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DOPAMINE-MEDIATED INCREASES IN NIGRAL SUBSTANCE P-LIKE IMMUNOREACTIVITY*

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Abstract—The results reported herein strongly suggest that increased dopaminergic and not increased serotonergic activity is responsible for methamphetamine-induced increases in the nigral concentration of substance P-like immunoreactivity (SPLI). Thus, treatment of rats with the specific dopamine (DA) uptake blockers amfonelic acid (AFA) and nomifensine caused elevations in the SPLI levels within the substantia nigra similar to that of methamphetamine (METH). In contrast, the specific serotonin uptake blockers citalopram and chlorimipramine were without significant effects on this substance P (SP) system. Additional studies revealed that the mechanisms whereby the DA uptake blockers and METH influence the striatonigral SP pathway are likely different. Specifically, AFA, unlike METH, altered the SP system without causing changes in the monoaminergic synthesizing enzymes tyrosine and tryptophan hydroxylase; in addition, pretreatment with reserpine abolished the AFA effect on nigral SPLI but did not interfere with METH-mediated changes in the SP system.

In the mammalian central nervous system, the highest concentration of substance P (SP) is found in the zona reticulata of the substantia nigra [1]. At this site, SP exists within vesicles associated with the terminals of axons which arise from the anterior neostriatum [2, 3]. *In vitro* release of nigral SP can be evoked by depolarization and is calcium-dependent, supporting the concept that SP acts as a neurotransmitter for this striatonigral pathway [4]. Furthermore, microiontophoretic studies have demonstrated that this peptide can evoke, after a characteristic delay, a prolonged excitation of neurons in the substantia nigra [5]. Specifically, biochemical [6–8] and behavioral studies [8, 9] have indicated that SP activates a population of nigral dopaminergic neurons which project to the striatum.

Evidence has been reported which suggests the existence of a dopaminergic influence on the SP striatonigral neurons. After chronic administration of the antipsychotic haloperidol, a potent dopamine (DA) receptor antagonist, nigral concentration of SP-like immunoreactivity (SPLI) is reduced substantially [10–12]. Similarly, destruction of the nigrostriatal DA projection either by 6-hydroxydopamine lesions [11] or associated with Parkinson's disease [13] also results in decreased nigral SPLI content. Consistent with these findings, the enhancement of DA activity with repeated administrations of the indirect DA agonist methamphetamine (METH) causes elevations of SPLI concentration in several structures associated with the basal ganglia including the substantia nigra [14]. This METH effect is blocked by concurrent administration of haloperidol, suggesting that the action of the drug on the striatonigral SP neurons is mediated through the DA system [14]. However, an apparent discrepancy has been observed following treatment with apomorphine. Our laboratory [12] and others [15] have reported significant decreases in nigral SP concentrations after an acute administration with this direct DA agonist, an effect which also is blocked by haloperidol coadministration.

Due to what appears to be conflicting findings, the mechanisms by which METH exerts its actions on the SP pathway are not clear. In addition, besides influencing dopaminergic activity, METH is also known to alter profoundly serotonergic activity [16–18]. The possibility that METH influences the SP system indirectly by altering serotonergic transmission is suggested by recent studies with *p*-chloroamphetamine. Increases in nigral SP content occur after treatment with this METH-related compound which possesses greater selectivity for serotonergic neurons [19]. In addition, haloperidol blocks striatal 5-hydroxytryptamine (5HT) receptors [20] as well as prevents METH-induced effects on several neurochemical parameters of the serotonergic system [18, 21]. Based on the above findings, the possibility that a serotonergic component has a role in mediating the increase in nigral SP levels elicited by METH cannot be excluded.

To elucidate the mechanism of METH on the striatonigral SP neurons, the present study examined agents which preferentially enhance either the activity of DA or 5HT striatal pathways and compared their actions on the striatonigral SP feedback system to that of METH. Specifically, the actions of the DA uptake inhibitors, amfonelic acid [22] and nomifensine [23], and the 5HT uptake blockers, chlorimipramine [24] and citalopram [25], were investigated and are discussed.

