

Effects of Long-term Cigarette Smoking on the Human Locus Coeruleus

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Background: It has been hypothesized that cigarette smoking among subjects with major depression is a form of self-medication. To explore a possible biological basis for this hypothesis, noradrenergic proteins in the locus coeruleus (LC) were measured in long-term cigarette smokers and in nonsmokers. The LC was studied because elevated amounts of α_2 -adrenoceptors and tyrosine hydroxylase have been observed postmortem in the LCs of subjects with major depression or who commit suicide, and because long-term administration of antidepressant drugs to rats down-regulates these proteins in the LC.

Methods: Postmortem LCs were obtained from long-term cigarette smokers (n=7) and from nonsmokers (n = 9), all of whom lacked diagnoses of major depression. Amounts of tyrosine hydroxylase immunoreactivity and radioligand binding to the norepinephrine transporter, monoamine oxidase A, and α_2 -adrenoceptors were measured.

Results: Amounts of tyrosine hydroxylase immunoreactivity and radioligand binding to α_2 -adrenoceptors were significantly lower (approximately 60% and 40%, respectively) along the axis of the LCs of long-term smokers compared with nonsmokers. Smoking had no statistically significant effects on binding to monoamine oxidase A or to the norepinephrine transporter.

Conclusion: This is the first demonstration that cigarette smoking affects noradrenergic proteins in the LC. The direction of these changes is opposite to that observed when comparing subjects who have major depression with normal controls and the same as that produced by long-term antidepressant treatment in animals. If the present observations reflect long-term effects of smoking on pre-mortem noradrenergic biochemistry, smoking-induced changes in LC biochemistry may strengthen the smoking habit among subjects with major depression.

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THE HIGH prevalence of cigarette smoking in persons with depression^{1,2} and difficulties with successful smoking cessation among depressed patients has led researchers to hypothesize that smoking is a type of self-medication. In a study involving 3200 community residents,³ among individuals with a history of major depression, 65% of the women and 80% of the men were regular smokers. Moreover, among those with a lifetime history of major depression, less than 14% of smokers were able to stop smoking and remain abstinent. Another study of 3023 individuals from the National Health and Nutrition Examination Survey also demonstrated a higher rate of smoking and a lower rate of smoking cessation with increasing levels of depression.⁴ Two biological actions of components of smoke provide further support for the self-medication hypothesis. Cigarette smoke contains a substance that inhibits

monoamine oxidase (MAO).^{5,6} It also contains nicotine, which is an agonist at nicotinic receptors.⁷ Interestingly, MAO inhibitors are effective antidepressants, and a recent study demonstrates antidepressant-like properties of nicotinic agonists in a rat behavioral model.^{8,9}

If tobacco has antidepressant properties, smoking might correct neurochemical deficits associated with depression; that is, the effects of smoking on brain biology might be similar to the effects of antidepressant drugs. Antidepressant drugs have profound effects on the neurochemistry of the noradrenergic locus coeruleus (LC). Long-term treatment of rats with antidepressant drugs of a variety of chemical and/or pharmacological classes down-regulates tyrosine hydroxylase in the LC.¹⁰ In contrast, repeated treatment of rats with psychoactive compounds lacking antidepressant activity does not affect LC tyrosine hydroxylase. Radioligand binding to α_2 -adrenoceptors is also reduced in the

