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Gi/Go Protein-Dependent Presynaptic Mechanisms Are Involved in Clozapine-Induced Down-Regulation of Tyrosine Hydroxylase in PC12 Cells

Purificación Tejedor-Real,[1,2] Roland Vogel,[1] Jacques Mallet,[1]* and Nicole Faucon Biguet[1]

[1]CNRS UMR 7091, L.G.N. Bâtiment CERVI, Hôpital de la Pitié Salpêtrière, Paris, France
[2]Department of Neuroscience (Pharmacology and Psychiatry), School of Medicine, University of Cadiz, Cadiz, Spain

Although the clinical effects of antipsychotics have been extensively studied, the molecular mechanisms underlying their antipsychotic activity are unclear. Chronic clozapine has been reported to reduce significantly the expression of tyrosine hydroxylase (TH) in the mesolimbic system. To characterize the mechanisms of action of clozapine on TH expression, PC12 cells turned out to be a useful model, being by far less complex than the entire brain. Both the quantity of TH protein and the amount of TH mRNA in PC12 cells were found to be decreased during incubation of the cells in the presence of clozapine. This decline was followed by a decrease in the enzymatic activity of TH. The effect of clozapine was blocked by preincubation with N-ethylmaleimide, a sulphydryl-alkylating reagent that interferes in Gi/o protein-mediated second messenger pathways. Clozapine may thus decrease TH expression by interacting with Gi/o protein-coupled receptors, such as D2 and 5HT1A. Knowledge of the molecular mechanisms underlying the clinical effects of established antipsychotics will promote the development of new and more efficient antipsychotic drugs.

Key words: antipsychotics; dopamine biosynthesis; gene expression; signal transduction; schizophrenia

Administration of antipsychotic drugs is the major treatment for schizophrenia. The clinical evidence is that early and prolonged interventions with these drugs improve long-term outcome. Although their clinical effects have been extensively studied, the molecular mechanisms underlying antipsychotic activity are still unclear. Exploring the pathobiochemical mechanisms involved in schizophrenia may contribute to the development of more effective treatments. In particular, analysis of changes in gene expression induced by antipsychotics may reveal new roles for known genes or identify novel functions associated with drug efficacy.

The dopaminergic theory of schizophrenia has been the most widely accepted explanation of the neurochemical abnormality underlying the disease. Consequently, the dopaminergic system has been one of the major targets in the treatment of this psychiatric illness (Meltzer and Stahl, 1976). It has been suggested that selectively reduced release of dopamine in the nucleus accumbens is required for the antipsychotic effect to be expressed (Chen et al., 1991). Dopamine production is regulated by tyrosine hydroxylase (TH; EC 1.14.16.2), the initial and rate-limitating enzyme of catecholamine biosynthesis (Nagatsu et al., 1964). In rats, chronic administration of clozapine, an atypical antipsychotic, significantly reduces both TH mRNA and protein in the accumbens, the striatum, the ventral tegmental area, and the substantia nigra (Tejedor-Real et al., 2003). This finding is of interest because the clozapine-induced reduction of dopamine in the mesolimbic system (Blaha and Lane, 1987; Lane et al., 1988; Chen et al., 1991; Kuroki et al., 1999 may result from the loss of TH. Understanding the mechanisms by which clozapine down-regulates TH might thus be the first step in elucidating the effect of antipsychotic drugs on the dopaminergic system. It will also help in identifying the signalling pathways that are abnormal in schizophrenia.

Clozapine displays significant affinities for several neurotransmitter receptors, including dopaminergic receptors, α-adrenergic receptors, and various serotonergic and cholinergic receptors; any of these activities may contribute to the clinical effectiveness of the drug

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*Correspondence to: J. Mallet, CNRS UMR 7091, L.G.N. Bâtiment Cervi, Hôpital de la Pitié-Salpêtrière, 83 Boulevard de l'Hôpital, 75013 Paris, France. E-mail: mallet@infobiogen.fr

