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Chronic Imipramine Administration Alters the Activity and Phosphorylation State of Tyrosine Hydroxylase in Dopaminergic Regions of Rat Brain

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In the present study the influence of imipramine, a tricyclic antidepressant, on the expression and function of tyrosine hydroxylase (TH) in dopaminergic rat brain regions was examined. Chronic administration of imipramine (18 days) decreased levels of TH enzyme activity in ventral tegmental area (VTA) and substantia nigra (SN), dopaminergic cell body regions, as well as in caudate-putamen (CP), nucleus accumbens (ACB), prefrontal cortex (PFC), and olfactory tubercle (OT), dopaminergic terminal fields. These effects were dependent on chronic drug treatment, as imipramine administration for 1 or 7 days did not significantly influence levels of TH activity in either SN or VTA. In contrast to drug regulation of enzyme activity, chronic imipramine treatment did not decrease levels of TH immunoreactivity in any of the dopaminergic cell body or terminal field regions studied, although levels of TH immunoreactivity were decreased in locus coeruleus (LC) as previously reported. However, imipramine treatment increased levels of TH back phosphorylation in VTA, suggesting that the antidepressant-induced decrease in levels of TH activity is a result of decreased phosphorylation of the enzyme. These results demonstrate that imipramine treatment regulates levels of TH enzyme activity in dopaminergic brain regions, and may account for some of the previously observed effects of these drugs on dopaminergic function. Finally, imipramine regulation of TH enzyme activity in VTA and immunoreactivity in LC was observed in Sprague Dawley, but not Wistar rats, demonstrating that different rat strains exhibit different biochemical responses to antidepressant treatment. ***[Neuropsychopharmacology 12:113–121, 1995]***

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Received February 22, 1994; revised July 26, 1994; accepted July 19, 1994.

KEY WORDS: *DA, dopamine; TH, tyrosine hydroxylase; LC, locus coeruleus; SN, substantia nigra; VTA, ventral tegmental area; CP, caudate-putamen; PFC, medial prefrontal cortex; OT, olfactory tubercles; ACB, nucleus accumbens*

Previous behavioral and neurochemical studies have reported that antidepressant treatments influence brain dopaminergic systems. Chronic administration of antidepressant drugs or electroconvulsive seizure increases psychostimulant- or apomorphine-induced locomotor activity, possibly as a result of a postsynaptic dopa-

NEUROPSYCHOPHARMACOLOGY 1995—VOL. 12, NO. 2

Published by Elsevier Science Inc.
655 Avenue of the Americas, New York, NY 10010

0893-133X/95/$9.50
SSDI 0893-133X(94)00066-9

minergic supersensitivity (Spyraki and Fibiger 1981; Wieslov 1981; Green et al. 1983; Martin-Iverson et al. 1983; Smialowski and Maj 1985; Spyraki et al. 1985; Jimerson 1987). In addition, chronic desipramine treatment increases intracranial self-stimulation of the A10 dopamine region in rats (Fibiger and Phillips 1981).

Antidepressant treatments are also reported to decrease presynaptic dopamine autoreceptor function. Serra and colleagues found that chronic administration of antidepressant drugs, electroconvulsive seizure, or deprivation of rapid eye movement sleep decrease the ability of low doses of apomorphine to produce sedation, an effect mediated by dopamine autoreceptors (Serra et al. 1979, 1980, 1981a, b). Antidepressants are reported to block the reduction in dopamine turnover in striatum in response to low doses of apomorphine (Serra et al. 1979, 1980; Holcomb et al. 1982; Jimerson 1987), and one study found that some antidepressants decrease basal levels of dopamine metabolites (Holcomb et al. 1982). Moreover, electrophysiological studies have provided direct evidence that antidepressant treatments decrease the sensitivity of dopamine autoreceptors (Chiodo and Antelman 1980a, b). Taken together, the literature suggests that chronic antidepressant treatments decrease the function of dopamine autoreceptors, although not all reports are in agreement with this conclusion (Spyraki and Fibiger 1981; Holcomb et al. 1982; Diggory and Buckett 1984; Spyraki et al. 1985).

