with the labelled 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 MP) for 20 days at −20°C. Different patterns of hybridization, found in the brain regions, fully agreed with the well known distribution of $D_2$ dopamine receptor mRNA, and provided support for the specificity of the probe under the present experimental conditions. The specificity of *in situ* hybridization was also assessed by pretreatment of some tissue sections with RNAse A (20 $\mu$g ml$^{-1}$) for 40 min at 30°C, which completely eliminated the hybridization signal with the cDNA probe.

Optical density measurements were made from autoradiograms corresponding to sections of the CP, by use of an image analysing system (Java: Jandel, Corte Madera CA, U.S.A.). The average optical density values were calculated after substraction of the film background density. The mean optical density values were obtained by averaging out the measurements from autoradiograms of the sections obtained from 6–8 animals per group.

### *[$^3$H]-N-0437 binding*

Binding of [$^3$H]-N0437 to the membranes prepared from rat CP was performed by the method described by Van der Weide *et al*. (1986). Briefly, 100 $\mu$l of membrane suspension (ca 2.5 mg of tissue) was incubated with various concentrations of [$^3$H]-N-0437 (spec. act. 100 Ci mmol$^{-1}$, Amersham, U.K.), ranging from 0.1 to 5 nM, in a final volume of 1 ml. Non-specific binding was determined in the presence of 1 $\mu$M (+)-butaclamol. The reaction was stopped after 45 min incubation of 25°C by the addition of 5 ml of ice-cold buffer, pH 7.4, and the mixture was filtered under vacuum through Whatman GF/B filters, which were then washed three times with 5 ml of ice-cold buffer, by use of a Brandell cell harvester.

### *Cyclic AMP measurements*

The measurements of cyclic AMP levels in the slices of the rat CP, *ex vivo* after administration of imipramine, were performed essentially as previously described (Dziedzicka-Wasylewska *et al*., 1995). Immediately after decapitation of the animals, the tissue was dissected, weighed and slices prepared with a McIlwain tissue chopper. Slices (350 $\mu$M) were placed at 35°C in a Krebs-Ringer bicarbonate-glucose buffer gassed continuously with 95%$O_2$/5%$CO_2$. The slices were incubated for 40 min before being individually placed into experimental tubes containing 1 ml of fresh Krebs buffer each. After 10 min test substances, i.e.: forskolin (10 $\mu$M), GppNHp (guanosine-5′-imidodiphosphate, 10 $\mu$M) or quinpirole (1 $\mu$M), were added to the assay tubes. Following a 15 min incubation, accumulation of cyclic AMP was stopped by rapid heating of the tubes to 95°C. The level of cyclic AMP was determined by radioimmunoassay with a commercially available kit (NEK-033, NEN DuPont, U.K.).

### *Drugs*

Imipramine HCl was obtained from Polfa (Poland); forskolin from Calbiochem (U.S.A.); quinpirole and (+)-butaclamol from Research Biochemicals International (U.S.A.); guanylyl-5′-imidodiphosphate (GppNHp) and terminal transferase from Boehringer Mannheim (Germany); 48-mer synthetic deoxyoligonucleotide probe complementary to the rat dopamine $D_2$ receptor as well as [$^{35}$S]-dATP and [$^3$H]-N-0437 from Amersham (U.K.); the RIA kit for cyclic AMP determinations was obtained from NEN DuPont (U.K.). All other chemicals were purchased from Sigma (U.S.A.).

### *Statistics*

The results were statistically assessed by one-way analysis of variance (ANOVA) and inter-group differences were analysed by Duncan's multiple range test.

## Results

Data obtained for the effect of imipramine on the level of mRNA coding for $D_2$ dopamine receptors in the rat CP is presented in Table 1. A single dose of imipramine (10 mg kg$^{-1}$, p.o.) produced a slight, but statistically significant increase in the level of $D_2$ dopamine receptor mRNA, at 72 h, but not 2 h after administration. Repeated, 14 days administration of imipramine resulted in a significant increase in the level of $D_2$ dopamine receptor mRNA, and the effect was more pronounced at 72 h than 2 h after the last dose of the drug.

