One stressful event blocks multiple actions of diazepam for up to at least a month

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Based on recent findings of this laboratory, the hypothesis was tested that a single stressful encounter might have a persistent antidiazepam influence. Our results indicate that one exposure to a brief stressful event up to at least one month earlier prevented completely the effect of diazepam on pentylenetetrazole-induced changes in dopamine in the rat frontal cortex, elevations of plasma corticosterone levels and seizures.

The potential long-term consequences of acutely traumatic events such as battle, natural and man-made disasters, severe accident, rape and hostage-taking have been well documented clinically. Despite its obvious importance and widespread nature, this problem has received little attention from most basic stress researchers who have instead chosen to concentrate on the immediate effects of acute treatments or the influence of long-term exposure to stressful conditions. By contrast, our own work has emphasized the long-term effect of brief stressful events.

During the course of studies on time-dependent sensitization after diazepam in the rat, it was discovered that an injection of isotonic saline, one month earlier, significantly diminished the influence of this benzodiazepine on pentylenetetrazole (PTZ)-induced changes in nucleus accumbens and frontal cortical dopamine (DA) concentrations. This finding was pursued on the assumption that it might have reflected a stress-mediated phenomenon. We now report that a single exposure to a stressor up to at least one month earlier, antagonizes the action of diazepam on PTZ-induced changes in DA concentrations in the frontal cortex, plasma corticosterone and seizures.

Male, Sprague–Dawley rats, weighing 200 g and double-housed with food and water available ad libitum were used in these studies. In the first experiment, we determined whether a 2-h period of immobilization stress 28 days earlier, or a single jab with an empty syringe needle 1 h to 28 days before, could change the action of diazepam on PTZ-induced alterations in frontal cortical DA and/or one of its principal metabolites, dihydroxyphenylacetic acid (DOPAC). Immobilization was induced by wrapping animals in muslin and a 26-gauge 0.5 in. syringe needle was used as the needle-jab stressor. Diazepam (0.5 mg/kg, i.p.) was administered an hour before PTZ (injected at the convulsive dose of 40 mg/kg, i.p.). Sacrifice by guillotine took place 10 min after PTZ injection, by which time all convulsive activity had ceased even in non-diazepam treated animals. Upon sacrifice, 10 ml of trunk blood was col-

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lected in heparinized beakers coated with 10,000 units/ml. The blood was centrifuged at 2000 rpm for 10 min and 0.5 ml of plasma saved and frozen at \(-70^\circ\text{C}\) for later measurement of plasma corticosterone by competitive protein binding radioassay. This assay requires only 25 \(\mu\)l of plasma and is sensitive in our hands to 0.2 \(\mu\)g/dl. Frontal cortices were dissected on an ice-cold “brain block” using the procedure of Heffner et al. frozen, and later assayed by high performance liquid chromatography according to an adaptation of the method of Reinhard and Roth. Data were analyzed by one-way analysis of variance followed by Duncan’s multiple range test to assess differences between groups.

Pretreatment with the needle-jab stressor failed completely to alter the effect of diazepam on brain DA (data not shown). In contrast, immobilization 28 days prior to diazepam and PTZ blocked the effects of diazepam; this is illustrated in Table I \((F_{3,23} = 6.63, P < 0.005)\). PTZ itself markedly and significantly elevated frontal cortical DA concentrations to 232% of control values without affecting DOPAC. Diazepam 1 h earlier significantly reduced the influence of PTZ back to 140% of control. Most importantly, however, pre-exposure to the stress associated with a 2-h immobilization a full month earlier (immobilization 28 days, diazepam 1 h, PTZ), overcame entirely the anti-PTZ action of diazepam and significantly reinstated DA levels to 243% of control values.

In contrast to the results obtained in the frontal cortex, both the needle-jab and immobilization stressors effectively prevented the action of diazepam on PTZ-induced elevations in plasma corticosterone \((F_{1,71} = 85.8, P < 0.0001)\). (The reader is reminded that corticosterone measurements and cortical DA determinations were done on the same animals.) PTZ itself induced more than a 12-fold increase in corticosterone compared to untreated controls (Table II). As opposed to its anti-PTZ action on DA concentrations (Table I), diazepam administered 1 h before PTZ resulted in a significant, 50% elevation in corticosterone levels. This effect of diazepam was completely obliterated by the stress of needle-jab administered once 1–28 days earlier (needle-jab 1–28 days, diazepam 1 h, PTZ). Up to a point, the influence of the needle-jab actually grew as the interval lengthened between this stressor and diazepam treatment. Thus, the effect observed with a one-week interval between needle-jab and diazepam not only differed significantly from diazepam 1 h before PTZ, but also relative to needle-jab itself given only 1 h before diazepam (Table II).

