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Chlordiazepoxide-Induced Released Responding in Extinction and Punishment-Conflict Procedures is not Altered by Neonatal Forebrain Norepinephrine Depletion

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BIALIK, R. J., B. A. PAPPAS AND W. PUSZTAY. *Chlordiazepoxide-induced released responding in extinction and punishment-conflict procedures is not altered by neonatal forebrain norepinephrine depletion.* PHARMAC. BIOCHEM. BEHAV. **16**(2) 279–283, 1982.—The effects of chlordiazepoxide (CDZ) in extinction and punishment-conflict tasks were examined in rats after neonatal systemic administration of 6-hydroxydopamine (6-OHDA) to deplete forebrain norepinephrine (NE). At about 70 days of age the rats were water deprived and trained for three days to drink in a novel apparatus. On the fourth day (test day) drinking was either extinguished by elimination of water from the drinking tube or punished by lick-contingent shock. Just prior to this test session half of the vehicle and half of the 6-OHDA treated rats were given an injection of CDZ (8 mg/kg IP). Both the injection of CDZ and forebrain NE depletion prolonged responding during extinction and reduced the suppressant effects of punishment in male rats, and these effects were of similar magnitude. Furthermore, CDZ was as effective in neonatal 6-OHDA treated male rats as in vehicle treated rats indicating that decreased transmission in ascending NE fibers caused by CDZ is not solely responsible for its behavioural effects in extinction and conflict tasks. Rather, these effects may involve cooperative mediation by both noradrenergic and serotonergic forebrain terminals. Unexpectedly, CDZ's anti-extinction effect was absent in female rats and its anti-conflict effect observed only in NE depleted females.

Chlordiazepoxide Extinction Conflict Neonatal 6-hydroxydopamine Norepinephrine

MINOR tranquilizing agents produce many effects on behaviour and on hippocampal electrical activity, specifically the 7.7 Hz theta rhythm, which are similar to effects caused by a reduction in forebrain norepinephrine (NE) transmission [6]. Both lesioning of the dorsal noradrenergic bundle (DNB) and administration of benzodiazepines eliminate the low threshold for activation of hippocampal 7.7 Hz theta by electrical stimulation of the septum [6]. Further, DNB lesions and benzodiazepine administration share some behavioural effects such as increased resistance to extinction of learned behaviours and increased resistance to the response-suppressive effects of punishment [1, 6, 7, 11, 19, 20, 23]. Since the 7.7 Hz hippocampal theta may mediate the behavioural inhibition necessary for normal responsiveness to extinction and punishment procedures [3, 4, 5], it follows that the reduced behavioural inhibition observed after benzodiazepine administration could be mediated through the DNB and its modulation of this theta rhythm. Recently, intravenous administration of the benzodiazepines, chlordiazepoxide (CDZ) and diazepam, have been reported to inhibit activity of cells in the locus coeruleus, the nucleus of origin of NE fibers ascending in the DNB, and thus a mechanism has been established by which benzodiazepines can inhibit transmission in the DNB [2].

Morris *et al.* [17] attempted to test this hypothesis by comparing the anti-extinction effect of CDZ in animals with and without radiofrequency lesions of the DNB. They reported that CDZ increased resistance to extinction in intact animals but not in animals which had received DNB lesions. The DNB lesions alone, however, did not increase resistance to extinction. To explain this result, Morris *et al.* claimed that their failure to observe increased resistance to extinction occurred because the lesioning technique caused only a 63% depletion of forebrain NE and there may have been enough intact DNB fibers (and/or supersensitive postsynaptic NE receptors in the hippocampus) to facilitate the 7.7 Hz hippocampal theta and inhibit the non-rewarded behaviour. Following this logic, however, would lead to the expectation

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that CDZ would reduce transmission in the remaining intact fibers in the DNB, thus preventing the facilitatory effect of these fibers on 7.7 Hz theta, thereby causing increased resistance to extinction. Therefore, this lack of effect of CDZ in animals with partial damage to the DNB may have been caused by some effect of the lesions other than damage to some of the DNB fibers, such as tissue damage not specific to noradrenergic neurons.