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METHODS AND MATERIALS

Animal treatment. Male Sprague–Dawley rats, 180–200 g (Sasco, Omaha, NE), were maintained for 1 week prior to testing under a 12-hr light–dark cycle (lights on 6:00 a.m. to 6:00 p.m.) and allowed free access to food and water. Rats generally received a subacute dosing regimen consisting of a dose of test drug every 6 hr for five doses, except in one experiment (Table 1) where animals received one, two or three doses of amfonelic acid (2.5 mg/kg) at 6 hr intervals. In another experiment (Fig. 3), rats received a single dose of reserpine (5 mg/kg, s.c.) 6 hr before treatment.

Drugs and drug vehicles. Methamphetamine hydrochloride, citalopram hydrobromide, chlorimipramine hydrochloride, nomifensine and amfonelic acid were provided by the National Institute on Drug Abuse, H. Lundbeck & Co., Ciba-Geigy, Hoechst Roussel, and Sterling Winthrop respectively. Reserpine was purchased from the Sigma Chemical Co. Drugs were administered in the following vehicles: 0.9% saline (methamphetamine, citalopram and chlorimipramine); 9:1 (v/v) propylene glycol: 2N K_2CO_3 (amfonelic acid); 4:1 (v/v) 1% citric acid: polyethylene glycol-400 (reserpine); and 3:1 (v/v) propylene glycol: 0.9% saline (nomifensine).

Dissection. All animals were decapitated 18 hr after the last treatment. Striata for tyrosine hydroxylase (TH) activity determinations were immediately dissected out and frozen on dry ice along with the remainder of the brain. For SPLI determination, substantia nigras were microdissected from 0.5-mm thick, frozen coronal sections using an extra fine dissecting scalpel. All tissues were stored at −80° until assayed.

SP radioimmunoassay. SPLI was determined by RIA. Details of this assay have been reported previously [26] and, therefore, will be described only briefly. Both substantia nigras from each animal were pooled and homogenized in 250 μl hydrochloric acid (0.01 N) using a polytron. After removing 20 μl for protein determination by the method of Bradford [27], the tubes containing homogenized samples were placed in boiling water for 10 min to denature proteases. Following centrifugation, supernatant fractions were frozen and lyophilized overnight. Samples were reconstituted with 0.5 ml of phosphate-buffered saline containing 0.1% gelatin, pH 7.4, and assayed for SPLI in triplicate after a final dilution (1:6). [^{125}I]SP (New England Nuclear) was used as the competitive label. The SP antiserum employed in this study could detect 10 pg of synthetic bovine hypothalamic SP at a 1:400,000 dilution. The specificity of this particular antiserum has been characterized and described [26] and displays less than 2% crossreactivity with the SP-like tachykinins, eledoisin and physalaemin.

Monoamine enzyme activity determinations. Neostriata were weighed and homogenized (1:3.5) in 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid buffer (Sigma Chemical Co.), pH 7.4, containing 0.2% Triton X-100 and 5 mM dithiothreitol. After centrifugation for 15 min at 27,000 *g*, duplicate 7.5-μl aliquots of supernatant fractions were taken for the tryptophan hydroxylase (TPH) assay, and similar aliquots diluted with 42.5 μl deionized water were used to assess TH activity. TPH activity was measured by a modified $^{14}CO_2$ trapping procedure [28, 29] as described by Hotchkiss *et al.* [30]. TH activity was determined by a tritium release assay of Nagatsu *et al.* [31].

Statistics. Differences between group means were analyzed by two-tailed Student's *t*-test and were considered significant when the probability that they were zero was less than 5%.

RESULTS

Effect of a DA uptake inhibitor on SPLI concentration in the substantia nigra. To examine the possibility that the effects of METH on nigral SPLI content are the result of increased DA activity, animals received the DA uptake blocker AFA (2.5 mg/kg) every 6 hr for one, two or three total doses. Table 1 shows that AFA, like METH, elevated nigral SPLI concentration; one, two and three injections of AFA resulted in increases in this variable of 37, 34 and 61%, respectively, above the control value 18 hr after treatment. Data in Table 1 also demonstrate that TPH activity was unaltered by AFA, while the activity of TH was depressed significantly (down 15%) only following three administrations of this uptake blocker. Thus, in contrast to METH, AFA can elevate nigral SPLI concentration without altering TH or TPH activity (one or two doses, Table 1).

