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The Inhibitory Effect of Diazepam on Defensive Burying: Anxiolytic vs. Analgesic Effects

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TREIT, D. *The inhibitory effect of diazepam on defensive burying Anxiolytic vs. analgesic effects* PHARMACOL BIOCHEM BEHAV 22(1) 47–52, 1985.—The hypothesis that analgesic mechanisms might account for the suppressive effect of diazepam on defensive burying was tested in four experiments In the first experiment, 1 mg/kg of diazepam had no appreciable effect on rat's latency to escape from a painful heat stimulus, but reliably suppressed defensive burying behavior There was no significant relationship between the diazepam-treated rats' latency to escape and their duration of burying. Rats in Experiment 2 were injected with diazepam during a delay between shock and testing, so that they could not be experiencing the putative analgesic effect of diazepam during the shock In spite of this, diazepam produced a significant suppression of burying compared to saline control In the next experiment, the effect of diazepam on defensive burying was assessed in the complete absence of painful stimulation by exposing the rats to a novel stimulus known to elicit burying behavior Diazepam suppressed burying behavior to the novel stimulus in a dose-dependent fashion. Finally, the ability of 10 mg/kg of naloxone to reverse the suppressive effect of 1 mg/kg of diazepam was assessed in Experiment 4. Nalxone failed to reverse the suppressive effect of diazepam and had no significant effect on defensive burying by itself, suggesting that the modulating influence of diazepam on rats' defensive burying behavior did not depend upon endogenous opiate mechanisms. Taken together, the results of the four experiments did not support the view that benzodiazepines produce their anxiolytic effects through analgesic mechanisms

Diazepam Anxiolytic Analgesic Animal models

TREIT, Pinel, and Fibiger recently showed that the rat's species-typical propensity to bury objects associated with aversive stimulation [18, 19, 29, 31] was suppressed by anxiolytic drugs in a dose-dependent manner [27,28]. Furthermore, they found that the relative potency of a number of known anxiolytics in suppressing the rat's "defensive burying" response was comparable to the relative potency of these anxiolytics in clinical settings. In contrast, nonanxiolytic drugs either had no reliable effect on defensive burying, or had effects that could be dissociated from those of known anxiolytics. Thus, the defensive burying test appeared to fulfill the pharmacological criteria of sensitivity, relative potency, and selectivity [10,16]

Because the defensive burying response can be reliably elicited by a single shock, the interpretation of drug effects in this paradigm is somewhat simplified compared to other, more complex paradigms. For example, in the Geller conflict test [4,9], animals first have to be pretrained on a task involving positive reinforcement, such as bar-pressing for food, which then serves as a behavioral baseline against which the effects of anxiogenic stimuli (e.g , punishing shocks) can be assessed. Anxiolytic effects in this paradigm are indicated by the "disinhibition" of punished responses. However, this combination of shock-motivated and food-motivated behaviors makes the interpretation of drug effects difficult because drugs such as the benzodiazepines have powerful effects on food-motivated behavior that are directionally the same as "anti-conflict" (i.e., anxiolytic) effects (e.g. [32]). How the effects of food-motivation can be separated in the conflict paradigms from their effects on fear-motivation is not entirely clear (cf [12,27]

It should be apparent that because the burying response can be produced without food reinforcement, anti-anxiety effects in this paradigm are not confounded with effects on appetitively motivated behaviours. However, there are other possible confounding factors that may make the interpretation of anxiolytic drug effects in the defensive burying paradigm difficult. For example, it is possible that the suppression of defensive burying produced by anxiolytics such as diazepam is due to analgesic effects rather than to anxiolytic effects (cf. [23]) According to this hypothesis, defensive burying would be suppressed because of a reduction in the animal's pain sensitivity, thereby reducing motivation to react to the shock source. This possibility is made plausible by several findings. First, it has been shown that anxiolytics such as the benzodiazepines do have some analgesic activity in a number of tests [2, 3, 13, 14, 15, 20]. Second, there is recent evidence that benzodiazepines mod-