SUBJECTS AND METHODS

Human brain tissue was obtained at autopsy at the Cuyahoga County coroner's office in Cleveland, Ohio, in accordance with an approved institutional review board protocol. Cadavers were refrigerated on arrival at the coroner's office and coded to protect identities. Causes of death were determined by the coroner (**Table 1**). Information on the lifetime and current (within the last month) psychiatric status, use of psychotropic medication, and illicit drug use of all subjects was obtained in structured clinical interviews^{14,15} with the next of kin. This information was used to identify smokers and to exclude a principal psychiatric diagnosis of major depression or schizophrenia. The interview used was the Schedule for Affective Disorders and Schizophrenia, Lifetime Version,¹⁶ supplemented with questions from the Diagnostic Interview Schedule¹⁷ to make diagnoses compatible with the *DSM-III-R*.¹⁸ Evaluation of drug and alcohol abuse and dependency was assessed using the Diagnostic Interview Schedule. Axis I diagnoses were made by a psychiatrist (H.Y.M.) and a clinical psychologist (J.C.O.) based on data gathered from the structured interview and, when available, hospital and physician's records. A toxicology screen of blood, bile, and urine from all of the subjects was performed by the Cuyahoga County coroner's office as described previously.^{19,20} Qualitative and quantitative assays were used to detect the following compounds or classes of compounds: ethanol, barbiturates, benzodiazepines, sympathomimetic drugs, and many antidepressant and antipsychotic drugs and their metabolites.

Information on smoking history was also collected in the interview (**Table 1**). Smokers were defined as people who smoked 20 or more cigarettes daily up until the time of death; nonsmokers were people with no history of cigarette smoking or tobacco chewing. Questions provided information about whether the subject was a cigarette smoker or chewing-tobacco user at the time of death, whether the subject had a history of smoking or chewing, whether the subject was exposed to secondhand smoke (including the nature and extent), the number of years since the subject had quit smoking or chewing (if a quitter), the number of cigarettes smoked per day, and the number of years that the individual smoked cigarettes or chewed tobacco.

rat LC following repeated antidepressant drug treatment.¹¹ These biological effects of antidepressants may be important for therapeutic efficacy because elevated levels of tyrosine hydroxylase¹² and increased radioligand binding to α_2 -adrenoceptors¹³ have been observed in the postmortem LCs of subjects diagnosed as having major depression, compared with psychiatrically healthy control subjects.

Because certain actions of the components of cigarette smoke resemble those of antidepressant drugs, the present study examined the possibility that long-term cigarette smoking might produce lower tyrosine hydroxylase and decreased α_2 -adrenoceptor binding in the human LC, similar to the effects of repeated antidepressant drug treatment on rats. Therefore, amounts of noradrenergic proteins in the LCs of subjects with a history of chronic smoking and of nonsmokers were compared.

Postmortem brain tissue samples containing the LC were obtained from 7 smokers and 9 nonsmokers (**Table 1**). One smoking and one nonsmoking subject had an adjustment disorder with depressed mood, and both had died of suicide. No other smoking or nonsmoking subjects had major psychiatric diagnoses. Ages ranged from 26 to 78 years (mean \pm SEM, 62 \pm 6 years) for nonsmokers and from 37 to 77 years (mean \pm SEM, 58 \pm 5 years) for smokers. Postmortem intervals were 6 to 24 hours (mean \pm SEM, 16 \pm 2 hours) for nonsmokers and 4 to 28 hours (mean \pm SEM, 18 \pm 3 hours) for smokers. Ages and postmortem intervals were not significantly different between smokers and nonsmokers. There were 8 men and 1 woman among the nonsmokers, and 4 men and 3 women in the group of smokers.

DISSECTION

Tissue blocks containing the LC were dissected as described previously^{19,20} and stored in an ultracold freezer (-83°C). Blocks were sectioned at 1-mm intervals (20- μm sections; -16°C) in a transverse plane perpendicular to the floor of the fourth ventricle, and sections were thaw-mounted onto gelatin-coated microscope slides. The LC was sectioned sequentially throughout its entire length beginning near its rostral end. The rostral border of the LC was defined by the frenulum, and the caudal border was the caudal extent of the LC (at the level of the motor nucleus of the trigeminal nerve), defined as the point at which neuromelanin-containing cells in the LC region were no longer visible.

