Cigarette Smoke and Dopaminergic System

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ABSTRACT: It has been reported that there is an ameliorative effect of cigarette smoking on certain neurological responses and neurodegenerative disorders. The purpose of this study was to examine the neurochemical and neurobehavioral response of cigarette smoke (CS) in the adult male guinea pig brain. Both acute and chronic CS exposure enhanced locomotor behavior and caused a decrease in midbrain dopamine (DA) levels and corresponding increase in 3,4-dihydroxyphenylacetic acid (DOPAC) levels. In addition, CS caused a significant increase in the protein levels of the dopamine D_1 and D_2 receptors. CS caused a significant increase in the binding capacity of the D_1 receptor and a significant decrease in the binding capacity of D_2. Furthermore, CS caused a significant increase in the binding capacity of the dopamine transporter (DAT). The mechanism by which cigarette smoke exposure increases locomotor activity remains to be elucidated but may include modulation of dopamine neuron activity that emerges after repeated direct smoke exposure. © 2007 Wiley Periodicals, Inc. J Biochem Mol Toxicol 21:325–335, 2007; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10:1002/jbt.20197

KEYWORDS: **Cigarette Smoke Exposure; Neurobehavioral Function; Dopaminergic Neuron System; Locomotor Activity; Guinea Pig**

INTRODUCTION

Cigarette smoking is an established risk factor for cancer and cardiovascular disease and is the leading cause of avoidable disease in most industrialized countries [1]. However, cigarette smoking also reduces anxiety, suppresses appetite, and enhances arousal [2]. Furthermore, cigarette smoke may exert a neuroprotective effect on neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) [3,4].

Cigarette smoke is composed of thousands of chemical compounds, including nicotine, the addictive substance of tobacco. Nicotine is the major pharmacologically active component of cigarette smoke. Locomotor activity is widely used to study nicotine's behavioral actions in rodents [5,6]. Furthermore, locomotor activity, especially horizontal activity, has been used to determine genetically based difference in nicotine's actions in various strains of rats and mice [7–11]. One study by Janhunen et al. reported that nicotine stimulates locomotor activity in rats [10]. Furthermore, nicotine has been shown to stimulate locomotor activity in habituated rats in a dose-dependent manner [12,13].

The control of motor behavior is regulated by a neurotransmitter network, which involves glutamatergic and GABAergic inputs from the motor cortex and striatum, respectively [14]. These pathways are modulated by D_1 and D_2 receptors innervated by the substantia nigra. Together, these pathways are responsible for controlling the outflow of the thalamus to the cortex and striatum, thereby controlling motor behavior [15]. This cortico-striatal-thalamo-cortical (CSTC) circuit is interchangeably referred to as the motor loop. Imbalances of the CSTC circuit are associated with neurological disorders, such as obsessive compulsive disorder (OCD), Parkinson's disease (PD), Huntington's disease (HD), and attention deficit hyperactivity disorder (ADHD) [16].

It is well established that the dopaminergic system is a target site of action for nicotine. Several studies have suggested that nicotine stimulates dopamine release [17–19]. For example, in response to 1, 5, and 10 μM nicotine, the dopamine output from superfused rat corpus striatal tissue increased [20]. Rahman et al. reported that acute nicotine injections produced an increase in extracellular dopamine levels in the rat nucleus accumbens [21]. However, the mechanism by

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which nicotine causes dopamine elevation within the brain remains to be fully elucidated.

The present study investigates the effects of cigarette smoke on locomotor activity and on the dopaminergic neuron system in a guinea pig model. Since the dopaminergic system is known to play an intricate role in the control of locomotion, we also examined the effects of cigarette smoke on dopamine and DOPAC levels; D_1, D_2, and dopamine transporter (DAT) protein levels and their ligand-binding capacity. The guinea pig model is used because consistent circadian rhythm has been established [22]. Furthermore, we have standardized earlier the dose used in this study [23]. We hypothesized that changes in locomotor behavior are indicative of alterations in the dopaminergic system. This is the first study to analyze locomotor behavior and the dopaminergic neuron system in a guinea pig model of exposure to cigarette smoke.

MATERIALS AND METHODS

Materials

Hartley strain male guinea pigs (275–300 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). All biochemicals were purchased from Sigma. [^{3}H]SCH23390, [^{3}H]spiperone, and [^{3}H]GBR12935 were purchased from DuPont New England Nuclear Research Products (Boston, MA). Nonradioactive SCH23390, butaclamol, and GBR 12909 were purchased from Tocris Bioscience (Ellisville, MS).

Exposure of Guinea Pigs to Cigarette Smoke

Adult male guinea pigs (Hartley strain), 4 weeks of age, weighing 275–300 g, were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Animals were divided into two groups of eight animals each: group I maintained as sham control (SH) and group II maintained as mainstream smoke exposed (MS). The animals were housed in individual stainless steel cages in a temperature-controlled (26°C) 12/12 h light/dark cycle room. They were fed a complete life-cycle diet (Purine diet #5025). Group II animals were exposed daily for 7 weeks to MS smoke from IR3F cigarettes in a University of Kentucky Tobacco and Health Research Institute (THRI) MS-SS smoke exposure system described in detail elsewhere [22]. The animals were exposed to smoke from three cigarettes per session (~1 hour/session) twice a day. The sham controls received an identical treatment to smoke-exposed groups but in the absence of cigarette smoke to simulate stress conditions. All animals received human care, and this study complies with the institutions guidelines and regulations regarding technical specifications for care and use of laboratory animals.

