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INVERSE RELATION OF SUBSTANCE P-LIKE IMMUNOREACTIVITY IN DORSAL RAPHE NUCLEUS TO SEROTONIN LEVELS IN PONS-MEDULLA FOLLOWING ADMINISTRATION OF COCAINE AND 5-HYDROXYTRYPTOPHAN

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Abstract—Changes in levels of substance P-like immunoreactivity (SPLI) were observed in the rat dorsal raphe nucleus after cocaine treatment and were compared with concurrent cocaine-induced changes in 5-hydroxytryptamine (5-HT) content in the pons-medulla (PM). Acute administration of cocaine (15–30 mg/kg) consistently resulted in increases of SPLI, specifically in the area of the dorsal raphe nucleus, which were accompanied by significant decreases of PM 5-HT levels. Treatment of rats with 5-hydroxytryptophan (5-HTP), the 5-HT precursor, along with RO4-4602, the decarboxylase inhibitor, caused increments in PM 5-HT content with concurrent decreases in dorsal raphe nucleus SPLI. When cocaine and 5-HTP were administered simultaneously the changes in serotonin and SPLI were attenuated, suggesting that each substance antagonized the effect of the other. These data suggest a specific interaction between substance P and 5-HT in the dorsal raphe nucleus area.

Substance P (SP), a neuropeptide, is receiving increasing attention as investigators attempt to clarify its physiological roles in the CNS. Results have been published that suggest important interactions between SP and other putative neurotransmitters such as the catecholamines [1–4] and enkephalin [5–8].

A particularly interesting and unusual relationship has been shown to exist with the serotonergic (5-HT) system. It has been reported that SP and 5-HT can coexist in the same neurons. Chan-Palay [9] used a combination of immunocytochemistry and autoradiography to demonstrate that soma and processes in raphe pallidus nucleus can contain both serotonin and substance P-like immunoreactivity (SPLI). In a complimentary study, Hökfelt *et al.* [10] presented immunohistochemical evidence showing the coexistence of SP and 5-HT in a population of neuronal cell somata in the rat medullary raphe nucleus, the nucleus interfascicularis hypoglossi, and an area immediately dorsal to the pyramidal tract. After injecting the serotonin-specific neurotoxins, 5′,6′- or 5′,7′-dihydroxytryptamine, Hökfelt and coworkers observed a substantial decrease in SP- and 5-HT-positive nerve terminals in the ventral horns of the spinal cord. These data were substantiated by Singer *et al.* [11] using quantitative biochemical methods. They observed approximately 50 per cent depletion of SP in the ventral horn of rat spinal cord following treatment with 5′,7′-dihydroxytryptamine. Under similar conditions, Björklund *et al.* [12] reported a 37 per cent reduction in SP content in the medullary raphe nuclei. The results of the above studies imply a close relationship between SP and 5-HT in some CNS regions.

In the present study, an attempt was made to determine if drugs that have been shown to alter serotonergic systems have an effect on SPLI levels in discrete CNS nuclei. It has been reported previously by Pradhan *et al.* [13, 14] that cocaine caused a significant reduction in the serotonin content of the pons-medulla (PM), which correlated with the onset of behavior effects such as stereotypy and increased motor activity. In contrast, the administration of 5-hydroxytryptophan (5-HTP), to animals that had been pretreated with RO4-4602, the decarboxylase inhibitor, substantially elevated levels of PM serotonin. Simultaneous, but separate, injections of cocaine and 5-HTP significantly attenuated the effect of each compound on the 5-HT level and reduced the cocaine-induced behavior phenomenon [15]. Experiments presented in this paper demonstrate that these two drugs not only affected the PM serotonergic system but, in a selective manner, induced concurrent but inverse changes in SPLI levels in the dorsal raphe nucleus (DRN).

MATERIALS AND METHODS

Treatment and dissection. Male Sprague–Dawley rats (220–250 g) were treated either acutely (a single injection 20 min prior to killing) or chronically (injections b.i.d. × 30 days) with doses of cocaine that have been shown to have both biochemical effects (15–30 mg/kg; Ref. 16). Other rats were treated with injections of 5-hydroxytryptophan

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(115 mg/kg) 50 min after giving a dose of the decarboxylase inhibitor RO4-4602 (50 mg/kg; Ref. 16) which has been shown to inhibit peripheral decarboxylase. In two experiments (Fig. 3), a single group of animals received acute, simultaneous doses of cocaine and 5-HTP 50 min after the administration of RO4-4602. The cocaine hydrochloride and the RO4-4602 were dissolved in saline, and the 5-HTP was dissolved in 0.1 M HCl and then neturalized with alkali. The animals were kept in standard laboratory conditions and given food and water *ad lib*.

