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EFFECTS OF CLONIDINE, PIPEROXANE AND LOCUS COERULEUS LESION ON THE SEROTONERGIC AND DOPAMINERGIC SYSTEMS IN RAPHE AND CAUDATE NUCLEUS

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Abstract—To assess the influences of central noradrenergic neurons on both serotonergic and dopaminergic systems, the neurochemical effects of clonidine, piperoxane, and 6-hydroxydopamine were examined. Using quantitative fluorescence histochemistry and high performance liquid chromatography, we have demonstrated that clonidine, much like apomorphine, preferentially augmented intracellular serotonin (5-HT) fluorescence in the dorsal raphe without affecting 5-HT cells in the median raphe nucleus. Clonidine also produced a significant decrease of extraperikaryal catecholamine (CA) fluorescence in the same region. Piperoxane, at a dose having no significant effect alone, antagonized the effects of clonidine on 5-HT and CA. 6-Hydroxydopamine lesions of the locus coeruleus produced a similar increase of 5-HT fluorescence in the dorsal raphe and decrease of CA fluorescence in both the dorsal and median raphe. Biochemically, clonidine decreased while piperoxane increased a measure of 5-HT turnover in the corresponding terminal region of the dorsal raphe, the striatum. Similarly, dopamine turnover was also decreased by clonidine and increased by piperoxane in the striatum. These effects may be mediated by noradrenergic projections from the locus coeruleus to both the dorsal raphe and the substantia nigra. These results support the hypothesis that the effects of clonidine on serotonergic and dopaminergic neurons are indirectly mediated through noradrenergic receptor stimulation.

We previously reported that the dopaminergic agonist apomorphine increases intracellular levels of serotonin (5-HT) in the dorsal raphe nucleus and 5-HT as well as its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the striatum, the major terminal area for dorsal raphe neurons [1]. These effects appear to be mediated through dopamine (DA) neurons since haloperidol and 6-hydroxydopamine (6-OHDA) pretreatments both antagonize the effects of apormorphine on 5-HT neurons [1, 2]. Furthermore, the dopaminergic agonist 3-3-hydroxyphenyl-*N*-*n*-propyl-piperidine mimicked the effects of apomorphine on 5-HT neurons [3]. Clonidine has been suggested to be a specific noradrenergic agonist in the central nervous system [4]. However, some biochemical studies indicate that clonidine also decreases 5-HIAA concentration without significantly altering 5-HT level in brain [4–6]. Clonidine has also been shown to decrease brain 5-HT synthesis [7]. On the other hand, Maj *et al.* [8] reported that clonidine (1 mg/kg) does not affect the levels of 5-HT and 5-HIAA in rat whole brain.

Recent studies using clonidine have also suggested interactions between central DA and norepinephrine (NE) containing neurons. In general, clonidine has been shown to reduce brain DA synthesis and utilization and these effects appear to be mediated through central noradrenergic neurons [4, 7, 9–11]. The noradrenergic antagonists yohimbine and phenoxybenzamine, conversely, appear to accelerate DA synthesis and turnover [9].

In the present study, central noradrenergic systems in rats were manipulated with clonidine, piperoxane and local infusions of 6-OHDA. Quantitative fluorescence measures of raphe cell bodies and biochemical measures of serotonergic and dopaminergic terminals were made to further investigate interactions between noradrenergic and serotonergic systems as well as noradrenergic and dopaminergic systems.

MATERIALS AND METHODS

Animals. Thirty-three male Sprague–Dawley rats (Charles River Breeding Laboratories) ranging from 250 to 275 g were used in the clonidine and piperoxane/clonidine interaction studies. Upon arrival, they were housed in pairs for at least 1 week prior to experimentation. The animals used in the 6-OHDA lesion study were the same animals whose biochemical and behavioral data have been published elsewhere [12]. In addition to five sham-lesioned animals, five 6-OHDA-treated animals having greater than a 70% depletion of hippocampal NE were selected for the present histochemical study. These animals were male Sprague–Dawley rats (Hilltop Laboratories, PA) ranging from 175 to 225 g and housed individually. Briefly, bilateral infusions (6 μg/0.6 μl/min) of 6-OHDA or vehicle were made stereotaxically into the locus coeruleus under Nembutal anesthesia 48 days prior to killing the animal

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[12]. All animals were located in a temperature-regulated animal room on a 12 hr/12 hr light–dark cycle. Food and water were available *ad lib*. Animals were decapitated at the appropriate time after drug administration.