We attempted to circumvent this difficulty in the present experiment by utilizing neonatal systemic 6-OHDA treatment to more selectively deplete forebrain NE [18, 19, 20]. To increase the generality of the results, animals depleted of forebrain NE were tested for their response to CDZ in two different behavioural paradigms in which CDZ affects performance. The first was the extinction paradigm, the second was the lick-suppression punishment-conflict procedure. It has been well established that minor tranquilizers release suppressed responding in this punishment-conflict paradigm [23] as does depletion of forebrain NE [19,20]. We expected that both 6-OHDA lesioned and CDZ treated rats would show increased resistance to extinction and attenuated responses to punishment, and that CDZ would be ineffective in the 6-OHDA treated rats.

METHOD

Subjects were 216 offspring delivered in this laboratory by Wistar mothers acquired approximately 14 days pregnant from Biobreeding Ltd., Ottawa. Animals were run in four replications. On the day of birth litters were culled to either 8 or 10 (equal number of males and females). Whole litters were assigned to treatments such that pups received either 100 mg/kg of 6-OHDA (Regis Chemical Co.) dissolved in 0.05 ml saline vehicle containing 0.2 mg/ml ascorbic acid or vehicle only. Injections were made on both the day of birth and the second day of life using a 30 gauge needle inserted subcutaneously at the middle of the back. Litters were maintained in opaque plastic breeding cages until weaning at 25 days of age, when they were sexed and housed doubly with a similarly treated mate in wire-mesh hanging cages. At 50 days all animals were singly housed in wire-mesh hanging cages. For their entire life, animals were maintained on a reversed 12 hour light-dark cycle (lights on at 2000 hours). All testing was conducted between 1500 and 1800 hours. Half of the males and half of the females from each of the two treatment groups (6-OHDA or control) were randomly chosen to receive CDZ on the day of testing (n=14 for each extinction group and n=13 for each punishment group).

Procedure

Behavioural testing began at approximately 70 days of age, following two days of water deprivation (10 minutes access to water in the home cage each day) and three days of adaptation in an apparatus similar to that described by Vogel *et al.* [23]. Adaptation involved placing the rats singly in a clear plexiglass chamber (25×26×60 cm) housed within a sound attenuating wooden compartment. A 15 W light illuminated the chamber and an exhaust fan provided a masking noise. Water was available through a spout protruding from one wall of the chamber. The only water animals received during the three-day adaptation period was from the 5 minute period in the apparatus and an additional 5 minute access to water each day in the home cage. The 5 minute adaptation session was initiated by manually starting a timer following the 35th lick at the water spout. (The 35th lick was used as the starting point in order to eliminate the possibility of the counter starting following incidental contacts with the spout, i.e. by the nose, paw or body of the rat.)

On the day of testing, animals were injected 30 minutes prior to being run with either 8 mg/kg CDZ IP or an equal volume of the saline vehicle (1 ml/kg). For extinction testing the water bottle was dry and the rats were placed in the apparatus for a 10 minute period beginning from the time the rat made its 35th lick at the spout. The total number of licks on the empty water spout for the 10 minute test period was recorded for each rat. For the punishment-conflict test the water bottle remained filled and animals were given shocks (1.2 mA for 2 seconds) at every twentieth lick, with the first shock delivered on the 35th lick. The shocks were delivered to the spout and routed through the grid floor of the chamber. The total number of licks during the 10 minute punishment phase was recorded.

Dissection and Assay Procedure

At approximately 100 days of age the rats were decapitated, brains were removed and dissected on saline-rinsed, ice-cooled plates following the procedure previously used in this laboratory [20]. The brainstem, hypothalamus, neostriatum and cortex-hippocampus were retained. Immediately after dissection, tissues were stored in liquid nitrogen until assayed for NE and DA by a modification of the fluorescence method of Jacobowitz and Richardson [9]. Each brain part sampled was used as its own blank in the fluorescence calculations.