Effect of multiple administrations of AFA and other DA- or 5HT-selective uptake inhibitors on nigral SPLI. To compare the METH-like elevations of nigral SPLI concentration induced by AFA with the effects of other specific inhibitors of the DA or serotonin reuptake carriers, a multiple dosing paradigm commonly used in our laboratory was utilized in which rats received either AFA (1 mg/kg), nomifensine (20 mg/kg), chlorimipramine (4 mg/kg) or citalopram (10 mg/kg) every 6 hr for five doses (Fig. 1). Increased nigral concentrations of SPLI were only observed following subacute administration of AFA or nomifensine, both of which potentiated DA activity by blocking its reuptake. However, there was no demonstrable effect on nigral SPLI levels with either 5HT blocker.

Effect of AFA on the response of the striatonigral SP and striatal DA systems to METH. Recently, it has been shown that AFA, like haloperidol, can block the long-term decreases observed in neostriatal DA concentration and in tyrosine hydroxylase activity following prolonged exposure to amphetamines [32–34]. Similar experiments were conducted to determine the effect of AFA on the METH-induced increases in the concentration of nigral SPLI.

As we previously demonstrated [14], multiple administrations of METH (15 mg/kg) resulted in an increase in the nigral concentration of SPLI (up 61%) and a corresponding reduction in the activity of tyrosine hydroxylase (down 40%, Fig. 2). While AFA alone, at this dose (0.15 mg/kg), had no significant effect on either of these variables, its combination with METH antagonized the METH-

Table 1. Effects of single and multiple doses of AFA on SPLI concentration in the substantia nigra*

Treatment	Substantia nigra	Striatum	
	SPLI (ng/mg protein)	TH (nmoles/g/hr)	TPH
Control	12.4 ± 0.7 (100 ± 6)	1318 ± 56 (100 ± 4)	32.0 ± 3.8 (100 ± 11)
1 Dose	17.0 ± 0.5† (137 ± 4)	1430 ± 84 (108 ± 6)	34.7 ± 2.7 (108 ± 8)
2 Doses	16.6 ± 0.9‡ (134 ± 7)	1249 ± 65 (95 ± 5)	28.0 ± 2.6 (87 ± 8)
3 Doses	20.0 ± 0.7† (161 ± 6)	1119 ± 57§ (85 ± 4)	29.5 ± 1.8 (92 ± 6)

* Rats were treated with one, two or three doses of AFA (2.5 mg/kg, every 6 hr) or vehicle alone, and were decapitated 18 hr after the final treatment. Nigral SPLI concentration and striatal monoamine enzyme activities were determined as described under Methods and Materials. Each value represents the mean ± S.E.M. of six animals, expressed in parentheses as a percentage of the corresponding control

†–§ Significant difference from respective control: † $P < 0.001$, ‡ $P < 0.01$, and § $P < 0.05$.

induced depression in TH activity in agreement with earlier findings; however, the effect of METH on the SP system was definitely not hindered by the presence of AFA.

Effect of reserpine on responses of the SP system to either AFA or METH. Some central stimulants believed to act through DA mechanisms can be pharmacologically subdivided on the basis of whether their stimulant-induced biochemical and behavioral effects can be inhibited by reserpine [35]. Whereas depletion of brain catecholamine stores with reserpine does not inhibit the central actions of METH, the actions of non-amphetamine stimulants such as AFA are substantially attenuated by pretreatment with this agent [36, 37]. Based on this reputed ability of reserpine to discriminate between these agents, we examined the effect of reserpinization on the changes in nigral SPLI induced by AFA or METH.

