

## Research report

# Blockade of $\alpha 1$ adrenoreceptors in the dorsal raphe nucleus prevents enhanced conditioned fear and impaired escape performance following uncontrollable stressor exposure in rats

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**Abstract**

Previous research has shown that the effect of exposure to uncontrollable stressors on conditioned fear responding and escape behavior in rats is dependent on serotonergic neural activity in the dorsal raphe nucleus (DRN). The role that norepinephrine released in the DRN plays in producing the behavioral consequences of exposure to inescapable tail shock in rats was investigated in the present study. The selective  $\alpha 1$  adrenoreceptor antagonist benoxathian was injected into the DRN before exposure to inescapable tail shock or before behavioral testing conducted 24 h later. Benoxathian prevented the impairment of escape responding produced by inescapable shock, but did not reverse this effect when given before testing. The enhancement of conditioned fear produced by prior inescapable shock was attenuated by benoxathian administered before inescapable shock or before behavioral testing. These results support the view that noradrenergic input to the DRN is necessary to produce the behavioral effects of inescapable tail shock. © 2002 Elsevier Science B.V. All rights reserved.

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The impact of a stressful situation often depends on whether or not the stressor can be predicted, avoided, escaped or otherwise controlled by an individual. Stressors that are not controllable have been shown to have greater negative impact on behavior compared to controllable stressors (see Maier, for a review [18]). Behavioral effects and physiological effects that occur only after uncontrollable, but not after controllable stressors, are referred to as learned helplessness effects [22].

Much work has been devoted to understanding the neural basis of learned helplessness. This extensive work has provided evidence for a role for acetylcholine [4], endogenous opioid peptides [9], norepinephrine

[25,31,40], dopamine [5], serotonin [24,30], gamma amino butyric acid [10,11], corticotropin releasing hormone [41], and adenosine [26] in the development and expression of learned helplessness behavior. This neurochemical evidence has often been characterized in combination with anatomical investigations, which have shown that the locus coeruleus [39,41], hippocampus [5,31], cortex [35], hypothalamus [5], and dorsal raphe nucleus [24] are important brain regions involved in learned helplessness behavior.

One recent hypothesis regarding the neural basis of learned helplessness is focused on the role of serotonin produced in the dorsal raphe nucleus (DRN) [18]. Maier et al. have provided substantial evidence that exposure to an uncontrollable stressor (a series of 100 inescapable tail shocks) increases the activity of serotonin neurons in the DRN to an extent such that they become transiently sensitized to subsequent input [24]. While the behavioral testing situation (a series of 30 mild escapable foot

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shocks) induces escape responses and conditioned fear responding in naïve rats, subjects exposed previously to an uncontrollable stressor show impaired escape responding [33] and exaggerated fear responses [17]. These learned helplessness effects are prevented and often reversed by manipulations that decrease serotonin neural activity in the DRN [14,20,21] and are produced by manipulations that increase serotonin neural activity in the DRN [13,19]. Thus, increased serotonin neural activity in the DRN appears to be necessary and sufficient to produce learned helplessness effects. Further evidence for this serotonin hypothesis has been provided by *in vivo* microdialysis experiments in which increased extracellular levels of serotonin are measured in the DRN [24] and in some of its projection regions [2,3] during and after the rat's exposure to an uncontrollable stressor. Lastly, direct evidence that an uncontrollable stressor, more than a controllable stressor, increases serotonin neural activity is provided by greater expression of the immediate early gene product Fos in serotonin neurons in the DRN under the uncontrollable stressor condition [15].

Given that increased serotonin neural activity in the DRN is important for producing and maintaining learned helplessness behaviors, we have attempted to identify potential sources of excitatory influence that are related to these behaviors produced by inescapable shock (IS). Thus, glutamatergic input to the DRN was demonstrated to be a source of excitation related to the effects of uncontrollable stressors [14]. Also, endogenous opiates within the DRN are involved in learned helplessness behavior [13,36]. The present study was focused on exploring a third source of excitation, that provided by norepinephrine. There is evidence that norepinephrine has an excitatory effect on DRN serotonin neurons [37], which depends on activation of  $\alpha_1$  adrenoreceptors [1]. This is especially interesting because there are many studies supporting the view that norepinephrine is important in learned helplessness behavior [5,31,41], and its influence within the DRN would help explain how norepinephrine and serotonin interact to produce learned helplessness behaviors.

