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Tyrosine Hydroxylase mRNA and Protein Are Down-Regulated by Chronic Clozapine in Both the Mesocorticolimbic and the Nigrostriatal Systems

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The dopaminergic system is one of the most important targets for pharmacological treatment of schizophrenia. Despite substantial work on mechanisms of action, it is not clear which dopaminergic pathways mediate the therapeutic effects of antipsychotic drugs. It has been shown that chronic clozapine, an atypical antipsychotic, decreases dopamine levels in the mesocorticolimbic system but not in the nigrostriatal system. Because tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of dopamine, we studied the effect of chronic clozapine in both dopaminergic systems. We demonstrated a decrease of tyrosine hydroxylase mRNA not only in the ventral tegmental area but also in the substantia nigra, the cell body areas of the mesocorticolimbic and the nigrostriatal systems, respectively. The reduced tyrosine hydroxylase mRNA level in these areas is accompanied by an ample reduction in the tyrosine hydroxylase protein level in their corresponding axonal terminal fields, the nucleus accumbens and the striatum. There was thus discordance between the clozapine-induced decrease of tyrosine hydroxylase mRNA and protein and the absence of an effect on dopamine levels in the nigrostriatal system. It has been suggested that reduced levels of dopamine in the mesocorticolimbic system are required for the antipsychotic effect of the drug. Therefore, the modulation of tyrosine hydroxylase gene expression by clozapine in the mesocorticolimbic system might be necessary for its antipsychotic effect; this effect might be of relevance when considering new atypical agents.

Key words: tyrosine hydroxylase; schizophrenia; clozapine; nigrostriatal system; mesocorticolimbic system

The dopaminergic theory of schizophrenia is the most widely accepted explanation for the underlying neurochemical abnormality in this psychiatric illness, and the dopaminergic system is the principal target of antipsychotic drugs (Meltzer and Stahl, 1976; Davis et al., 1991; van Kammen and Kelley, 1991; Rao and Moller, 1994). Studies of dopaminergic receptor occupancy and regulation are providing new leads for investigations of the pathophysiology and causes of schizophrenia and may contribute to the development of more effective therapeutic agents.

Despite the substantial work on the mechanisms of action of antipsychotics, it is not yet clear which dopaminergic brain regions, receptor subtypes, or metabolic pathways specifically mediate the therapeutic effects of antipsychotic drugs. There is a large body of preclinical and clinical evidence, mostly pharmacological, that only particular populations of dopaminergic neurons are involved in the antipsychotic action of neuroleptics, and this may provide a clue to the pathogenesis of schizophrenia. Long-term administration of haloperidol, a typical antipsychotic, decreases dopamine release in the striatum and the nucleus accumbens, whereas administration of clozapine, an atypical neuroleptic, decreases dopamine release in the nucleus accumbens and the prefrontal cortex but not in the striatum (Blaha and Lane, 1987; Chen et al., 1991; Hernandez and Hoebel, 1995). In addition, it has been suggested that the selectivity of clozapine for the mesolimbic as opposed to the nigrostriatal dopaminergic system explains its minimal, or absent, extrapyramidal side effects (Chiodo and Bunney, 1985; Hand et al., 1987). These observations implicate the mesocorticolimbic system as the target for the antipsychotic effect. However, this idea has recently

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been dismissed by several authors, who have failed to find any mesocorticolimbic selectivity of clozapine (Kinon and Lieberman, 1996; Melis et al., 1998).

Tyrosine hydroxylase (TH; EC 1.14.16.2) is the rate-limiting enzyme in the biosynthesis of dopamine, and its level is regulated by various transcriptional and post-transcriptional mechanisms (Kumer and Vrana, 1996). This regulation makes a major contribution to controlling the amount of catecholamines. Thus, modulation of TH gene expression may be implicated in reducing dopamine levels after chronic clozapine treatment. To examine this question and to assess whether clozapine has an effect in the nigrostriatal system, we investigated the effect of chronic treatment on TH mRNA and protein levels in both the nigrostriatal and the mesocorticolimbic systems. Because clinical observations have shown that prolonged antipsychotic treatments—2 weeks or more—are necessary for implementing the antipsychotic effect (Chouinard and Annable, 1976), long-term administration was used in this study. Clozapine has been intensively studied as a reference standard for atypical antipsychotics, but, to date, its effect on TH gene expression has not been investigated. We show that chronic clozapine reduces the TH mRNA level not only in the ventral tegmental area but also in the substantia nigra. The decrease of TH mRNA is accompanied by a reduction of the TH protein level in the terminal fields projecting from both areas, the nucleus accumbens and the striatum. These findings provide new insights into the (already very complex) mechanism of action of this atypical antipsychotic.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (Iffa Credo, France), weighting between 300 and 350 g, were housed in groups of three per cage. Animals were maintained at 22°C on a 12-hr light cycle, with water and food ad libitum. Animal experiments were conducted in accordance with the French "Ministère de l'Agriculture et de la Forêt" and European Community guidelines for the care of laboratory animals.

Drug Treatment

Clozapine (Tocris Bioblock Scientific) was dissolved in diluted glacial acetic acid and saline solution, adjusted to pH 6.0–6.2 with 1 N sodium hydroxide, and brought to a final concentration of 10 mg/ml. To minimize nonspecific effects, the lowest effective dose of clozapine was selected within a range of doses (from 0.1 to 30 mg/kg) that in rats effectively alter behavior (Manzaneque et al., 2002; Lee and Clifton, 2002), receptor occupancy (Di Matteo et al., 2002; Suhara et al., 2002; Sebban et al., 2002), firing rates (Melis et al., 1998, 1999), and biochemical parameters (Volonté et al., 1997; Kuroki et al., 1999; Freeman et al., 2001; Dwivedi et al., 2002). A group of rats ($n = 8$ for TH mRNA in situ hybridization and $n = 6$ for TH protein immunochemistry) received a dose of 10 mg/kg of clozapine s.c. daily for 21 days, and a control group for each experiment was similarly injected with the vehicle solution.

The animals were killed by fast decapitation 24 hr after the last administration of clozapine, taking into account the half-life of clozapine (Baldessarini et al., 1993), in order to avoid the acute effect of the last dose. The brains were rapidly removed, frozen in chilled isopentane, and stored at −80°C until use.