Down-regulation of autoreceptors suggests that antidepressant treatments influence presynaptic dopaminergic function. One potential target is tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine synthesis. This possibility is studied by examining the influence of chronic imipramine treatment on levels of TH enzyme activity, immunoreactivity, and phosphorylation state in dopaminergic cell nuclei and terminal fields. The results demonstrate that chronic, but not acute, imipramine treatment decreases levels of TH enzyme activity in dopaminergic brain regions and that this effect is mediated via phosphorylation of the enzyme and not down regulation of total amounts of TH protein.

MATERIALS AND METHODS

In Vivo Drug Treatments and Sample Preparation

Male Sprague-Dawley rats (Camm; 150 to 200 g) received daily IP injections of imipramine (Sigma) at a dose of 15 mg/kg for 1 to 18 days. Where indicated, Wistar rats (150 to 250 g) were treated with imipramine exactly as described for Sprague Dawley rats. In all cases, control animals were treated with saline for a period of time equivalent to that of experimental subjects. In one experiment, rats were subjected to cold stress for 5 days as described (Melia et al. 1992a,b). The influence of antidepressant treatments on animal behavior was determined using a standard predictor of antidepressant activity, the Porsolt swim test, as described (Porsolt et al. 1977). Animals were sacrificed by decapitation 18 hours after the last treatment. The brain was rapidly dissected on ice using previously published methods (Deutch et al. 1985) for all regions except LC, substantia nigra (SN), and ventral tegmental area (VTA), which were dissected as described by Beitner-Johnson et al. (1992). Isolated brain regions were immediately prepared for Western blot and phosphorylation experiments or frozen on dry ice and stored at −70°C for analysis of TH activity. All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Yale Animal Care Committee.

Assay of Tyrosine Hydroxylase Activity

Tyrosine hydroxylase activity was measured in soluble fractions of isolated brain regions using a modification (Rosin et al. 1992) of the previously described coupled decarboxylase assay (Waymire et al. 1971; Kapatos and Zigmond, 1979). Briefly, a 10-µl aliquot of tissue supernatant was assayed at pH 6.8 with either a subsaturating (0.25 mmol/L) or saturating (1.0 mmol/L) concentration of the natural pterin cofactor [6R]-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (BH_4; Dr. B. Schirks Laboratories, Jona, Switzerland) or at pH 6 with 0.75 mmol/L BH_4. All samples were assayed in the presence of 0.1 µCi L-[1-^{14}C]tyrosine (Amersham Corp.) with a final concentration of 100 µmol/L tyrosine. The reaction product, $^{14}CO_2$, was trapped on methylbenzathonium hydroxide-soaked paper wicks, and radioactivity was quantitated by liquid scintillation spectrometry. Protein was measured as described (Lowry et al. 1951). With the use of this microassay for TH activity, we were able to measure enzyme activity in tissue samples of less than 0.5 mg of wet weight.

Immunoblotting of Tyrosine Hydroxylase

Isolated brain regions were homogenized in 2% (wt/vol) sodium dodecyl sulfate (SDS) and normalized for protein content. The resulting samples were then subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis for TH as previously described (Guitart et al. 1990), using an affinity-purified rabbit polyclonal antiserum (a gift from Dr. John W. Haycock, Louisiana State University) and [^{125}I]-labeled goat anti-rabbit IgG (New England Nuclear). Resulting blots were dried and subjected to autoradiography using an intensifying screen (DuPont). Levels of immunolabeling were quantitated by densitometry or by counting radioactivity in excised bands; the two methods gave equivalent results.