Since a broad variety of stressors have been shown to induce the synthesis of new protein, this could, at least in theory, relate to the phenomenon reported here. We therefore ran additional groups with a 4-week interval between needle-jab and diazepam, which were also pretreated with an effective dose of the protein synthesis inhibitor, anisomycin (25 mg/kg, i.p.) or vehicle, either 30 min before or 24 h after needle-jab in order to determine whether the anti-diazepam effect of needle-jab could be prevented. Anisomycin did not alter the effect of the stressor and therefore these groups serve as additional support for the long-lasting anti-diazepam influence of needle-jab (Table II). As noted above, immobilization stress one month earlier also reversed.

**Table I**

<table>
<thead>
<tr>
<th></th>
<th>DA (ng/g)</th>
<th>DOPAC (ng/g)</th>
<th>DOPAC/DA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>32.7 ± 3.7</td>
<td>50.4 ± 4.6</td>
<td>1.59 ± 0.16</td>
</tr>
<tr>
<td>PTZ</td>
<td>76.0 ± 7.5*</td>
<td>65.6 ± 10.0</td>
<td>0.88 ± 0.12*</td>
</tr>
<tr>
<td>Diazepam 1 h, PTZ</td>
<td>45.9 ± 3.1*</td>
<td>45.4 ± 2.1</td>
<td>1.02 ± 0.09*</td>
</tr>
<tr>
<td>Immobilization 28 days, Diazepam 1 h, PTZ</td>
<td>79.6 ± 12.7*</td>
<td>71.0 ± 11.9</td>
<td>0.89 ± 0.08*</td>
</tr>
</tbody>
</table>

* P < 0.01 relative to no treatment.  
* P < 0.05 relative to PTZ.  
* P < 0.05 relative to diazepam 1 h, PTZ.
TABLE II
The effects of pre-exposure to a needle-jab (NJ) or immobilization (IMM) on diazepam alteration of PTZ-induced changes in plasma corticosterone levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Corticosterone (μg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.9 ± 0.9</td>
</tr>
<tr>
<td>PTZ</td>
<td>35.8 ± 3.9*</td>
</tr>
<tr>
<td>NJ 1 h diazepam 1 h, PTZ</td>
<td>53.7 ± 3.6*</td>
</tr>
<tr>
<td>NJ 1 day diazepam 1 h, PTZ</td>
<td>46.9 ± 4.6*</td>
</tr>
<tr>
<td>NJ 14 days diazepam 1 h, PTZ</td>
<td>38.7 ± 3.7*</td>
</tr>
<tr>
<td>NJ 28 days diazepam 1 h, PTZ</td>
<td>25.5 ± 2.2*</td>
</tr>
<tr>
<td>Vehicle 28 days, diazepam 1 h, PTZ</td>
<td>25.5 ± 2.2*</td>
</tr>
<tr>
<td>IMM 28 days, diazepam 1 h, PTZ</td>
<td>34.5 ± 2.1*</td>
</tr>
<tr>
<td>Anisomycin 30 min, NJ 28 days, diazepam 1 h, PTZ</td>
<td>37.5 ± 4.4*</td>
</tr>
</tbody>
</table>

ANOVA: $F_{11, 71} = 85.8, P < 0.0001$.
Duncan’s multiple range test, $n = 6–9$.
* $P < 0.01$ relative to no treatment.
* $P < 0.01$ relative to PTZ.
* $P < 0.01$ relative to diazepam 1 h, PTZ
* $P < 0.01$ relative to NJ 1 day, diazepam 1 h, PTZ.

the effects of diazepam on plasma corticosterone concentrations (Table II).

We next inquired whether pre-exposure to a stressor could similarly alter behavioral effects of diazepam. The ability of diazepam to prevent PTZ-induced seizures was the behavior examined. To increase the difficulty of antagonizing this action, the dose of diazepam was increased to 1 mg/kg. PTZ (40 mg/kg) administered 14 days earlier also served as the prestressor in this experiment. As shown in Table II, it causes a pronounced activation of the hypothalamic–pituitary–adrenal axis, evidenced by a marked rise in corticosterone concentrations, a principal criterion for defining an agent as stressful. We have also found that it induces a very marked elevation in cortical norepinephrine metabolism, a frequent concomitant of exposure to stressors (unpublished observations). In addition, it is thought to be a prototypical anxiogenic agent in rats and is known to induce feelings of catastrophic anxiety in humans. It might be argued that the convulsions induced by our dose of PTZ confound its use as a stressor. However, similar problems exist with most commonly employed laboratory stressors, e.g. the pain and/or startle which accompanies electric shock and the muscular exertion seen with immobilization.

As in the previous experiments, the anticonvulsant influence of diazepam was also markedly diminished in animals exposed to the stressor 2 weeks earlier (Table III). PTZ alone caused convulsions in 100%
of controls. Pretreatment with diazepam 1 h earlier or diazepam preceded by PTZ vehicle or an injection 2 weeks earlier, significantly diminished the incidence of convulsions down to 20% of controls. However, when PTZ itself was administered 2 weeks before diazepam (PTZ 2 weeks, diazepam 1 h, PTZ), the incidence of seizures rose significantly to 67%, a level not different from PTZ alone. The ability of a brief stressful event to influence the actions of drugs administered weeks later has been demonstrated for compounds as diverse as amphetamines, haloperidol, and tetracaine (submitted for publication), among others.