Locomotor Activity

Animals were individually placed in activity cages ($42 \times 42 \times 30$ cm Digiscan Animal Activity System, Omnitech Electronics, Columbus, OH), for a 7-day 2-h habituation period. Thereafter, the animals underwent a daily 1-h habituation period (basal) before exposure to cigarette smoke and the activity during the habituation period was recorded. Immediately after each smoke exposure, the animals were returned to the activity cages for monitoring locomotor behavior. Animals were individually placed in photocell cages, and locomotor activity was measured. The cages were equipped with 32 photobeams (16 front to back and 16 side to side, every 2.5 cm) located above the floor to measure locomotor activity (horizontal, vertical, total distance). Horizontal activity was measured with 16 photocells located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 2 cm above the cage floor. Vertical activity was measured by another set of 16 horizontal beams, 4 cm above the cage floor. Experimental data were collected using a microprocessor equipped with software and processed by the Digiscan Analyze and Digiscan Digipro System (Digipro, Columbus, OH). Locomotor behavior was recorded for 60 min at 15 min intervals. Effect of acute exposure is defined as locomotor behavior on test day 1; and chronic exposure defined as locomotor behavior over the 7-week period. The results are expressed as shown minus basal activity.

High-Performance Liquid Chromatography

Guinea pig brain was rapidly removed, dissected, and stored at −80°C until assayed for dopamine and DOPAC according to Lowry et al. [23]. The tissue from the midbrain region was homogenized in 3 volumes (w/v) of ice-cold 0.4 M perchloric acid, using a PRO 200 double insulated homogenizer (Pro Scientific, Monroe, CT). The homogenates were centrifuged (9000g for 20 min at 4°C); the supernatant layer was removed into a 1-mL syringe and then filtered through a 0.22-μm Millipore filter. Thirty microliters of each sample was injected into a hypersil ODS C-18 reverse phase column connected to the HPLC-EC system (5 μm, 250 mm × 4.6 mm, Whatman EQC). A dual electrochemical detector (Coulochem II, ESA) was set with 350 mzv at the guard cell and $E_1 = -50$ mV, $E_2 = 500$ mV at the analytical cell. The mobile phase consisted of a 0.1 M sodium acetate, 60 mM citric acid, 0.6 mM octanesulfonic acid sodium salt, 0.5 mM disodium EDTA, in 15% methanol in water, adjusted

to pH 3.5 and pumped at a rate of 1.1 mL/min. Chromatographic data were analyzed with a data module integrator and quantified using the peak area of ratio of the internal standard.

SDS–PAGE Electrophoresis and Western blot Analysis

Frozen samples of brain tissue from the midbrain region (~100 mg) were thawed and homogenized in 1 mL of phosphate buffer saline (PBS), pH 7.4 containing 0.1 EDTA, 0.1 mM EGTA, 0.5% SDS, 0.1 mM DTT containing 2 μg/mL of the protease inhibitors, leupeptine, aprotinin, and pepstatin for 2 min. Protein concentrations of the tissue lysates were determined according to the modified Lowry method [24]. Twenty micrograms of protein were resolved under a reducing condition on a 12% SDS-polyacrylamide gel. After electrophoresis, the proteins were transblotted to nitrocellulose membranes (Hybon ECL, Amersham Pharmacia Biotech, Amersham, Piscataway, N.J.). Non-specific-binding sites were blocked with 5% nonfat dry milk and then incubated at 4°C overnight with either goal polyclonal antibody against either D_1 or D_2 dopamine receptor (Santa Cruz Inc, Santa Cruz, CA). After washing with Tris-buffered saline-Tween buffer, the membrane was incubated with horseradish peroxidase donkey anti-goat IgG (1:5000) for 1 h at room temperature. The membranes were subsequently washed twice and developed with enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Amersham) according to the instructions of the manufacturer. The membranes were then exposed to X-ray film (Phenix, Hayward, CA), which was subsequently developed. The image was digitalized with a Biorad digital camera and quantified the surface and intensity of each band. The membrane was re-probed for β-actin protein as positive control and results were expressed in relative units.

Receptor-Binding Assay

D_1 Receptor Binding Assay

Frozen samples of midbrain tissue were thawed and homogenized in ice cold 50 mM Tris–HCl buffer at pH 7.4. The ligand-binding assay for D_1 dopamine receptors was performed according to the method of Billard et al. [25]. The homogenates were centrifuged at 20,000*g* for 10 min at 4°C. The pellet was washed and suspended in 50 mM Tris–HCl buffer, pH 7.4 containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$. The resulting crude membrane preparation was used for binding assays. The binding of [^{3}H]SCH23390 was determined in a volume of 0.25 mL, containing 10 μL of 4 nM [^{3}H]SCH23390, 10 μL of SCH23390 (10 μM or buffer), and 210 μL of assay buffer (50 mM Tris buffer, pH 7.4, containing 1 mM EDTA, 0.1% ascorbic acid, and 1 μM ketanserine). The reaction was initiated by the addition of 20 μL crude brain membrane (50–100 μg protein) and incubated at 37°C for 30 min. The reaction was terminated by rapid filtration over Whatman GF/B strips using Brandel cell harvestor (Biomedical Research and Development Laboratories, Gaithersburg, MD) and washed twice with 2 mL of ice cold 50 mM Tris–HCl buffer. The radioactivity retained by the filters was measured in a Beckman S-355 (Beckman Coulter, Fullerton, CA) ligand scintillation counter using 5 mL of UniverSol (ICN, Costa Mesa, CA) as a scintillant. The specific binding of [^{3}H]SCH23390 was defined as the differences in the binding obtained in the presence and absence of SCH23390.