Back-Phosphorylation and Immunoprecipitation of TH

Cyclic AMP-dependent protein kinase back phosphorylation was conducted exactly as previously described (Guitart et al. 1990). Briefly, isolated punches of VTA were homogenized in 150 µl of 20 mmol/L citric acid containing 0.01% NP-40 (final pH = 2.8 to 3.0) and then centrifuged at 10,000 × g for 15 minutes at 4°C. The supernatant was collected and neutralized by addition of 200 mmol/L Na_2HPO_4 (330 µl/ml, final pH = 6.0 to 6.3). Aliquots of neutralized VTA supernatants were back phosphorylated with cyclic AMP-dependent protein kinase (Sigma) and γ-[^{32}P]-ATP (30 Ci/mmol; New England Nuclear); incubations were performed for 30 minutes at 30°C and terminated by addition of 1% SDS. Immunoprecipitation of TH was performed using anti-TH antibody and *Staphylococcus aureus* cells as previously described for synapsin I (Nestler and Greengard 1980). The resulting immunoprecipitates were resuspended in 2% SDS, boiled for 2 minutes, and subjected to electrophoresis as described above. This procedure has been shown to result in specific and quantitative precipitation of TH from brain extracts (Guitart et al. 1990). Levels of immunolabeling were quantitated by densitometry or by counting radioactivity in excised bands; the two methods gave equivalent results.

Receptor Ligand Binding Analysis

Levels of β-adrenergic and 5-HT_2 receptor ligand binding were determined using the radioligands [3H]-CGP 12177 and [3H]-ketanserin, respectively, according to published procedures (Hosoda and Duman 1993; Butler et al. 1993).

Statistical Analysis

For in vivo TH activity experiments, data were analyzed by analysis of variance and subsequent post hoc comparisons were made using the Scheffe test. Alternatively, data from multiple experiments, when pooled, were analyzed using the Wilcoxon sign test. For TH back-phosphorylation studies the χ^2 test was used.

RESULTS

Effects of Chronic Imipramine Treatment on TH Activity

The influence of chronic imipramine treatment on levels of TH enzyme activity was examined in several brain areas enriched in dopamine. Tyrosine hydroxylase activity was measured under different assay conditions optimized for estimating changes in either enzyme K_m for pterin cofactor or total activity (V_{max}): conditions optimized to reflect a change in K_m are pH 6.8 and cofactor (BH_4) concentrations of either 0.25 or 1.0 mmol/L; conditions optimized to reflect a change in total activity are pH 6.0 and cofactor concentration of 0.75 mmol/L (Simon and Roth 1979; Goldstein and Green 1987). Imipramine treatment for 18 days significantly decreased levels of TH activity in both VTA and SN at pH 6.8 but not pH 6.0 assay conditions (Figure 1). Regulation of TH activity was found to be dependent on chronic treatment, as 1 or 7 days of imipramine treatment did not significantly alter levels of activity in either brain region (data not shown). Chronic imipramine administration (18 days) also significantly decreased lev-