Drugs. Clonidine hydrochloride, piperoxane chlorohydrate, and 6-OHDA hydrobromide (Sigma) were used. Doses refer to the salt form. Clonidine and piperoxane were freshly dissolved in 0.9% isotonic saline and were administered intraperitoneally with a volume of 1 ml/kg. 6-Hydroxydopamine was dissolved in an ice-cold 0.1% solution of ascorbic acid in isotonic saline immediately before infusion.

Apparatus. The microspectrofluorimeter is described in detail elsewhere [13]. Briefly, a 1000 Watt Xenon lamp and grating monochromater (Schoeffel) provide 410 nm excitation light that is admitted to the specimen under computer control through an electronic shutter. A Leitz Orthoplan microscope and variable-aperture optical system with sub-stage phase-contrast red light and a Ploem epi-illuminator allow for alignment of the specimen in phase-contrast and the isolation of a 5 μm circular area of the cytoplasm adjacent to the nucleus for fluorescence measurement. Alternatively, the measurement aperture is aligned in the regions between cell bodies. This extraperikaryal measure has been found to respond to some drugs independently of changes in the intracellular measures [13]. A small grating monochromater is used to select the optimal emission wavelength (512 nm) for biogenic amines to be detected by a Schoeffel M460 photometer. After alignment of each cell, readings are taken automatically at one per second and stored on magnetic tape for subsequent analyses. Both model droplet analyses and *in vitro* studies with pargyline have shown that the fluorescence intensity remaining after 14 sec of excitation is proportional to the concentration of CAs, while a fading measure—the difference in fluorescence intensities from sec 1 to sec 14 of excitation—is the best predictor of 5-HT concentration and independent of CA levels. To allow reliable comparisons among a large number of tissue samples, the formaldehyde histochemical method has been modified so that all samples within an experiment are batch-processed together from sacrifice until sectioning [13].

Microscopy. Using red-light phase-contrast, the slides containing the regions of interest were selected by reference to predetermined landmarks. In these experiments, one rostrocaudal plane through the midbrain was selected for the dorsal and median raphe cell measures. For each region, at least six intracellular and six extraperikaryal readings were taken from two adjacent slides. Four background readings were taken from non-fluorescent cell bodies in the lateral reticular formation. Since cells were selected by phase-contrast, no experimeter bias could contribute to the individual cell readings, as discussed previously [13]. Furthermore, tissue blocks and slides were coded by number so that the experimenter was blind to the treatment condition when conducting the microscopy.

Tissue preparation. At the appropriate time after injection, animals were decapitated, and their brains were removed within 90 sec. After dissection, as described previously [13], brain samples were frozen in propane, freeze-dried over phosphorous pentoxide for 4 weeks at −60°, treated with gaseous formaldehyde, embedded in paraffin, and sectioned at eight μm on a rotary microtome. Sections were mounted on slides in Entellan (Merck).

High performance liquid chromatography (HPLC). The chromatographic system used was the Bioanalytical Systems LC-17 equipped with a "μBondapack" C-18 reverse-phase column (3.9 × 30 cm; Waters Associates), an Altex pump, and an LC-4 amperometric detector coupled to a TL-5 glassy carbon electrode (Bioanalytical Systems) and an Ag/AgCl reference electrode. Stainless steel tubing was used throughout. Output from this system was recorded with a Shimadzu integrator and a Houston dual-pen recorder. Water for the solvent was deionized using a Millipore "Milli-Q" water purification system. Amines and their metabolites were estimated according to the methods of Mefford [14]. As described earlier, slight modifications have been made for 5-HT and 5-HIAA assays [1] and for DA and 3,4-dihydroxyphenylacetic acid (DOPAC) assays [2] respectively.