Biochemical verification of the 6-OHDA treatment was done once, with a representative sample of 10 (five 6-OHDA and five vehicle) rats from both the extinction and punishment groups. Sex of the animal was not taken into account since previous reports from this laboratory have indicated no differences in brain catecholamine levels between males and females following neonatal systemic 6-OHDA treatment [20].

RESULTS

Assays

The mean and standard errors of vehicle (VEH) and 6-OHDA injected groups for both NE and DA are presented in Table 1 for each of the four brain parts assayed. The results obtained with neonatal 6-OHDA treatment are consistent with previous reports from this laboratory [18,20]. The mean level of forebrain NE was 20% and that of hypothalamic NE was 81% of vehicle control levels. Conversely, brainstem NE was increased by 85%. There was no alteration of NE in the caudate or of DA in any of the four brain areas assayed.

Extinction:

Analysis of variance performed on these data revealed significant main effects due to CDZ administration, $F(1,104)=15.31$, $p<0.001$, 6-OHDA treatment, $F(1,104)=11.95$, $p<0.001$, and sex, $F(1,104)=10.99$, $p<0.001$, as well as, a significant drug by sex interaction, $F(1,104)=10.16$, $p<0.005$. Figure 1 (upper panel) shows the mean (±s.e.) numbers of licks during extinction for the various groups.

Rats administered CDZ prior to testing made more licks on the empty water spout ($\bar{x}=996\pm57.0$) than animals given

TABLE 1

REGIONAL BRAIN LEVELS (μg/g) OF NE AND DA FOR NEONATAL 6-OHDA AND VEHICLE (VEH) INJECTED RATS

	NE (μg/g)		DA (μg/g)	
Tissue	VEH	6-OHDA	VEH	6-OHDA
Cortex and hippocampus	0.184 ± 0.008	0.036 ± 0.004*	0.374 ± 0.011	0.354 ± 0.025
Hypothalamus	0.933 ± 0.037	0.752 ± 0.057*	0.728 ± 0.152	0.621 ± 0.070
Brainstem	0.350 ± 0.016	0.649 ± 0.018*	0.178 ± 0.012	0.151 ± 0.012
Neostriatum	0.219 ± 0.015	0.186 ± 0.007	5.91 ± 0.322	6.11 ± 0.157

Values shown are means ± S.E.M. Asterisks indicate significant differences between 6-OHDA and vehicle groups as determined by t-tests ($p<0.01$).

saline ($\bar{x}=727\pm51.4$) and animals depleted of forebrain NE made more responses ($\bar{x}=980\pm64.3$) than intact animals ($\bar{x}=742\pm43.6$). Also, in general, males ($\bar{x}=975\pm63.9$) tended to make more responses during extinction than females ($\bar{x}=748\pm44.7$). Analysis of the drug by sex interaction using the Newman-Keul's procedure revealed that CDZ increased resistance to extinction in males, but not in females.

Because the main hypothesis stated that CDZ would not be behaviourally active in 6-OHDA treated animals, a priori t-tests were used to analyze the treatment x drug interaction even though the overall ANOVA indicated that this interaction was not significant, $F(1,104)=0.43$, $p<0.50$. Since there was no drug effect in females, the treatment by drug interaction was analyzed for males and females separately.

In males, both 6-OHDA treatment and CDZ increased resistance to extinction. The effect of CDZ appeared to be greater than that due to 6-OHDA treatment but this comparison did not quite reach significance, $t(13)=1.74$, $p<0.10$. Most importantly, in 6-OHDA treated animals, CDZ increased responding during extinction to a level significantly higher than that caused by 6-OHDA treatment alone, $t(13)=3.24$, $p<0.01$. The effects of 6-OHDA treatment and of CDZ were additive although responding during extinction was not significantly greater in 6-OHDA-CDZ animals ($\bar{x}=1323\pm30.8$) than in control-CDZ animals ($\bar{x}=1116\pm86.8$), $t(13)=1.51$, $p<0.10$. This lack of an effect may be due to a ceiling effect on the 6-OHDA-CDZ animals induced by the arbitrary 10 minute test period.