QUANTITATIVE AUTORADIOGRAPHY

The specific binding of [³H]Ro41-1049 to MAO-A,²¹ p-[¹²⁵I]iodoclonidine ([¹²⁵I]PIC) to α_2 -adrenoceptors,²² and [³H]nisoxetine to the norepinephrine transporter¹⁹ was measured by quantitative receptor autoradiography. Tyrosine hydroxylase immunoreactivity was measured using a tissue transfer method.²³

Binding of [³H]Ro41-1049 to MAO-A

Sections were incubated for 60 minutes at 37°C with 20nM [³H]Ro41-1049 (18.5 Ci [68.45 \times 10¹⁰ Bq]/mmol) in a Tris

RESULTS

Amounts of tyrosine hydroxylase immunoreactivity in the LC, measured autoradiographically, were significantly lower in individuals with long-term smoking histories compared with nonsmoking controls. A repeated-measures ANOVA of 3 anatomically distinct levels of the LC revealed a significant difference at the middle level, with a trend toward significance at the rostral and caudal levels (**Table 2; Figures 1A and 2A**). Analysis of curves modeled to measurements of tyrosine hydroxylase at 1-mm intervals (**Figure 2A**) did not demonstrate a significant difference in quadrature between smokers and nonsmokers, but did demonstrate a significant difference between amounts of tyrosine hydroxylase ($t_{13} = 2.75$; $P = .02$). Using a quadratic model to compute differences, the maximum difference between smokers

buffer (pH, 7.4; Sigma Chemical Co, St Louis, Mo) containing 50mM Tris, 120mM sodium chloride (NaCl), 5mM potassium chloride (KCl), 14mM magnesium chloride (MgCl₂), and 0.5mM ethyleneglycotetraacetic acid. Sections were then washed 3 times (2 minutes each) in the ice-cold buffer. Nonspecific binding was determined with 1μM clorgyline.

Binding of [¹²⁵I]PIC to α₂-Adrenoceptors

Sections were pre-incubated in a Tris-magnesium (Tris-Mg) buffer (170mM Tris, 10mM MgCl₂; pH, 7.6) at 23°C for 60 minutes. Sections were then incubated for 90 minutes at 23°C with 300pM of [¹²⁵I]PIC (2200 Ci [8140 × 10¹⁰ Bq]/mmol) in the Tris-Mg buffer. Sections were then washed once for 10 minutes in the ice-cold buffer. Nonspecific binding of [¹²⁵I]PIC was determined with 10μM *l*-norepinephrine. The use of norepinephrine to define nonspecific binding eliminated the influence of imidazoline sites in the calculation of specific binding to α₂-adrenoceptors.

Binding of [³H]Nisoxetine to the Norepinephrine Transporter

Sections were incubated at 4°C for 4 hours with 3.0nM [³H]nisoxetine (82 Ci [303.4 × 10¹⁰Bq]/mmol) in a Tris buffer (pH, 7.4) containing 50mM Tris, 300mM NaCl, and 5mM KCl. Sections were then washed 3 times (5 minutes each) in the ice-cold buffer. Nonspecific binding was determined with 1μM mazindol.

Sections and brain mash-calibrated [³H] standards were apposed to [³H]-Hyperfilm (Amersham, Piscataway, NJ) and exposed in x-ray cassettes at room temperature for 20 hours for [¹²⁵I]PIC, 2 weeks for [³H]Ro41-1049, and 4 weeks for [³H]nisoxetine. Film was processed with the GBX developer and fixer (Eastman Kodak, Rochester, NY) at 17°C. After autoradiography, the same sections were lightly stained with cresyl violet for aid in LC identification. Densitometric measurements of autoradiograms were made using the Microcomputer Controlled Imaging Device (MCID M2; Imaging Research Inc, St Catharines, Ontario). Locus coeruleus autoradiograms were analyzed by simultaneously overlaying the image of the autoradiogram with the image of

the same histologically stained section. The smallest region encompassing all neuromelanin-containing cell bodies was outlined. Specific binding was defined as the difference between total and nonspecific binding. The binding of radioligands to the left and right sides of the LC was measured independently. Right- and left-side binding density was averaged because no significant difference in binding between sides was observed.