Statistics. In cytofluorimetric studies, separate analyses of variance (ANOVAs) were done for both intracellular and extraperikaryal measures from each brain region examined. The appropriate values from each animal were averaged after subtraction of the corresponding blank value so that each animal contributed only one value to each ANOVA. Specific comparisons between each treatment group and a common control group were made with Dunnett's *t*-test [15]. The same analysis of variance and Dunnett's *t*-test (tD) were also used to analyze biochemical data.

Experiment 1. The effects of clonidine on dorsal and median raphe cells were examined in this experiment. Nine animals were randomly divided into two groups. Group 1 (N = 5) received a single intraperitoneal (i.p.) injection of saline; group 2 (N = 4) received a single i.p. injection of 50 μg/kg clonidine. Animals were killed 45 min after injection. Raphe brain slices were processed for fluorescence histochemistry.

Experiment 2. To test the hypothesis that the effects of clonidine on 5-HT neurons are mediated through the noradrenergic system, the alpha-adrenergic antagonist piperoxane was used in this experiment. Twenty-four animals were randomly assigned to four groups. Group 1 (N = 6) received a single i.p. injection of saline; group 2 (N = 6) received an i.p. injection of 50 μg/kg clonidine; group 3 (N = 6) received an i.p. injection of 10 mg/kg piperoxane; and group 4 (N = 6) received an i.p. injection of the combination of 50 μg/kg clonidine and 10 mg/kg piperoxane. Animals were killed 45 min after injections. Raphe brain slices of these animals were processed for fluorescence histochemistry, and caudate nucleus of all animals for biochemistry of 5-HT, 5-HIAA, DA and DOPAC.

Experiment 3. To verify the specificity of noradrenergic mediation of the observed effects of clonidine on 5-HT neurons, 6-OHDA was directly infused into the locus coeruleus in five animals. The other five animals received sham operations. De-

tailed information regarding surgery, drug infusion, and lesion verification is described elsewhere [12].

RESULTS

Experiment 1. As shown previously for apomorphine [1], clonidine preferentially elevated intracellular 5-HT fluorescence in the dorsal raphe (saline: 1608 ± 68; clonidine: 1975 ± 97; $F = 10.18$, $P < 0.05$) without affecting 5-HT cells in the median raphe significantly (saline: 2604 ± 72; clonidine: 2526 ± 136; NS). Extracellular CA fluorescence was also decreased by clonidine in the dorsal raphe which was confirmed by the final fluorescence intensity measure (saline: 2961 ± 144; clonidine: 2529 ± 43; $F = 6.66$, $P < 0.05$). Neither 5-HT nor CA fluorescence measure was altered significantly by clonidine in the median raphe nucleus.

Experiment 2. As displayed in Fig. 1, clonidine consistently elevated the 5-HT fading measure intracellularly in the dorsal raphe ($F = 6.32$, $P < 0.05$). Piperoxane at 10 mg/kg had no significant effect by itself, yet completely antagonized the effect of clonidine on this measure. As shown in Fig. 2, piperoxane also antagonized the clonidine-induced reduction of extracellular CA fluorescence in the dorsal raphe ($F = 3.90$, $P < 0.05$). Consistent with the results of experiment 1, none of the treatments had any significant effect on 5-HT or CA fluorescence in the median raphe nucleus.

Biochemically, as shown in Table 1, clonidine also significantly elevated 5-HT concentration in the striatum ($F = 4.93$, $P < 0.05$). Piperoxane alone produced a non-significant decrease of 5-HT in this region but completely antagonized the effect of clonidine on 5-HT. Neither drug altered 5-HIAA level significantly; however, when a measure of 5-HT turnover (5-HIAA/5-HT) was considered, clonidine decreased while piperoxane increased this ratio in the striatum ($tD = 2.98$, $P < 0.01$ and $tD = 2.42$, $P < 0.05$).

