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Clonidine-Induced Sedation is not Modified by Single or Combined Neurochemical Lesions of the Locus Coeruleus, the Median and Dorsal Raphe Nuclei

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NASSIF-CAUDARELLA, S., E. KEMPF AND L. VELLEY. *Clonidine-induced sedation is not modified by single or combined neurochemical lesions of the locus coeruleus, the median and dorsal raphe nuclei.* PHARMACOL BIOCHEM BEHAV **25**(6) 1211–1216, 1986.—Single or combined neurochemical lesions of the locus coeruleus, the dorsal and the median raphe nuclei were performed on different groups of rats. Starting 10 days after the lesion, the locomotor activity of all rats was measured for 5 min every day in an open-field. For the first 21 days all lesioned rats, independently of the lesion site, were significantly less active than controls, but from the 11th to the 16th day the locomotor activity of lesioned animals increased progressively and, thus on days 15 and 16, the mean activity of all lesioned groups was not significantly different from that of the controls. From the 17th day onwards the sedative effect of small doses of clonidine (5–100 μg/kg) was measured. Neither single nor combined lesions modified the response to clonidine and the linear decrease of activity produced by increasing doses of clonidine was the same in all groups, lesioned or not. Biochemical assays showed a marked loss of corresponding amines as a result of the lesions in cortex, hippocampus and the brainstem. These results suggest that the α_2-receptors involved in clonidine-induced sedation are located neither on noradrenergic fibers coming from the locus coeruleus, nor on serotoninergic fibers originating in the median and dorsal raphe nuclei.

Neurochemical lesions Locus coeruleus Median and dorsal raphe nuclei Clonidine Sedation Open-field Rats

IN spite of many studies, the mechanism underlying the well-known sedative effect of small doses of the α_2-receptor agonist clonidine is not clearly understood. According to some authors, clonidine produces sedation by activating α_2-receptors located presynaptically on noradrenergic terminals and cell bodies and consequently mediating a feed-back inhibition of noradrenaline (NA) release [11, 12, 20, 39, 40, 50]. If this hypothesis is correct, the specific destruction of noradrenergic neurons by 6-hydroxydopamine (6-OHDA) would reduce the clonidine-induced sedation as a result of the loss of the presynaptic α_2-binding sites. However, binding studies have shown that after central noradrenergic denervation the number of the α_2-binding sites is either increased or not modified in most of the brain regions analysed [7, 16, 28, 31, 38, 42–45, 48], suggesting a postsynaptic location of these receptors (review in [35]). In agreement with these findings we showed recently [29] that the sedative effect of small doses of clonidine was not modified after a 6-OHDA lesion of the locus coeruleus (LC) in spite of an almost total loss of NA in the cortex and hippocampus and a 33% loss of NA in the brainstem.

However, neither the binding data nor the behavioral results are easy to explain since the possible loss of presynaptic α_2-adrenoceptors might have been masked by an increased number of the α_2-binding sites located postsynaptically. The purpose of the present study was an attempt to test this possibility. Recently, α_2-adrenoceptors have been reported to be located on serotoninergic neurons in the rat cerebral cortex and hippocampus [13, 15, 25, 34]. Consequently, we decided further to investigate the effects of

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single or combined lesions of brain noradrenergic and serotoninergic neurons on clonidine-induced sedation.

METHOD

Animals

Male rats of the Sprague-Dawley strain were individually housed in wire-mesh cages and maintained on a regular 12 hr/12 hr light-dark cycle in a temperature-regulated (21–23°C) animal room. Food and water were provided ad lib. Surgery was performed when the rats were one month old.