TYROSINE HYDROXYLASE IMMUNOREACTIVITY

Adjacent sections (in duplicate) at the same LC levels from the same subjects were transferred to an Immobilon-P membrane (Millipore Corp, Bedford, Mass), immunoblotted for tyrosine hydroxylase, and quantified autoradiographically using the MCID M2 as previously described.¹² Nissl-stained sections were used to identify borders of the cellular region of the LC. Optical densities of autoradiograms were standardized using an optical density step-wedge (Imaging Research Inc).

STATISTICAL ANALYSIS

Repeated-measures analysis of variance (ANOVA) models were used to investigate differences between smokers and nonsmokers. Measures of specific proteins were obtained at 1-mm intervals of the LC to assure anatomical alignment along its rostral-caudal axis for all subjects. The focus was the potential differences in the rostral, middle, or caudal portions of the LC because of their different projections, as noted in rat studies.²⁴⁻²⁶ Hence, measurements of the distance from the frenulum were averaged for the rostral (0.5-2.5 mm), middle (3.5-6 mm), and caudal (7-9 mm) portions for each subject. Mean values of these regions were compared for smokers and nonsmokers at each of the 3 positions, with adjustments for multiple testing using the Bonferroni adjustment. In addition to analyses of the 3 positions, we considered quadratic modeling of measures from all 1-mm intervals to determine where the maximum difference along the axis of the LC occurred between smokers and nonsmokers. To facilitate statistical analysis, measures were transformed to a natural logarithmic scale. Summary statistics are reported as the mean ± SEM for the transformed data. *P* < .05 was considered significant.

and nonsmokers appeared at a distance of 4 to 5 mm from the frenulum, where the mean tyrosine hydroxylase amount for nonsmokers was 4.7 times higher than for smokers. Traditional Western blot analysis was performed in homogenates of LC punches from some of the same subjects at the same LC levels to confirm that tyrosine hydroxylase content was lower in samples from smokers compared with those from nonsmokers (**Figure 3**).

The specific binding of [¹²⁵I]PIC to α₂-adrenoceptors was also significantly lower in the LCs of smokers compared with nonsmokers. Repeated-measures ANOVA of the 3 anatomically distinct levels of the LC revealed significant differences in amounts of [¹²⁵I]PIC binding between smokers and nonsmokers at rostral and middle levels, and a trend toward significance at the caudal level (**Table 3**; Figures 1B and 2B). Analysis of amounts of

[¹²⁵I]PIC binding at 1-mm intervals (Figure 2B) demonstrated a significant difference in quadrature (*P* < .001) and in amounts (*t*₁₄ = 2.93; *P* = .01) between smokers and nonsmokers. The maximum difference between smokers and nonsmokers, computed using quadratic modeling, appeared at a distance of 5 and 6 mm from the frenulum, where the mean amount of [¹²⁵I]PIC binding in nonsmokers was 1.8 times higher than that of smokers.

The binding of [³H]nisoxetine to the norepinephrine transporter (Figure 2C) and of [³H]Ro41-1049 to MAO-A (Figure 2D) in the LC was not significantly different between smokers and nonsmokers.

COMMENT

Our findings demonstrate a statistically significant association between long-term smoking and low levels of ty-

Table 1. Psychiatric Information on Study Subjects Obtained Through Psychiatric Autopsy*

Subject/Age, y/Sex	PMD, h	Smoking, y (No. of Packs per Day)	Cause of Death
NS-1/44/M	6		Myocardial infarction†
NS-2/71/M	23		Hypertensive sclerotic heart disease‡
NS-3/26/M	13		Homicide by gunshot
NS-4/78/F	11		Hypertensive cardiovascular disease, diabetes
NS-5/73/M	22		Myocardial infarction, cardiomegaly
NS-6/51/M	16		Coronary sclerotic heart disease
NS-7/77/M	24		Hypertensive cardiovascular disease, diabetes
NS-8/69/M	18		Hemopericardium by aortic aneurysm
NS-9§/73/M	8		Suicide by hanging
S-1/70/M	4	50 (1)	Pulmonary embolism
S-2/58/F	12	37 (1-2)	Hypertrophic cardiomyopathy
S-3/67/F	28	45 (1)	Myocardial infarction
S-4/37/M	26	27 (5)	Myocardial fibrosis, coronary atherosclerosis¶
S-5/47/M	17	29 (1)	Coronary sclerotic heart disease
S-6/50/F	27	37 (2)	Myocardial infarction
S-7§/77/M	14	40 (1)	Suicide by gunshot, chest