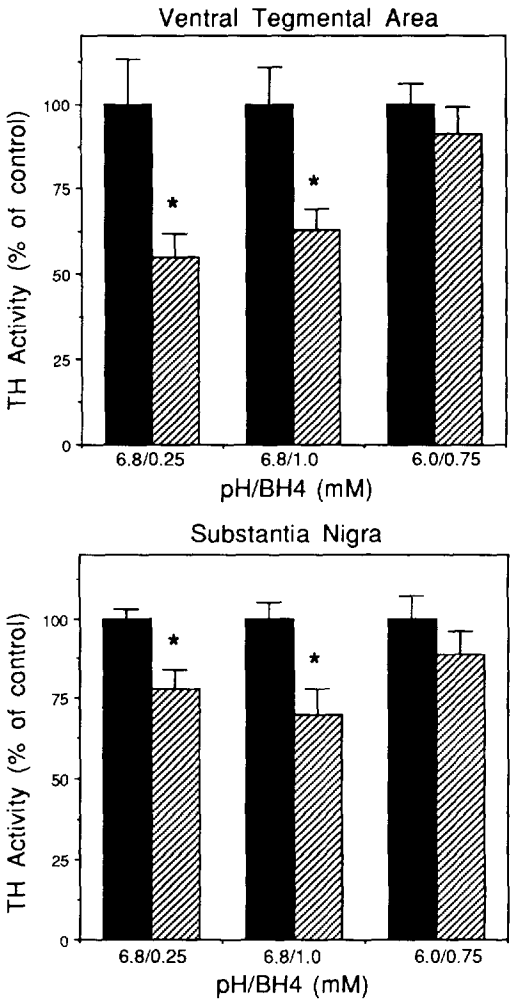

Figure 1. Influence of imipramine treatment on levels of TH activity in dopamine cell body regions. Sprague Dawley rats were treated with imipramine for 18 days, and TH activity was analyzed in VTA and SN as described in Materials and Methods. The results are expressed as percent of control and are the mean ± SEM of 12 separate determinations, each performed in triplicate. The levels of enzyme activity in control VTA and SN were similar to those listed in Table 1. * $p < 0.05$ compared to controls. Control rats (*solid bars*) and imipramine-treated rats (*striped bars*).

els of TH activity in several dopaminergic terminal fields including PFC, ACB, CP, and OT at the high, but not low, pH conditions (Figure 2). The observed effect at pH 6.8 but not at pH 6.0 suggests that imipramine treatment influences the affinity of the enzyme for cofactor, but not the maximal amount of enzyme activity.

Regulation of TH Immunoreactivity by Chronic Imipramine

To directly examine whether decreases in TH activity are associated with changes in the amount of TH protein, levels of TH immunoreactivity were analyzed by Western blot analysis using an affinity-purified TH antibody. Chronic imipramine treatment did not significantly influence levels of TH immunoreactivity in any of the dopaminergic brain regions examined, including VTA, SN, OT, or ACB (Figure 3). These findings suggest that the decrease in levels of TH activity does not result from decreased expression of TH protein. In contrast, levels of TH immunoreactivity in LC were significantly decreased by chronic imipramine treatment as shown previously (Nestler et al. 1990).

Regulation of TH Phosphorylation by Chronic Imipramine

Another possibility that could explain the regulation of enzyme activity by imipramine treatment is a change in TH phosphorylation. Phosphorylation of TH by sev-