The binding of [$^3$H]-N-0437 to $D_2$ dopamine receptors in the CP following single and repeated administration of imipramine is shown in the Table 2. There was no change in the value of $B_{max}$ in all groups studied, i.e. after either a single dose (10 mg kg$^{-1}$) or repeated (10 mg kg$^{-1}$, twice a day, 14 days)

**Table 1** The effect of imipramine (Imip) on the level of mRNA coding for $D_2$ dopamine receptors in the rat caudate putamen

| *Treatment* | *Level of $D_2$ dopamine receptor mRNA* (optical density arbitrary units [% control]) |
|---|---|
| Imip single, 72 h | 113.0±5.6* |
| Imip repeated, 2 h | 112.3±5.8* |
| Imip repeated, 72 h | 126.4±1.0** |

Imipramine (10 mg kg$^{-1}$, p.o.) was administered as a single dose (Imip single) or twice a day for 14 days (Imip repeated). The rats were decapitated either 2 or 72 h after the last dose of the drug. The mean optical density values were obtained from 5–6 animals, and calculated as percentage of the control level (100±2.4%). Statistical significance was determined by ANOVA followed by Duncan's test; *$P < 0.01$; **$P < 0.001$ vs the control level.

**Table 2** Parameters of [$^3$H]-N-0437 binding to homogenate of rat caudate putamen following treatment with imipramine (Imip)

| *Treatment* | $B_{max}$ (pmol g$^{-1}$ tissue) | $K_D$ (nM) |
|---|---|---|
| Control | 1.08±0.05 | 0.88±0.02 |
| Imip single, 72 h | 0.97±0.14 | 0.56±004 |
| Imip repeated, 2 h | 0.97±0.42 | 0.43±0.01* |
| Imip repeated, 72 h | 1.21±0.15 | 0.46±0.08* |

Imipramine (10 mg kg$^{-1}$, p.o.) was administered as a single dose (Imip single) or twice a day for 14 days (Imip repeated). Results are the mean±s.e.mean from eight determinations per group. Statistical significance was determined by ANOVA followed by Duncan's test; *$P<0.001$ vs the control level.

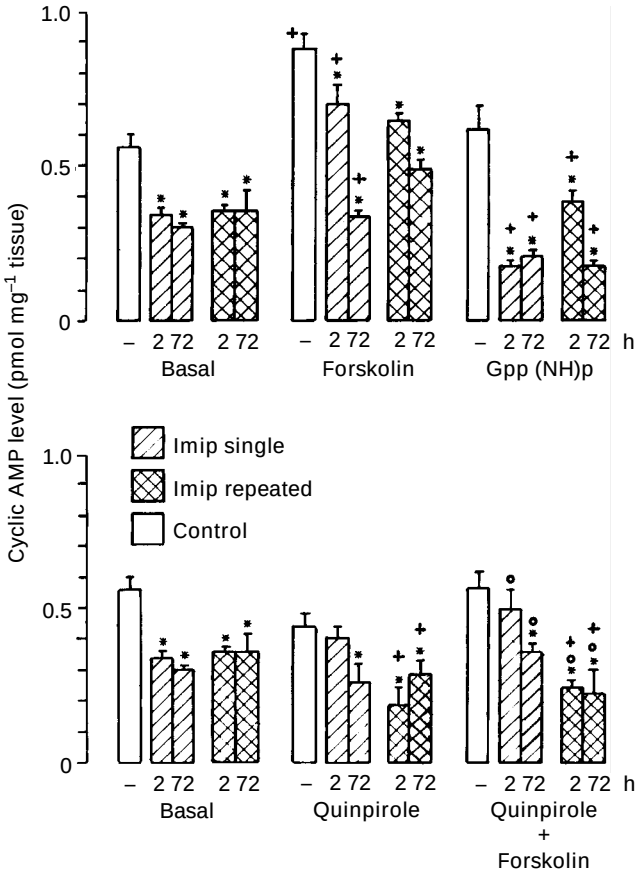


**Figure 1** The effect of imipramine (Imip; 10 mg kg$^{-1}$, p.o.) *ex vivo* on the cyclic AMP level in the slices of rat caudate putamen. The tissue for further *in vitro* experiments was obtained 2 and 72 h after the single or repeated (twice a day for 14 days) administration of Imip. Slices (350 $\mu$m thick) obtained from the experimental groups of animals were incubated in the presence of forskolin (10 $\mu$M), GppNHp (10 $\mu$M) or quinpirole (1 $\mu$M). Results are the mean± s.e.mean from eight determinations per group. Statistical significance was determined by ANOVA followed by Duncan's test. *$P<0.001$ vs the control level in each group; $^+P<0.001$ vs the control level in

treatment with imipramine, 2 h or 72 h after treatment. However, significant attenuation in the $K_D$ value was observed after acute (only 72 h) and repeated (2 h and 72 h) administration of imipramine, indicating an increase in the affinity of the $D_2$ dopamine receptor for the agonist.