*PMD indicates postmortem delay; NS, nonsmoking subjects; and S, smoking subjects.

†Toxicology screen revealed ephedrine, phenylpropranolamine, and chlorpheniramine.

‡Toxicology screen revealed chlorpheniramine.

§Diagnosed as having adjustment disorder with depressed mood.

||Toxicology screen revealed codeine, lidocaine, and cyclobenzaprine.

¶Toxicology screen revealed ethanol and lidocaine.

Table 2. Analyses of Tyrosine Hydroxylase at 3 Anatomical Levels Along the Rostral-Caudal Axis of the Locus Coeruleus*

Subjects	Rostral LC	Middle LC	Caudal LC
Smokers	-0.11 ± 1.22	0.11 ± 1.10	0.27 ± 1.39
Nonsmokers	1.51 ± 1.14	1.84 ± 1.23	1.85 ± 1.12
<i>t</i> (<i>df</i> = 13)	2.65	2.86	2.44
<i>P</i>	.02	.01	.03
Adjusted <i>P</i>	.06	.04	.09

*Values are presented as mean ± SEM of the natural logarithms of amounts of tyrosine hydroxylase for smokers (*n* = 7) and nonsmokers (*n* = 8). Raw *P* values are based on 3 independent sample *t* tests, and *P* values are adjusted for multiple testing using the Bonferroni adjustment. LC indicates locus coeruleus.

rosine hydroxylase and α_2 -adrenoceptor binding in the human LC. One interpretation of these data is that long-term smoking, either through direct or indirect effects of components of tobacco smoke, down-regulates tyrosine hydroxylase and α_2 -adrenoceptors in the LC. The putative effects of smoking appear to be relatively specific to these 2 proteins; smoking did not affect radioligand binding to the norepinephrine transporter or MAO in the same subjects. Furthermore, putative smoking-induced effects appear to be widespread in the LC and are not limited to a single LC subregion. Because the LC is topographically organized with respect to its projections,²⁴⁻²⁶ this latter finding implies widespread effects of cigarette smoking on noradrenergic activity in the central nervous system.

A limitation of this study is that it is not possible to determine whether cigarette smoking causes reductions in levels of tyrosine hydroxylase and α_2 -adrenoceptors or, alternatively, if this biochemical phenotype predisposes an individual to the acquisition of a smoking habit.