In Situ Hybridization Experiments

Coronal sections 16 μm thick were cut in a cryostat at −25°C throughout the substantia nigra and the ventral tegmental area. The sections were thaw mounted on clean superfrost slides and stored at −80°C until use. A 24-base oligonucleotide probe complementary to nucleotides 45–69 in exon 1 of the rat TH mRNA (Grima et al., 1985; 5′-CCTGCTTGGAATCCTG-CTCTGAGA-3′) was synthesized and purified by Genset (France). It was 3′-end labeled with [^{35}S]dATP (Amershan Biosciences, Arlington Heights, IL) using terminal deoxyribonucleotidyl transferase (Amersham Biosciences) at a specific activity of 8×10^8 c.p.m./μg. The probe was purified on a P10 column (Bio-Rad, Hercules, CA) prior to use and stored at −20°C in TE buffer (Tris 10 mM, pH 7.4, EDTA 1 mM) containing 100 mM dithiothreitol (DTT). Frozen sections were first warmed to room temperature and then fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 10 min. The sections were then rinsed three times with 1× phosphate-buffered saline (PBS) for 10 min each, then for 5 minutes in a bath of absolute ethanol, and air dried.

Sections were next hybridized with the ^{35}S-labeled TH oligonucleotide probe as described elsewhere (Dumas et al., 1996). The sections were coverslipped with parafilm, placed in humid metacrilate boxes, and incubated at 45°C for 15 hr. The sections were then washed in two baths of 1× SSC/10 mM DDT at 53°C and rinsed twice in 0.5× SSC/10 mM DDT at 53°C and once in 0.5× SSC/10 mM DDT at room temperature. The samples were then rapidly washed in water and dehydrated in absolute ethanol (5 min). No signal was obtained from tissue sections in control experiments performed either by displacing specific mRNA hybridization with a 50-fold excess of unlabeled oligonucleotide or by using a sense oligonucleotide.

For an overall analysis, autoradiograms of brain were generated by apposition of ^{35}S-labeled sections to β-max hyperfilm (Amersham Biosciences) for 7 days. Sections were then dipped in nuclear emulsion (Ildford K5 diluted in 2× SSC) for estimation of cellular expression in the substantia nigra and the ventral tegmental area. Sections were exposed for 5 weeks, then developed and counterstained with Cresyl violet.

Image Analysis

For in situ hybridization, signals on autoradiograms (240 sections; an average of five per rat and per level) was quantified using an image-analysis system (Samba) with a camera coupled to a computer. The optical density in both the substantia nigra and the ventral tegmental area as a whole was quantified. Values are expressed as optical density units/pixel, indicative of the intensity of TH mRNA labeling.

For further comparison with previous autoradiography density measures of TH mRNA, cellular expression was analyzed at level 1 of the ventral tegmental area and the substantia nigra (from bregma: −4.52 to −5.00; the level of the maximal TH concentration) in three randomly chosen animals per group. TH mRNA was quantified at the cellular level both by counting the number of TH mRNA-labeled neurons and by determining

the density of silver grains over selected cell profiles overlying a given Cresyl violet-stained nucleus. The number of TH mRNA-labeled cells was determined in the entire bilateral area of both the substantia nigra and the ventral tegmental area. Cell profile counts were determined under a microscope (Zeiss Axioskope) at ×40, with the aid of an eyepiece grid. Cells were considered as TH $mRNA^+$ only when higher magnification clearly indicated that silver grains were associated with discrete cell bodies. The number of TH $mRNA^+$ cells in treated animals was calculated as a percentage of that in control animals.

Computer-assisted image analysis (Histo200; Biocom) was used for quantification of silver grain density as described elsewhere (Vila et al., 1997). The density of silver grains was assessed by measuring the surface area associated with the grain cluster under polarized light. A standard curve for optical density was obtained with known numbers of silver grains. Grain density (number of silver grains per surface area of the neuron) was then calculated.

Immunochemistry Experiments

Tissues were punched out from frozen 200-μm-thick serial sections of brain cut in a cryostat at −25°C throughout the prefrontal cortex, the nucleus accumbens, the striatum, the amygdala, the substantia nigra, and the ventral tegmental area and stored at −80°C until use. Because these structures have a bilateral localization, punches from the two sides were pooled.

Western Blot Analysis

Tissue homogenates from the ventral tegmental area, the prefrontal cortex, the nucleus accumbens, the amygdala, the substantia nigra, and the striatum were prepared by brief sonication in 10 mM phosphate buffer, pH 7, with 0.5% Triton X-100 containing a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany), followed by centrifugation at 4°C for 5 min at 13,000*g*. Protein concentrations of the supernatants were measured by the method of Bradford (1976) using bovine serum albumin as a standard. Samples (25 μg protein/lane for the ventral tegmental area, the substantia nigra, the nucleus accumbens, the amygdala, and the striatum; 50 μg protein/lane for the prefrontal cortex), corresponding to individual rats, were resolved by electrophoresis on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel in the presence of 50 mM DTT and transferred to a Protran nitrocellulose membrane (Schleicher and Schuell, Keene, NH; Towbin et al., 1992). The blots were blocked with Blotto-Tween (PBS, 0.1% Tween 20, and 5% fat-free milk powder) for 1 hr and incubated overnight at 4°C with agitation with a polyclonal anti-TH antibody (Jacques Boy, France) at a final dilution of 1:1,000. Before incubation with the antibody, the total amount of blotted protein was estimated by Ponceau red staining to verify that an identical quantity of proteins had been loaded in all the lanes.

After washing in Blotto-Tween, membranes were incubated for 30 min with the secondary antibody, anti-rabbit IgG coupled to horseradish peroxidase (Amersham Biosciences). Membranes were washed five times (for 5 min each) in Blotto-Tween and once in PBS for 1 min. The blots were normalized using actin, as a marker, detected with a polyclonal antiactin antiserum (Sigma, France) at a dilution of 1:100. The antibody-antigen complexes were detected using a chemioluminescence system (ECL+) according to the manufacturer's instructions (Amersham Biosciences) and the results collected by phosphoimager analysis. The immunoreactivity was quantified by fluorescent image analyzer FLA-2000 Fuji AIDA software. Values are reported in linear arbitrary units (LAU), which give a direct measure of fluorescence. Autoradiograms of all the membranes were also generated by apposition of treated membranes to Hyperfilm MP (Amersham Biosciences) for various times depending on the signal in each structure. Each experiment was performed at least three times.