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ulate the release of endogenous opiates [6, 7, 33, 34] which might have the effect of dampening the animals' responses to painful stimulation Third, there are a number of reports that the anti-conflict effects of benzodiazepines can be blocked by administering the opiate antagonist, naloxone [1, 5, 24] Taken together, these results suggest that benzodiazepines may produce some of their effects through an analgesic mechanism. Therefore, any model of anti-anxiety actions that involves painful stimuli, such as the defensive burying test, must dissociate possible analgesic effects from actual anxiolytic effects [23]

The general purpose of the present investigations was to try to rule out the possibility that analgesic mechanisms might account for the suppressive effect of diazepam on defensive burying In the first experiment, the effects of 1 mg/kg of diazepam was assessed in both a standard animal test of analgesia as well as in the defensive burying test Since the same animals served in each of these two kinds of test, it was also possible to determine the correlation between "analgesic" reactions and "anxiolytic" reactions. In the next two experiments, the suppressive effect of 1 mg/kg of diazepam on defensive burying was assessed in the absence of painful stimulation. In one experiment, rats were shocked first and then injected with diazepam for a burying test that occurred 15 min later, and in the other experiment, the effect of diazepam was assessed on defensive burying elicited by a novel but non-painful stimulus. Finally, in the last experiment, the ability of naloxone to counteract the suppressive effect of 1 mg/kg of diazepam was assessed

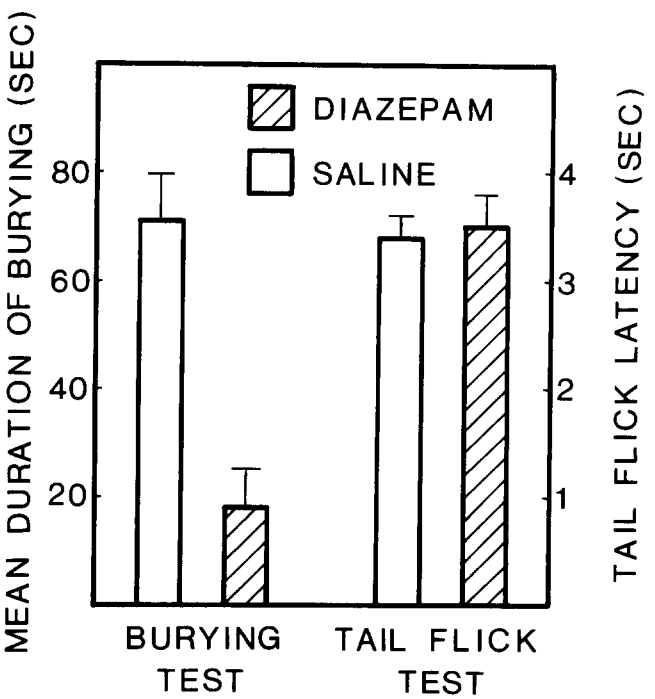

FIG 1 Mean (±SEM) duration of burying (left side) and mean tail-flick latency (right side) in diazepam and saline-treated rats in Experiment 1

GENERAL METHOD

The general methods used in the present experiments were similar to those used in previous investigations (cf [27,28])

Subjects

The subjects were 156 naive, 250–450 g male hooded rats purchased from Canadian Breeding Farm and Laboratories, La Prairie, Quebec The rats were housed in groups of four or five in wire-mesh cages, with food and water available ad lib A 12 hr light/dark cycle was in effect throughout the experiments (light on 7.00 a.m)

Apparatus

The test apparatus was a 40×30×40 cm Plexiglas chamber, with bedding material spread evenly over the floor of the chamber. Two, 1 cm diameter holes were centered on the end walls of the apparatus, 2 cm above the level of the bedding material. On test days a 6 5×0.5×0 5 cm wire-wrapped prod was inserted through one of the two holes In one experiment a 9.8×4.5×0 5 cm wooden mouse trap was attached to the inside of the chamber instead of the prod.

Procedure

Habituation On each of four consecutive days before a test day the rats were placed in the test chamber in groups of four or five for a period of 30 min.