Forty-five male naïve Sprague–Dawley rats obtained from Harlan Laboratories were used as subjects. They were approximately 100 days old at the time of surgery and were housed individually under a 12-h light/dark cycle with free access to standard rodent pellets and water. The experiment was conducted with the approval of the University of Colorado Institutional Animal Care and Use Committee. IS or restraint was administered to rats in Plexiglas tubes measuring 17.5 cm in length and 7 cm in diameter. Two clip electrodes were positioned on the distal portion of the tail. Behavioral testing occurred in shuttle boxes measuring 46 × 20.7 × 20 cm (L × W × H). Foot shocks were delivered through a grid floor. Each shuttle box included a stainless steel archway in

the center to allow the subject to pass from one side to the other. Bright illumination was provided by a 28 V bulb, and background noise was provided by ventilation fans. The clear Plexiglas front and top of each shuttle box allowed for behavioral observation.

Rats were implanted with a guide cannula approximately 2 weeks prior to each experiment. The rats were anesthetized with 60 mg/kg ketamine and 13 mg/kg xylazine. A 13 mm 26 ga stainless steel cannula was positioned 1 mm above the DRN using coordinates from interaural reference at AP +0.7 mm, ML 0.0 mm, DV +4.5 mm according to the Paxinos and Watson atlas [29]. The cannula was secured with dental acrylic applied to the cannula and screws anchored in the surrounding skull. The cannula was kept patent by a stainless steel stylet. Animals were monitored daily and handled twice after surgery.

Injections were made by removing the stylet and replacing it with a 14 mm microinjector made from 33 ga stainless steel tubing. A total volume of 1  $\mu$ l was injected slowly over a period of 30 s and the microinjector was left in place for 2 min while the rat was gently restrained in a towel. Cannula placements were verified histologically at the completion of the experiment. Six subjects were excluded either because dye injected through the cannula was evident in the ventricles or in brain sites other than the region of the DRN.

IS or restraint was applied approximately 10 min after drug injections. IS consisted of 100 1.0 mA 5 s tail shocks delivered on average every 60 s while the control condition was restraint without shocks. Rats were returned to their home cage immediately after the 2-h session. Conditioned fear and shuttle box escape performance was assessed in the same subjects 24 h after stressor pretreatment in the shuttle boxes. Conditioned fear was measured as the occurrence of freezing, defined as the complete lack of movement except that required for respiration. Rats were placed individually in the shuttle box and freezing observed for 10 min. The subjects were observed every 8 s by an observer unaware of group membership and scored as freezing or not freezing. Each rat then received two 0.8 mA foot shocks through the grid floor of the box, each of which could be terminated by crossing to the other side of the box through an archway. Freezing behavior was then assessed for an additional 20 min as above. After the observations were complete, the rats received three further single-crossing escape trials (fixed ratio-1; FR-1) followed by 25 trials in which a back-and-forth crossing (FR-2) was required to terminate shock. Trials occurred on average every 60 s and shock intensity continued at 0.8 mA. Escape latency was recorded automatically and each trial was terminated at 30 s if no response was made.