D_2 Receptor Binding Assay

Frozen samples of midbrain tissue were thawed and homogenized in ice cold 50 mM Tris–HCl buffer at pH 7.4. The ligand-binding assay for the D_2 receptor was performed according to Pugsley et al. [26]. The homogenates were centrifuged at 20,000*g* for 10 min at 4°C. The pellet was washed and suspended in 50 mM Tris–HCl buffer containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$ at pH 7.4. The resulting crude membrane preparation was used for binding assays. The binding of [^{3}H]spiperone was determined in a volume of 0.25 mL containing 10 μL of 4 nM [^{3}H]spiperone, 10 μL butaclamol (5 μM or buffer), and 210 μL of assay buffer (50 mM Tris–HCl buffer containing 1 mM EDTA, 0.1% ascorbic acid, and 1 μM ketanserine). The reaction was initiated by the addition of 20 μL crude brain membrane (50–100 μg protein) and incubated at 37°C for 30 min. The reaction was terminated by rapid filtration over Whatman GF/B strips using Brandel cell harvestor (Biomedical Research and Development Laboratories, Gaithesburg, MD) washed twice with 2 mL of ice cold 50 mM Tris–HCl buffer. The radioactivity retained by the filters was measured in a Beckman S-355 as described above. The specific binding of [^{3}H]spiperone was defined as the differences in the binding obtained in the presence and absence of butaclamol.

Dopamine Transporter Binding Assay

Frozen samples of midbrain tissue were thawed and homogenized in 0.32 M sucrose at pH 7.4. The ligand-binding assay for DAT binding was performed according to the method of Akunne et al. [27]. The homogenates were centrifuged at 20,000*g* for 10 min at 4°C. The pellet was washed and suspended in 55.2 nM $NaPO_4$ at pH 7.4. The resulting crude membrane preparation was used for binding assays. The binding of

[^{3}H]GBR12935 was determined in a volume of 0.25 mL, containing 10 μL of 4 nM [^{3}H]GBR12935, 10 μL GBR 12909 (10 μM or buffer), and 210 μL of assay buffer (55.2 nM $NaPO_4$). The reaction was initiated by the addition of 20 μL crude brain membrane (50–100 μg protein) and incubated for 2 h at 25°C. The reaction was terminated by rapid filtration over Whatman GF/B strips (presoaked in 0.5% polyethylemine for at least 1 h) using a Brandel cell harvester and washed twice with 2 mL of ice-cold 55.2 nM sodium phosphate buffer, pH 7.4 and radioactivity was measured as described above. The specific binding of [^{3}H]GBR12935 was defined as the differences in binding obtained in the presence or absence of GBR 12909.

Statistical Analysis

The mean and standard error of the mean (SEM) were determined for each set of data and one-way analysis of variance (ANOVA) or student *t*-Test for statistical analysis. Differences were considered statistically significant when $p \leq 0.05$.

RESULTS

Locomotor Activity

Figure 1 shows the acute effects of cigarette smoke on locomotor activities (horizontal activity, vertical