Statistical Analysis

For in situ hybridization experiments, parametric values of quantitative autoradiographic and silver grain density (density units/pixel) results were analyzed using the *t*-test for individual comparisons between control and treated animal groups. Values are expressed as mean ± SEM. For immunohistochemical analysis, values are expressed in phosphoimager LAU ± SEM. A *t*-test was performed for each structure to determine whether there was a significant difference between protein levels in treated animals and those in control animals. The significance level for *t*-test analyses was set at $P < 0.05$.

RESULTS

Expression of the TH Gene After Chronic Clozapine Treatment in the Ventral Tegmental Area and Substantia Nigra

Rat TH mRNA was investigated in sections of the rat ventral tegmental area and substantia nigra after chronic clozapine treatment (10 mg/kg, s.c., one dose/day for 21 days) by in situ hybridization with an oligonucleotide probe complementary to nucleotides 45–69 in exon 1. The pattern of autoradiographic TH mRNA labeling (Fig. 1) was consistent with previous descriptions of the rat substantia nigra and ventral tegmental area (Weiss-Wunder and Chesselet, 1991; Leviel and Faucon-Biguet, 1992). In each animal, the TH mRNA labeling was stronger in the substantia nigra than in the ventral tegmental area and denser in the rostral than in the caudal regions of both structures. Neighboring regions were not labeled, confirming the specificity of the probe. Because there are unavoidable ambiguities in distinguishing the A8, A9, and A10 dopaminergic neuron groups at the most posterior part of the brain (German and Manaye, 1993), we did not analyze the sections posterior to −6.04 from Bregma (Paxinos and Watson, 1982).

Chronic administration of clozapine for 21 days caused a decrease in TH gene expression in the ventral tegmental area and substantia nigra. TH mRNA density as assessed by autoradiography was significantly lower in treated than in control animals along the whole structure of the ventral tegmental area ($P < 0.0004$) and substantia nigra ($P < 0.0001$).

In view of the heterogeneity within of the ventral tegmental area and substantia nigra, the brain sections were classified into three consecutive rostrocaudal levels such that data were obtained for each of the three anatomical subareas. The anatomical subpopulations of dopaminergic

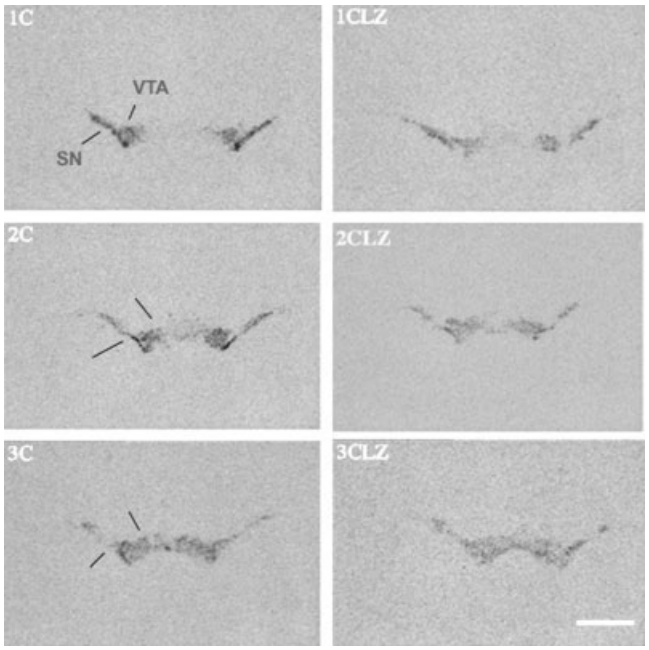

Fig. 1. Labeling of TH mRNA observed at low magnification at three different levels of the substantia nigra and ventral tegmental area in coronal sections (16 μm thick) of rat brain as revealed by in situ hybridization. Brain sections were assigned to three consecutive rostrocaudal levels (five sections per level): level 1, from −4.52 to −5.00; level 2, from −5.00 to −5.60; and level 3, from −5.60 to −6.04 from bregma (approximately according to Paxinos and Watson, 1982). 1C, 2C, 3C, autoradiograms at levels 1, 2, and 3 of control animals, respectively. 1CLZ, 2CLZ, 3CLZ, autoradiograms at levels 1, 2, and 3 of chronic clozapine-treated animals, respectively. These photoghaphs are from a single control and from a single clozapine-treated rat. Scale bar = 1 mm.

neurons are not well defined in the ventral tegmental area and substantia nigra. Therefore, to measure the optical densities, the substantia nigra was rigorously demarcated from the ventral tegmental area according to the anatomical limits described elsewhere (Halliday and Törk, 1986; Weiss-Wunder and Chesselet, 1991; German and Manaye, 1993; Adelbrecht et al., 1996). Sections at the same coronal levels from all the animals were used: level 1, from −4.52 to −5.00; level 2, from −5.00 to −5.60; and level 3, from −5.60 to −6.04, from bregma, corresponding to the Paxinos and Watson (1982) rat brain atlas (Fig. 1).

To identify any neuron subpopulations within the ventral tegmental area and substantia nigra, sections from each of the three levels (defined above) were analyzed. *t*-Test analysis showed a significantly lower TH mRNA levels in treated than in control animals at levels 1 and 2 of the ventral tegmental area, but not at level 3, the most caudal level (level 1, $P < 0.01$; level 2, $P < 0.02$; level 3, NS; Fig. 2A). Chronic clozapine induced a significant TH mRNA decrease at all three levels in the substantia nigra (level 1, $P < 0.0002$; level 2, $P < 0.0002$; level 3, $P < 0.05$; Fig. 2B).