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(Bymaster et al., 1999). Many of the transmembrane signalling processes of clozapine are mediated by interaction between receptors and heterotrimeric guanine nucleotide binding proteins (G-protein; Gilman, 1987). Heterotrimeric G proteins are pivotal in postreceptor information transduction. They act as switches regulating information processing circuits that connect the cell surface to a variety of intracellular effectors. Investigations of the G protein-associated receptors are pertinent to the mechanism of action of antipsychotic drugs because they are potential targets of most psychotropic compounds (Avissar et al., 2001; Dwivedi et al., 2002). The affinity of these receptors for their activating ligands is low when they are dissociated from G protein. Gi/o proteins are uncoupled from receptors by N-ethylmaleimide (NEM), a sulphydryl-alkylating reagent that alkylates the cysteine residue of Gi/o proteins that is ADP-ribosylated by pertussis toxin (Winslow et al., 1987). NEM has been extensively used to inhibit signalling pathways mediated by Gi/o protein-coupled receptors (Olianas and Onali, 1996; Gonzalez-Maeso et al., 2000, 2002; Ikeda et al., 2001), reducing their activity (Pineyro and Blier, 1996).

The general aim of this work was to investigate the mechanisms by which clozapine affects the amount of TH in target cells. We used PC12 cells as a model. The cells were incubated with clozapine under various conditions, some including preincubation with NEM, to study the involvement of G protein-associated receptors in the modulation of TH expression. The amount of TH in PC12 cells was lower when the cells were incubated in the presence than in the absence of clozapine, demonstrating that clozapine may act directly on dopaminergic cells. This effect of clozapine on TH was abolished by preincubation with NEM. Thus, this presynaptic mechanism of clozapine action presumably involves Gi/o protein signalling pathways. These findings contribute to elucidating how antipsychotic drugs affect the expression of the TH gene in vivo.

MATERIALS AND METHODS

Cell Culture Experiments

Rat pheochromocytoma (PC12) cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal calf serum, under a humidified atmosphere containing 5% CO_2 at 37°C. Cells were plated in 100-mm dishes and used after 5 days in culture, when a density of about 10^6 cells/dish was reached. Twelve hours prior to the administration of clozapine, the culture medium was removed and replaced by incubation medium (RPMI 1640 containing 5% heat-inactivated horse serum). On the day of the experiment, half of the medium in each dish was replaced with incubation medium to which stock solutions of Clozapine (Tocris-Bioblock Scientific, Illkirch, France) and/or NEM (Sigma-Aldrich, St. Quentin Fallavier, France) had been added. In control dishes, half of the medium was replaced with incubation medium containing appropriate amounts of the diluents used to prepare the stock solutions of the drugs (vehicle treatment). For chronic treatments, every day half of the medium was replaced in each dish with incubation medium containing the necessary amounts of clozapine to reach the appropriate concentration; control cell cultures were subjected to the same manipulation using only diluents. After incubation with clozapine and/or NEM, the cells were scraped into ice-cold phosphate-buffered saline (PBS) and centrifuged at 1,000*g* and 4°C for 5 min. The cells were resuspended once in PBS and centrifuged again as described above. Finally, the cell pellets were either stocked at –80°C for Western blot analysis or used immediately for TH enzyme activity assays. For inactivation of the Gi/o proteins, we used NEM, because its action is rapid and specific at micromolar concentrations (Shapiro et al., 1994).

Western Blot Analysis

The effect of clozapine on TH protein content of PC12 cells was investigated by Western blot analysis of cell extracts. The cell pellets were lysed by brief sonication in 10 mM sodium-phosphate buffer, pH 7, containing 0.5% Triton X-100 and the Protease Inhibitor Set (Roche, Meylan, France). The homogenates were centrifuged at 10,000*g* and 4°C for 5 min, and the protein content in the cleared extracts was quantified according to the method of Bradford (1976) by using the Bio-Rad protein assay (Bio-Rad, Munich, Germany) and bovine serum albumin as standard. Samples of the cleared extracts containing 15–50 μg protein were resolved by electrophoresis on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel in the presence of 50 mM dithiothreitol. The proteins were then transferred to a Protan nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) in an electroblotting apparatus, using standard procedures (Towbin et al., 1979). Nonspecific binding sites on the membranes were blocked by a 1-hr incubation with PBS containing 0.1% Tween 20 and 5% skimmed milk powder as blocking reagent (''Blotto-Tween'' solution). A polyclonal anti-TH antibody (Institute J. Boy, Strasbourg, France) was diluted 1:1,000 in ''Blotto-Tween'' solution and then added to the membranes to detect TH protein. After an overnight incubation at 4°C with shaking, the membranes were washed five times for 5 min each in Blotto-Tween solution. The membranes were then incubated for 30 min with an anti-rabbit Ig-horseradish peroxidase conjugate (Amersham Biosciences Europe, Orsay, France) diluted 1:5,000 in Blotto-Tween solution. The membranes were washed as described above and then rinsed briefly with PBS. Finally, the antibody-antigen complex was detected by using the ECL^+ chemoluminescence system according to the manufacturer's instructions (Amersham Biosciences). Chemoluminescence was analyzed with a FLA 2000 phosphoimager (Fujifilm, Tokyo, Japan) and quantified with the Fujifilm-AIDA software that provides linear arbitrary units (LAU) correlated with chemoluminescence intensity. The values reflected both the intensity and the surface area of the bands. For documentation, autoradiograms of all the membranes were also generated by exposure of treated membranes to hyperfilm (Amersham Biosciences).