The effects of clonidine and piperoxane on the dopaminergic system are summarized in Table 2. As shown in this table, striatal DA was not altered significantly by either manipulation. However, clonidine caused a decrease and piperoxane produced a marked increase of DOPAC concentration in this region ($tD = 2.89$, $P < 0.05$ and $tD = 12.12$, $P < 0.01$). Therefore, clonidine decreased and piperoxane increased DA turnover, as measured by the ratio of DOPAC to DA. The combination of these two drugs still maintained a significant elevation of DOPAC level in the striatum ($tD = 5.74$, $P < 0.01$).

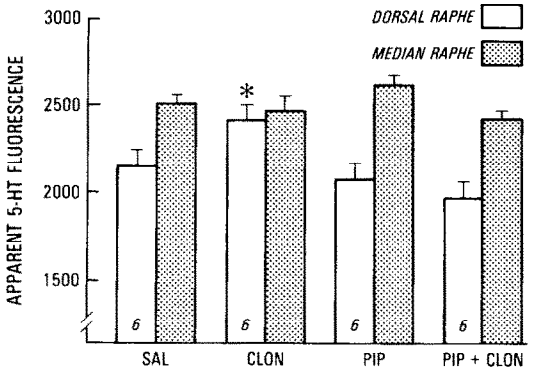

Fig. 1. Effects of clonidine (50 μg/kg) and piperoxane (10 mg/kg) on intracellular 5-HT fluorescence in the raphe nuclei. Each bar represents the mean ± S.E.M. of six animals. Key: (*) $P < 0.05$.

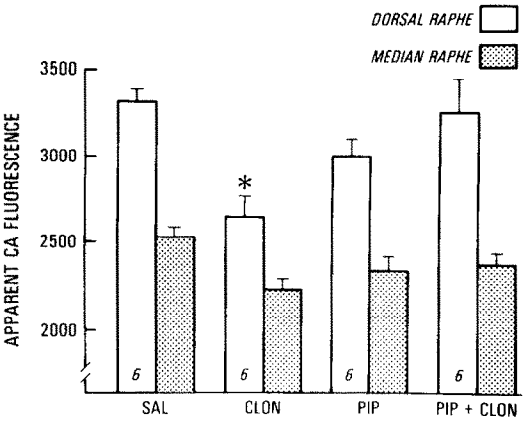

Fig. 2. Effects of clonidine (50 μg/kg) and piperoxane (10 mg/kg) on extraperikaryal CA fluorescence in the raphe nuclei. Each bar represents the mean ± S.E.M. of six animals. Key: (*) $P < 0.05$.

Table 1. Effects of clonidine and piperoxane on the striatal serotonergic system*

Treatment	N	5-HT	5-HIAA	5-HIAA/5-HT
SAL	6	1019 ± 22	895 ± 37	0.88
CLON (50 μg/kg)	6	1134 ± 27†	853 ± 23	0.75‡
PIP (10 mg/kg)	6	960 ± 44	975 ± 65	1.01†
PIP + CLON	6	968 ± 44	911 ± 63	0.94

* Values are means ± S.E.M. and are not corrected for recovery. Data are expressed as ng/g tissue.
† $P < 0.05$.
‡ $P < 0.01$.

Table 2. Effects of clonidine and piperoxane on the striatal dopaminergic system*

Treatment	N	DA	DOPAC	DOPAC/DA
SAL	6	5728 ± 323	273 ± 24	0.048
CLON (50 μg/kg)	6	5923 ± 188	196 ± 28†	0.033‡
PIP (10 mg/kg)	6	5339 ± 534	515 ± 83‡	0.096†
PIP + CLON	6	6032 ± 630	465 ± 35‡	0.077

* Values are means ± S.E.M. and are not corrected for recovery. Data are expressed as ng/g tissue.
† $P < 0.05$.
‡ $P < 0.01$.