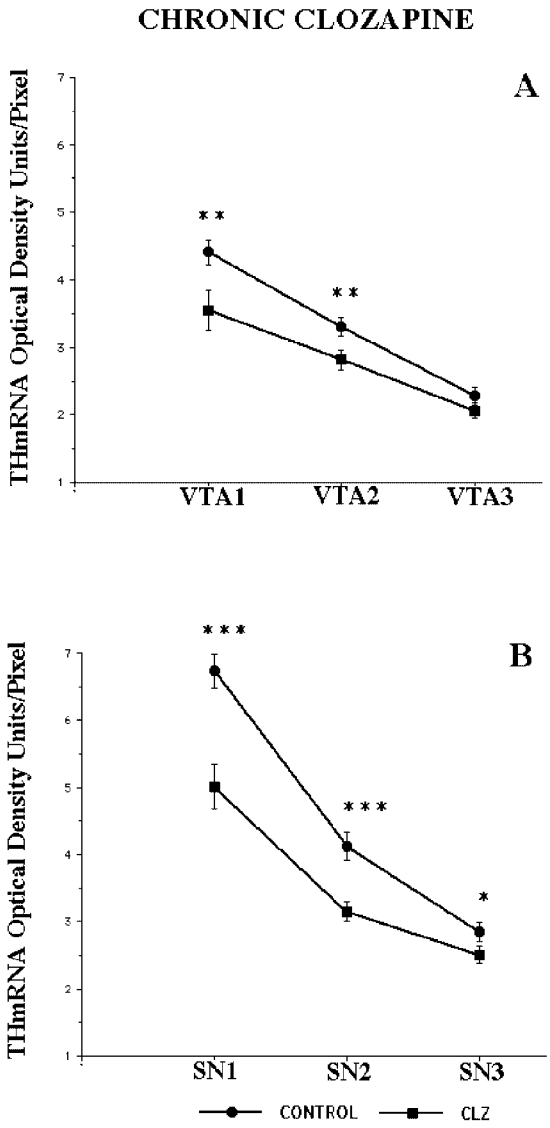

Fig. 2. Effect of chronic clozapine (CLZ; 10 mg/kg, s.c., daily for 21 days) on TH mRNA levels as revealed by in situ hybridization. Signals on autoradiograms (an average of four or five per level and per eight rats) were quantified using an image-analysis system (Samba) with a camera coupled to a computer to determine the optical density in the substantia nigra (SN) and ventral tegmental area (VTA). The bars represent mean ± SEM labeling of TH mRNA, expressed as TH mRNA optical density units/pixel, at three different rostrocaudal levels of the ventral tegmental area (**A**) and the substantia nigra (**B**) in control and chronic clozapine-treated animals (CLZ). Level 1 (n = 37/33), from −4.52 to −5.00; level 2 (n = 40/34), from −5.00 to −5.60; level 3 (n = 40/35), from −5.60 to −6.04 from bregma (approximately according to Paxinos and Watson, 1982). ⋆$P < 0.05$; ⋆⋆$P < 0.02$; ⋆⋆⋆$P < 0.001$ vs. control, by Student's *t*-test.

The effects of clozapine on TH mRNA levels as determined by autoradiography were analyzed on the cellular level (level 1, the level of the highest TH concentration). Polarized, high-power, darkfield and brightfield

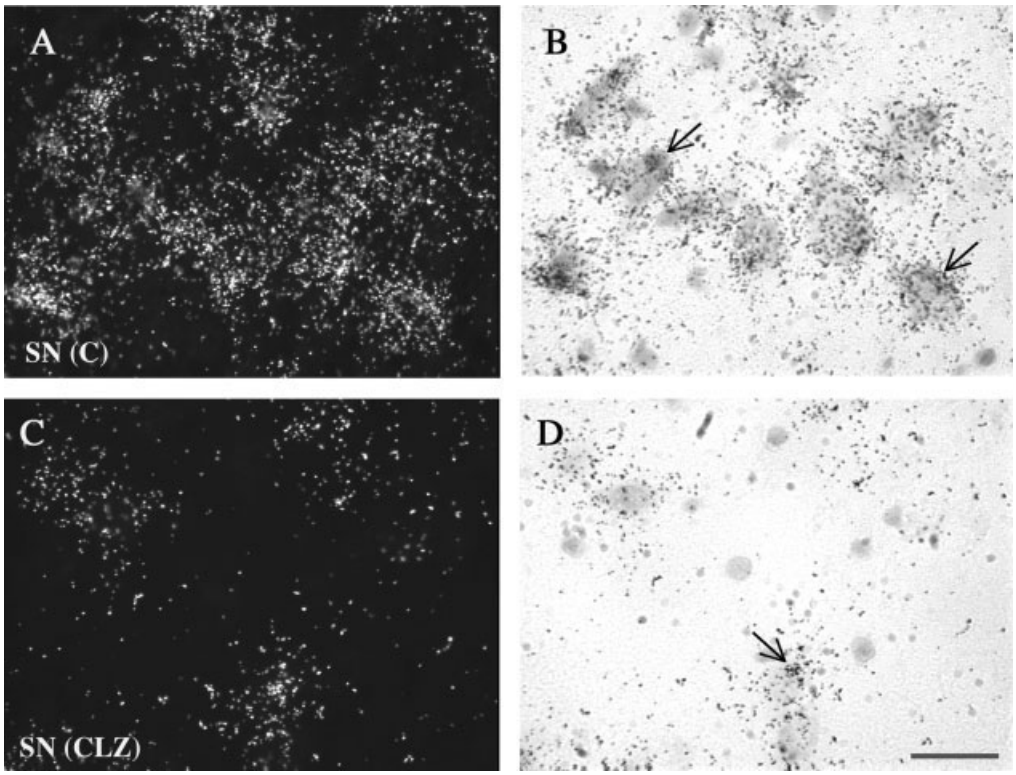

Fig. 3. Emulsion-coated tissue sections of the substantia nigra of a control [SN (C)] and a chronic clozapine-treated [SN (CLZ)] animal at level 1 (from −4.52 to −5.00 from bregma; approximately according to Paxinos and Watson, 1982). High-power darkfield (**A,C**) and bright-field (**B,D**) photomicrographs illustrating TH mRNA labeling in control (A,B) and clozapine-treated (C,D) rats. Fresh-frozen rat brain sections 16 μm thick were processed for the detection of TH mRNA by in situ hybridization. They were first hybridized with the ^{35}S-labeled TH oligonucleotide probe, and then the sections were exposed for 5 weeks for silver grain detection on autoradiographic emulsion. The dots correspond to the silver grains in the emulsion, and their density is an indicator of the amount of TH mRNA. Arrows indicate single TH mRNA-positive cells. Note that TH mRNA abundance appears to be lower in the dopaminergic cells of clozapine-treated animals. Scale bar = 20 μm.