TH Enzyme Activity Assay

TH enzyme activity was determined by monitoring the release of tritiated H_2O from L-[3,5-^{3}H]tyrosine (Amersham

Biosciences) as described elsewhere (Reinhard et al., 1986). In brief, cells (1×10^6) were resuspended and vortexed in 100 µl ice-cold lysis buffer (100 mM Na Hepes, 02% Triton X-100, pH 7.0), and the homogenate was centrifuged for 10 min at 4°C and 15,000*g*. A 30-µl aliquot of the cleared lysate was added to 45 µl of reaction cocktail containing 56 µM L-[3,5-^{3}H]tyrosine (0.08 µCi/nmol), 1.1 mg/ml catalase, 0.2 mM $FeSO_4$, and 222 mM Na Hepes, pH 7.0. The enzyme reaction was started by adding 25 µl of a 2 mM solution of tetrahydrobiopterin (Sigma-Aldrich) in 20 mM dithiothreitol. After a 15-min incubation at 37°C, the reaction was stopped by the addition of 1 ml of a 7.5% suspension of charcoal in 1 N HCl. The samples were centrifuged for 10 min at 15,000 *g*. Aliquots (100 µl) of the supernatants were transferred to scintillation vials, and 7 ml of scintillation liquid (Ready Solv HP) were added to each. The number of decays per minute was measured in an LS 6500 liquid scintillation counter (Beckman Coulter, Roissy, France). For the determination of specific enzyme activity, the protein content of the lysate was determined according to the method of Bradford 1976 as described above.

Northern Blot Analysis

TH mRNA was assayed by a Northern blot technique according to Faucon-Biguet et al. (1986), with some modifications. Briefly, total cellular RNA was isolated with the RNeasy Qiagen kit (Qiagen, Courtaboeuf, France) Total RNA (3 µg) was denatured by heating at 65°C for 15 min in the presence of 6% formaldehyde and 50% formamide and then subjected to horizontal electrophoresis on 1% agarose gels containing 6% formaldehyde. The RNA was blotted to nitrocellulose membranes (Hybond N^+; Amersham Biosciences) by a capillary procedure using 20× SSC buffer for 18 hr. Then, the RNA was fixed with an UV Stratalinker and hybridized to the label. The resulting ^{32}P-labelled RNA-DNA hybrids were detected with the FLA 2000 phosphoimager (Fujifilm) and quantified with the Fujifilm-AIDA software that provides LAU.

Statistical Analysis

Unpaired Student's *t*-tests were used to determine the significance of the differences between untreated and clozapine-treated PC12 cells. $P < 0.05$ was considered statistically significant.

RESULTS

Chronic administration of clozapine leads to reduced levels of TH mRNA and TH protein in the mesocorticolimbic and nigrostriatal systems of rats (Tejedor-Real et al., 2003). To investigate whether the drug acts directly on dopaminergic cells, PC12 cells were incubated with four different concentrations of clozapine for 1 hr. This incubation led to a dose-dependent decrease in TH protein content as assessed by Western blot analysis (Fig. 1) Cells treated with either 10 µM or 100 µM clozapine contained significantly less TH protein than control cells ($P < 0.05$). The effect of both concentrations was similar, so 10 µM clozapine was applied in subsequent experiments.