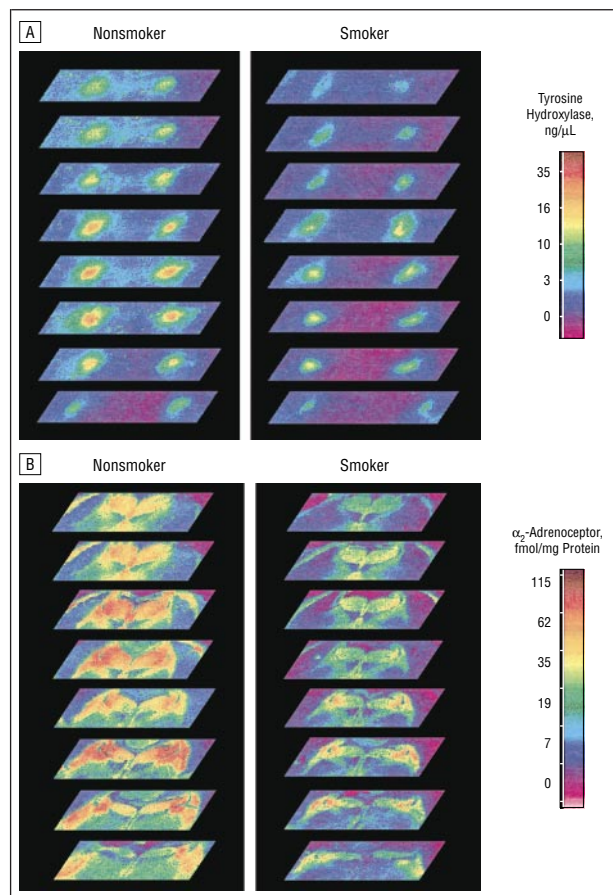


Figure 1. Digitized autoradiograms of tyrosine hydroxylase immunoreactivity (A) and of the specific binding of *p*-[¹²⁵I]iodoclonidine to α_2 -adrenoceptors (B) at multiple levels along the rostral-caudal axis of the locus coeruleus (LC) from a nonsmoking subject (left panel, subject NS-2) and from an age-matched subject who was a cigarette smoker (right panel, subject S-1). Panels are oriented so that the top is rostral and the bottom is caudal along the LC axis.