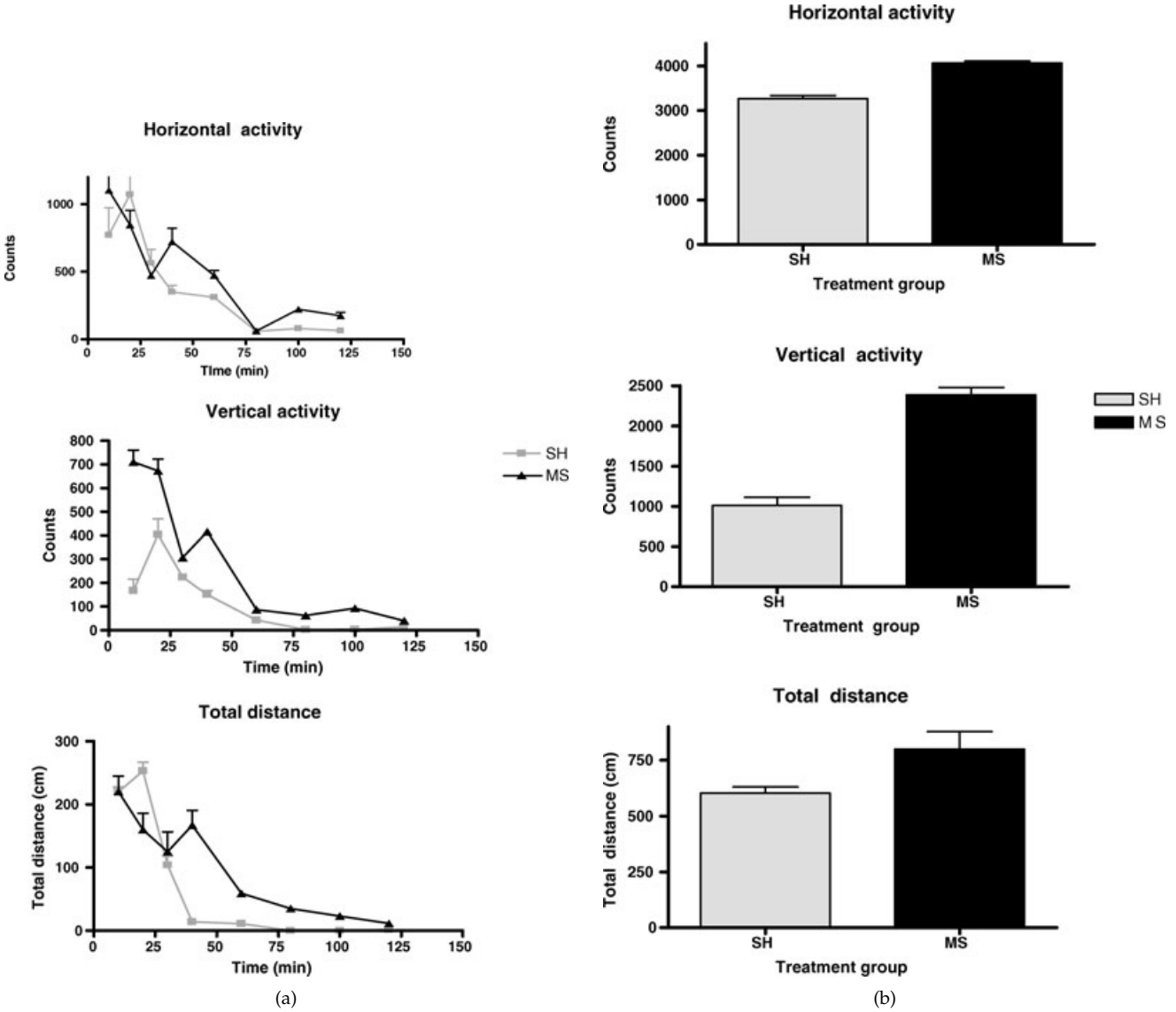

FIGURE 1. Acute effects of cigarette smoke exposure on locomotor activities in male guinea pig brain on day 1. (a) Locomotor activities (horizontal activity, vertical activity, and total distance) were measured at 15 min intervals and (b) total locomotor activities (horizontal activity, vertical activity, and total distance). Values are expressed as means ± SEM ($N = 8$). One-way ANOVA followed by student *t*-Test was used for statistical analysis to compare different groups. $^*p < 0.05$ indicates significance compared to the control. Abbreviations: SH, sham; MS, mainstream.

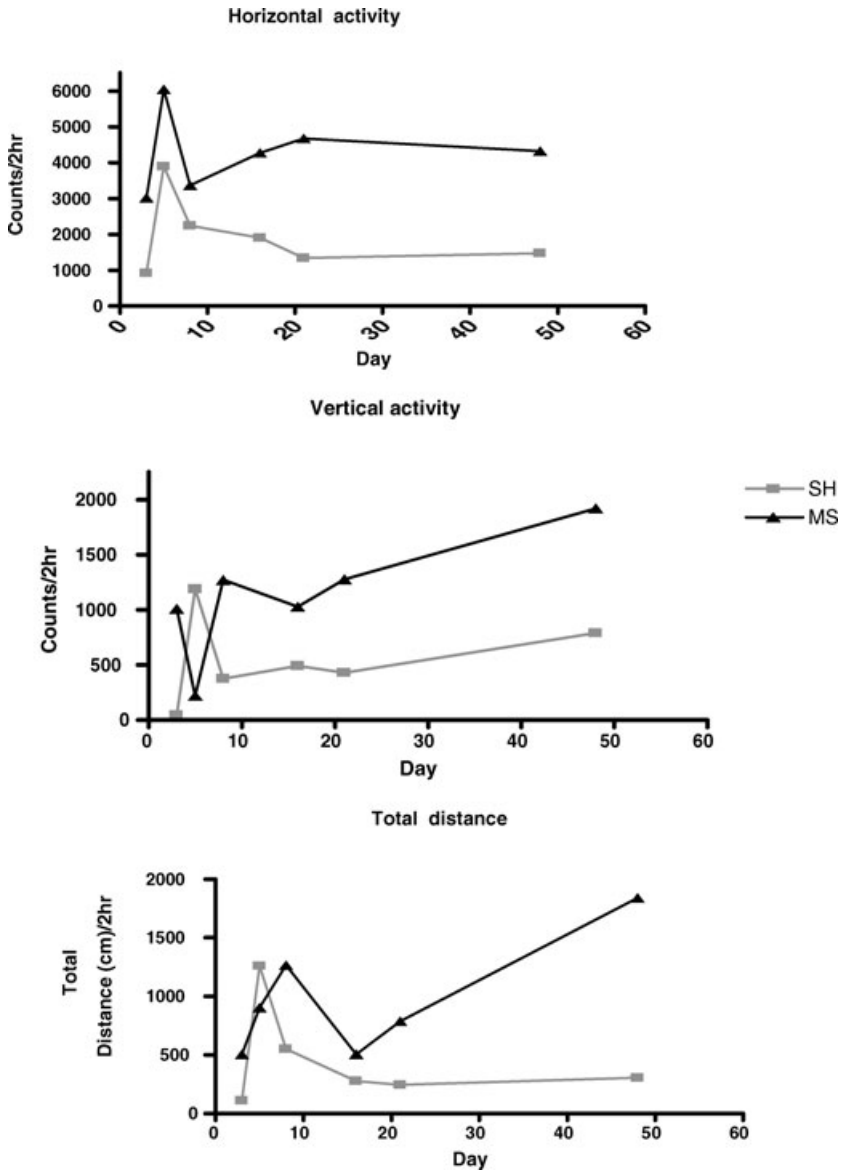

FIGURE 2. Chronic effects of cigarette smoke exposure on locomotor activities in male guinea pig brain after 6 weeks twice daily treatment, locomotor activities (horizontal activity, vertical activity, and total distance) were measured. Values are expressed as means ± SEM ($N = 8$). One-way ANOVA followed by student t-Test was used for statistical analysis to compare different groups. $^{*}p < 0.05$ indicates significance compared to the control. Abbreviations: SH, sham; MS, mainstream.

activity, and total distance) on test day 1. In this experiment, a 7-day 2-h habituation period was utilized to acclimatize the animal to its new environment and stress due to handling. Furthermore, a 1-h habituation period was used prior to each cigarette smoke exposure. Acute cigarette smoke exposure increased the locomotor activities when compared to the sham group. However, the greatest activity counts were observed during the first 30 min and gradually declined over the 2-h sampling period.