Drug administrations On the fifth day of each experiment, the rats were randomly assigned to treatment conditions The rats in drug conditions received an intraperitoneal injection of a drug in solution, whereas those in control conditions received an injection of saline. Diazepam (Roche) was dissolved in a commercial vehicle of 40% propylene glycol and 10% ethanol, while naloxone (Sigma) was dissolved in 0 9% saline

Shock administration Immediately before the test session on day 5, the shock prod was inserted 6 cm into the test chamber and when each individually tested rat first touched the prod with a forepaw it received a brief electric shock from a 1000 V power source In some cases, the rats received a relatively mild shock (approximately 1 mA), whereas in other cases they received a more intense shock (approximately 6 mA) Current intensity was varied using a variable resistor, and current duration was determined by the latency for the rat to withdraw its paw (typically 30–35 msec, cf [18, 26, 29])

Behavioral observation. Immediately after shock administration, the behavior of each rat was viewed for 15 min from a separate room through one-way glass (Experiments 1, 2, 4) or via closed circuit television (Experiment 3) The duration of burying behavior (i e , the total duration of the rapid, alternating thrusts of the forepaws by which the rats directed bedding material toward or over the prod or the trap during the 15 min test) was recorded on an electronic counter

EXPERIMENT 1

The purpose of Experiment 1 was to assess both the analgesic and the anxiolytic effects of 1 mg/kg of diazepam Because the same subjects served in both the analgesic and the anxiolytic test, direct comparisons could be made of the effects of diazepam in the two tests. If diazepam was producing its effects in the two tests through an analgesic mechanism, then (1) there should be a significant difference in the analgesic test between diazepam-treated and saline-treated

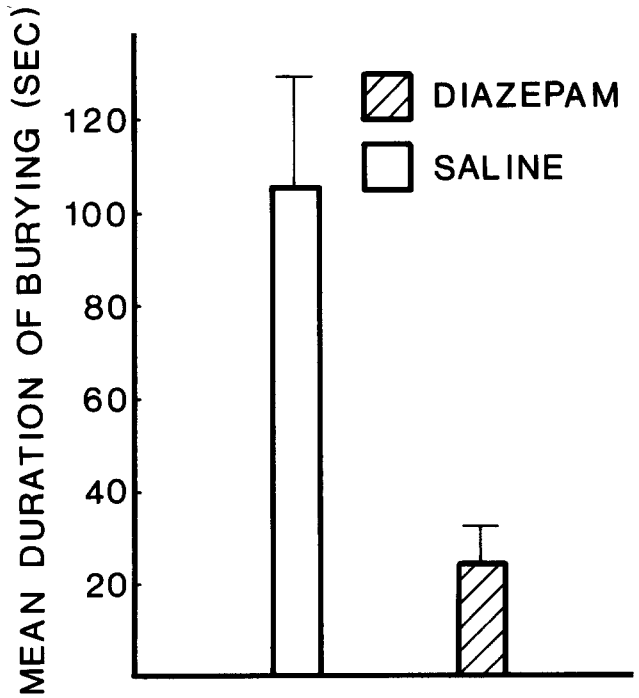

FIG 2 Mean (±SEM) duration of burying in the diazepam and saline-treated rats of Experiment 2