All animals were decapitated and the brains were rapidly removed. In experiments where SPLI was to be assayed, the brains were immediately frozen on dry ice. The brain nuclei were localized according to Ljungdahl *et al.* [3, 17] and dissected from 500 μM thick frozen sections, using either an extra fine dissecting scalpel for the substantia nigra zona reticulata or a 19 gauge needle punch for raphe nuclei, and placed in liquid nitrogen until assayed. The raphe nuclei consisted of dorsal and medullary (magnus and obscurus) raphe areas. When 5-HT was measured, the brain was sectioned at 4° immediately after decapitation and the pons-medulla was homogenized (100 mg/ml) in ice-cold 0.4 M perchloric acid as described by Pradhan *et al.* [14] and assayed.

Biochemical assays. The serotonin levels were measured according to the method of Snyder *et al.* [18]. The method used for protein determinations is described by Bradford [19].

SPLI was assayed in tissue samples pooled from two animals. These samples were homogenized in a volume of 250 μl of 0.01 M HCl. The homogenate was heated by placing it in boiling water for 10 min, after which the samples were centrifuged at 5000 g for 20 min and the resulting supernatant fraction was lyophilized. Samples were reconstituted by adding 0.5 ml of phosphate buffered saline with gelatin (0.1 M monobasic sodium phosphate, 0.9 sodium chloride, and 0.1% gelatin; pH 7.4) and were centrifuged for 20 min at 5000 g to remove insoluble material. The supernatant fraction was assayed for substance P-like immunoreactivity.

The antiserum was prepared by Dr. William Campbell (University of Texas, Dallas) according to the procedure of Nilsson *et al.* [20] and could reliably detect 10 pg of synthetic bovine hypothalamic SP at a $1:2 \times 10^5$ dilution. The antiserum showed less than 2 per cent cross-reactivity with eledoisin and physalemin. However, SP-fragments greater than the C-terminal pentapeptide did significantly cross-react with the antiserum. Thus, it is possible that the radioimmunoassay detected not only intact SP but also certain SP metabolites as reported by Ben-Ari *et al.* [21]. Consequently, all results are expressed in terms of substance P-like immunoreactivity.

The radiolabeled SP was prepared by iodinating tyrosine-8 substance P with high specific activity iodine-125 according to the procedure of McConahey and Dixon [22], after which the reaction mixture was passed over an anion exchange resin (Bio-Rad AG 1×8, 20–50 chloride form) and eluted with 0.2 M acetic acid (pH 5.0). The eluate was collected in 0.5 ml increments. The high activity fraction was purified using a K 9/60 column of 56 MM Sephadex G-25, fine grade, and eluted by acetate buffer (pH 3.5). The fraction containing the initial peak of radioactivity was retained and diluted to an activity of approximately 1.2×10^6 dpm/0.5 ml with 2.0 mM Tris–0.2% lysozyme–0.1 M glycine buffer (pH 5.0) and stored at −80°.

Supernatant fractions from all tissue sites were assayed at multiple dilutions; the slopes of their dose–response curves were not significantly different from that of synthetic SP. In all experiments, the SPLI was routinely assayed, in two dilutions of each supernatant sample, by preincubating the antiserum with the duplicates of each supernatant dilution for 2 hr at room temperature. The radiolabeled SP (6000–8000 dpm) was added to each incubation mixture and allowed to incubate for an additional 18 hr. Antibody-bound and free iodine-125 SP were then separated by mixing the reactant with dextran-coated charcoal slurry according to the procedure of Herbert *et al.* [23]. SPLI was determined by comparing the ratio of bound to total radiolabeled peptide for each sample to a standard curve and was expressed in units of ng of SPLI/mg of protein.

The recovery of SPLI from tissue samples was determined according to the techniques described by Jessell *et al.* [24]. In control experiments, recovery of iodine-125 SP, added to tissue homogenates and carried through the assay procedure, usually approximated 80 per cent.

Chemicals. Substance P was purchased from Beckman (Silver Spring, MD) and the iodine-125 (16.5 mCi/μg of iodine) used for protein iodination was purchased from Amersham/Searle (Arlington Heights, IL). RO4-4602 and 5-HTP were obtained from Hoffmann-La Roche, Inc. (Nutley, NJ) and the Sigma Chemical Co. (St. Louis, MO) respectively.

Statistics. All values reported in this paper are means ± S.E.M. Differences between means were analyzed by Student's t-test and were considered significant when the probability that they were zero was equal to, or less than 0.05.