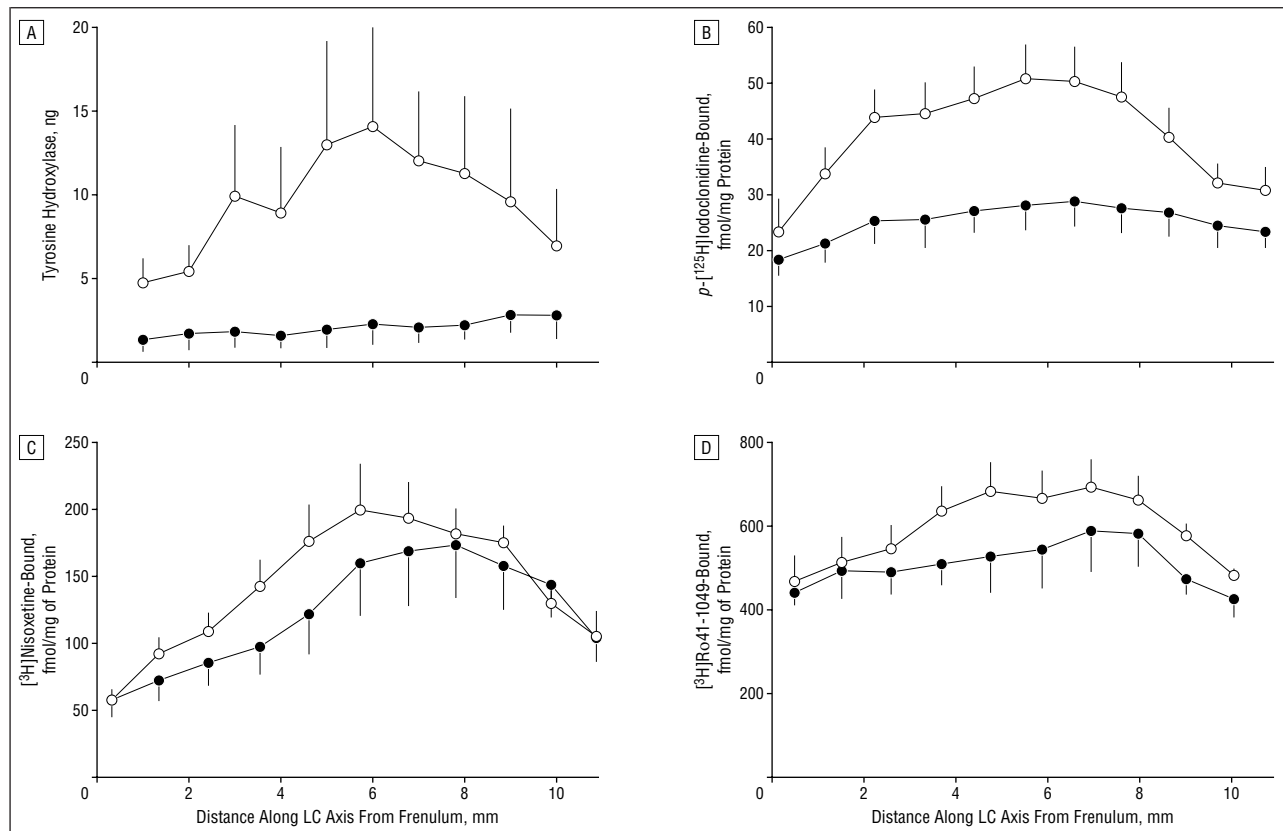


Figure 2. The distribution of tyrosine hydroxylase immunoreactivity (A), specific binding of p -[125 I]iodoclonidine to α_2 -adrenoceptors (B), specific binding of [3 H]nisoxetine to the norepinephrine transporter (C), and specific binding of [3 H]Ro41-1049 to monoamine oxidase A (D) along the rostral-caudal axis of the human locus coeruleus (LC) of smokers (solid circles; $n=7$) and nonsmokers (open circles; $n=8$ or 9). Values are the means of amounts of each group determined by the average of 4 estimations (left and right sides of the LC, both in duplicate) made for each subject. The abscissa is the distance from the frenulum along the rostral-caudal axis of the LC. Density readings of tyrosine hydroxylase were transformed to natural logarithms because of the departure of their distributions from normality ($P<.05$).

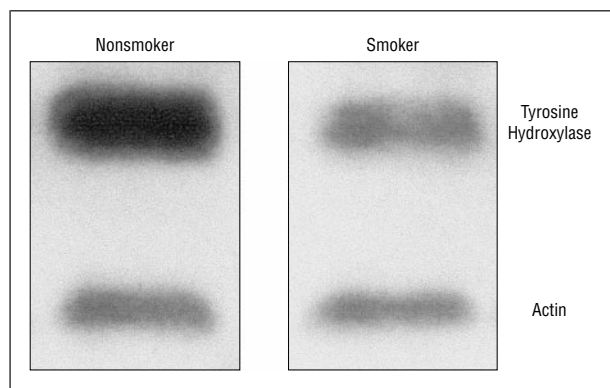


Figure 3. Western blotting of tyrosine hydroxylase immunoreactivity in locus coeruleus (LC) tissues from a nonsmoker and a smoker (same subjects as shown in Figure 1). For Western blotting, each well was loaded with 50 μ g of total protein from the middle level of the rostral-caudal extent of the LC. The bottom panel (left and right) shows immunoreactive actin probed with antiactin antibody on the same blots as a control for protein loading and transfer.

Further studies in humans with smoking histories and in rats exposed to long-term smoke are required to determine whether there is a cause or effect relationship. Another limitation of the study is that there were more women in the smoking group than in the nonsmoking group. To date, a relationship between sex and levels of tyrosine hydroxylase or α_2 -adrenoceptors in the LC has

Table 3. Analyses of α_2 -Adrenoceptor Binding at 3 Anatomical Levels Along the Rostral-Caudal Axis of the Locus Coeruleus*

Subjects	Rostral LC	Middle LC	Caudal LC
Smokers	3.04 \pm 0.49	3.26 \pm 0.42	3.26 \pm 0.40
Nonsmokers	3.63 \pm 0.36	3.83 \pm 0.37	3.75 \pm 0.34
t ($df = 14$)	2.76	2.86	2.62
P	.02	.01	.02
Adjusted P	.05	.04	.06

*Values are presented as mean \pm SEM of the natural logarithms of amounts of p -[125 I]iodoclonidine binding to α_2 -adrenoceptors for smokers ($n = 7$) and nonsmokers ($n = 9$). Raw P values are based on 3 independent sample t tests, and P values are adjusted for multiple testing using the Bonferroni adjustment. LC indicates locus coeruleus.

not been observed, although studies have not been specifically designed to address these potential effects.