In females, CDZ had no effect in control or 6-OHDA lesioned animals. Thus, whether forebrain NE depletion abolishes the anti-extinction effects of CDZ could not be tested in females since CDZ had no effect on females in this testing paradigm. While CDZ had no effect, 6-OHDA increased responding by females, $t(1,13)=1.99$, $p<0.05$.

Punishment

Analysis of variance performed on the punishment-conflict data revealed significant main effects due to CDZ administration, $F(1,96)=34.8$, $p<0.001$, 6-OHDA treatment, $F(1,96)=29.71$, $p<0.001$, and sex of the animal, $F(1.96)=25.24$, $p<0.001$, as well as a significant 3-way interaction (treatment×drug×sex), $F(1,96)=3.86$, $p=0.05$. Consistent with the extinction results, rats administered CDZ during testing responded more often (and thus received more shocks) during the punishment test period

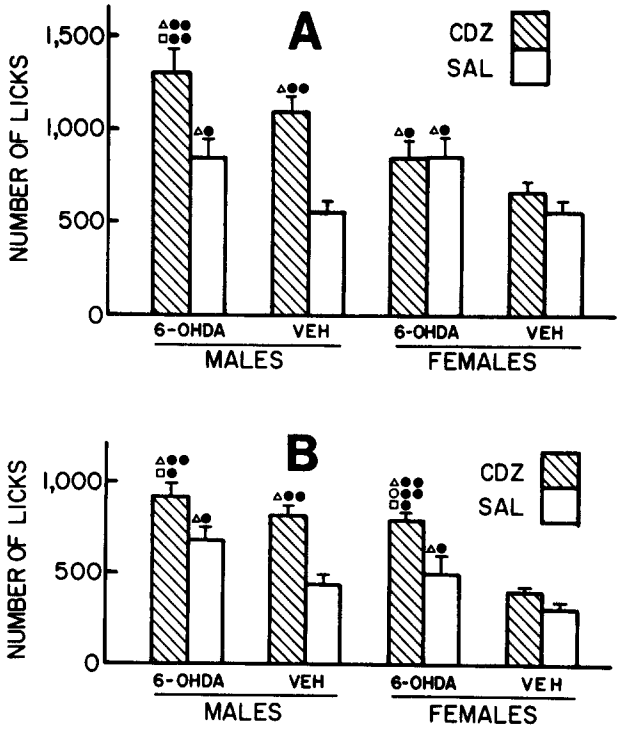

FIG. 1. (Upper Panel) Mean (±s.e.) total licks during extinction session for neonatal vehicle (VEH) or 6-OHDA treated rats who were injected with saline (SAL) or chlordiazepoxide (CDZ) prior to testing. Data are shown separately for males and females. An open triangle denotes significant difference (t-test) from VEH-SAL, an open circle from VEH-CDZ and an open square from 6-OHDA-SAL. One symbol indicates $p<0.05$, two symbols indicate $p<0.01$. (Lower Panel) Mean (±s.e.) total licks during punishment session for neonatal vehicle or 6-OHDA treated rats who were injected with saline or chlordiazepoxide prior to testing. Data are shown separately for males and females. The interpretation of legend and symbols is the same as in the upper panel.

($\bar{x}$=729±37.7) than animals given saline ($\bar{x}$=475±38.1) and animals depleted of forebrain NE made more responses ($\bar{x}$=719±41.7) than vehicle animals ($\bar{x}$=485±35.1). Also, overall, males made more responses during the punishment test ($\bar{x}$=710±39.2) than females ($\bar{x}$=494±38.9). The treatment by drug interaction was analyzed separately for males and females, as was done for the extinction data. The Newman-Keul's procedure for post-hoc analysis was used to make these comparisons. The results are summarized in Fig. 1 (lower panel) which also shows the mean scores (±s.e.) for the various groups.

For males, both 6-OHDA treatment and CDZ increased responding during the punishment test period and the effects of both treatments were of similar magnitude. The most important comparison to note is that in animals treated with 6-OHDA, CDZ increased punished responding to a level significantly higher than that produced by the 6-OHDA treatment alone. Again, it must be pointed out that a ceiling effect caused by the arbitrary 10 minute test-period may have prevented the 6-OHDA-CDZ group ($\bar{x}$=918±69.9) from responding significantly more often than the control-CDZ group ($\bar{x}$=816.6±52.4).