After the guinea pigs were repeatedly exposed to cigarette smoke, its effects on the locomotor activities were similar to those after acute cigarette smoke exposure. Furthermore, relative to the sham group, there was an overall significant increase in locomotor activity following 7 weeks of smoke exposure (Figure 2). Thus, chronic cigarette smoke exposure caused a significant increase in locomotor activities (horizontal activity, vertical activity, and total distance) in the adult male guinea pig. Furthermore, Figure 2 also demonstrates that the stimulant effects of cigarette smoke are significantly enhanced after repeated exposure in comparison to Figure 1.

It has been shown that cigarette smoke exposure stimulates locomotor activity. We hypothesize that the stimulating effect of cigarette smoke is due to its effect

on the dopaminergic neuron system. To explain our hypothesis, we further examined the effect of CS on the dopaminergic neuron system.

Effects of Cigarette Smoke Exposure on the Dopamine Turnover Rate in the Adult Male Guinea Pig

Figure 3 depicts the effect of cigarette smoke exposure on dopamine and DOPAC levels in the midbrain region of the guinea pig. After 7 weeks of cigarette smoke exposure, the animals were sacrificed and the midbrain region isolated. Cigarette smoke exposure decreased dopamine (DA) levels in the midbrain region of the adult male guinea pig by 69.6%. Furthermore, cigarette smoke exposure increased 3,4-dihydroxyphenylacetic acid (DOPAC) levels within the midbrain region by 22% (Figure 3). Thus, cigarette smoke exposure increased dopamine turnover significantly, as evidenced by an increase in the ratio of DOPAC/DA. The ratio was 0.16 ± 0.03 and 0.63 ± 0.03, for SH and MS groups, respectively, resulting in an increase in the turnover rate following repeated exposure to smoke. The *t*-test revealed a significant effect of cigarette smoke on DA and DOPAC levels ($p < 0.05$). To determine the specificity of the effects of cigarette smoke exposure on the midbrain region, other regions of the guinea pig brain were also tested. The results revealed that cigarette smoke did not cause a significant change on the levels of DA and its metabolite DOPAC in the other regions of the brain (data not shown).

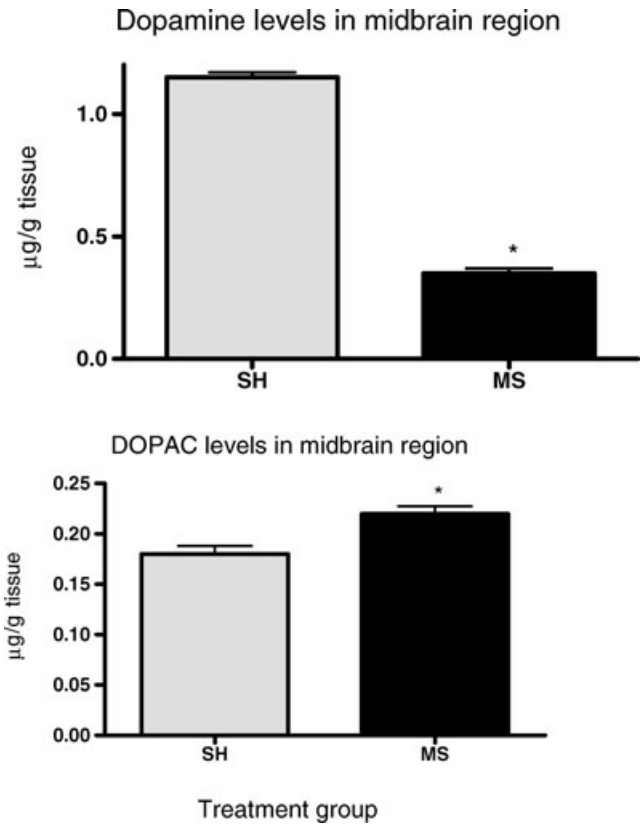

FIGURE 3. Effect of cigarette smoke exposure on dopamine and DOPAC levels in the midbrain of the guinea pig brain. Chromatographic data were analyzed with a data module integrator and quantified using the peak area ratio of the internal standard. Error bars represent SEM, $n = 8$ per group. Student *t*-test was used for statistical analysis to compare control and treatment group. Significance compared to the control indicated as $^{*}p < 0.05$. Abbreviations: SH, sham; MS, mainstream; DOPAC, 3,4-dihydroxyphenylacetic acid.

Effects of Cigarette Smoke Exposure on the Dopamine D_1, D_2 Receptor, and DAT

Western blot analysis was used to evaluate the level of dopamine D_1 and D_2 receptor proteins in the midbrain region after cigarette smoke exposure. Specifically, the D_1 and D_2 signal was seen as a band of approximately 74 and 48 kDa, respectively (Figures 4 and 5). Cigarette smoke exposure caused an increase in the protein levels of both D_1 and D_2 dopamine receptors. We do not know at this time whether an increase in the levels of DOPAC is causal for the increases in the protein levels of the dopamine receptors.

Furthermore, to determine whether there was a change in the uptake or binding of dopamine to its receptors after cigarette smoke exposure, we measured the binding characteristics of both receptors (D_1 and D_2) as well as dopamine transporter after 7 weeks of cigarette smoke exposure. The Scatchard analysis for D_1 indicates a K_d of 1.53 nM and a B_{max} of 1.86 pmol/mg of protein for the SH group and a K_d of 1.39 nM and a B_{max} of 2.88 pmol/mg of protein for the MS group (Table 1). However, for D_2, a K_d of 5.42 nM and a B_{max} of 5.68 pmol/mg of protein for the SH group and a K_D of 3.81 nM and a B_{max} of 2.57 pmol/mg of protein for the MS group was observed (Table 2). The results reveal that while the binding capacity of the D_1 receptor was increased by 54.8%, D_2 receptor binding capacity was decreased by 54.8% (Figure 6).