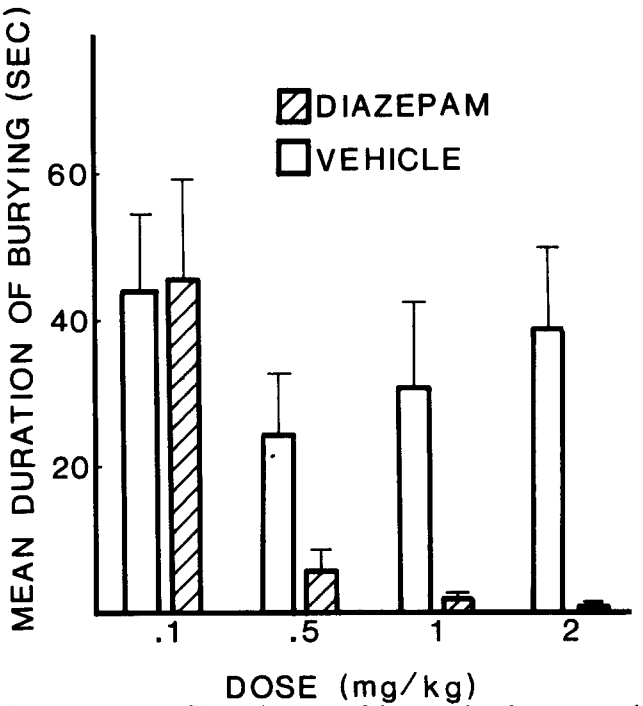

FIG 3 Mean (±SEM) duration of burying by diazepam and vehicle-treated rats exposed to the novel stimulus in Experiment 3

rats, and (2) the effect of diazepam in the analgesic test should account for a significant portion of the variance of the effect of diazepam in the burying test

METHOD

On the test days, half of the 32 rats serving as subjects were randomly assigned to receive 1 mg/kg of diazepam, while the other half received an equivalent volume of saline, 30 min before either a tail-flick test or a burying test. The order in which the two tests were administered was counter-balanced across the 32 rats, with a one week delay between the two tests to minimize possible tolerance effects (cf [30]). In the tail-flick test, rats were immobilized in a cylindrical restrainer and their tails immediately placed 10 cm into a water bath that was maintained at 55° centrigrade [11]. The latency for the rat to curl its tail up out of the water served as the index of analgesia. In the burying test, each rat was placed individually into the center of the Plexiglas test chamber with the prod attached to one wall. A fifteen min test session began immediately after the rat had been shocked with 1 mA on the forepaw, and the duration that the rat sprayed bedding material toward or over the prod was recorded on an electronic counter.

RESULTS

It can be seen from Fig 1 (right side) that 1 mg/kg of diazepam had no apparent effect on the latency of rats to remove their tails from a heat stimulus, whereas the same dose of diazepam had a substantial suppressive effect on the duration that rats buried the shock-source (left side). A priori orthogonal comparisons confirmed that 1 mg/kg of diazepam significantly suppressed burying behavior compared to saline, $t(30)=4.55$, $p<0.001$, but had no significant effect on tail-flick latencies, $t(30)=0\ 27$, $p>0.5$ Furthermore, there was no significant relationship between effect of diazepam on rats' duration of burying and its effect on their latency to escape the heat stimulus, r= 108, $p>0.5$.

EXPERIMENT 2

Taken together, the results of Experiment 1 suggested that analgesia, as measured by the tail-flick test, could not account for the suppressive effect of 1 mg/kg of diazepam on the rats' defensive burying behavior. Nevertheless, it could still be argued that the tail-flick test, or the particular version of it employed in Experiment 1, was not a sensitive enough assay of the analgesic effect of low doses of diazepam, and hence no significant relation was found between the effect of diazepam in the tail-flick test and its effect in the burying test. Such an argument is difficult to refute empirically because it can be applied to any assay of analgesia in which a particular drug has no significant effect. In order to circumvent this problem, rats in Experiment 2 were shocked before they were injected with diazepam and then tested in the defensive burying paradigm. If diazepam still suppressed defensive burying under these conditions, it would be more difficult to argue that the drug effect was due to a reduction in the rats' sensitivity to painful electric shock (i.e., analgesia).