Surgical Procedures

Each rat was anaesthetized with 40 mg/kg of Nembutal and placed in a stereotaxic apparatus (Kopf Instruments). The incisor bar was level with the interaural line. The nucleus locus coeruleus (LC) was bilaterally destroyed in 6 rats by local injection of 6-hydroxydopamine (4 μg expressed as weight of the salt, dissolved in 1 μl of 0.9% saline containing 0.2 mg/ml of ascorbic acid). The injections were made through two cannulae (external diameter 240 μm) connected to a micropump. The co-ordinates were: 1.7 mm posterior to the ear bars, $\pm$1.2 mm lateral to the sagittal suture and 6.5 mm ventral to the top of the skull. The injections were made over 6 min 30 sec and 8 more min elapsed before removal of the cannulae. The dorsal raphe nucleus (DR, n=6) or the median raphe nucleus (MR, n=8) were lesioned by local injection of 5-7 dihydroxytryptamine (5-7 DHT: 10 μg expressed as weight of the salt, dissolved in 2 μl of 0.9% saline containing 0.2 mg/ml ascorbic acid). Only one cannula implanted in the sagittal plane, 0.3 mm anterior to the ear bars was used to inject the neurotoxin in the DR or in the MR. For the DR the cannula was implanted 6.2 mm ventral to the top of the skull and for the MR 8 mm ventral to the top of the skull. For the combined lesions, the same co-ordinates as those indicated above were used. Each double lesion (LC+MR, n=8 and DR+MR, n=8) and the triple lesion (LC+MR+DR, n=8) was performed during the same operation. For the two kinds of lesion including the destruction of the MR and the DR, the MR nucleus was lesioned first. Twenty minutes before the neurotoxin injections the rats received IP a dose of 50 mg/kg of pargyline. All co-ordinates were previously determined on rats of the same age. Moreover, a preliminary neurochemical analysis showed that with the injection procedures described above, 6-OHDA and 5-7 DHT produced a significant decrease of noradrenaline and serotonin respectively in the cortex, the hippocampus and the brainstem. Two groups of rats injected with pargyline were used as controls: one group of non-operated rats (group C, n=6) and one group of rats injected in the LC (n=3) or in the MR (n=3) with the vehicle (group V).

Behavioral Test

The locomotor activity of all rats was tested 21 days later in a square wooden open-field that measured 100×100×50 cm [29]. The floor was white and divided into 25 squares of equal size. The open-field was evenly lit by overhead lights (1100 lux at the floor). The activity of each rat was recorded by means of a closed-circuit television coupled with a video-recorder. Each day, the rat was placed in the center of the open-field and the number of squares crossed was recorded for a 5 min period. During the first 16 consecutive days, no drug or vehicle was injected before the test. This preliminary period was necessary since, at the beginning of testing, the locomotor activity of lesioned rats was significantly different from the activity of control rats (see the Behavioral Results section). On days 17, 20, 23 and 27 all rats received IP injections of increasing doses of clonidine HCl, 30 min before testing, 5 μg/kg on day 17, 10 μg/kg on day 20, 50 μg/kg on day 23 and 100 μg/kg on day 27. The drug was dissolved in 0.9% NaCl and all doses were injected in a volume of 1 ml/kg body weight. On all other days, the locomotor activity of each rat was measured but no drug was injected. The experiments were carried out between 10 a.m. and 2 p.m. Behavioral testing ended on day 30 after the beginning of the experiment.

Biochemical Assays

At the end of the experiment, all rats were decapitated, the brains were quickly removed and dissected on ice into the following regions: hippocampus, cerebral cortex and brainstem. The different brain areas were stored in liquid nitrogen until use. Noradrenaline (NA) and serotonin (5-HT) were determined simultaneously utilizing a reverse phase chromatography procedure coupled with an electrochemical detection. On the day of analysis, frozen samples of cortex, hippocampus and brainstem were weighed and homogenized in $HClO_4$ 0.1 N containing Na metabisulfite 6 mM and EDTA 1 mM. The homogenates were centrifuged at 10,000 g for 20 min at 4°C. Aliquots of the supernatant were transferred into the LCEC system with a Wisp automatic injector (Waters). The LCEC system consisted of a Bioanalytical systems LC4 amperometric detector with a glassy carbon working electrode and a pump (Waters). The potential was set at 800 mV (vs. Ag-AgCl reference electrode). The column, a Bondapak phenyl column (10 μm particle size, 300×3.1 mm i.d.) was purchased from Waters Assoc. The flow rate was 1.4 ml/min, and the sensitivity was set at 5 nA/V (1 volt full scale). The mobile phase consisted of 3% methanol in 0.1 M Na-phosphate buffer pH 2.5, Na^2 EDTA 0.1 mM, and 1-octane sulfonic acid Na salt (BDH) 2 mM. 3,4-Dihydroxyhydrocinnamic acid (Aldrich) was used as internal standard.