RESULTS

Effects of acute and chronic cocaine. The level of SPLI in dorsal raphe nuclei was elevated in rats treated acutely with cocaine. This group of animals was killed 20 min after a single injection of cocaine (15 mg/kg) and was found to have a significant, 21 per cent increase in the level of SPLI in the dorsal raphe nuclei (Fig. 1). The SPLI in the dorsal raphe nuclei of animals treated chronically with cocaine (15 mg/kg, b.i.d. × 30 days), although still somewhat higher than controls (9 per cent) was significantly lower than the acutely treated group. Other raphe nuclei (pallidus, magnus and obscurus) and the substantia nigra zona reticulata were also assayed for SPLI and were unaffected by either the acute or chronic cocaine treatment (data not shown). Thus, acute treatment with cocaine caused a specific elevation of SPLI in the dorsal raphe nucleus, whereas long-term exposure to cocaine appeared to result in a significant reduction of this effect.

Dose-dependency of the cocaine effect. To determine if the cocaine-induced changes in DRN were

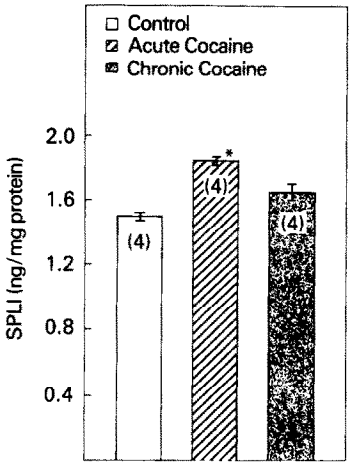

Fig. 1. Cocaine effects on SPLI in dorsal raphe nuclei after acute and chronic administration. Rats were treated with cocaine (15 mg/kg, i.p.) administered acutely (single injection) or chronically (b.i.d. × 30 days). The animals were killed 20 min after the final injection. Control animals were injected with saline in a manner identical to the cocaine-treated groups. In preliminary experiments, animals receiving chronic and acute injections of saline were found to have identical levels of SPLI in the DRN, so control animals in this study were treated with single saline injections. An asterisk (*) indicates that the acute group differed significantly from the control ($P < 0.001$) and the chronic ($P < 0.05$) groups. Bars represent means ± S.E.M. (N = 4).

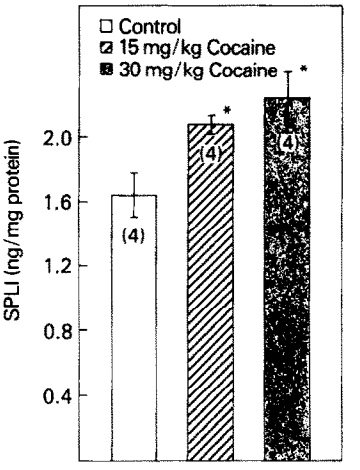

Fig. 2. Elevation of SPLI in dorsal raphe nuclei following acute administration i.p. of 15 or 30 mg/kg of cocaine. Twenty minutes after drug treatment the rats were killed, and the DRN were removed and assayed for SPLI as described in Materials and Methods. Controls were injected in the same way with saline. An asterisk (*) indicates that the labeled value is significantly different from the corresponding control group ($P < 0.05$). Bars represent mean ± S.E.M. (N = 4).