METHOD

Twenty naive rats served as subjects. On the test day, rats were randomly assigned to receive either 1 mg/kg of diazepam (n=10) or an equivalent volume of saline (n=10). Unlike rats in the previous experiment, however, rats in Ex-

periment 2 were not injected until they had been shocked (6 mA) from the prod. Immediately after the shock, the rats were removed from the test chamber, injected with either saline or diazepam, placed in a holding cage for 15 min, and then returned to the test apparatus for a 15 min test. The duration that rats buried the prod over the 15 min test was recorded on an electronic counter

RESULTS

Figure 2 shows that rats shocked from the prod and then injected with diazepam buried the prod substantially less than controls treated in the same manner but injected with saline The reliability of this difference was assessed with a t test which showed that diazepam still produced a significant suppression of defensive burying, in spite of the fact that rats in this experiment did not experience the effects of diazepam during the shock, only during the test, $t(18)=3.24$, $p<0.005$

EXPERIMENT 3

Because diazepam suppressed the defensive burying of rats that were not under the influence of diazepam at the time that they were shocked, it is difficult to attribute the suppressive effect of diazepam found in previous experiments [27,28] to putative analgesic mechanisms. The plausibility of this argument, however, depends on the assumption that there are no long-lasting pain sensations that follow a 6 mA shock to the forepaw. If there were painful aftereffects of this intensity of electric shock, it is not unlikely that they would be suppressed by an analgesic drug, and therefore the results of Experiment 2 do not totally rule out the possibility that diazepam might suppress defensive burying through an analgesic mechanism. In order to provide further evidence that diazepam can suppress defensive burying in the absence of painful stimulation, rats in Experiment 3 did not receive any form of painful stimulation during the experiment; instead, they were simply exposed to a novel stimulus that had been shown to elicit defensive burying in previous studies [26] If diazepam suppressed defensive burying to a novel stimulus that produced no obvious pain to the animal, it would be even more difficult to attribute the suppressive effect of diazepam to an analgesic action.

METHOD

On the test day, after the 4 days of habituation, 80 naive rats were randomly assigned to one of four diazepam conditions (n=40) or four control conditions (n=40) Rats in each of the diazepam conditions (n=10) received a 0.1, 0.5, 1, or 2 mg/kg injection of diazepam 30 min before being individually placed into the Plexiglas test chamber. Fixed to the wall at one end of the test chamber was an unset, wooden mousetrap The test session began immediately after the rats were placed in the chamber and the amount of time they spent spraying the bedding material toward or over the trap was recorded on an event recorder. Rats in each of the control conditions (n=10) were treated in the same manner, except that they received a volume of the ethanol-propylene glycol vehicle that was equivalent to the volume of fluid received by rats in the appropriate drug condition This design was adopted because pilot work had suggested that rats' burying behaviour under these conditions was particularly variable, so that an accurate assessment of the effects of each dose might be facilitated if it could be compared to its own control Furthermore, unlike the case for burying elicited by prod-shock, there were no dose-response data on the effects of diazepam on burying elicited by a novel stimulus

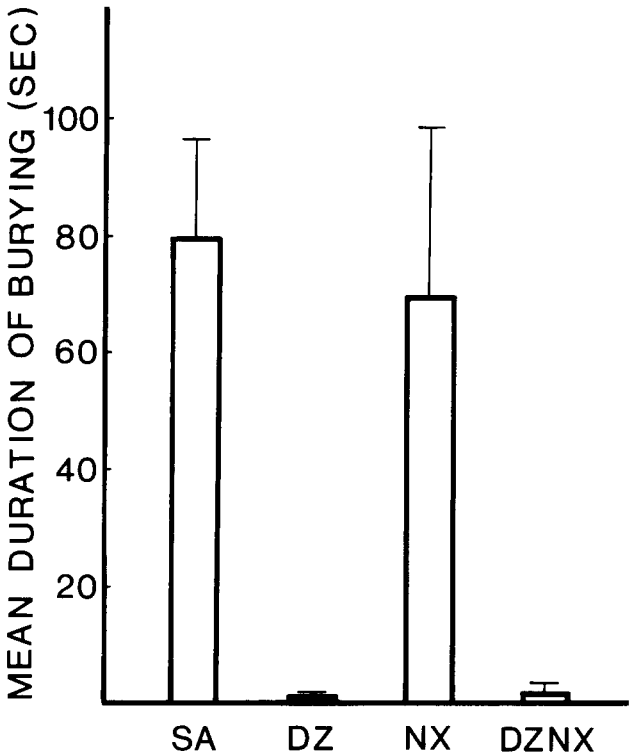

FIG 4 Mean (±SEM) duration of burying by rats given saline (SA), diazepam (DZ), naloxone (NX), or diazepam plus naloxone (DZNX) in Experiment 4