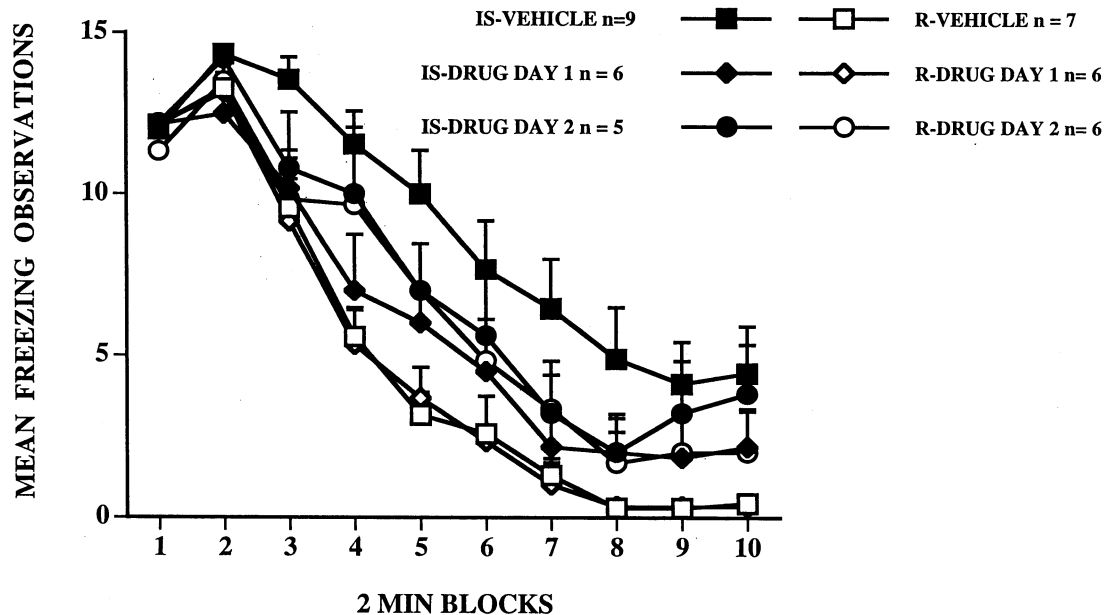


Fig. 1. Freezing was measured every 8 s for 20 min (15 observations in each 2-min block) following the presentation of two 0.8 mA scrambled foot shocks. Pretreatment with IS led to enhanced freezing (IS-Vehicle vs. R-Vehicle). Benoxathian ( $4 \mu\text{g}/\mu\text{l}$  per rat) or saline ( $1 \mu\text{l}/\text{rat}$ ) was administered before stressor pretreatment (filled symbols) or before behavioral testing conducted on the second day of the experiment (open symbols). Benoxathian attenuated the effect of IS if it was injected either before stressor pretreatment or before behavioral testing.

No freezing was observed during the first 10 min of shuttle box exposure (data not shown). As shown in Fig. 1, the two foot shocks produced freezing which extinguished across the 20 min observation period. IS produced enhanced freezing, compared to restraint. Benoxathian administered before IS dampened the enhanced freezing effect. The drug administered 24 h after IS, just prior to behavioral testing, also attenuated freezing. Benoxathian administered before behavioral testing produced enhanced freezing in the restraint group. Differences were not evident early in the observation period, when freezing was maximal in all groups, but emerged between groups as the observation period elapsed. A 3-way analysis of variance (ANOVA) revealed a reliable main effect of stressor,  $F(1,33) = 7.079$ ;  $P < 0.05$ , the 2-min blocks of observations,  $F(9,297) = 222.438$ ,  $P < 0.0001$ , and the stressor  $\times$  observations interaction,  $F(9,297) = 2.594$ ;  $P < 0.01$ . Post-hoc analysis (Newman–Keuls,  $P < 0.05$ ) performed on a one-way ANOVA using the average number of freezing observations across the 20 min period indicated that the IS group that did not receive drug (IS-Vehicle) showed more freezing behavior than any other group. In addition, the IS group that received benoxathian on day 1, on day 2, and the restraint group that received drug on day 2 (IS-Drug Day 1, IS-Drug Day 2, R-Drug Day 2) were not different from each other, but were different from all remaining groups. The same post-hoc analysis confirmed that the restraint group that received drug on

day 1 (R-Drug Day 1) and the restraint group that received vehicle on both days (R-Vehicle) did not differ from each other.

Fig. 2 shows that IS led to impaired escape responding compared to restraint and that benoxathian delivered before IS completely prevented this effect of IS. The drug did not reverse the effects of IS on escape responding when administered before behavioral testing and had no effect of its own in restraint controls. The 3-way ANOVA revealed a significant effect of stressor,  $F(1,33) = 7.398$ ;  $P < 0.01$ , the trials  $\times$  stress interaction,  $F(4, 132) = 4.262$ ;  $P < 0.01$ , and the 3-way interaction between trials, stressor and drug,  $F(8,132) = 3.016$ ;  $P < 0.01$ . A one-way ANOVA was performed on the average escape latencies over 30 trials and Newman–Keuls post-hoc analysis ( $P < 0.05$ ) indicated that the IS group given drug before testing (IS-Drug Day 2) and the IS-Vehicle group differed from the remaining four groups, which did not differ from each other.

The results of this study suggest that norepinephrine released into the DRN is necessary to produce enhanced conditioned fear responses and impaired escape behavior resulting from rats' exposure to a series of inescapable tail shocks. The influence of norepinephrine is likely served by  $\alpha_1$  receptors as benoxathian is characterized as being selective for that receptor subtype pharmacologically [12,23,28] and functionally [6,16]. Benoxathian administered before testing produced a slight increase in freezing responses in a restraint group,