For females, CDZ had no effect in the vehicle animals but significantly increased punished responding in 6-OHDA treated animals. Interestingly, the 6-OHDA-CDZ group emitted significantly more punished responses than any of the other three groups (i.e. vehicle-vehicle, 6-OHDA-vehicle, vehicle-CDZ).

DISCUSSION

For male rats, the results show unequivocally that in two different behavioural paradigms, extinction and punishment-conflict, CDZ is as behaviourally active in rats depleted of forebrain NE as in control rats. Further, CDZ alone and depletion of forebrain NE alone produced comparable behavioural effects in both test situations. Several additional aspects of the results warrant comment. First, rats treated with 6-OHDA made more licks at an empty water spout where water was previously available, whereas Mason [12, 13] failed to observe increased resistance to extinction in DNB lesioned rats trained to leverpress for a water reward. Second, consistent with recent results reported by Koob *et al.* [10], the punishment data indicate that the DNB does not have to be intact for CDZ to have its anti-conflict effect. These authors found that 6-OHDA lesions of the DNB in the adult rat failed to alter the release of responding by CDZ in a modified Geller-Seifter paradigm. They also reported no effect of their DNB lesions alone in this variant of the conflict paradigm, whereas, consistent with previous findings from this laboratory [19,20], we found that destruction of forebrain NE neurons in infancy reduced the response suppressant effects of punishment in the adult rat. This discrepancy may be due to the specific tasks used in each experiment or the different means of producing forebrain NE depletion used in the two studies. It should also be noted that while our finding of retarded extinction in the 6-OHDA treated rats supports the observation of Mason and co-workers that lesion of the DNB retards extinction [11, 12, 13, 14, 16], the effects of forebrain NE depletion were not restricted to the extinction procedure in the present experiment.

The present findings and those of Koob *et al.* [10] are inconsistent with the hypothesis that the response suppressive properties of CDZ are mediated solely through facilitation of septal-driving of the 7.7 Hz hippocampal theta rhythm by fibers of the DNB [4]. However, Gray has proposed that possibly the critical feature of anti-anxiety agents is that they eliminate the low threshold for driving hippocampal theta with 7.7 Hz septal stimulation [4, 5, 15]. Inhibition of DNB function has such an effect [6]. Inhibition of serotonin (5-HT) synthesis, however, eliminates this low threshold for activation at 7.7 Hz but in a different manner—it increases the likelihood of septal-driving of hippocampal theta by stimulation frequencies other than 7.7 Hz [15]. Thus, the effects of minor tranquilizers on hippocampal theta and on behaviour may be due to combined effects mediated by both NE and 5-HT neurons.

Consistent with this suggestion, inhibition of 5-HT synthesis by PCPA [22] or destruction of central 5-HT neurons by injection of 5,7-dihydroxytryptamine (5,7-DHT) into the ventromedial tegmentum of rats [21] can release punished responding in a conflict situation. Also, CDZ has an attenuated, but significant anti-conflict effect in 5,7-DHT injected rats, indicating that intact 5-HT neurons are not essential for this drug effect [21]. Since both forebrain NE and 5-HT systems may be involved in the response-suppressive effects of benzodiazepines, if one system is lesioned the other may assume the mediation of this behavioural effect. Thus, it would be worthwile examining the effects of combined NE and 5-HT lesions on the response to CDZ.

Finally, it was clear that females were not affected by CDZ to the same extent as males were in the present experiments. We know of no studies directly investigating differential responses to CDZ by males and females. However, in an early study [8] it was reported that CDZ increased the preference to explore a novel environment in male rats but had no effect on this behaviour in female rats. Thus, the present is at least the second report that CDZ alters the behaviour of male rats more so than female rats. It is possible that female rats are simply less sensitive to CDZ than male rats and therefore a higher dose in female rats might produce behavioural effects similar to those found with a lower dose in male rats. Future research should further explore this phenomenon with a wider range of behaviours and appropriate drug doses.

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