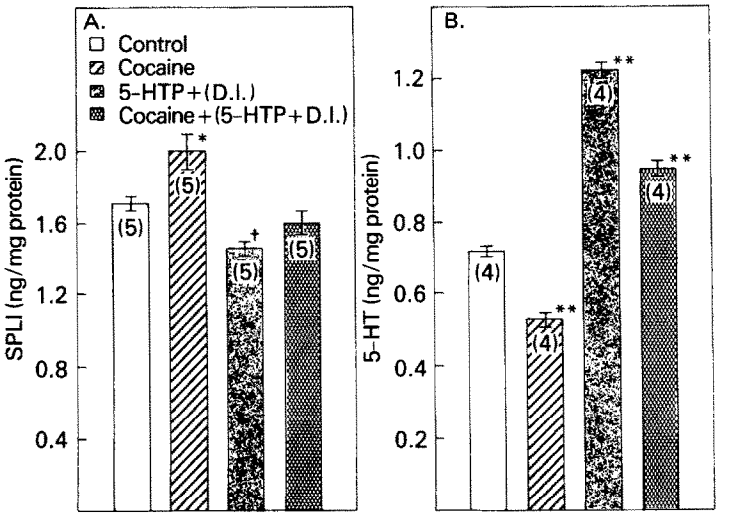

Fig. 3. Inverse effects of cocaine and 5-HTP on SPLI and 5-HT levels in dorsal raphe nuclei and pons-medulla regions respectively. The cocaine (20 mg/kg)-treated animals were killed 20 min after drug injection. The 5-HTP-treated animals were first given a decarboxylase inhibitor (RO4-4602, 50 mg/kg)and after 50 min were treated with 5-HTP (115 mg/kg). The combination groups received both cocaine and 5-HTP simultaneously, but separately, preceded by RO4-4602 treatment. Panels A and B represent two identical but different experiments. In panel A the dorsal raphe nuclei were dissected, removed, and assayed for SPLI as described in Materials and Methods. In panel B the pons-medulla was used to measure serotonin levels as described in Materials and Methods. Control animals received a single saline injection 20 min prior to killing. An asterisk (*) indicates that the value differs significantly ($P < 0.025$) from all other values. A dagger (†) indicates that the value differs significantly ($P < 0.01$) from the corresponding control. Double asterisks (**) indicate that these values differ significantly ($P < 0.001$) from their respective control groups and from each other. Values are means ± S.E.M. Sample numbers are in parentheses.

dose-dependent, rats were treated acutely with one of two doses of the drug. The elevation of SPLI in DRN was 27 and 36 per cent following 15 and 30 mg/kg injections of cocaine respectively (Fig. 2). The changes of the SPLI were significant in both drug-treated groups compared to controls. Although there was a suggestion of dose-dependency in the changes of SPLI in DRN, the difference between the treated groups was not statistically significant.

Antagonism of cocaine effect by 5-HTP. The cocaine-induced elevation of SPLI in DRN was correlated with concurrent changes of 5-HT in the pons-medulla. The results in Fig. 3 show that the level of 5-HT following acute administration of cocaine (20 mg/kg) was decreased by 26 per cent compared to a 17 per cent elevation of SPLI (compare panel A to panel B). When 5-hydroxytryptophan (115 mg/kg), the serotonin precursor, was administered with a decarboxylase inhibitor, however, the results were reversed; there was a 14 per cent reduction in SPLI and a concurrent 71 per cent elevation of 5-HT. All changes in the levels of SPLI and 5-HT following both drug treatments were significantly different from control groups.

To determine if the actions of 5-HTP could antagonize the cocaine-induced effects, these two drugs were administered simultaneously. The results presented in panels A and B of Fig. 3 demonstrate that 5-HTP completely blocked the cocaine-mediated elevation and decrease of SPLI and 5-HT respectively. These observations suggest that cocaine and 5-HTP exert their opposite effects through a common system.

Specificity of SPLI changes in DRN. The effects of cocaine and 5-HTP on SPLI in CNS nuclei were tested to determine the extent to which these drugs could alter SPLI in the brain. The substantia nigra zona reticulata and medullary raphe nuclei were studied because of their demonstrated involvement with both the 5-HT and the SP systems. Following treatment (with either cocaine or 5-HTP) identical to that used for Fig. 3, there were no changes in SPLI in either the substantia nigra zone reticulata or medullary raphe nuclei (Table 1). These results suggest a uniqueness in the SP system in DRN which makes it responsive to the action of these serotonin-active drugs.

DISCUSSION

This paper presents evidence which suggests that a unique relationship exists between the SP and 5-HT systems in the dorsal raphe area. This conclusion is supported by several lines of evidence. First, the acute cocaine-induced elevation of SPLI in the DRN (Figs. 1–3) is accompanied by a depression of 5-HT levels in the same general area (Fig. 3). Although 5-HT level has not been estimated specifically from DRN, it is expected that 5-HT content in the DRN will follow the same trend of change as in the PM as a whole following cocaine treatment. Second, administration of 5-HTP with a decarboxylase inhibitor caused a decrease in dorsal raphe SPLI with a concomitant rise in PM 5-HT (Fig. 3). Third, simultaneous injections of cocaine and 5-HTP attenuated the effects of these substances on both the SPLI and 5-HT levels in DRN and PM respectively (Fig. 3). Fourth, chronic administration of cocaine resulted in an attenuation of the effect on both the SP (Fig. 1) and 5-HT [16] systems in the DRN and PM respectively. Fifth, changes in SPLI were observed only in the dorsal raphe nucleus which is a primary center for the CNS serotonergic system [25]. While these results demonstrate that concurrent variations are occurring in the regulation of the steady-state level of SP and 5-HT, they do not explain the relationship of these changes or their underlying mechanisms.