RESULTS

Neither single nor combined lesions produced visible behavioral debilitation. The day before the beginning of the test, namely 20 days after the operation, the mean body weight of the non-operated rats did not differ from the mean body weight of the vehicle-injected animals [$F(1,10)=0.13$, n.s.]. Likewise the mean body weight of these two pooled groups was not different from the mean body weights of all lesioned groups of rats [$F(6,49)=0.74$, n.s.]. Results showing the locomotor activity over the first 16 days of testing without drug are shown in Fig. 1. No significant difference was observed between the activity of non-operated rats and that of vehicle-injected rats [analysis of variance with repeated measures: $F(1,10)=0.29$, n.s.]; the data from these two groups were thus pooled (Fig. 1, top, C+V). All lesions either single or combined produced a significant decrease of locomotor activity. The top part of Fig. 1 shows the evolution of locomotor activity of the 3 groups of rats with a single lesion of LC, DR or the MR nucleus. No significant difference was observed between the 3 groups, $F(2,17)=1.28$, $p=0.30$). However, the activity of the MR lesioned group was significantly lower than the activity of the C+V group,

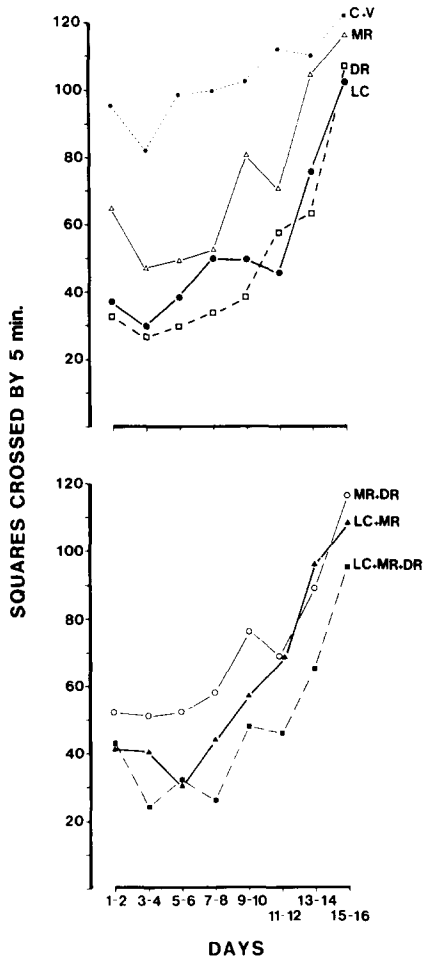

FIG. 1. Open-field locomotor activity during the first 16 days of testing. Top: activity of the C+V group and of the 3 groups with an isolated lesion. Bottom: activity of the 3 groups of rats with a combined lesion. Each point represents the mean number of squares crossed. For clarity the individual values of two consecutive days have been pooled and the S.E.M.s (between 6.5 and 22.3) are not shown.