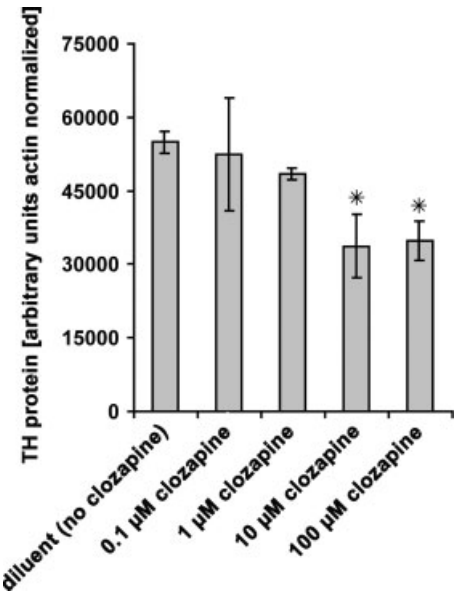

Fig. 1. Quantification of TH protein by densitometric analysis of Western blots. Protein extracts from PC12 cells incubated for 1 hr with various concentrations of clozapine were analyzed. The data represent means ± SD of values from four independent experiments that were each normalized to actin protein. The difference in TH protein between untreated and clozapine-treated cells was significant (*$P < 0.05$) for both 10 µM and a 100 µM concentration of the drug.

The decrease in the amount of TH protein was accompanied by a decrease in the specific enzymatic activity of TH in the cell lysates (Fig. 2) The activity was significantly lower ($P < 0.05$) after 1 hr of incubation of the cells with the drug but returned to control levels 5 hr after the removal of clozapine by exchange of the culture medium. The effect of 10 µM clozapine on the activity of TH was thus reversible.

Chronic administration of clozapine is required for clinical antipsychotic effects. Therefore, PC12 cells were treated with the drug for longer periods, to determine whether the down-regulation of TH protein persists. An even more marked decrease in TH protein was obtained when PC12 cells were incubated with 10 µM clozapine for 24 and 72 hr. The amount of TH protein was reduced by 25% after 24 hr and by 61% after 72 hr as compared with untreated control cells, respectively (Fig. 3A). At the same time points, decreased levels of TH mRNA were also observed. Northern blotting of RNA fractions revealed that there was about 50% of TH mRNA in PC12 cells incubated with 10 µM clozapine for 24 or 72 hr compared with untreated controls (Fig. 3B).

We used NEM to investigate which second messenger pathway and therefore which receptor family type is involved in the down-regulation of TH protein expression. Micromolar concentrations of NEM selectively uncouple receptors from Gi and Go (Jakobs et al., 1982; Shapiro et al., 1994), probably by alkylation of the cysteine residue in the G protein carboxyl termini (Win-

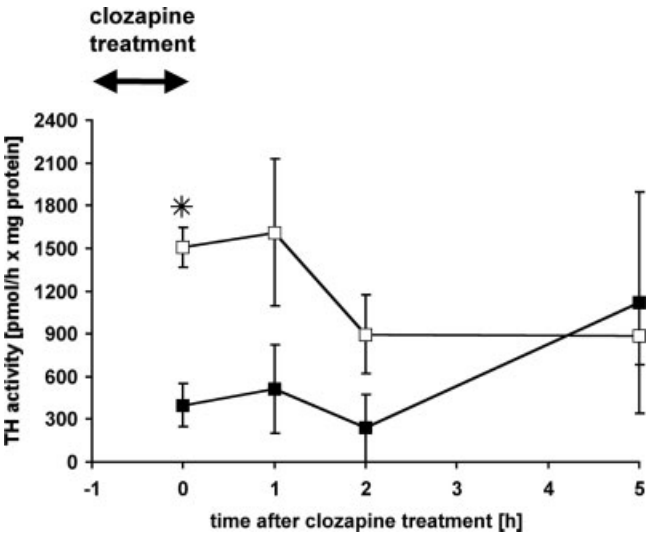

Fig. 2. Effect of 10 μM clozapine on the activity of TH in PC12 cells. The cells (1×10^6) were incubated for 1 hr with medium containing either the vehicle alone or clozapine in vehicle. Then, the medium was removed, and the incubation of the cells was continued with RPMI medium containing 10% horse serum and 5% fetal calf serum. The time points refer to the end of drug treatment and indicate when cells were lyzed and the specific activity of TH was determined. Open squares represent activity values from cells treated with vehicle alone; solid squares correspond to activity values from cells incubated with clozapine. Values are means ± SEM, n = 3. The difference in TH activity between cells incubated in the absence of clozapine and cells incubated in the presence of 10 μM clozapine was significant ($^*P < 0.05$) at the end of the 1-hr incubation with clozapine.