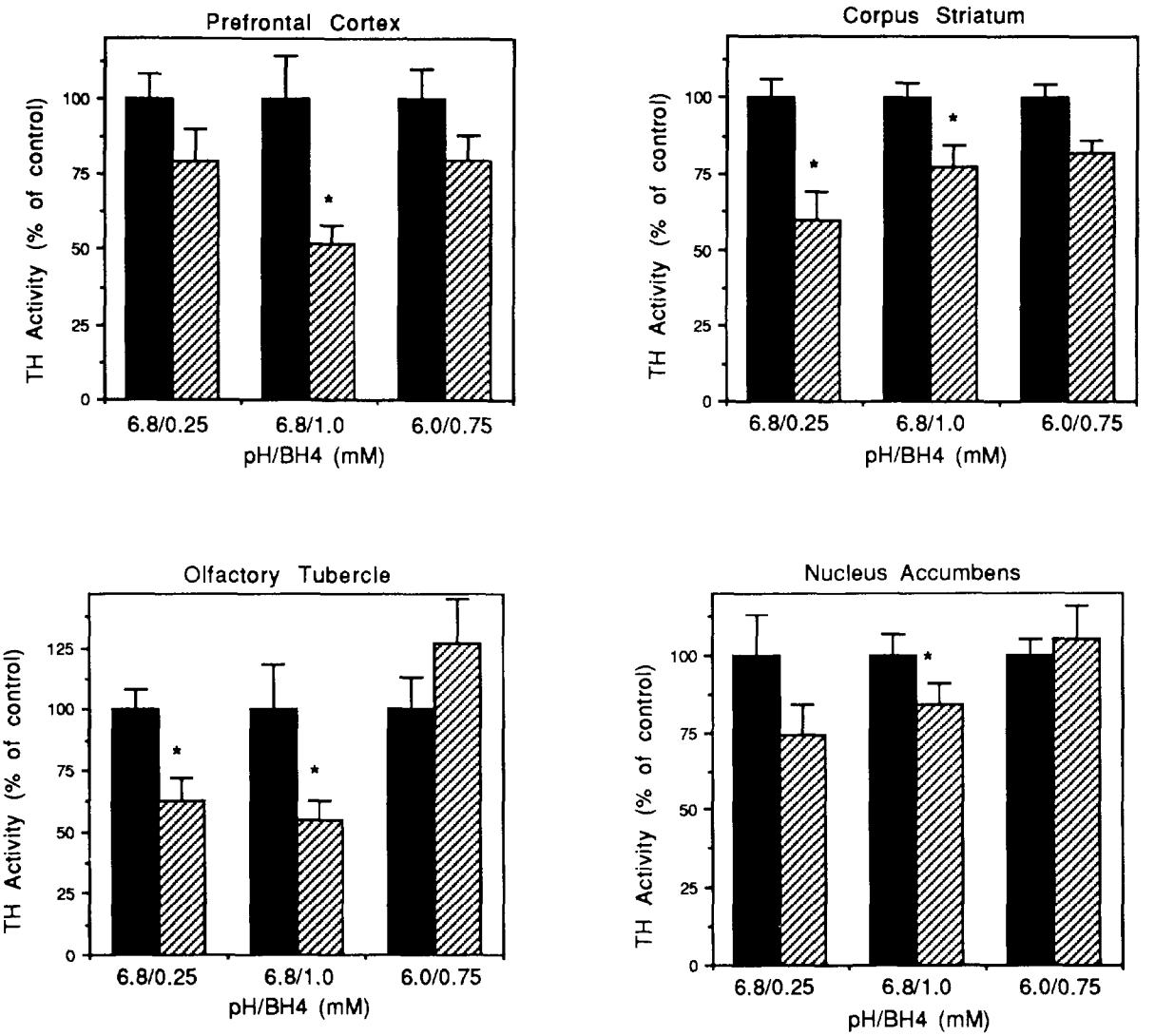

Figure 2. Influence of imipramine treatment on levels of TH activity in dopamine terminal fields. Sprague Dawley rats were treated with imipramine for 18 days, and TH activity was analyzed in CP, ACB, PFC, and OT as described in Materials and Methods. The results are expressed as percent of control and are the mean ± SEM of 6 to 12 separate determinations, each performed in triplicate. * $p < 0.05$ compared to controls. Control rats (*solid bars*) and imipramine-treated rats (*striped bars*).

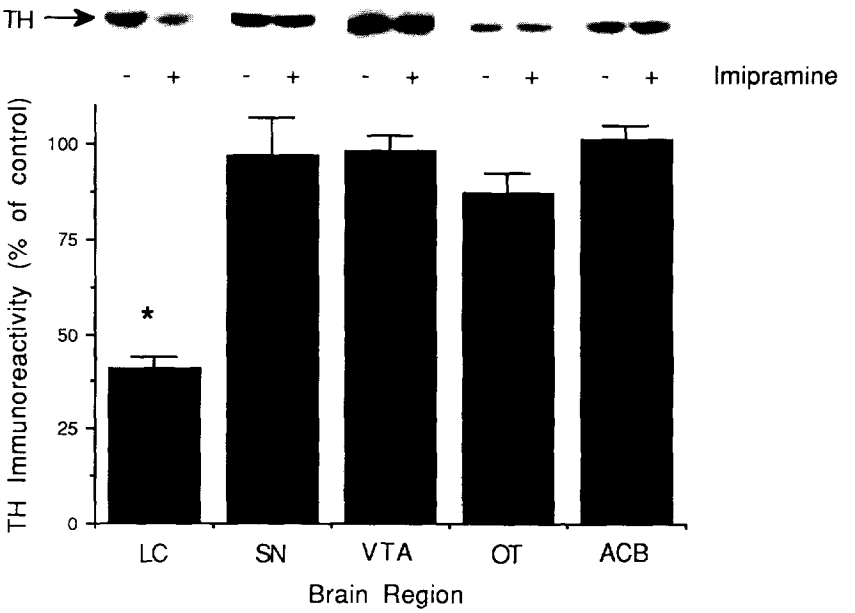

Figure 3. Influence of imipramine treatment on levels of TH immunoreactivity in dopamine and norepinephrine brain regions. Sprague Dawley rats were treated with imipramine (21 days), and levels of TH immunoreactivity were determined in LC, SN, VTA, OT, and ACB by Western blot analysis as described in Materials and Methods. Representative autoradiograms of TH immunoreactivity in the different brain regions are shown above. For the bar graph, levels of TH were quantitated as described in Materials and Methods and are expressed as percent of control (mean ± SEM of 6 of 11 separate determinations). * $p < 0.05$ compared to control.