microscopic examination showed that the labeling of TH mRNA was concentrated as silver grains over cell bodies (Figs. 3, 4). The silver grain concentration was estimated by the method of Vila et al. (1997). In clozapine-treated animals, there were fewer silver grains in clusters than in controls in the substantia nigra (0.40 ± 0.08 vs. 0.79 ± 0.03; $P < 0.002$) and ventral tegmental area (0.25 ± 0,01 vs. 0.77 ± 0.10; $P < 0.02$; clozapine-treated vs. untreated ± SEM, arbitrary units). The total number of TH mRNA$^+$ neurons as assessed under the microscope was also lower in both the substantia nigra and the ventral tegmental area of chronic clozapine-treated animals than in controls (substantia nigra, 50% of the control value; ventral tegmental area, 52% of the control value) at the level studied. Therefore, emulsion-coated tissue sections and autoradiographic analysis yielded similar results.

Effect of Chronic Clozapine on TH Protein Levels in the Cell Body Nucleus and Terminal Fields of Both the Mesocorticolimbic and the Nigrostriatal Systems

Effects in the mesocorticolimbic system. Samples from the ventral tegmental area, a dopaminergic cell body-containing region, and from its dopaminergic terminals in the nucleus accumbens, the prefrontal cortex and amygdala, were investigated by immunoblotting with anti-TH antibodies. An immunoreactive band with an apparent molecular weight of 52 kDa corresponding to the TH protein was found in all cases (Fig. 5). Clozapine treatment reduced TH protein levels in the nucleus accumbens by 24% of that in the corresponding control ($P < 0.05$; Fig. 5). In the amygdala, clozapine did not have a significant effect on the amount of TH protein. Similarly, in the cell bodies of the ventral tegmental area, clozapine did not cause any detectable change in TH protein. Under the same experimental conditions, only barely detectable and nonquantifiable bands of TH enzyme were observed in the prefrontal cortex, another terminal field originating from the ventral tegmental area. The relative amounts of TH protein that were observed in these different brain regions (striatum ≥ nucleus accumbens > substantia nigra ≥ ventral tegmental area > amygdala > prefrontal cortex) are consistent with the concentrations of dopamine reported by other authors (striatum > nucleus accumbens > prefrontal cortex; Hernandez and Hoebel, 1995).

Effects in the nigrostriatal system. TH immunoblots of the substantia nigra and striatum samples showed, as expected, very pronounced TH protein bands. Phosphoimager quantification of the protein bands indicated that the amount of TH in the striatum of clozapine-treated animals was substantially (45%) and significantly (t-test, $P < 0.04$) lower than that in controls (Fig. 6). In the dopaminergic cell bodies of the substantia nigra, clozapine did not affect TH levels.

DISCUSSION

Extensive biochemical and neurophysiological studies indicate that atypical antipsychotics, such as clozapine, in contrast to typical antipsychotics, may act preferentially

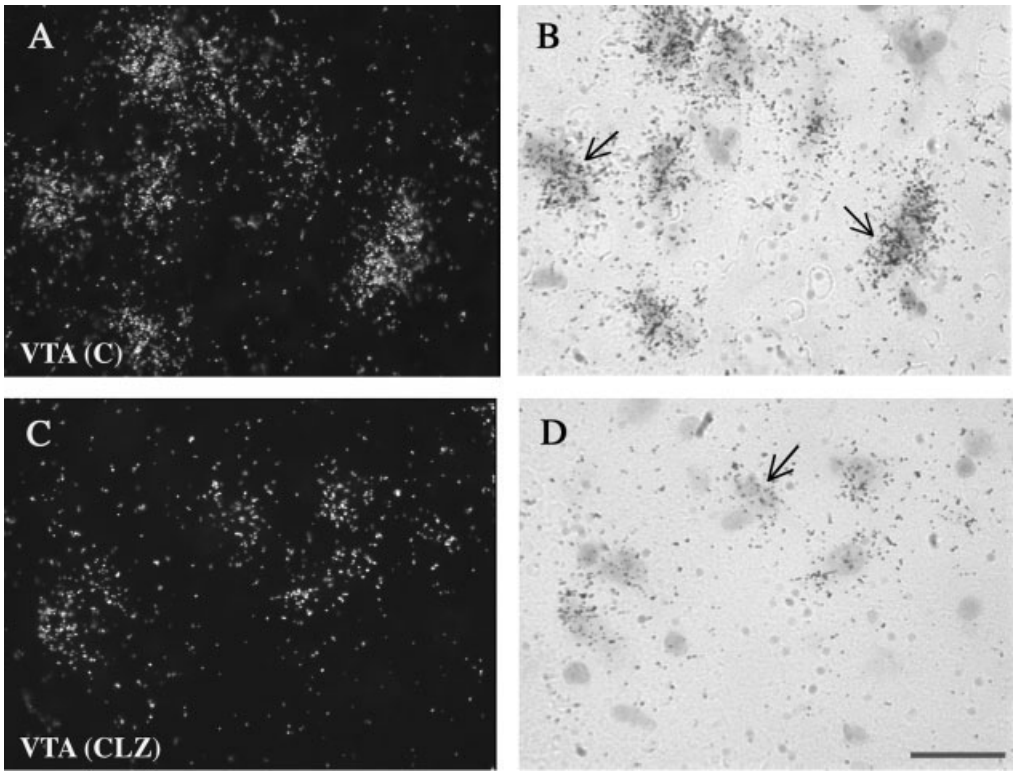

Fig. 4. Emulsion-coated tissue sections of the ventral tegmental area of a control [VTA (C)] and a chronic clozapine-treated [VTA (CLZ)] animal at level 1 (from −4.52 to −5.00 from bregma; approximately according to Paxinos and Watson (1982). High-power darkfield (**A,C**) and brightfield (**B,D**) photomicrographs illustrating TH mRNA labeling in control (A,B) and clozapine-treated (C,D) rats. Arrows indicate single TH mRNA-positive cells. Scale bar = 20 μm.