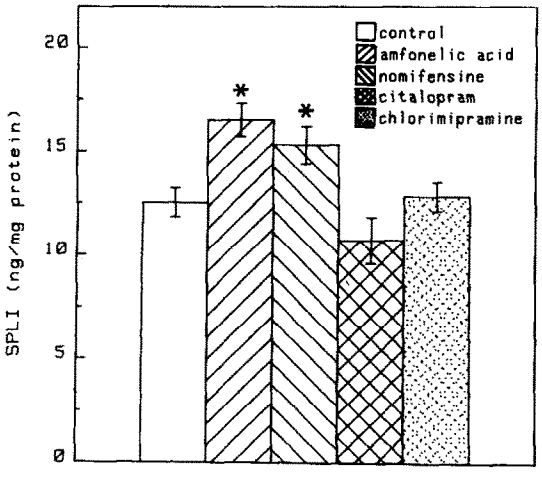

Fig. 1. Effects of subacute administrations of selective monoamine uptake inhibitors on nigral SPLI content. Rats were given five sequential injections (one every 6 hr) of AFA (1.0 mg/kg), nomifensine (20 mg/kg), citalopram (10 mg/kg), or chlorimipramine (4 mg/kg). Respective control groups received injections of the appropriate vehicle. Eighteen hours after the final dose, animals were killed, and substantia nigras were dissected and assayed for SPLI concentration. Values from each control group did not differ significantly from each other; they were therefore pooled and are represented by the control entry in the table. Bars represent the mean ± S.E.M. of six to seven determinations. An asterisk indicates that the value differs significantly from control ($P < 0.05$).

Interestingly, we observed that a single dose of reserpine alone (5 mg/kg) significantly reduced nigral SPLI levels (down 45%, Fig. 3). This decrease is consistent with similar reductions in nigral SPLI which occur after DA activity is antagonized by chronic haloperidol administration or a 6-hydroxydopamine lesion of the nigrostriatal DA pathway [10–12].

Animals which were treated with only AFA (1.0 mg/kg) had a 30% elevation in nigral SPLI content, whereas AFA had no significant effect on SPLI levels in the substantia nigras of reserpine-pretreated animals. In comparison, METH, administered in a manner like that of AFA, also increased nigral SPLI concentration above control. However, in contrast to AFA, when METH was administered to reserpinized animals, a significant 54% increase in nigral SPLI concentration compared to reserpine alone was observed.

DISCUSSION

Data presented herein demonstrate that the elevation in nigral SPLI elicited by METH [14] likely is due to the potentiating actions of this agent on DA, and not 5HT, neurotransmission. This conclusion is based on two observations. First, administrations of specific inhibitors of DA uptake, AFA (Table 1, Figs. 1 and 3) or nomifensine (Fig. 1), caused

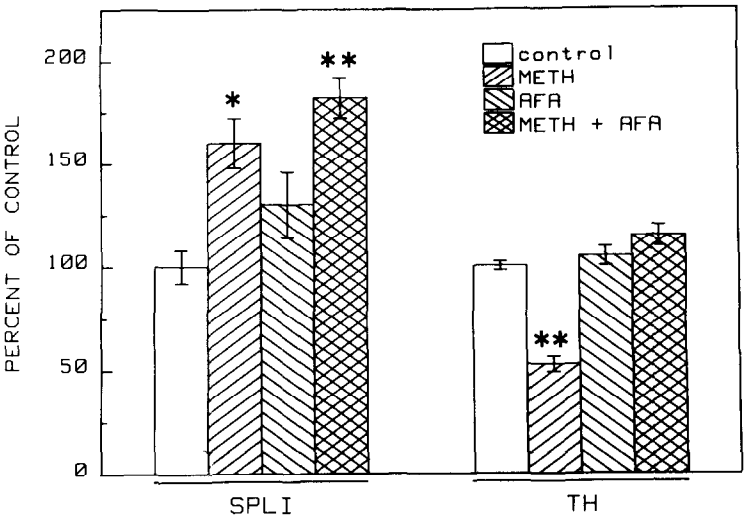

Fig. 2. Effect of AFA on METH-induced changes in nigral SPLI concentration and striatal TH activity. Animals received METH (15 mg/kg every 6 hr for five doses) with a vehicle plus or minus AFA (0.15 mg/kg) and were killed 18 hr after the last dose. Substantia nigras and striata were assayed for SPLI concentration and TH activity, respectively, as described under Methods and Materials. Each bar represents the mean ± S.E.M., expressed as percentage of control, of five to seven determinations. Asterisks denote a value significantly different from control (* $P < 0.01$; ** $P < 0.001$). Control SPLI concentration and TH activity values were 11.1 ± 0.4 ng/mg protein and 3045 ± 64 nmoles/g/hr respectively.