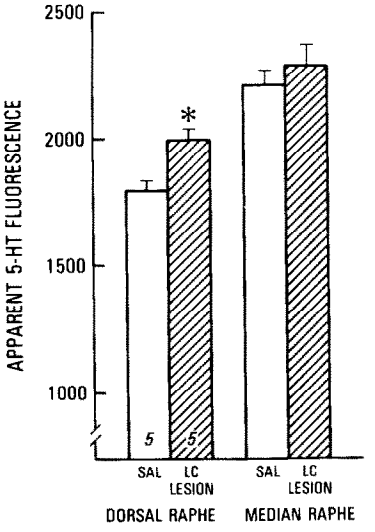

Fig. 3. Effects of 6-OHDA lesion of locus coeruleus on intracellular 5-HT fluorescence in the raphe nuclei. Each bar represents the mean ± S.E.M. of five animals. Key: (*) $P < 0.05$.

Experiment 3. As shown in Fig. 3, similar to the effects of clonidine, 6-OHDA lesions of the locus coeruleus also elevated intracellular 5-HT fluorescence in the dorsal raphe without influencing 5-HT cells in the median raphe significantly ($F = 11.62$, $P < 0.05$). A detailed investigation further revealed that this manipulation in general elevated cellular 5-HT concentrations in each subdivision (dorsal, ventral, medial and lateral parts) of the dorsal raphe (data not shown). Catecholamine fluorescence was decreased significantly by the same manipulation in both the dorsal and median raphe nuclei, as illustrated in Fig. 4 ($F = 20.57$, $P < 0.01$ and $F = 5.18$, $P < 0.05$ respectively).

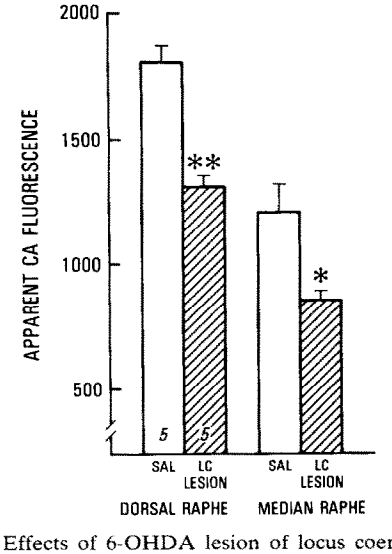

Fig. 4. Effects of 6-OHDA lesion of locus coeruleus on extraperikaryal CA fluorescence in the raphe nuclei. Each bar represents the mean ± S.E.M. of five animals. Key: (*) $P < 0.05$ and (**) $P < 0.01$.

DISCUSSION

Systemic administration of clonidine at low doses has been shown to suppress NE neuron firing in the locus coeruleus and 5-HT neuron firing in the dorsal raphe nucleus [16]. The action of clonidine on 5-HT neurons has been suggested to be mediated through the noradrenergic system since administration of reserpine, which impairs noradrenergic transmission, also suppresses 5-HT cell firing [17]. In contrast, administration of amphetamine, which releases CAs [18, 19], restores 5-HT cell firing. These studies suggest that the firing activity of 5-HT containing neurons in the dorsal raphe is dependent upon a tonically active noradrenergic input. Furthermore, clonidine has weak or variable effects on 5-HT cell activity when applied microiontophoretically to raphe neurons [16].

Anatomical evidence has also indicated a noradrenergic–serotonergic interaction between the locus coeruleus and the dorsal raphe. Fluorescence histochemical studies have suggested that CA terminals innervate 5-HT cells in the dorsal raphe [20]. Horseradish peroxidase study also indicates that the dorsal raphe nucleus receives direct projections arising from the locus coeruleus [21]. Noradrenergic terminals in the dorsal raphe have also been identified by biochemical [16, 22, 23] and immunocytochemical [24, 25] methods.

The present results indicate that clonidine selectively augmented intracellular 5-HT level in the dorsal raphe without significantly affecting 5-HT cells in the median raphe nucleus. Clonidine also decreased a measure of 5-HT turnover in the striatum. The effects reported here may indeed be underestimates, since the influence of clonidine on the serotonergic system reportedly peaks at 3 hr [7] whereas our animals were killed 45 min after injection. In any case, these results are consistent with the hypothesis that noradrenergic innervation to the dorsal raphe mediates a tonically active influence on 5-HT cells in this area. Clonidine, by inhibiting NE neuron firing in the locus coeruleus, decreased NE release and thus removed the tonic excitatory input to dorsal raphe neurons. Decreased serotonergic activity was indicated by an apparent reduction in 5-HT turnover, and the elevated intracellular 5-HT content may have resulted from a decrease of 5-HT release from cell bodies. This hypothesis is further supported by the finding that CA concentration in the dorsal raphe was also reduced after clonidine administration. Piperoxane, possibly by exerting an opposite action on NE neurons in the locus coeruleus, antagonized the effect of clonidine on raphe neurons and increased a measure of 5-HT turnover in the striatum. These results are consistent with most of the biochemical data suggesting that clonidine decreased brain 5-HT synthesis and turnover [5–7].