Measurements of cyclic AMP production in the slices of rat CP, *ex vivo*, following single and repeated imipramine administration, 2 h and 72 h after withdrawal, were performed in order to characterize further the functional state of $D_2$ dopamine receptors in this brain region. The data are presented in Figure 1. The basal level of cyclic AMP was decreased in the slices obtained from all groups of imipramine-treated rats in comparison to the control. The forskolin (10 $\mu$M) stimulation of cyclic AMP production was attenuated following a single dose or repeated administration of imipramine. Quinpirole (1 $\mu$M), like GppNHp (10 $\mu$M), did not significantly change the level of cyclic AMP in the slices of control animals. However, following the administration of imipramine, a significant attenuation of cyclic AMP production was apparent after *in vitro* addition of quinpirole (except for a single dose of imipramine, 2 h after the administration), as well as of GppNHp.

## Discussion

Recently it has become clear that long-term changes in neuronal synaptic function are correlated with, and in some cases shown to be dependent on, the induction of new programmes of gene expression (Karin, 1992; Sheng & Greenberg, 1990). Successful cloning of the genes for the dopamine receptors (Bunzow *et al*., 1988; Zhou *et al*., 1990; Civelli *et al*., 1991) has allowed the study of the modulation of the dopamine system at the level of gene expression. It also becomes apparent that receptor activity may be coupled to receptor biosynthesis, thus maintaining dopamine homeostasis in the brain. It has been shown that dopamine receptor mRNA is regulated by dopamine agonists, neuroleptics and 6-hydroxydopamine lesions (Savasta *et al*., 1988; Coirini *et al*., 1990; Gerfen *et al*., 1990; LeMoine *et al*., 1990; Angulo *et al*., 1991; Chen *et al*., 1991; 1993; Jongen-Relo *et al*., 1994). Regulation of receptor expression by agonists has been recently described for other receptors (Hadcock & Malbon, 1991).

The present study provides data indicating that pharmacological intervention with imipramine, the mechanism of action of which is not directly linked to dopaminergic transmission, also profoundly influences the level of mRNA coding for $D_2$ dopamine receptors in the rat brain.

Treatment with imipramine resulted in an increase in the level of mRNA coding for $D_2$ dopamine receptors in the rat CP. This effect is very interesting, since many behavioural studies have already indicated the functional up-regulation of $D_2$ dopamine receptors (see Introduction).

With the recent availability of [$^3$H]-N-0437, a $D_2$ dopamine receptor agonist radioligand (Van der Weide *et al*., 1986), we performed further experiments in order to determine whether it is possible to observe any difference in binding parameters of [$^3$H]-N-0437, in comparison to the previously used $D_2$ dopamine receptor antagonist, [$^3$H]-spiperone, following prolonged treatment with imipramine. Some previous studies have already indicated that antagonist ligands do not always provide full information concerning the state of the endogenous receptor and the binding of agonists was often necessary to elucidate more precisely any subtle, but important alterations at the level of the receptors (Seeman & Grigoriadis, 1987). Indeed, the results obtained in the present study, with [$^3$H]-N-0437, show a significant increase in the affinity of this radioligand towards $D_2$ dopamine receptors, following administration of imipramine. This finding is in line with the previously published results showing a significant increase of quinpirole affinity to $D_2$ dopamine receptors induced by antidepressant treatment (Klimek & Maj, 1989).