increased concentrations of nigral SPLI. These alterations are comparable in magnitude to the increases which occur after repeated treatments with METH and, like the "METH effect", are probably due to feedback depression of the excitatory striatonigral SP pathway and accumulation of this transmitter in the nigral terminals [14]. Second, pharmacological enhancement of brain serotonergic activity with 5HT uptake blockers citalopram or chlorimipramine [38] consistently failed to alter SPLI content (Fig. 1). This indicates that 5HT likely is not the mediator of the effect of METH on the SP system. In further support of the involvement of DA, preliminary lesion studies indicate that destruction of the nigrostriatal

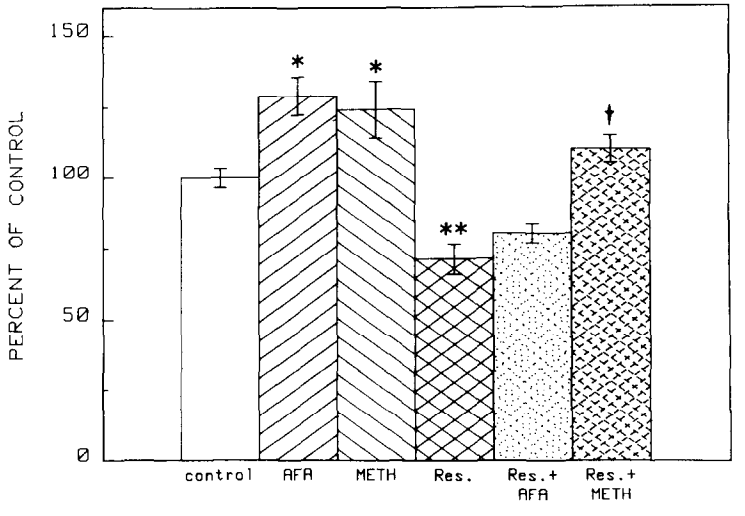

Fig. 3. Effect of reserpine pretreatment on responses of striatonigral SP system to AFA and METH. Reserpine (5 mg/kg, s.c.) or vehicle was administered to rats 6 hr before the initiation of a subacute regimen consisting of either AFA (1 mg/kg) or METH (10 mg/kg), for five doses (one every 6 hr). Rats were decapitated 18 hr after the fifth dose, and substantia nigras were assayed for SPLI content. Bars represent the mean ± S.E.M. for five determinations. Symbols indicate significant differences as follows: * $P < 0.05$ and ** $P < 0.01$ with respect to control; † $P < 0.01$ with respect to reserpine alone.

DA pathway blocks the ability of METH to increase SPLI concentration in this tissue.*

Consequently, the differences in the response of the striatonigral SP system to METH and apomorphine [12, 15] cannot be attributed to METH-induced enhancement of serotonergic activity. It is likely that both of these agents influence this SP pathway due to their actions on the DA system. Support for this conclusion was reported recently by Sonsalla *et al.* [39], who found that differential stimulations of DA receptor subtypes D_1 and D_2 cause decreases and increases, respectively, in nigral SPLI content. These observations, with the data presented herein suggest that both METH and apomorphine mediate their effects through the DA system, but preferentially activate different DA receptor subtypes, which in turn have opposite effects on the SP striatonigral pathway.