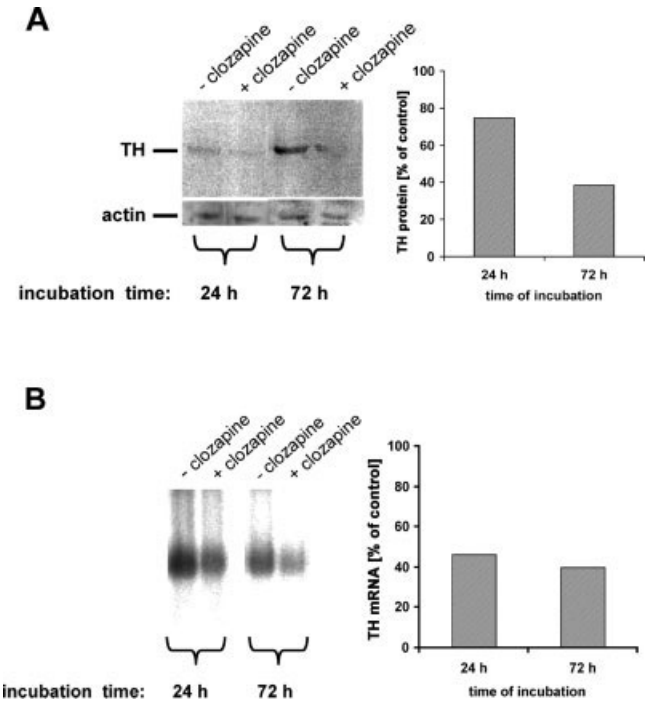

Fig. 3. Representative analyses demonstrating the down-regulation of TH protein and TH mRNA in PC12 cells after chronic treatment with clozapine. **A:** Western blot analysis of protein extracts from cells incubated for 24 hr and for 72 hr in the presence and in the absence of 10 μM clozapine. TH protein and actin were both detected, the latter as internal standard for the densitometric quantification. **B:** Northern blot analysis of total RNA preparations from the same cells. The diagrams at right refer to the blot analyses displayed and give the percentages of TH protein and TH mRNA in clozapine-treated cells relative to the respective quantities in the vehicle-treated control cells. Overall, three independent treatments of PC12 with clozapine for 24 hr and 72 hr were performed.

slow, 1987). To our knowledge, no experiments have been reported in which PC12 cells were treated with NEM. Consequently, a set of assays was designed in accordance with conditions that have turned out to be efficient in vitro (Ueda et al., 1990; Shapiro et al., 1994; Gonzalez-Maeso et al., 2000; Momiyama and Koga, 2001). PC12 cells were incubated with various concentrations of NEM for 30 min, and the viability of the cells was verified every 5 min. Based on these assays, a preincubation for 15 min with a 100 μM concentration of NEM was selected. This concentration of NEM is within the concentration range that allows sufficient and specific action on Gi/o proteins (Larsen et al., 1981; Jakobs et al., 1982; Kilpatrick et al., 1982; Smith and Harden, 1984; Asano and Ogasawara, 1986; Kitamura and Nomura, 1987; Ueda et al., 1990; Takats et al., 1990; Shapiro et al., 1994). Moreover, 10–15 min of NEM treatment are sufficient to modify G proteins completely (Von Euler et al., 1987), without the induction of other unwanted effects. PC12 cells were preincubated with 100 μM NEM for 15 min and then treated with clozapine for 1 hr. Cellular proteins were then extracted and analyzed via Western blotting. Preincubation with NEM completely blocked the effect of clozapine on TH protein content (Fig. 4) The amounts of TH protein in cells treated with NEM and clozapine and those treated with neither clozapine nor NEM were indistinguishable. The down-regulation of TH protein induced by clozapine may thus be mediated by a receptor interacting either with a Gi or a Go protein.

DISCUSSION

The molecular mechanisms underlying the clinical effects of antipsychotics in schizophrenia are largely unknown. Indeed, although the dopaminergic system has always been the most important target for pharmacological treatment of schizophrenia, and antipsychotics have been in clinical use for more than 4 decades, the dopaminergic molecular mechanisms involved are poorly characterized.