Furthermore, a long-term depletion of central noradrenergic neurons by 6-OHDA 48 days prior to sacrifice yielded similar results. Specifically, bilateral 6-OHDA-lesions of the locus coeruleus also augmented intracellular 5-HT fluorescence in the dorsal

raphe preferentially and decreased CA fluorescence in both raphe nuclei. The decreased CA concentration in the dorsal raphe is consistent with the hypothesis of a noradrenergic innervation from the locus coeruleus to the dorsal raphe. The same finding in the median raphe may have been due to a depletion of the ascending medial forebrain bundle or it may indicate the existence of a noradrenergic input to the median raphe as well.

The differential effects of clonidine on 5-HT in the dorsal versus median raphe may be related to the differential amounts of catecholaminergic innervation to these two nuclei [26, 27]. We previously reported that the dopaminergic agonists apomorphine and amphetamine both had selective effects on 5-HT fluorescence in the dorsal versus median raphe, although the effect of amphetamine was to decrease rather than increase intracellular 5-HT [28]. Additionally, the different phenomena observed with clonidine and 6-OHDA on dorsal and median raphe neurons further suggest that clonidine influences the serotonergic system indirectly through noradrenergic mediation. These results also support the anatomical data that the dorsal and median raphe comprise two distinctive cell groups with distinctive projections to the forebrain [29–31].

Accumulating evidence has also indicated noradrenergic–dopaminergic interactions between the locus coeruleus and the substantia nigra [32]. Diffuse NE containing nerve endings in the neuropil of the substantia nigra zona reticulata have been demonstrated [33]. Both neurochemical and electrophysiological evidence also suggest that a pathway exists from the locus coeruleus to the substantia nigra which utilizes NE as a neurotransmitter and that stimulation of the locus coeruleus exerts an excitatory effect on cells in the substantia nigra [34]. Further, the nigral neurons excited by locus coeruleus stimulation have been identified as being within the zona compacta and to have firing rates and action potentials characteristic of DA neurons [35].

In the present study, clonidine reduced the apparent turnover of DA in the striatum, the projection site of the substantia nigra, whereas piperoxone increased the same measure; the effect of a combination of these two drugs did not differ from control effects. These results are consistent with the hypothesis that noradrenergic systems in the locus coeruleus facilitate DA cell activity in the nigrostriatal pathway, since suppression of locus coeruleus neuron firing by clonidine decreased the activity and metabolism of dopaminergic neurons. This hypothesis is further supported by other biochemical data showing that clonidine and phenoxybenzamine decelerate the synthesis and utilization of brain DA [36]. Conversely, the noradrenergic antagonist yohimbine accelerates these measures [9, 10]. Furthermore, clonidine has been shown to decrease tyrosine hydroxylase activity in mouse brain [11]. Opposite results indicating increased DA synthesis following clonidine administration have also been reported [37]; however, this might have been due to the use of different doses of the drug. Additionally, NE concentration in the substantia nigra was also increased by clonidine (unpublished observations). The elevated NE in the substantia nigra might have been a buildup of NE in presynaptic terminals, resulting from reduced impulse flow along the locus coeruleus–substantia nigra pathway after clonidine. An alternative possibility is that the dopaminergic effects of clonidine were mediated indirectly through the dorsal raphe, which projects to the substantia nigra [38]. However, this more circuitous route would not explain the elevation of nigral NE by clonidine. Clearly, further study is required to clarify the nature of the interactions among the monoaminergic systems in brain.

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