on mesocorticolimbic but not nigrostriatal dopaminergic pathways. Chronic haloperidol, a typical antipsychotic, reduces the dopamine release (Blaha and Lane, 1987; Lane et al., 1988) and electrophysiological activity in dopaminergic neurons (Hand et al., 1987) in both systems, whereas chronic clozapine does so only in the mesocorticolimbic system (Chiodo and Bunney, 1985; Hand et al., 1987; Blaha and Lane, 1987; Chen et al., 1991; Hernandez and Hoebel, 1995). Dopamine is the first catecholaminergic neurotransmitter the biosynthesis of which is known to be regulated by TH, the rate-limitating enzyme of the catecholaminergic pathway. We studied the effect of chronic clozapine on TH expression and demonstrated a decrease of TH mRNA not only in the ventral tegmental area but also in the substantia nigra, the cell body dopaminergic areas in the mesocorticolimbic and the nigrostriatal systems, respectively. The lower TH mRNA is accompanied by a reduction in the TH protein level in the nucleus accumbens and the striatum, the terminal fields of axonal projections from the ventral tegmental area and substantia nigra. In contrast, no variations in the TH protein content were found in the amygdala, although this structure receives, as the nucleus accumbens, dopaminergic input from the ventral tegmental area. The effect of clozapine on TH gene expression might be mediated by an intraneural signal transduction mechanism induced by receptors blockade (Leveque et al., 2000). These intracellular signals that regulate TH mRNA concentrations in response to clozapine are still unknown, but a common mechanism of action is presumably involved in both dopaminergic systems. We demonstrate, in contrast to the classic hypothesis of the mesocorticolimbic selectivity of atypical antipsychotics, that clozapine also produces a pharmacological effect in the molecular processes occurring in the nigrostriatal system. This result further highlights the atypical profile of clozapine, insofar as some other pharmacological treatments interfering with the dopaminergic neurotransmission do not modify TH mRNA levels in the substantia nigra (Cottingham et al., 1990; Pasinetti et al., 1990).

Modulation of TH Gene Expression by Clozapine in the Mesocorticolimbic System Might Be Necessary for Its Antipsychotic Effect

The mechanisms by which the direct action of antipsychotics is translated into therapeutic effects remain unknown. Although a few authors have reported a lack of effect of chronic clozapine on extracelular dopamine in the nucleus accumbens (Ichikawa and Meltzer, 1991; Invernizzi et al., 1995), most of data in the literature demonstrate that chronic clozapine reduces dopamine levels selectively in this nucleus in the rat (Blaha and Lane, 1987; Lane et al., 1988; Chen et al., 1991; Kuroki et al., 1991). Moreover, the same treatment decreseas the number of active dopaminergic neurons in the ventral tegmental area (Skarsfeldt, 1988). Clinical studies have shown that chronic treatment with antipsychotics is needed for the antipsychotic effect to be expressed (Chouinard and Annable, 1976). In that chronic treatment by both typical and atypical antipsychotic reduces dopamine release in the