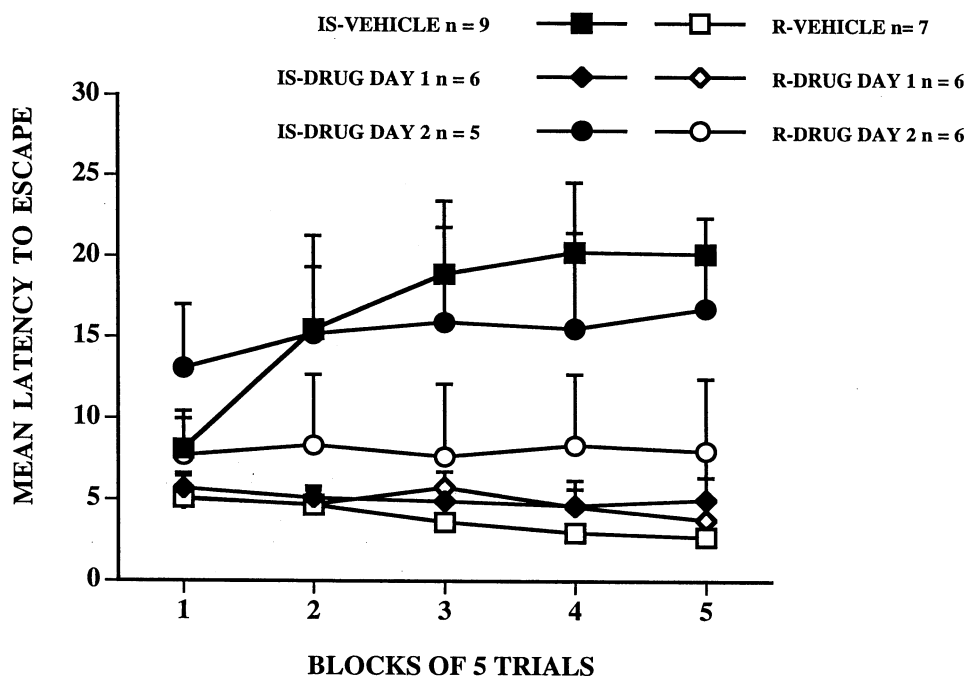


Fig. 2. Latency to escape foot shock was measured for 25 trials (maximum latency was 30 s) in which two crossings of the shuttle box were required to terminate shock. IS produced an escape deficit, shown as longer latencies to escape over five blocks of five trials in the IS-Vehicle group compared to the R-Vehicle group. Benoxithian (4  $\mu$ g/ $\mu$ l per rat) or saline (1  $\mu$ l/rat) was administered before stressor pretreatment (filled symbols) or before behavioral testing conducted on the second day of the experiment (open symbols). Benoxithian prevented the effect of IS if it was injected before stressor pretreatment but had no effect if injected before behavioral testing.

suggesting a potential inhibitory role for norepinephrine in fear responding. No other overt behaviors were noted as a consequence of benoxathian administration.

Activation of  $\alpha_1$  receptors in the DRN causes depolarization of serotonin neurons [1,8,27,37,44]. Assuming that norepinephrine has an excitatory influence on serotonin neurotransmission in the DRN, these results are consistent with other studies conducted in our laboratory in which blocking excitatory input to serotonin neurotransmission in the DRN was sufficient to prevent the development of learned helplessness behaviors. The timing of drug administration in these studies is important. If the excitation of serotonin neurons caused by inescapable tail shock is prevented (drug administration before IS) then the behavioral consequences of inescapable tail shock are prevented. It is less effective to reduce serotonin activation at the time of testing. This pattern of effects was evident in the present study, in which benoxathian was effective in preventing, but not reversing, the effects of inescapable tail shock. The present results provide further evidence that the effects of inescapable tail shock on escape responding and conditioned fear are mediated by several excitatory influences on serotonin neurons in the DRN, which include that provided by glutamate, opioid peptides and norepinephrine.

While there is ample evidence that norepinephrine influences serotonin neurons in the DRN, the source of

norepinephrine to this brain region is not clear. The locus coeruleus has been shown to project to the DRN [32] and it has been argued that this represents its primary source of norepinephrine [8]. However, norepinephrine could also be derived from the A5 cell group [7], the A1 cell group [43], or from the lateral tegmentum [32]. Interestingly, it has been demonstrated that the lateral tegmentum is involved in mediating the effects of stressors on drug-seeking behavior [34], which could be related to the potentiated rewarding properties of morphine that have been linked to the effects of inescapable tail shocks [42]. However, Weiss and his colleagues have shown that inescapable, relative to escapable shocks, activates norepinephrine neurons in the locus coeruleus [39] and that this leads to changes within the locus coeruleus that are critical in the mediation of learned helplessness [38,39]. It may be that the locus coeruleus is critical because it provides input to the DRN with the locus coeruleus then being on the afferent and the DRN on the efferent end of the 'learned helplessness circuit'.

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