However, in the present study we were not able to establish any significant changes in the density of $D_2$ dopamine receptors following administration of imipramine, which apparently contrasts with the results indicating the increase in the biosynthesis of $D_2$ dopamine receptor mRNA. This discrepancy might be explained if one takes into account that the *in situ* hybridization technique and classic binding studies actually make apparent different populations of $D_2$ dopamine receptors. Namely, at the level of the forebrain, the specific receptors and their corresponding mRNA are only coincidentally co-localized (Mansour *et al*., 1990). The CP is a good example of such a relationship, since there are high levels of $D_2$ receptor mRNA as well as a high density of $D_2$ receptors, and yet the binding sites in this structure are primarily a reflection of translocation of mRNA that occurs outside of this structure, followed by transport of the receptor to a distal terminal site. While some are intrinsic to the CP, most of the cells synthesizing $D_2$ dopamine receptors in this brain region are located in the midbrain, thalamus and cortex, which is where the corresponding $D_2$ dopamine receptor mRNA is visualized. Therefore the density of $D_2$ dopamine receptors observed in the binding studies with [$^3$H]-N-0347 in the homogenate is not the best method for resolution of cellular versus terminal localization of the $D_2$ dopamine receptors. The lack of changes in the density of $D_2$ dopamine receptors might well reflect contradictory effects of prolonged treatment with imipramine on the biosynthesis of $D_2$ dopamine receptor mRNA in the postsynaptic cells intrinsic to the CP, in comparison to the cells originating in the midbrain. However, such a notion, which needs further experimental verification, might be justified in the light of evidence that antidepressant treatments decrease the function of dopamine autoreceptors (Chiodo & Antelman, 1980).

Another interpretation of the results could be based on the possibility that imipramine induces a more rapid turnover of $D_2$ dopamine receptor protein, as a need for higher synthesis of the $D_2$ receptor and elevated mRNA levels. The change of $K_D$ for an agonist binding is indeed consistent with the insertion of newly synthesized and immature receptor proteins into the membrane. However, it does not seem to be the case, since it has already been found (Nowak & Żak, 1991) that repeated administration of imipramine does not change the recovery after N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)-induced inactivation of $D_2$ receptors.

On the other hand, the measurements of the level of cyclic AMP in the slices of the CP obtained from rats treated with imipramine, strengthen the hypothesis that there is indeed an up-regulation of postsynaptic $D_2$ receptors following administration of this drug, since these are receptors coupled to the adenylate cyclase in this brain region (Weiss *et al*., 1985; Thomas *et al*., 1992). The basal level of cyclic AMP is lower in the slices obtained from treated rats, also the forskolin stimulation is attenuated following administration of imipramine. The non-hydrolyzable analogue of GTP, GppNHp, possesses different affinities towards various populations of G proteins (Cooper, 1983; Onali *et al*., 1985; Ahlijanian *et al*., 1987). The lack of significant alterations in the level of cyclic AMP in the slices of CP obtained from control animals induced by 10 $\mu$M GppNHp may result from mixed effect on different populations of G proteins. On the other hand, following administration of imipramine, such changes take place either in the fluidity of the cell membrane — as described previously by Melzacka and Nocoń (1991) — or at the very level of $G_i$ proteins themselves, that inhibition of adenylate cyclase becomes apparent. Indeed, *ex vivo* (i.e. following prolonged administration of imipramine) addition of GppNHp, acting at the G protein level, as well as of quinpirole, a $D_2$ receptor agonist, both resulted in the inhibition of cyclic AMP production, which was more pronounced in slices obtained from rats treated with imipramine. Such results indicate that following administration of imipramine, a certain up-regulation of factors inhibiting cyclic AMP production develops.

Even though G proteins (inhibitory in this case) are important for transducing the signals in the cell membrane from the receptor to the effector system (unequivocally documented by Birnbaumer, 1990 and Gilman, 1987) and are also involved in the mechanism of action of antidepressant drugs (Menkes *et al*., 1983; Ozawa & Rasenick, 1989), it is most probably the $D_2$ dopamine receptor which undergoes functional up-regulation, resulting from the enhancement of its biosynthesis.

As already discussed by others (e.g. Mansour *et al*., 1990; Civelli *et al*., 1991; Jongen-Relo *et al*., 1994), between the receptor synthesis and the availability of the functional receptors in the neuronal membrane, a complex series of events takes place. This includes post-transcriptional, translational and post-translational processes, the subsequent incorporation of the receptor protein into cell membrane, coupling through G proteins with the effector systems and eventually degradation of the receptors.

The precise relationships between alterations in gene transcription, mRNA stability, translational processes and dopamine receptor binding remain to be clarified. Nevertheless our study demonstrates, for the first time, that the steady state level of dopamine receptor mRNA — which is the net result of gene transcription and degradation of the messenger — is susceptible to treatment with imipramine, the widely used antidepressant drug. It has become increasingly apparent that the alterations in the expression of genes coding for neurotransmitter receptors is the level where one should search for the mechanism of action of drugs, which are therapeutically effective only after prolonged administration.

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