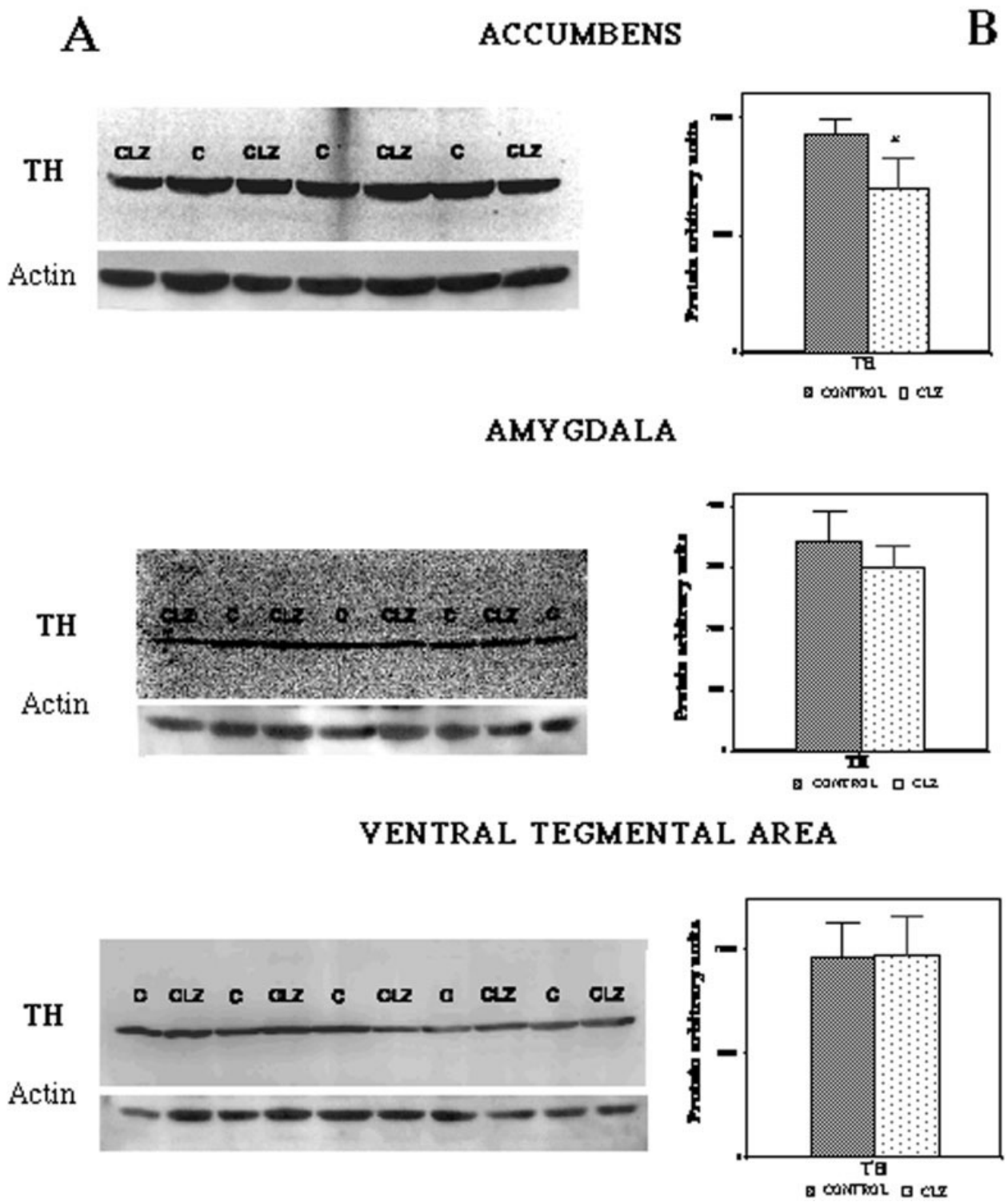

Fig. 5. Effect of chronic clozapine on TH protein level in the mesocorticolimbic system, the ventral tegmental area and its projecting terminal fields, the accumbens and amygdala. **A:** Analysis by phosphoimager of images obtained by chemiluminiscence of typical Western blots obtained using a polyclonal antibody raised against TH and a secondary antibody conjugated to horseradish peroxidase. **B:** Bar graphs show the immunoreactivity corresponding to TH bands quantified by phosphoimager analysis, normalized to the actin signal and expressed in arbitrary units. Data are mean ± SEM. $\star P < 0.05$ vs. control animals (n = 5) by Student's *t*-test. To verify equal protein loading, membranes were probed with an antiactin antibody.

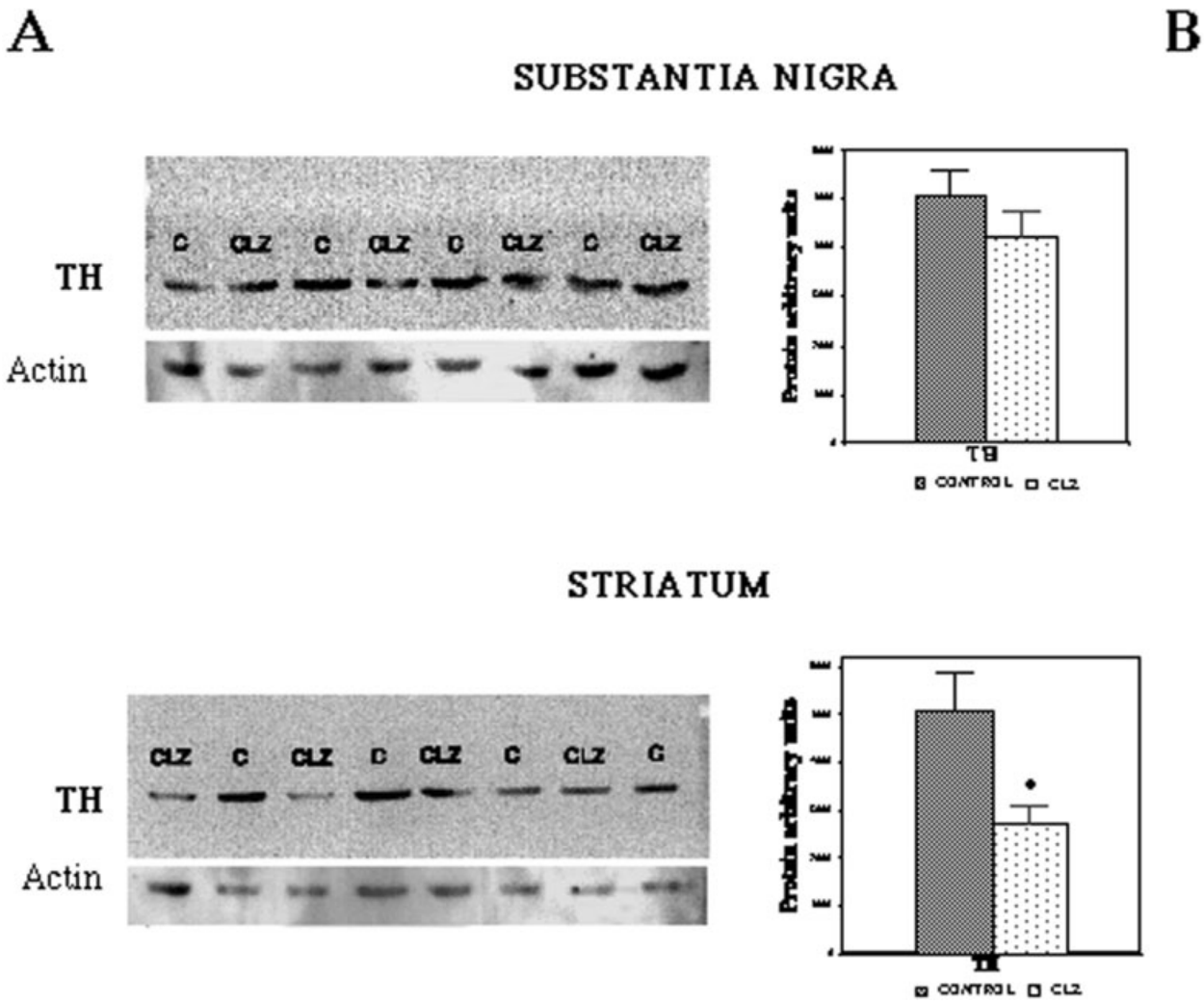

Fig. 6. Effect of chronic clozapine on TH protein level in the nigrostriatal system, the sustantia nigra, and its projecting terminal field in the striatum. **A:** Analysis by phosphoimager of typical Western blots. **B:** Bar graphs corresponding to TH bands quantified by phosphoimager analysis, normalized to actin and expressed in arbitrary units. Data are means ± SEM. $\star P < 0.05$ vs. control animals (n = 5) by Student's *t*-test.

mesolimbic system (Blaha and Lane, 1987; Chen et al., 1991; Chesi et al., 1995; Feasey-Truger et al., 1995), this effect could be necessary for the therapeutic benefits of this drug. Therefore, the clozapine-reduced level of TH mRNA observed in the ventral tegmental area and the decrease of TH protein in the nucleus accumbens might be necessary for the dopamine level to be reduced and, therefore, for the antipsychotic effect of this drug to occur. Further studies on the TH mRNA or protein levels in the same areas of the brain of schizophrenic patients would be instrumental in assessing the functional relevance of these results.