eral protein kinases has been shown to result in its activation (Goldstein and Greene 1987; Griffith and Schulman 1988; Haycock et al. 1988; Waymire et al. 1988; Zigmond et al. 1989). To examine this possibility, extracts of VTA were back phosphorylated with cyclic AMP-dependent protein kinase and then immunoprecipitated. Chronic imipramine treatment significantly increased back phosphorylation of TH in VTA by ~45% (Figure 4). Back phosphorylation provides a measure of the dephospho form of a phosphoprotein. Thus, an increase in back phosphorylation, with no change in the total amount of the protein, would suggest a decrease in its state of phosphorylation (see Guitart et al. 1990; Beitner-Johnson et al. 1992). Such a decrease in phosphorylation state could underlie the observed decrease in TH activity (see Discussion).

Regulation of TH by Imipramine Treatment in Wistar versus Sprague Dawley Rats

In contrast to the observed decrease in TH activity in the VTA (Figure 1), a preliminary report found that chronic imipramine treatment significantly increased levels of TH activity and TH mRNA in this and certain other dopaminergic regions (Leviel et al. 1990). Although not identical, the treatment regimens and biochemical assays were comparable to those used in the present study. However, the Wistar strain of rat was used by Leviel and colleagues, whereas Sprague Dawley rats were used in the present study. In an attempt to resolve this discrepancy, the influence of chronic imipramine treatment on levels of TH was examined in Wistar rats. Surprisingly, levels of TH activity were not decreased in the VTA of Wistar rats as was observed for Sprague Dawleys. In fact, there was a

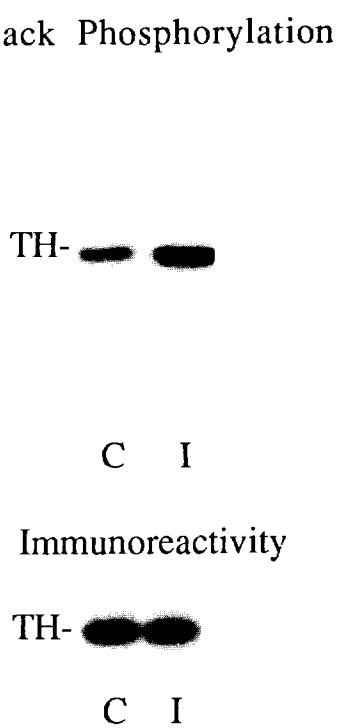

Figure 4. Influence of imipramine treatment on TH phosphorylation and immunoreactivity in VTA. Extracts of VTA from control (C) and chronic imipramine (I) treated Sprague Dawley rats were subjected to back phosphorylation in the presence of cyclic AMP-dependent protein kinase, and then to TH immunoprecipitation as described in Materials and Methods. An autoradiogram representative of the influence of chronic imipramine treatment is shown. Chronic imipramine treatment significantly increased levels of TH back phosphorylation in VTA [145 ± 17 percent of control ($p < 0.05$ by Chi square test, $n = 12$)]. Also shown is an autoradiogram of TH immunoreactivity illustrating that this increase in TH back phosphorylation was not associated with a change in the amount of TH.

small, although nonsignificant, increase in levels of TH activity in the VTA of the Wistars (Table 1).