RESULTS

Figure 3 shows that burying that is elicited in the absence of painful stimulation is also suppressed by diazepam Planned orthogonal comparisons confirmed that the amount of burying behavior displayed by diazepam-treated rats was significantly lower than the amount displayed by vehicle-treated rats at every dose except 0 1 mg/kg (0 1 mg/kg, $t(18)=1.01$, $p>0$ 5, 0.5 mg/kg, $t(18)=2.77$, $p<0$ 01, 1 mg/kg, $t(18)=2$ 48, $p<0.02$, 2 mg/kg, $t(18)=3$ 21, $p<0$ 04).

EXPERIMENT 4

The results of the first 3 experiments provided no support for the view that the suppressive effect of diazepam on defensive burying is due to a reduction in the rat's pain sensitivity However, these results do not rule out the possibility that the effect of diazepam is indirectly mediated through an endogenous opiate mechanism There is some evidence that benzodiazepines modulate the release of enkephalins [6, 7, 33, 34] and there are some studies that show that the anti-conflict effects of benzodiazepines can be blocked by administering the opiate antagonist naloxone [1, 5, 24] Why these apparent relationships between benzodiazepines and endogenous opiate mechanisms would not be expressed in terms of an obvious reduction in pain-sensitivity is not, at present, clear Nevertheless, one tactic that might be used to assess the involvement of opiate mech-

anisms in anxiolytic drug effects would be to determine whether naloxone can block the suppressive effect of diazepam on defensive burying. Experiment 4 was designed to assess this possibility.

METHOD

On the test day, the 24 naive rats were randomly assigned to one of four conditions (n=6). Rats in the three drug conditions received intraperitoneal injections of either diazepam (1 mg/kg), naloxone (10 mg/kg), or diazepam plus naloxone (1 mg/kg; 10 mg/kg). Diazepam was injected 30 min before the burying test, whereas naloxone was injected 10 min before the test. The time of naloxone injection and the dose of naloxone were the same as those that had previously been shown to reverse the "anticonflict" effects of benzodiazepines [5], and are within the range known to reliably reverse the antinociceptive effects of a variety of drugs [21]. Rats in the control condition received an intraperitoneal injection of saline (1 ml/kg) 30 min before the test. The rats were placed individually into the center of the Plexiglas chamber and shocked once (1 mA) when they first touched the prod with a forepaw. During the next 15 min, the duration that each rat buried the prod was recorded on an electronic counter.

RESULTS

As can be seen in Fig. 4, diazepam produced a substantial suppression of defensive burying, regardless of whether it was injected alone or in combination with naloxone, whereas naloxone by itself had no obvious effect on defensive burying. These results were analyzed with a one-way ANOVA, which showed that the main effect of drug condition was significant, $F(3,20)=6.24$, $p<0.003$. Subsequent pair-wise analysis (Newman-Keuls, $p=0$ 05) confirmed that diazepam, alone or in combination with naloxone, produced a significant suppression of rats' defensive burying behavior compared to saline controls, whereas when naloxone-injected animals were compared with the saline controls, there was no significant difference in the duration of burying behavior. Finally, there was no significant difference in the suppression of defensive burying in the two groups given diazepam. These results show that the suppressive effect of diazepam on defensive burying was not influenced by the opiate antagonist naloxone, and thus, the possible involvement of endogenous opiate mechanisms in the anxiolytic effect of diazepam was not indicated