There is overwhelming evidence that the prefrontal cortex is an important site of abnormal function in schizophrenia, probably involved in the harmful symptoms, and is a relevant drug target for antipsychotic activity (Meltzer and Stahl, 1976; Weinberger, 1987; Gessa et al., 2000; Weinberger et al., 2001). A hypodopaminergic function has been suggested for the prefrontal cortex in schizophrenia that may give rise to the deficit in executive function (Carter and Pycock, 1980). Thus, an increase in cortical dopamine would be favorable for drug antipsychotic activity (Deutch, 1992; Yamamoto and Cooperman, 1994). Accordingly, Youngren et al. (1994) have observed that chronic clozapine produces a sustained enhancement in dopaminergic tone in the prefrontal cortex. TH protein variations in the prefrontal cortex were not quantifiable, but, taking into account this hypothesis, it is difficult to assess the functional role of the clozapine-induced decrease in TH mRNA level in the ventral tegmental area, containing the cell body of dopaminergic neurons, which project to the prefrontal cortex.

Discordances Between the Decreases of TH Protein and Dopamine Levels in the Nigrostriatal System After Chronic Clozapine

The reduced level of TH protein after chronic clozapine would be expected to reduce dopamine levels in the nigrostriatal dopaminergic system. However, clozapine has only a small or no effect on the dopamine level in this dopaminergic system (Lane et al., 1988; Chen et al., 1991; Ichikawa and Meltzer, 1992; Hernandez and Hoebel, 1995). The dissociation between the down-regulated expression of TH and the unaltered dopamine level in the nigrostriatal system, in contrast to the mesocorticolimbic system, indicates a regional specificity of regulation. Various potential mechanisms may be involved. 1) The first is the affinity of clozapine for different specific serotonergic receptors and their different anatomical distribution. 5-HT2 antagonists suppresses the inhibitory effect of 5-HT on dopamine release in the striatum via the inhibition of 5-HT binding at the 5-HT2 receptor on the nerve terminal of the dopaminergic neuron (Muramatsu et al., 1988); as a consequence, when 5-HT2 receptors are blocked by clozapine, dopamine release increases and cancels out the dopamine blockade effect at postsynaptic receptor sites (Glazer, 2000). Furthermore, this effect may be amplified by differences in the 5-HT2/D2 ratio that underlie the atypical properties of clozapine (Meltzer et al., 1989). 2) Second is the differential distribution of D2 receptors in the ventral tegmental area and substantia nigra. The differences between the effects of clozapine and haloperidol on dopamine levels in the two dopaminergic systems has been previously ascribed to the different somatodendritic D2 autoreceptors in the ventral tegmental area and substantia nigra, in the different presynaptic autoreceptors in the axon terminals, and in the different inhibitory potencies of clozapine and haloperidol for these receptors (Cragg and Greenfield, 1997). 3) Third is the differences in dopamine reuptake in both dopaminergic systems. The dopamine reuptake is greater in the nigrostriatal than in the mesolimbic system (Giros et al., 1996), probably as a consequence of a different distribution of dopamine uptake sites in the two regions. Moreover, dopamine reuptake appears to be modulated by presynaptic D2-like autoreceptors (Garris et al., 1994). Thus, a different distribution of these receptors may be implicated in this phenomenon (see point 2). 4) Fourth is the different dynamic regulation of catecholamine biosynthesis through reversible phosphorylation of TH, differing between cell subpopulations, and modifying the activity of the enzyme (Zigmond et al., 1989). Acute clozapine increases the activity of TH in striatum (Rayevsky et al., 1978; el Mestikawy et al., 1986) and nucleus accumbens (Zivkovic et al., 1975; Hetey et al., 1985) neurons, whereas administration of clozapine for 8 days fails to modify TH activity (Rayevsky et al., 1978). The heterogeneity in the level of expression of GTP cyclohydrolase I in the mesocorticolimbic and nigrostriatal systems may also play an important role in determining steady-state levels of tetrahydrobiopterin (the cofactor for TH) and, consequently, the regulation of dopamine biosynthesis (Lentz and Kapatos, 1996; Hirayama and Kapatos, 1998).

These mechanisms may allow different controls of dopamine in the two dopaminergic systems after chronic clozapine in spite of the similar reduction of TH mRNA and TH protein levels. All or some of these mechanisms may be associated with the nondevelopment of extrapyramidal symptoms of clozapine. In addition, the modulation by clozapine of both the γ-aminobutyric acid (GABA) turnover (Mao et al., 1977) and the concentrations of allopregnanolone and allotetrahydroxycorticosterone (Barbaccia et al., 2001) may also be relevant to the mechanisms of action of this antipsychotic drug and in particular the absence of extrapyramidal side effects. Thus, Mao et al. (1977) suggested that an increase of GABA turnover and perhaps of GABA release in the striatum and substantia nigra might account for the lack of tardive dyskinesia and extrapyramidal side effects of clozapine. Furthermore, Barbaccia et al. (2001) suggested that the clozapine-induced increases in neuroactive steroid concentrations in the brain may contribute to the atypical pharmacological profile of this antipsychotic drug.

Decreases of TH mRNA Levels Under Clozapine in the Ventral Tegmental Area and Substantia Nigra Are Accompanied by a Reduction of TH Protein Concentration in the Terminal Fields, Striatum and Nucleus Accumbens but Not in the Dopaminergic Cell Body Regions

The chronic clozapine treatment significantly decreases the amount of TH protein in the striatum and in the nucleus accumbens but has no significant effect either in the amygdala or in the substantia nigra and ventral tegmental area. Thus, although the TH protein depletion in the striatum and the nucleus accumbens may occur as a consequence of the reduced TH mRNA level in the dopaminergic cell body-containing regions, compensatory mechanisms appear to preserve dopaminergic function in the substantia nigra, ventral tegmental area, and amygdala. These mechanisms, which may involve, for example, the axonal transport of TH, could differentially regulate the amount of protein in the main projection areas of the dopaminergic neurons. This, in turn, may provide a mean for the fine tuning of dopamine turnover via local mechanisms that may be relevant for the antipsychotic effect of clozapine.

CONCLUSIONS

The down-regulation of TH gene expression in the mesocorticolimbic system after chronic clozapine that we describe could be crucial to its antipsychotic effect. There is a clear need for new antipsychotic compounds that are more effective and better tolerated. Specific modulation of TH gene expression (transcription and/or translation) in the mesocorticolimbic system may be a pertinent new criterion for assessing the potential of new atypical agents.

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