Chronic clozapine administration in rats (Tejedor-Real et al., 2003) reduces TH gene expression, as indicated by amounts of mRNA and protein. TH is the rate-limitating enzyme in the biosynthetic pathway of dopamine. Therefore, clozapine may decrease the dopamine level in the central nervous system by down-regulation of TH enzyme. To identify the type of signalling

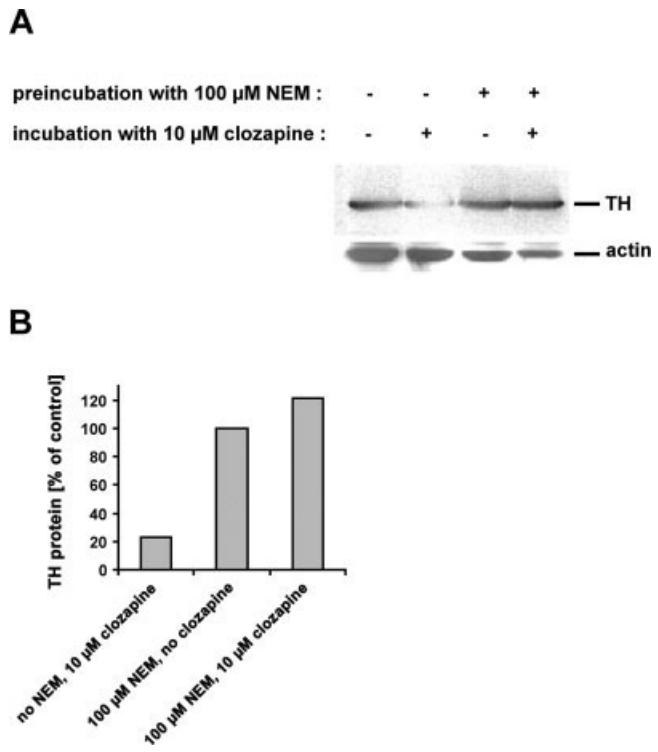

Fig. 4. Effect of NEM on clozapine-mediated down-regulation of TH protein as determined by Western blot analysis. Before the 1 hr incubation with 10 µM clozapine, PC12 cells were preincubated or not with 100 µM NEM for 15 min. Cells only preincubated with 100 µM NEM (no application of clozapine) and cells incubated neither with NEM nor with clozapine (no drug treatment) served as controls. **A:** Immunoblot detection of TH protein and actin. **B:** Quantification of TH protein by densitometric analysis and normalization of the signals to those for actin. The data refer to the representative analyses displayed in A and give the percentages of TH protein relative to the quantity in the vehicle-treated control cells. Overall, three independent treatments of PC12 cells with NEM/clozapine were performed.

transduction pathways involved and to clarify whether this antipsychotic acts via a pre- or a postsynaptic mechanism, we have used the PC12 cell line as a model. We found that clozapine reduced the TH protein level in PC12 cells, as it does in the rat brain (Tejedor-Real et al., 2003). This TH decrease was dose and time dependent. Chronic treatment (72 hr) produced a robust TH reduction, which is in line with clinical observations showing that prolonged antipsychotic treatments are necessary for implementing the antipsychotic effect (Chouinard and Annable, 1976).

Clozapine also reduced the level of TH mRNA, showing that the decrease in TH protein is at least partially a consequence of transcriptional effects. The reduced TH protein level coincided, as would be expected, with a decrease in the enzymatic activity of TH. The lower activity of TH might also have been caused by posttranslational modification of the enzyme, such as phosphorylation. Indeed, El Mestikawy et al. (1986) found that dopaminergic agonists and antagonists control TH activity via presynaptic dopamine autoreceptors associated with modulations of the cyclic AMP-dependent phosphorylation of the enzyme.

An understanding of the pathways of signal transduction that underlie the reduced expression of TH by clozapine would be valuable for the development of new antipsychotics and their clinical application. Clozapine shows affinity for dopaminergic, serotoninergic, cholinergic, histaminergic, and adrenergic receptors, but not all of them are implicated in its antipsychotic properties. Insofar as dysregulation of dopamine transmission may contribute to the pathology of schizophrenia (Meltzer and Stahl, 1976), dopaminergic receptors have been the main target for antipsychotic drugs. Indeed, it has been observed that TH activity can be modulated by a D2 receptor pathway (Booth et al., 1994), and D2-like autoreceptors regulate dopamine release by regulating TH activity (Pothos et al., 1998). If the reduced TH activity observed is the consequence of the reduced TH mRNA and protein, clozapine may exert its action through, among others, D2 receptor family.