TABLE 1. Kinetic Parameters of [^{3}H]23390 Binding to Sham and Mainstream Smoke-Exposed Guinea Pig Brain

Group	K_D *(nM)*	B_{max} *(pmol/g tissue)*
Sham	1.529 ± 0.3348	1.862 ± 0.1223
Mainstream	1.394 ± 0.5273	$2.883 \pm 0.3193^{*}$

B_{max} represents mean values ± SEM from three separate experiments performed in triplicate. $^{*}p \leq 0.05$ compared to control; ($n = 6$ animals).

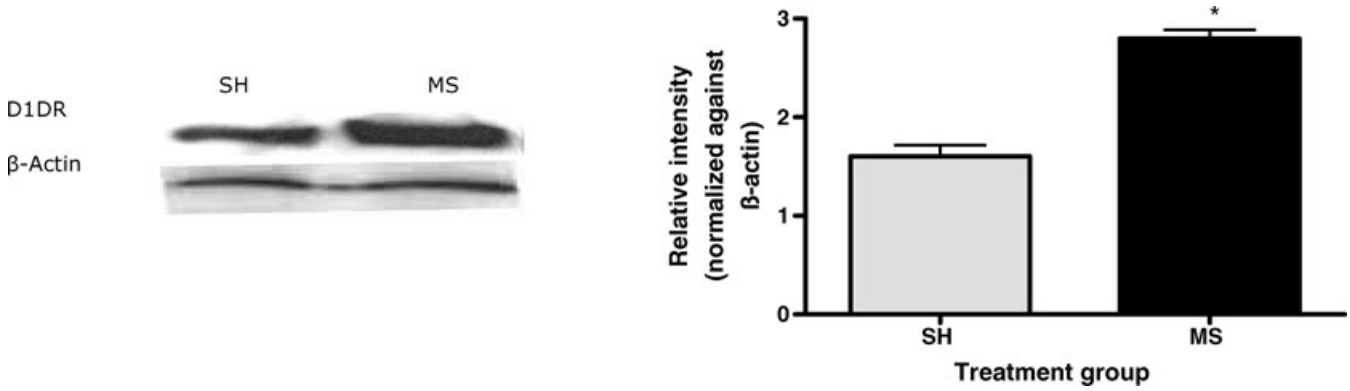

FIGURE 4. Effect of cigarette smoke exposure on the protein levels of dopamine D_1 receptor band intensity is expressed as a ratio relative to the corresponding β-actin band. Error bars represent SEM, $n = 8$ per group. Student t-Test was used for statistical analysis to compare different groups. $^*p < 0.05$ indicates significance compared to the control. Abbreviations: SH, sham; MS, mainstream.

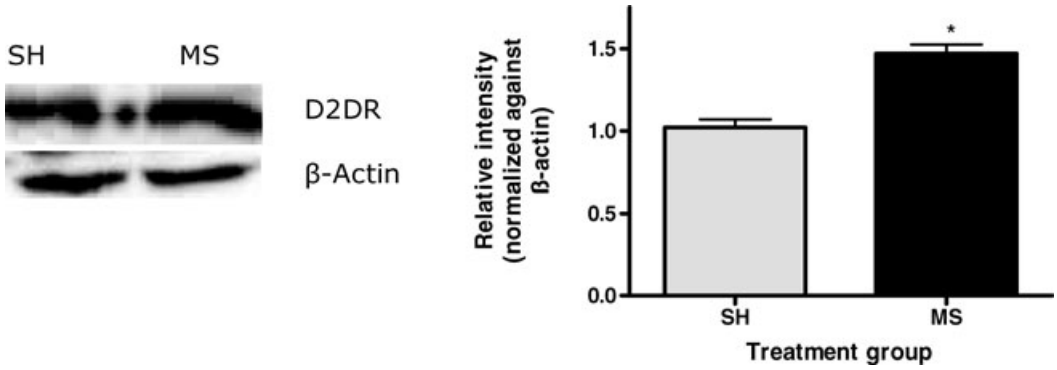

FIGURE 5. Effect of cigarette smoke exposure on the protein levels of dopamine D_2 receptor band intensity is expressed as a ratio relative to the corresponding β-actin band. Error bars represent SEM, $n = 8$ per group. Student t-Test was used for statistical analysis to compare different groups. $^*p < 0.05$ indicates significance compared to the control. Abbreviations: SH, sham; MS, mainstream.

The DAT capacity binding was also increased by 52.7% due to smoke exposure (Figure 7). The Scatchard analysis for DAT indicates a K_d of 10.80 nM and a B_{max} of 0.78 pmol/mg of protein for the SH group and a K_d of 11.28 nM and a B_{max} of 1.65 pmol/mg of protein for the MS group (Table 3).

These findings confirm that cigarette smoke alters dopaminergic neuron activity by increasing dopamine turnover and altering the receptor level and/or binding capacity in the midbrain region of the guinea pig.

DISCUSSION

Acute and chronic exposure to cigarette smoke induced an increase in locomotor activity in guinea pigs habituated to the test environment. The present study demonstrates that the enhancement of locomotor activity in the adult male guinea pig occurs due to daily cigarette smoke exposure. Furthermore, cigarette smoke exposure caused an increase in dopamine turnover, by modulating dopamine metabolism. Cigarette smoke exposure also caused a

TABLE 2. Kinetic Parameters of [^{3}H]spiperone Binding to Sham and Mainstream Smoke Exposed Guinea Pig Brain

Group	K_D *(nM)*	B_{max} *(pmol/g tissue)*
Sham	5.415 ± 1.317	5.682 ± 0.5887
Mainstream	3.805 ± 0.214	2.572 ± 0.3139*

$B_{max\ represents}$ mean values ± SEM from three separate experiments performed in triplicate. $^*p \leq 0.05$ compared to control; ($n = 6$ animals).