Additional studies were conducted to determine if there are other differences between Sprague Dawley and Wistar rats. Imipramine treatment, which decreases levels of TH immunoreactivity in LC of Sprague Dawley rats (Nestler et al. 1990), had no effect on levels of TH immunoreactivity in LC of Wistar rats (Table 1). In addition, chronic stress, which increases levels of TH in LC of Sprague Dawley rats (Melia et al. 1992a), did not influence levels of TH in LC of Wistar rats (data not shown). We have also found a behavioral difference between Wistar and Sprague Dawley rats in the Porsolt swim test, which is used as an animal model predictor of antidepressant actions (Porsolt et al. 1977): imipramine treatment decreased immobility on the test day in Sprague Dawleys, as previously reported, but not in Wistar rats, which also displayed lower levels of immobility in the absence of drug treatment (Table 1). These results demonstrate that imipramine regulation of TH and behavioral immobility differ in Sprague Dawley and Wistar rats. In contrast to these differences, chronic imipramine treatment down-regulated levels of β-adrenergic and 5-HT_2 receptor ligand binding in both strains of rat (Table 1), effects that are consistently reported in the literature (for review see Heninger and Charney 1987).

Table 1. Influence of Chronic Imipramine Treatment on TH, Swim Immobility, and β-Adrenergic and 5-HT_2 Receptor Ligand Binding in Sprague Dawley and Wistar Rats

	Rat	
Treatment	**Sprague Dawley**	**Wistar**
TH Activity in VTA (% of control)		
Control	100 ± 12	100 ± 12
Imipramine	63 ± 6*	129 ± 9
TH Immunoreactivity in LC (mean densitometric units)		
Control	2.7 ± 0.3	2.7 ± 0.3
Imipramine	1.5 ± 0.2*	2.7 ± 0.4
Porsolt swim test: immobility (sec per 5 min)		
Control	132 ± 28	50 ± 12**
Imipramine	59 ± 7*	51 ± 11
β-Adrenergic receptor ligand binding in frontal cortex (fmol/mg protein)		
Control	22 ± 3	25 ± 2
Imipramine	15 ± 2*	13 ± 2*
5-HT_2 receptor ligand binding in frontal cortex (fmol/mg protein)		
Control	76 ± 4	82 ± 4
Imipramine	48 ± 10*	60 ± 6*

Sprague Dawley and Wistar rats were administered imipramine for 18 days (15 mg/kg, IP), except for the Porsolt swim test where rats were administered 3 imipramine treatments (15 mg/kg, IP 24, 5 and 1 hr before the test). Analysis of TH (at pH 6.8, 1 mM BH_4), receptor ligand binding, and behavioral tests were conducted as described in Materials and Methods. Each point is the mean ± SEM of 6 to 12 separate determinations.

* $p < 0.05$ compared to control.

** $p < 0.05$ compared to Sprague Dawley rats.

DISCUSSION

Chronic administration of the tricyclic antidepressant imipramine was found to regulate TH activity in dopaminergic brain regions, including the cell body and terminal field regions of the mesolimbic and nigrostriatal pathways. This regulation of TH activity could reflect an alteration in expression of TH protein or could result from covalent modification of a constant amount of the enzyme. To examine the first possibility, the influence of imipramine treatment on levels of TH immunoreactivity was determined. Imipramine treatment did not significantly influence levels of TH immunoreactivity in any of the dopaminergic regions examined, although levels of TH were decreased in LC, as previously reported (Nestler et al. 1990; Melia et al. 1992a). In contrast, the phosphorylation of TH in VTA was altered by chronic imipramine treatment. Back phosphorylation of TH in VTA by cyclic AMP-dependent protein kinase was increased by imipramine treatment, which is indicative of a decrease in the phosphorylation state of the enzyme. Because the activity of TH is positively correlated with its phosphorylation state (Goldstein and Greene 1987; Griffith and Schulman 1988; Haycock et al. 1988; Waymire et al. 1988; Zigmond et al. 1989), decreased phosphorylation of TH would be consistent with, and could account for, the decreased enzyme activity observed after chronic imipramine treatment. Taken together, the results are consistent with the hypothesis that chronic imipramine treatment decreases levels of TH enzyme activity in VTA via phosphorylation-dependent regulation of enzyme affinity for its pterin cofactor. Future studies will directly examine this possibility by analysis of the Km for TH. Given that levels of TH enzyme activity but not total amounts of protein immunoreactivity were decreased in the other dopaminergic regions, it is possible that a change in phosphorylation similar to that observed in VTA could account for the effects of imipramine.