It has recently been assumed that the antipsychotic effect of clozapine is due to the antagonism of not only dopaminergic receptors but also serotoninergic receptors in the brain (Seeman, 1994; Meltzer, 1999). Several authors have reported that the ability of clozapine to modulate the concentration of dopamine in the prefrontal cortex involves the stimulation of 5-HT1A somatodendritic autoreceptors in the dorsal raphe, because there is a reduction in serotonergic input from the dorsal raphe nucleus (Hagino and Watanabe, 2002). Furthermore, 5-HT1A agonists consistently increase dopamine release in the prefrontal cortex in rodents, which is an effect that might be predicted to counteract negative symptoms (Bantick et al., 2001).

Here we show that the dissociation of Gi/o proteins from the Gi/o protein-coupled receptors by NEM abolished the effect of clozapine, demonstrating that this drug may use a Gi/o protein-coupled receptor-signalling pathway to reduce the expression of TH. D2-like and 5HT1A receptors are associated with Gi/o proteins (Senogles et al., 1987; Ohara et al., 1988; Sidhu and Niznik, 2000), and both have been strongly implicated in schizophrenia (Bantick et al., 2001; Potkin et al., 2003). The D2-like receptors are well expressed in PC12 cells (Courtney et al., 1991), specifically D2 and D4 (Inoue et al., 1992; Pothos et al., 1998), and we have observed the presence of 5-HT1A receptors in PC12 cells (unpublished data). Consistently with clozapine acting directly on dopaminergic cell bodies, both types of receptors have somatodendritic localization in animals (Mercuri et al., 1997; Pickel et al., 2002; Hagino and Watanabe 2002). Therefore, D2-like and 5HT1A receptors are potential targets for clozapine to reduce TH expression. Muscarinic M4 receptors, at which the clozapine behaves like a partial agonist (Olianas et al., 1997), also fulfill some of the D2-like and 5HT1A characteristics described above. They are also associated with Go/i proteins (Migeon, 1995), and the dopamine release-stimulating M4 receptors are probably located on neuronal

cell bodies (Zhang et al., 2002). In that M4 receptors are expressed in PC12 cells (McClatchy et al., 2002), they might also be, to some extent, a relevant mechanism for the clozapine-reduced TH level. However, to date, M4 receptors have not been associated with the antipsychotic effect of this drug (Bymaster and Felder, 2002).

Heterotrimeric G proteins play a pivotal role in postreceptor information transduction. Receptor-transduced extracellular stimuli might be converted into changes in gene expression through specific nuclear transcription factors. D2 and 5HT1A, both Gi/o protein-coupled receptors, activate the same intracellular signalling pathway involving cyclic AMP-dependent protein kinase (PKA). The signal results in the nuclear phosphorylation and activation of the transcription factor cyclic AMP response element-binding protein (CREB). CREB recognizes and activates the cAMP response element (CRE), which is present in the TH gene proximal promoter and is involved in the control of gene expression. CRE is essential for both basal and cAMP-inducible transcription of the TH gene. Interestingly, clozapine reduces CREB phosphorylation (Pozzi et al., 2003). Moreover, Dwivedi et al. (2002) observed that chronic but not acute treatment with clozapine significantly decreased cAMP binding to the regulatory subunit of PKA as well as the catalytic activity of PKA in subcellular fractions of the rat cortex, the hippocampus, and the striatum. In these fractions, significantly decreased quantities of selective RII alpha-, RII beta-, and Cat beta-subunit isoforms of PKA were recorded after clozapine treatment. These decreases were accompanied by decreased quantities of the respective mRNA. Thus, clozapine might reduce TH expression via the signalling pathway involving Gi/o proteins, cAMP, PKA, and CRE regulating the transcription of the gene.

Understanding the mechanisms of TH regulation is essential to clarify the action of clozapine and the basic mechanisms of schizophrenia. Gene expression profiles may be altered by long-term treatment, and this may be the basis of the clinically beneficial antipsychotic effect. Studies of expression profiles by using functional genetic methods will be very useful in investigating changes in gene transcription induced by drug treatments and could contribute to the discovery of new potential drug targets.

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