Although inhibitors of DA uptake share with METH the ability to induce increases in nigral SPLI concentration, several observations indicate that these agents alter the striatonigral SP pathway by different mechanisms. Our finding that a single injection of AFA (2.5 mg/kg) elevated nigral SPLI content (Table 1), in contrast to METH which requires multiple treatments [14] for this change to be manifest in nigral tissue, supports this conclusion. Furthermore, AFA-induced effects on the SP system were inhibited by reserpine, whereas those of METH were not (Fig. 3). This finding is consistent with other behavioral and biochemical evidence that reserpine differentially affects the central stimulatory actions of METH and AFA [36, 37] and supports the current view that AFA and METH act on different neuronal storage pools of DA [40]. AFA is speculated to potentiate the activity of DA which originates from a reserpine-sensitive vesicular pool that is normally released by impulse-propagated exocytosis. In contrast, METH is thought to release DA directly from a nonvesicular, cytoplasmic pool that is resistant to the actions of reserpine. Perhaps selective action on one or the other DA pools by METH or AFA accounts for the different time courses of response by the SP system to these compounds. Thus, the results presented in Fig. 3 also support the hypothesis that increases of SPLI in nigral SP terminals occur in response to enhanced DA activity regardless of its source.

We previously suggested that a correlation exists between increased nigral SPLI concentration and decreased striatal tyrosine hydroxylase activity resulting from multiple administrations of METH [14]. This was supported by the finding that haloperidol was able to antagonize the effects of METH on both the SP and DA systems concomitantly. Although the changes in these two transmitter systems are often parallel, data in Table 1 and Fig. 2 suggest that they can respond independently. Similar to haloperidol, AFA antagonized the long-term decreases in DA, its metabolites, and in tyrosine hydroxylase activity following multiple exposures to METH but, in contrast to haloperidol, AFA did not block the increase in nigral SPLI concentration induced by METH. It is also worth noting that, in contrast to haloperidol, which blocks METH-induced stereotypy and increased locomotor activity, AFA actually exacerbated the behavioral response to METH. Thus, alterations occurring in nigral SPLI concentration following administration of indirect DA agonists appear to correlate more closely with behavioral responses than do changes in tyrosine hydroxylase activity.

Reduced nigral concentrations of SPLI have been reported previously under several conditions in which nigrostriatal DA activity was antagonized, including 6-hydroxydopamine or electrolytic lesions of the nigrostriatal pathway [12], chronic haloperidol administration [10–12], and, more recently, in humans with Parkinson's disease [13]. Consequently, it is not surprising that reserpine administration decreased nigral SPLI levels (Fig. 3) by a comparable magnitude. This agent, which nonselectively depletes vesicular stores of monoamines, reduces dopamine turnover and metabolism.

It has been postulated that these reductions in nigral SPLI may be due to a depletion of terminal SP stores caused by a compensatory increase in the level of activity of the excitatory feedback striatonigral SP neurons in response to the decrease in postsynaptic DA activity [10–12]. This hypothesis is supported by two recent findings. First, Bannon and Goedert [41] observed that protein synthesis inhibition by cycloheximide accelerated the rate at which haloperidol depleted nigral SP levels, suggesting an enhanced utilization of striatonigral SP following haloperidol. Next, Melis and Gale [42] observed that infusion of SP antagonists into the substantia nigra blocked the haloperidol-induced activation of striatal tyrosine hydroxylase, manifested as an increase in the affinity of the enzyme for its cofactor, tetrahydrobiopterin. This suggests that haloperidol does evoke an increase in the nigral release of SP which may account for the reduction of SP observed in this tissue after haloperidol treatment. It would be interesting to examine whether reserpine which, like haloperidol, elicits an activation of striatal tyrosine hydroxylase [43] also does so by an SP-mediated mechanism, especially in light of our observation that reserpine decreased nigral SPLI concentration.

In summary, we have utilized selective inhibitors of DA and 5HT uptake to demonstrate that pharmacological enhancement of DA, and not 5HT, activity resulted in elevations of SPLI concentration in the rat substantia nigra. These observations demonstrate that the effect of METH previously reported to cause increases in the concentration of SPLI in the substantia nigra is likely DA-mediated. We postulate that this increase, as well as those occurring after AFA and nomifensine, reflect a decrease in the activity of the striatonigral SP feedback mechanism and represent an accumulation of SP within the nigral terminals of this pathway.

* P. Sonsalla and G. Hanson, unreported observation.

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