Studies in peripheral tissues and in the central nervous system have demonstrated that TH activity is regulated by nerve impulses and neurotransmitter stimulation, and that these effects appear to be achieved via changes in enzyme protein phosphorylation (Goldstein and Green 1987; Zigmond et al. 1989; c.f. Nestler and Greengard 1989). The decrease in TH activity and phosphorylation observed in the present study could result from decreased activity of dopaminergic neurons,

which would thereby decrease the requirement for dopamine synthesis. One study has reported that chronic antidepressant treatment decreases basal levels of dopamine metabolites (Holcomb et al. 1982), a finding consistent with decreased dopamine synthesis and release. This decrease in dopaminergic activity could result from elevation of synaptic levels of dopamine in response to antidepressant treatment, although the tricyclic antidepressants have low affinity for the dopamine transporter relative to that for the norepinephrine or serotonin transporters. Alternatively, antidepressants may indirectly inhibit the activity of dopaminergic neurons via effects on the serotonin and norepinephrine neurotransmitter systems. In either case, the results of the present study are consistent with the reports that chronic antidepressant treatments decreased dopamine autoreceptor function, an effect which would also occur when synaptic levels of dopamine are elevated.

Imipramine regulation of TH activity in VTA was found to be dependent on the strain of rat, with chronic treatment decreasing enzyme activity in Sprague Dawley rats but slightly increasing enzyme activity in Wistar rats. This is consistent with the results of an earlier study on imipramine regulation of TH in Wistar rats (Leviel et al. 1990). Although the reason for this strain difference is not known, the inconsistent effect of antidepressants on dopamine systems in the literature could arise from the use of different strains of rats. At least two studies that failed to observe an attenuation of apomorphine-induced sedation by antidepressants utilized Wistar rats (Spyraki and Fibiger 1981; Spyraki et al. 1985). Although some negative studies did use Sprague Dawley rats, it is conceivable that different colonies of the same rat strain could respond differently. These observations demonstrate that large individual differences exist in biochemical responses to antidepressant treatments, consistent with clinical observations of individual differences in the therapeutic responsiveness of depressed patients to these drugs.

Some of the behavioral abnormalities of major depression, such as changes in activity, cognition, and the ability to experience pleasure (i.e., anhedonia), are thought to be influenced by the mesolimbic dopamine system. Imaging studies have also demonstrated that depressed patients display differences in blood flow and glucose metabolism in prefrontal cortex (Baxter et al. 1989; Drevets et al. 1992). Studies in rats have demonstrated that some antidepressants increase locomotor activity in response to psychostimulants or dopamine agonists suggesting increased postsynaptic sensitivity (Green and Deakin 1980; Spyraki and Fibiger 1981; Wieslov 1981; Green et al. 1983). Some of these treatments also reportedly increase self-stimulation of A10 dopamine neurons (Fibiger and Philips 1981). Regulation of locomotor and reward behaviors via effects on dopaminergic systems could contribute to the therapeutic actions of some antidepressant treatments. The study of antidepressant regulation of TH and dopaminergic function will help elucidate the neurochemical mechanisms which underlie these behavioral effects of antidepressant treatments.

ACKNOWLEDGMENTS

The authors express their gratitude to Dr. John W. Haycock (Louisiana State University) for generously supplying tyrosine hydroxylase antibody. We also wish to thank Kati Piros, Chen Pun, and Eva Beckman for expert technical assistance and critically reviewing the manuscript. This work was supported by USPHS grants MH 14092 (RHR), MH45481 (RSD), DA07359 (EJN), Program Project grant 2 P01 MH25642, the Abraham Ribicoff Research Facilities of the State of Connecticut, and by a Veterans Administration National Center Grant for PTSD, VA Hospital in West Haven, Connecticut.

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