The data presented in these studies graphically illustrate some of the extremely long-term biochemical, endocrinological and behavioral sequelae of brief stressful experiences. How can such remarkable findings be understood? It seems unlikely that they could be due simply to increased degradation of diazepam resulting from prestress since, up to a point, the anti-diazepam influence actually grows as time increases between prestress and drug (Table II). One, not unreasonable possibility, is that even a single stressful experience weeks earlier can directly antagonize the neurochemical substrate through which diazepam exerts many of its actions (presumably GABA). Very recent findings suggest that stressors can modify the benzodiazepine/GABA receptor coupled chloride ionophore, but whether the present data might be explained in this way, remains to be determined. At this point, it cannot be ruled out. A second, though not necessarily mutually exclusive hypothesis, is that experience with the prestressor sensitizes various levels of the organism's functioning (biochemical, hormonal and behavioral) to subsequent stressors — in this case PTZ — with the result that any agent sandwiched between, like diazepam, is "victimized". Since all groups received the last PTZ injection on the same day, it is unlikely that these results could be due to day-to-day fluctuations in the effectiveness of PTZ. Also relevant to the present finding, we have recently obtained evidence for significant sensitization of the hypokinetic effect of clonidine up to 2 weeks following a single injection of PTZ (in preparation). We have previously provided evidence for stressor-induced sensitization to a subsequent stressor over time periods similar to those used here. However, while this is a plausible and even likely hypothesis to account for our biochemical (Table I) and behavioral data (Table III) where stressors overcame anti-PTZ actions of diazepam, it is not obvious how it might explain the hormonal findings of Table II in which prestress also reversed diazepam's potentiation of the rise in corticosterone seen after PTZ. Although at this point there is no work other than our own which demonstrates that acute exposure to a stressor can have a very long-lasting influence on benzodiazepine actions, there are several reports of an anti-benzodiazepine effect of stress in the short term.

The finding that diazepam further increased plasma corticosterone concentrations elevated by PTZ requires some comment since at the dose used it typically blocks stressor-induced changes in corticosterone. Since diazepam was not tested without PTZ, it could be argued that it alone might have increased corticosterone concentrations for some reason idiosyncratic to our setting. Although possible, this seems unlikely since we have examined the corticosterone response to the same dose of diazepam as part of another experiment and have not obtained any difference from controls (unpublished observations). A more likely possibility is that the low dose of diazepam synergized with the very stressful influence of PTZ to produce the significantly enhanced corticosterone concentrations. Precedent for such a suggestion can be seen in the often-reported finding that benzodiazepines can facilitate aggressive behavior. Whatever the explanation of our findings, they are important for a number of reasons: (1) in once again demonstrating the long-term effects of acute stressors, they provide a feasible animal model for studying the apparent clinical analogue of this phenomenon, post-traumatic stress disorder. (2) Together with our earlier finding with a different stressor, they furnish the first experimental evidence that even remote stressful events can markedly influence the efficacy of benzodiazepines such as diazepam. In so doing, they suggest that benzodiazepines may not be very useful in situations where the individual is sensitized to stress. They could also provide a model for clarifying the controversy surrounding the effectiveness of these compounds in the treatment of panic disorder, which may, in some cases, represent a state of sensitization to stress. (3) By clearly showing that the effects of just a single needle-jab can per-
sist for extraordinary periods and even sensitize with time (Table II), our findings point up the dangers of assuming (as is now done) that this, one of the most widely used of laboratory procedures, is an innocent event, free of long-term consequences. As a caution, our data suggest the need for additional, un.injected control groups wherever possible. (4) The finding that diazepam can have both anti- (Table I) and pro (Table II)-PTZ actions simultaneously in the same animals suggests the possibility that anxiolytic and anxiogenic effects of these agents can occur in the same organism at the same time. (5) Our biochemical data (Table I) suggest that an acute stressor (immobilization) can have an extremely long-lasting influence on the mesocortical DA system and that this influence contrasts with the increase in DA activity seen in this region in experiments using only a brief period between stressor and sacrifice. (6) The failure of needle-jab to antagonize the influence of diazepam on PTZ-induced changes in frontal cortical DA, coupled with its success in overcoming diazepam's action on PTZ-induced alterations in plasma corticosterone levels, suggests that the pituitary–adrenocortical axis is more sensitive to stressors than are brain DA systems. This conclusion is reinforced by other similar findings from our laboratory. (7) Finally, our data suggest the need for basic and clinical researchers alike to begin to consider the possible long-term effects on syndrome development or drug action of acutely stressful events.

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