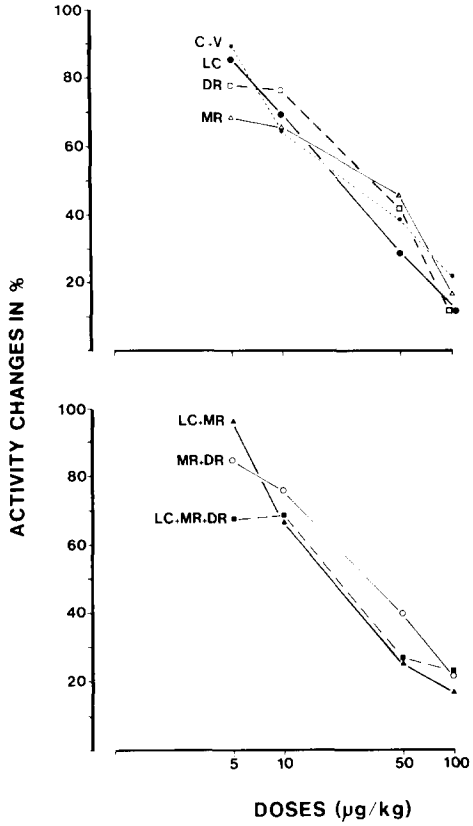

FIG. 2. Effect of increasing doses of clonidine on the open-field activity of the different groups of rats. Top: C+V group and the 3 groups with an isolated lesion. Bottom: groups of rats with a combined lesion. Abscissae: doses of clonidine (log. scale). Ordinates: activity changes calculated as percentages of the mean activity levels of each group of rats during the last two days before the first injection of clonidine (days 15 and 16, see Fig. 1). For clarity S.E.M.s (between 2.7 and 18.4) are not shown.

$F(1,18)=5.45$, $p=0.03$. The bottom part of Fig. 1 summarizes the effect of the double and triple lesions. The activity of these 3 groups was not different, $F(2,21)=1.06$, $p=0.36$, but the activity of the MR+DR lesioned rats was significantly different from the activity of the C+V rats, $F(1,18)=5.8$, $p=0.03$. Moreover, no significant difference was observed between the groups with a single lesion and those with a combined lesion. For example, the comparison between the 3 groups with a single lesion and the group with the triple lesion was not significant, $F(3,24)=1.23$, $p=0.32$. Lastly, the activity of all operated groups increased progressively from the days 7 to 10 and on the last two days of preliminary testing, the mean values of these groups no longer differed from the values of the C+V group [$F(6,49)=0.69$, n.s.].

Figure 2 shows the effect of increasing doses of clonidine on the locomotor activity of the different groups. The decreases produced by the drug were the same in the vehicle and control groups. Consequently these two groups were pooled (C+V). A 7×4 (group × clonidine) ANOVA was conducted to test the interaction between the lesions and the effect of increasing doses of clonidine. This analysis showed no significant group effect, $F(6,49)=1.39$, $p=0.23$, while the drug effect was significant, $F(3,147)=166.6$, $p<0.001$. However, the interaction term (lesion/drug) was not significant, $F(18,147)=1.41$, $p=0.13$, showing that whatever the lesion performed the sedative effect of increasing doses of clonidine was the same.

Neurochemical Analysis

The results of this analysis are summarized in Table 1. As for the behavioral data, the results of the non-operated rats and those of the vehicle-injected rats were pooled since no significant difference for noradrenaline and serotonin contents was observed in any of the 3 structures analysed (p between 0.30 and 0.98 for the 6 determinations).

The LC lesion did not modify serotonin levels; likewise the MR lesion had no influence on the level of noradrenaline. However a small but significant loss of noradrenaline in the hippocampus was observed after the DR lesion. The combined LC+MR lesion produced a significant loss of the two

TABLE 1

EFFECT OF THE DIFFERENT NEUROCHEMICAL LESIONS ON NORADRENALINE AND SEROTONIN CONTENT IN CORTEX (Cx), HIPPOCAMPUS (Hpc) AND THE BRAINSTEM (Bs)