GENERAL DISCUSSION

The results of the present investigations strongly suggest that diazepam does not suppress defensive burying behavior through a simple analgesic mechanism (cf [23]). In the first experiment, 1 mg/kg of diazepam had no appreciable effect on the rat's latency to escape from a painful heat stimulus, but reliably suppressed defensive burying behavior More importantly, there was no apparent relationship between the effect of diazepam on rats' pain sensitivities and the duration that they buried the prod Because these negative results could have been due to an insensitive test of analgesia, the next two experiments examined whether diazepam would have a suppressive effect in the absence of an obvious pain stimulus If diazepam produced its effects exclusively through an analgesic mechanism, then it should have no effect when injected in the absence of painful stimulation. Thus, rats in Experiment 2 were shocked first, and then injected with diazepam for a burying test that occurred 15 min later. Diazepam still produced a significant suppression of defensive burying compared to saline controls In order to control for the possibility that painful aftereffects of electric shock actually had been the locus of the suppressive effect of diazepam in Experiment 2, rather than fear of shock source, rats in Experiment 3 were tested for the effects of diazepam in the complete absence of painful stimulation. These rats were simply exposed to a novel stimulus that had been shown to elicit defensive burying [26]. Diazepam suppressed the burying behavior that was elicited by this non-painful novel stimulus in a dose-dependent fashion.

Since it is clear from these results that diazepam can suppress defensive burying behavior in the absence of painful stimulation, the hypothesis that anxiolytics produce their effects exclusively through an analgesic mechanism was contradicted. The present results also failed to provide any support for the possibility that endogenous opiate mechanisms were involved in the anxiolytic effects of diazepam. Naloxone, an opiate antagonist, did not block the suppressive effect of diazepam on defensive burying, and naloxone administered by itself had no significant effect on the rats' defensive burying.

The apparent discrepancies between the present results and previous demonstrations that diazepam can have analgesic effects may be due to number of factors. For example, the 1 mg/kg dose of diazepam used in Experiments 1, 2, and 4 is well below the range that produces obvious motor impairments [16]. This factor is significant because although benzodiazepines have been shown to be active in standard analgesic tests, these 'analgesic' effects are typically restricted to doses that produce gross motor impairments, and thus may not reflect 'pure' analgesic action [20]. In view of these observations, it may not be too surprising that 1 mg/kg of diazepam had no apparent effect in the tail-flick test used in Experiment 1. Nevertheless, the 1 mg/kg dose of diazepam has repeatedly been shown to reliably suppress defensive burying without any obvious side-effects, and a number of experiments have provided evidence that defensive burying fulfills the pharmacological criteria of a model of anxiolytic drug effects [27,28] Furthermore, morphine, a prototypic analgesic, does not have a significant effect on the amount of burying displayed by animals shocked from a prod [27]. Taken together, these results suggest that benzodiazepines reduce responsiveness in the defensive burying test through mechanisms other than simple analgesia, and that analgesia may not account for their effects at high doses in standard analgesic tests

Another apparent discrepancy is the finding that naloxone, an opiate antagonist, did not reverse the suppressive effect of diazepam on defensive burying, even though it has repeatedly been shown to reverse the "anti-conflict" effect of diazepam [1, 5, 24]. However, it has been argued [24] that because naloxone depresses food intake under a variety of conditions [17, 22, 24, 25], it might 'reverse' the anti-conflict effects of benzodiazepines though this side-effect on appetite, rather than through a specific interaction with the mechanisms underlying the anxiolytic effect of benzodiazepines. Since the defensive burying response does not depend upon food motivation, this interpretational problem is avoided in the present experiment. Moreover, there is good evidence that naloxone can selectively block opiate systems (cf. [8,21]), and therefore the results of Experiment 4 are relatively clear: they do not support the view that endogenous opiate mechanisms are involved in the anxiolytic effects of benzodiazepines

In conclusion, the present experiments have provided a number of lines of evidence against the hypothesis that anxiolytics such as diazepam act in the defensive burying paradigm by suppressing the animals' pain sensitivities (i.e., analgesia). Instead, the results of these studies are consistent with the view that anxiolytics suppress defensive burying by reducing the animals' fear-motivation, and thus they provide further support for defensive burying as a simple animal model for studying the anxiolytic actions of drugs

ACKNOWLEDGEMENTS

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