It is interesting to compare the putative effect of long-term smoking on human LC biochemistry with effects produced by drugs known to modulate LC biochemistry in rats. Repeated treatment of rats with antidepressant drugs of multiple chemical classes down-regulates tyrosine hydroxylase in the LC, an effect not observed following treatment with nonantidepressant compounds.¹⁰ Long-term treatment of rats with the smoking cessation and antidepressant drug bupropion also

down-regulates LC tyrosine hydroxylase. Repeated treatment of rats with antidepressant drugs also reduces α_2 -adrenoceptor binding in the LC.¹¹ In contrast to the effects on tyrosine hydroxylase and α_2 -adrenoceptors, repeated treatment of rats with the antidepressant desipramine or repeated electroconvulsive shock has no effect on [³H]nisoxetine binding to the norepinephrine transporter in the LC.²⁷ The known effects of antidepressant drug treatment on rat LC biochemistry are remarkably similar to the putative effects of smoking on human LC biochemistry.

Recently, elevated levels of tyrosine hydroxylase¹² and higher amounts of α_2 -adrenoceptor binding^{13,28} have been observed in the LCs of subjects with major depression and who commit suicide compared with normal control subjects. The present association of long-term smoking with reduced levels of LC tyrosine hydroxylase and α_2 -adrenoceptor binding is opposite to changes in the levels of these proteins in major depression in humans. Together, preclinical and clinical findings suggest that cigarette smoking produces antidepressant-like effects on central noradrenergic neurons.

One of the known biological actions of tobacco that links smoking to depression is the inhibition of MAO. MAO inhibitors, particularly MAO-A inhibitors, are effective antidepressant drugs.²⁹ The activities of MAO-A and MAO-B are inhibited when rats are exposed to tobacco smoke but not when they are exposed to nicotine alone.³⁰ Similarly, an aqueous extract of cigarette smoke, or saliva obtained after smoking, can irreversibly inhibit the action of MAO on a variety of the enzyme's substrates in rat lung tissue.⁵ Significant decreases in MAO-A and MAO-B in the brains of smokers relative to nonsmokers or former smokers has recently been demonstrated using in vivo positron emission tomography imaging with radiotracers specific for MAO-A and MAO-B.^{31,32} In our study, radioligand binding to MAO-A was reduced modestly but not significantly in the LCs of smokers relative to nonsmokers. The lack of significant changes in MAO-A binding could be a function of an insufficient sample size. Also, the time of death relative to the last cigarette smoked was not known for the subjects included in this study and would probably be highly variable, possibly contributing to variability in MAO binding levels.

Long-term inhibition of MAO by components of cigarette smoke could produce effects similar to those observed in our study, particularly given that long-term treatment of rats with MAO inhibitors down-regulates LC tyrosine hydroxylase.¹⁰ Another biological component of tobacco that could potentially induce changes in LC biochemistry is nicotine. Nicotine increases the LC firing rate and stimulates the release of norepinephrine from LC neurons.^{33,34} A single dose of nicotine increases tyrosine hydroxylase messenger RNA in the LC.³⁵ Nicotinic agonists have demonstrated antidepressant-like effects in an animal model of depression.^{8,9} Unfortunately, the long-term effects of nicotine on LC activity and biochemistry have not been studied.

This report is the first direct observation in the human brain (postmortem) that long-term cigarette smoking is associated with neurochemical abnormalities in the

noradrenergic LC. These changes are opposite to those observed in the LC in major depression^{12,13} and are similar to the effects observed in animals repeatedly treated with antidepressant drugs. Such data argue that the high incidence of smoking in patients with major depression and the difficulty with smoking cessation among this group might be a partial result of smoking-induced neurochemical "corrections" of biological abnormalities associated with this disorder. This evidence cannot justify the use of tobacco in these individuals because of the adverse effects of smoking. However, a thorough understanding of the neurochemical basis for the high incidence of smoking in people with depression is needed to develop better therapies for smoking cessation, particularly among those with major depression.

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