		NA			5-HT		
Groups		Cx	Hpc	Bs	Cx	Hpc	Bs
Control	(6)	299 ± 30	248 ± 25	678 ± 42	264 ± 14	518 ± 58	764 ± 48
Vehicle	(6)	250 ± 32	210 ± 35	653 ± 30	277 ± 20	598 ± 67	766 ± 31
LC	(6)	28 ± 8‡	62 ± 12‡	449 ± 22‡	263 ± 17	583 ± 36	781 ± 35
	%	10	27	67	97	104	102
MR	(8)	243 ± 23	205 ± 29	609 ± 43	183 ± 25†	305 ± 66†	623 ± 67*
	%	89	90	92	68	55	81
DR	(6)	225 ± 7	130 ± 24*	613 ± 57	113 ± 32‡	276 ± 30‡	563 ± 58†
	%	82	57	92	42	49	73
LC+MR	(8)	54 ± 13‡	68 ± 14‡	427 ± 27‡	126 ± 12‡	134 ± 19‡	500 ± 30‡
	%	20	30	64	47	24	65
MR+DR	(8)	208 ± 5	159 ± 6	570 ± 26*	88 ± 16‡	179 ± 26‡	476 ± 45‡
	%	76	69	86	33	32	62
LC+MR+DR	(8)	ND	68 ± 4 ‡	477 ± 34‡	110 ± 27‡	258 ± 62‡	464 ± 30‡
	%		30	72	41	46	61

Values in ng/g ± SEM. Statistical significances: *t*-test was used to compare each mean value of each lesioned group with the corresponding mean value of the pooled group C+V: *$p<0.05$; †$p<0.01$; ‡$p<0.001$. %: residual content expressed as percentage of the corresponding values of the C+V group. ND: not determined.

amines in the 3 structures. After the combined lesion of the two raphe nuclei, besides the expected loss of serotonin, a significant decrease of noradrenaline was observed in the brainstem.

DISCUSSION

The present study was primarily designed to test if the sedative effect of small doses of clonidine could be suppressed or attenuated by single or combined lesions of the dorsal and median raphe nuclei or by a lesion including, in addition, the nucleus locus coeruleus. Our pharmacological data show clearly that whatever the lesion performed, the sedation produced by the drug was the same and did not differ from the sedation observed in normal or vehicle injected rats. Furthermore, the linear decrease of activity with increasing doses of clonidine was the same in all groups lesioned or not.

In accordance with our previous data [29] the LC lesion did not modify the response to clonidine, showing that the presynaptic α_2-receptors located on the coerulean cell bodies and terminals are not involved in the sedative effect of the drug. As already indicated, however (see the Introduction section), the increase in postsynaptic α_2-receptors due to the 6-OHDA treatment might have masked a loss of presynaptic binding sites. In fact some authors observed a small decrease of the α_2-binding sites some days after noradrenergic denervation [7,42], but this finding was not confirmed by others [16]. Thus far, presynaptic and postsynaptic α_2-receptors are pharmacologically indistinguishable ([42]; see however [5,32]) and it is not known if the function of these two kinds of receptors is different or not. With regard to the sedative effect of clonidine, if the activation of the pre- and postsynaptic α_2-receptors produces the same behavioral action, the lack of effect of the LC lesion might be explained by an increase of the postsynaptic binding sites which would compensate for the loss of presynaptic receptors. However, the present results show that neither a single lesion of either the DR or the MR, nor a combined lesion of these two nuclei modified the sedative effect of clonidine, suggesting that α_2-receptors located on the fibers coming from the DR and the MR are not implicated in the behavioral action of this drug.

Our findings are indirectly confirmed by recent data showing that the number of ^{3}H-clonidine binding sites in cortical membranes was neither increased nor decreased after intraventricular injection of 5-7 DHT [16]. Likewise, the same injection did not modify the number of ^{3}H-para-amino-clonidine binding sites in the cortex and the hypothalamus, while a small but significant increase of these receptors was observed in the hippocampus, suggesting a postsynaptic location of these sites [33]. By contrast, our behavioral data do not confirm the results of Kostowski *et al.* [23], indicating that the neurochemical lesion of the median raphe suppressed the sedative action of clonidine. This discrepancy may be explained by some differences in the procedures used. In particular, the neurotoxin used by these authors was 5-6 dihydroxytryptamine and the histological analysis showed an extensive damage of the median raphe region, suggesting that the drug has produced a non-specific lesion. To explain their observations these authors proposed that the destruction of serotoninergic neurons makes noradrenergic neurons hyperactive and, consequently, less sensitive to clonidine. This hypothesis is not confirmed by the present results, since the combined lesion including the LC and the MR, as well as the triple lesion did not change the response to clonidine, despite a significant loss of the two amines in cortex and hippocampus (Table 1).