TABLE 3. Kinetic Parameters of [^{3}H]GBR12935 Binding to Sham and Mainstream Smoke Exposed Guinea Pig Brain

Group	K_D *(nM)*	B_{max} *(pmol/g tissue)*
Sham	10.80 ± 1.11	0.784 ± 0.34
Mainstream	11.28 ± 0.854	1.648 ± 0.54*

B_{max} represents mean values ± SEM from three separate experiments performed in triplicate. $^*p \leq 0.05$ compared to control; ($n = 6$ animals).

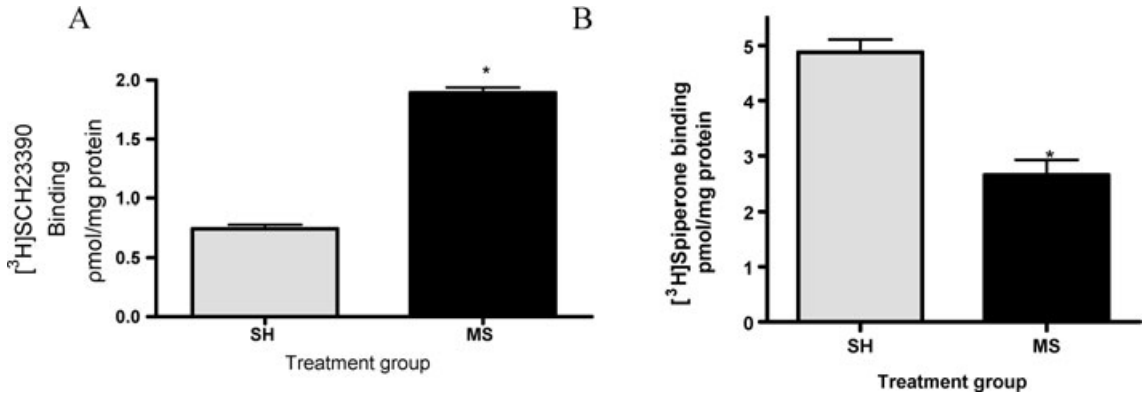

FIGURE 6. Effect of cigarette smoke exposure on the ligand-binding capacity of dopamine receptors (D_1 and D_2) in the guinea pig midbrain region. Error bars represent SEM, $n = 8$ per group. Student t-Test was used for statistical analysis to compare different groups. $^* p < 0.05$ indicates significance compared to the control. Abbreviations: SH, sham; MS, mainstream.

significant increase in the D_1 and D_2 protein levels. In addition, cigarette smoke increased the binding capacity of D_1 dopamine receptor and dopamine transporter by 54.8% and 52.73%, respectively, and caused a 54.8% decrease in the binding capacity of the D_2 dopamine receptor. This is the first study to analyze the effects of direct cigarette smoke exposure on the dopaminergic neuron system in a guinea pig model of exposure to cigarette smoke.

This study revealed that cigarette smoke exposure increased locomotor activities (horizontal, vertical, total distance) in the adult male guinea pig, and that chronic cigarette exposure further stimulated motor activity. There have been several studies on the acute and chronic effects of nicotine on locomotor activity, and most have revealed that small doses of nicotine have a stimulating effect that is enhanced by chronic administration [17]. However, one study by Saunders et al. reported that benzo(a)pyrene, a major component of cigarette smoke, elicited dose and time dependent suppression of locomotor activity [28]. The results shown by Saunders group may be due to the high doses administered. Our studies are on the overall effect of cigarette smoke, rather than a single component. In the present study, cigarette smoke exposure caused a significant increase in locomotor activities after acute and chronic exposure.

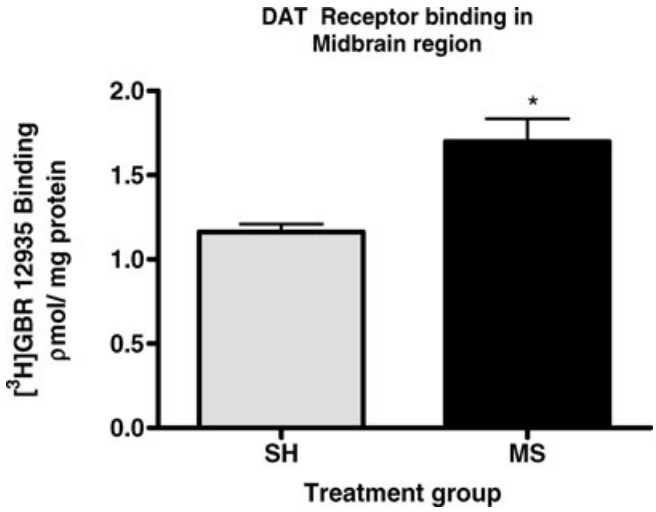

FIGURE 7. Effect of cigarette smoke exposure on the ligand-binding capacity of DAT in the guinea pig midbrain region. Error bars represent SEM, $n = 8$ per group. Student t-Test was used for statistical analysis to compare different groups. $^* p < 0.05$ indicates significance compared to the control. Abbreviations: SH, sham; MS, mainstream.

Cigarette smoke exposure caused an increase in all locomotor activities: horizontal activity, vertical activity, and total distance on day 1. The greatest activity counts were observed during the first 30 min and gradually declined over the 2-h sampling period. Furthermore, we have shown that acute cigarette smoke exposure causes a significant increase (Figure 1B) in total locomtor activity (parameter study). Arousal, learning, memory, motivation, and anxiety are characteristics of rodent response to a new environment. In this case, a 1-h habituation period was sufficient to reduce such characteristics in unexposed animals to basal levels. Therefore, variation of activity between the SH and MS groups are not due to the habituation rate, but rather, to the acute effects of cigarette smoke exposure (Figure 1).