One possible explanation for the observations described above has been suggested by Chan-Palay *et al.* [26]. If SP and 5-HT coexist in a fraction of DRN neurons, as has been described in other CNS regions [9, 10, 26], it may be that within these neurons the amounts of the two transmitters are inversely proportional to each other and that their levels fluctuate depending on the physiological demands of the system. Thus, pharmacologically induced variations in the 5-HT system caused by agents such as cocaine and 5-HTP could be expected to result in inverse changes in the SP system. It is important to note, however, that no one has reported any histochemical evidence supporting SP and 5-HT coexistence in the ascending pathways, that originate in the dorsal raphe nucleus, and the levels of SPLI in the medullary raphe nuclei, an area where coexistence of SP and 5-HT has been demonstrated [9, 26],

Table 1. Lack of effect of cocaine and 5-HTP on SPLI in substantia nigra zona reticulata or medullary raphe nuclei*

	SPLI (ng/mg protein)	
	Substantia nigra (zona reticulata)	Medullary raphe nuclei
Control	13.0 ± 1.0	1.65 ± 0.12
Cocaine	14.0 ± 0.5	1.76 ± 0.09
5-HTP	13.6 ± 1.4	1.69 ± 0.13
Cocaine + 5-HTP	13.4 ± 1.0	1.53 ± 0.04

* Tissues were dissected as described in Materials and Methods from four groups of rats acutely treated in a manner identical to the groups described in Fig. 3. The samples were assayed for SPLI. Values are means ± S.E.M. (N = 4).

were unchanged following acute treatment with either 5-HTP or cocaine (Table 1).

A second possibility is that the changes observed in transmitter levels are a consequence of interactions between separate 5-HT and SP pathways. Distinct pathways may act on a common physiological function and modulate its activity by exerting either an excitatory (SP; Refs. 4, 27–29) or inhibitory (5-HT; Ref. 30) influence. As part of this regulation there could exist feedback mechanisms which mediate opposite changes in the SP and 5-HT pathways according to the needs of the system. For example, under conditions that supress the function of the system, the excitatory input (SP pathway) would be activated and the inhibitory input (5-HT pathway) blocked in order to return the system to a normal level of activity. If cocaine and 5-HTP altered the activity of this system in opposite directions, these agents might activate the feedback mechanism and thus induce the inverse changes observed in the SPLI and 5-HT levels (Fig. 3).

The physiological roles of a possible SP and 5-HT interaction are unclear. These two transmitter systems might function together to help regulate specific DRN activities such as pain modulation [31, 32] or blood pressure regulation [33]. Supplementary studies are necessary, however, to assign any definitive function to this putative relationship.

It should be noted that the behavior-altering doses of cocaine and 5-HTP administered in this study are known to influence neurotransmitters other than 5-HT in rats. Pradhan *et al.* [14, 15] observed recently that administration of these compounds altered dopamine levels in the caudate nucleus and diencephalon-midbrain in a manner similar to the changes in SPLI of the DRN observed in the current study (Fig. 3). The similarity of change in dorsal raphe SPLI and midbrain dopamine levels in response to cocaine and 5-HTP suggests that some interaction between these two transmitter systems may occur. These observations, however, argue against the possibility that changes in SPLI levels in DRN are due to interactions with the dopamine system. First, acute (1/2 hr) and chronic (daily × 10 days) subcutaneous injections of the dopamine agonist apomorphine (1 mg/kg) had no appreciable effect on SPLI levels in DRN (unpublished results). Second, cocaine and 5-HTP had no apparent effect on SPLI in a dopamine center such as the substantia nigra (Table 1). Third, changes in SPLI levels following cocaine and 5-HTP administration were observed only in the DRN, an area with little known dopamine input. The results, however, do not exclude the involvement of other neurotransmitter systems in the drug-mediated effects described herein.

It is interesting that cocaine-induced changes in the SPLI of the DRN also appear to correspond to some aspects of cocaine-related behavioral variations described by Pradhan *et al.* [14, 16]. First, acute cocaine administration significantly elevated spontaneous motor activity and stereotypic behavior as well as increased dorsal raphe nuceli levels of SPLI (Figs. 1–3). Second, concomitant administration of cocaine and 5-HTP greatly reduced the cocaine-induced hyperactivity and stereotypy [15] while completely eliminating the SPLI increases in DRN. Perhaps the SP system in the dorsal raphe nucleus plays some role in mediating the behavioral alterations that typically accompany cocaine administration.

In summary, the data presented in this paper demonstrate that the SP system in the DRN is susceptible to pharmacologically induced changes by cocaine and 5-HTP. These changes could be the result of dynamic interactions between SP and 5-HT or other neurotransmitters. Until additional work is completed, however, the significance of these results can be only speculative.

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