However, our presently available data are not sufficient to disprove the hypothesis that the sedative action of

clonidine would be due to activation of α_2-receptors located on noradrenergic fibers and/or on serotoninergic fibers. First, neither 6-OHDA nor 5-7 DHT lesions produced total loss of NA and 5-HT in cortex and the hippocampus. Furthermore, we can suppose that denervation induced an increase in the number of α_2-receptors located postsynaptically. Thus, the possibility that these two associated modifications would be sufficient to maintain the sedative effect of clonidine cannot be excluded. However, this hypothesis is unlikely, since, given the significant loss of the two amines in cortex and the hippocampus, one would expect a modification of the dose-response relationship; such a modification was not observed. Second, our biochemical results show that the loss of NA and 5-HT in the brainstem, although significant, was small (Table 1). This result could be expected since the lateral and dorsal tegmental NA cell groups (review in [26]) as well as the 5-HT cell groups of the caudal medulla (review in [9]) were spared by our local lesions. Thus, given the widespread distribution of the α_2-receptor in the brain, especially in the spinal cord and in the medulla oblongata [41], the sedation produced by clonidine could be due to activation of α_2-binding sites located on terminals or cell bodies of these undamaged aminergic systems. Behavioral data showed that clonidine depresses acoustic startle in acutely decerebrate rats, indicating that the action of the drug was due to the activation of the α_2-receptors located in the caudal brainstem and/or in the spinal cord [8]. However, changes in startle do not correlate with changes in locomotor activity [20], showing that the regulation of these two behaviors is not the same. Thus, further experiments are necessary in order to investigate the possible role of NA and 5-HT neurons located in the caudal brainstem on the clonidine-induced sedation.

Lastly, a comment is in order with regard to the effects of the various lesions on the locomotor activity. In agreement with previous data [49] our results show that the locomotor activity of the control rats declined slightly during the first 4 days of testing, then increased between days 5 and 16. The injection of vehicle in the LC or in the MR did not modify this evolution. By contrast and in accordance with our previous findings [29], neurochemical lesion of the LC significantly decreased locomotor activity during the first 12 days of testing. This observation agrees with some previous findings [4,17] but differs from other results [6,36]. This discrepancy may be explained by certain procedural differences: a locomotor activity deficit was observed only if the open-field was the first test used after the lesion ([4, 17, 29] and the present data), whereas if the activity was measured after other behavioral tasks, decrease of activity was not observed [6,36]. The fact that this deficit disappeared with repeated testing confirms this possibility and suggests in agreement with the hypothesis of Amaral and Sinnamon [1] that a major function of the LC is to dampen the organism's response to stressors and especially to novelty [4,47].

In our experimental conditions a single lesion of the DR or the MR produced the same deficit as the LC lesion. The role of these serotoninergic nuclei in the regulation of locomotor activity is controversial. In contrast with electrolytic lesion of the MR which, in general, produced hyperactivity [21] the neurochemical lesion of this nucleus by 5-7 DHT either did not modify the activity level [2, 18, 24] or decreased it [37]. With regard to the DR nucleus, neither the electrolytic nor the neurochemical lesion modified the locomotor activity [14, 21, 24] while the combined lesion of the two nuclei by 5-7 DHT produced significant hypoactivity [10,19]. The results of our combined serotoninergic lesions are in agreement with these last data.

The combined lesions including on the one hand the LC and the MR, and on the other hand the LC and the two raphe nuclei did not significantly increase the deficit observed after a single lesion, showing that these noradrenergic and serotoninergic nuclei are coupled together with regard to the regulation of locomotor activity. It was suggested that noradrenaline and serotonin are mutually antagonistic systems with opposed behavioral functions [9, 22, 27]. However, it is noteworthy that these two systems have many characteristics in common [1, 3, 46]. Thus the possibility that in particular behavioral situations, as those used in the present experiments, the lesion of these two systems induces the same deficit, cannot be excluded.

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