In addition, cigarette smoke exposure enhanced the locomotor activity of guinea pigs after daily exposure as compared to the sham control group over the 7 week period. Clark et al. reported a direct excitatory action of nicotine on dopaminergic neurons of the substantia nigra pars compacta [29]. Therefore, our data suggest that cigarette smoke exerts its action on the dopaminergic neurons resulting in enhancements in locomotor

activity. Moreover, exposure to nicotine was reported to excite dopaminergic neurons by acting on the postsynaptic alpha-$_7$ and alpha$_4$-beta$_2$-type nicotine acetylcholine receptors [30]. Therefore, our data also suggest that cigarette smoke can cause alterations in locomotor by modulating the dopaminergic neuron system.

Studies linking the neurobehavioral effects of cigarette smoke to the distribution of dopamine and its metabolites are limited [31,32]. It has been reported that nicotine alters dopamine uptake, release, and metabolism. In addition, it is believed that nicotine causes increases in locomotor activity by stimulating the dopaminergic neuron system. Previous studies have also shown that behavioral sensitization after chronic nicotine exposure results in DA release specifically in the prefrontal cortex [33]. The prefrontal cortex has interconnections between the brainstem and limbic system. Therefore, we assume that the dopamine released from the prefrontal cortex causes downstream signaling within the midbrain region. The dopaminergic system has also been found to be involved in morphine-induced locomotor response [34] and in the locomotor activity enhancing effects of nicotine [35–37].

In the present study, cigarette smoke exposure caused decreases in the DA levels and increases in the levels of DOPAC (Figure 3). The DOPAC/DA ratio is used as an indicator of dopaminergic neuron activity. The decrease in the parent compound and accumulation of its metabolite within the midbrain region posttreatment corresponded with the behavioral effects. This suggests that dopamine metabolism plays an important role in enhancing the effects of cigarette smoke on locomotor behavior. Several studies have also shown that acute nicotine treatment enhances the striatal and limbic dopamine metabolism [38,39]. This suggests that cigarette smoke exposure causes uptake of dopamine and rapid conversion of dopamine to DOPAC. Therefore, we can assume that cigarette smoke enhances the dopamine metabolism within the midbrain region. Corrigall et al. [35] have shown that both nicotine and amphetamine evoke [^{3}H]dopamine release from superfused rat striatal slices, via different mechanisms. Therefore, our results suggest that cigarette smoke exposure may exert its effect on locomotor activity via a DA-dependent or DA-independent mechanism.

The basal ganglia are a group of nuclei in the brain associated with motor function. Within this corticostriatal-thalamo-cortical (CSTC) circuit, activation of the direct pathway is involved in facilitation of desired motor behaviors, whereas activation of the indirect pathway is involved in suppression of undesirable motor behavior. Dopamine has opposing effects on the direct and indirect pathways of the basal ganglia [40]: via dopamine D_1 receptors a stimulatory effect on the direct pathway, whereas via D_2 receptors an inhibitory effect on the indirect pathway.

In the present study, it was observed that D_1 and D_2 receptor protein levels were significantly increased within the midbrain region of the adult guinea pig due to chronic cigarette smoke exposure (Figure 6). This result does not explain the changes seen in locomotor activity. Therefore, to elucidate the role of D_1 and D_2 receptors in locomotor behavior within the guinea pig model of exposure to cigarette smoke, we determined the functional activity of these receptors.

Cigarette smoke exposure altered the binding capacity of the D_1 and D_2 receptors, as well as, the dopamine transporter in the guinea pig midbrain region (Figures 6 and 7). Most interestingly, while the binding capacity of D_1 receptor was increased (54.8%), the binding capacity of the D_2 receptor was equally decreased (54.8%) in the midbrain region after chronic smoke exposure. The increases in the capacity binding of the D_1 receptor may be a feedback mechanism to counter the decreases in dopamine levels (Figure 3). Furthermore, the decreases in the D_2 receptor binding capacity may lead to the enhancements in locomotor activity (Figures 1 and 2). As noted above, enhances in locomotor activity were observed after chronic cigarette smoke exposure. This suggests that the dopaminergic activities in the midbrain of the guinea pig are associated with locomotor behavior. Therefore, the present observation that cigarette smoke exposure causes an increase in the binding capacity of D_1 and decreases in binding capacity of D_2 receptors in the midbrain region suggest that cigarette smoke may have an effect on cognitive deficits associated with neurological dysfunction. Further evidence may be obtainable by using a primate model with neurological damage for behavioral testing.

Furthermore, the binding capacity of the dopamine transporter increased (52.7%) as a result of chronic cigarette smoke exposure (Figure 7). Synaptic dopamine concentrations are regulated by the dopamine transporter, and DAT is a major target for psychostimulant drugs, for example, cocaine and amphetamine [41]. Therefore, our results suggest that cigarette smoke exposure may also target DAT and lead to alterations in DAT function.

In conclusion, cigarette smoke exposure enhanced locomotor activity as compared to the controls, suggesting that cigarette smoke has an effect on the dopaminergic neuron system. In addition, the resulting increase in the concentrations of DOPAC confirms that cigarette smoke exposure increased dopamine turnover and metabolism. Cigarette smoke also altered the protein levels and binding capacity of both the D_1 and D_2

receptors. This suggests that the mechanism by which cigarette smoke exerts its enhancing effects on locomotor behavior is through modulation of the dopaminergic neuron system. Our novel findings, on the binding capacity of the dopamine transporter, suggest that cigarette smoke may exert its effect on locomotor activity by recycling the functional dopamine. The mechanism that underlies the effect of cigarette smoke on behavior and dopaminergic neuron system function remains to be fully elucidated. However, it is possible that the locomotor sensitization is most closely associated with the motivational, addictive properties of cigarette smoke.

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