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Rat Pup Ultrasonic Isolation Calls: Possible Mediation by the Benzodiazepine Receptor Complex

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INSEL, T. R., J. L. HILL AND R. B. MAYOR. *Rat pup ultrasonic isolation calls: Possible mediation by the benzodiazepine receptor complex.* PHARMACOL BIOCHEM BEHAV **24**(5) 1263–1267, 1986.—Rat pups, while separated from their littermates and placed in a novel environment, emit ultrasonic isolation calls. These ultrasonic calls decrease in number, power, and frequency following administration of the anxiolytic, diazepam (0.5 mg/kg). Pentylenetetrazol (20 mg/kg), which has been reported to be clinically anxiogenic, increases the number and the power of these calls. These changes following diazepam and pentylenetetrazol administration are dose dependent and do not appear to be secondary to nonspecific effects of these drugs on arousal or thermoregulation. The benzodiazepine receptor antagonist RO 15-1788, which has generally been reported to lack intrinsic activity at low doses, also decreases the number of rat pup isolation calls. These findings suggest that the benzodiazepine-GABA receptor-chloride channel complex may play a role in the physiologic mediation of the rat pup isolation call.

Isolation calls Distress vocalizations Ultrasonic calls Benzodiazepine receptor Benzodiazepine antagonist Penetylenetetrazol

ISOLATION calls or "distress vocalizations" are the cries emitted by infants separated from their parents and littermates. Although these cries are nearly universal among mammalian infants, in many species they are ultrasonic and therefore go unnoticed by human observers. In the rat pup, for instance, isolation calls range from 30 to 50 kHz [27,41]. Previous studies with rodent pups have demonstrated that these ultrasonic calls are laryngeal in origin [38], are affected by ambient temperature [1, 31, 42], and are associated with maternal retrieval [2, 13, 29, 44]. In the rat, these calls can be demonstrated within hours after birth, but they are most intense from postnatal days 6 to 12 [28].

Pharmacologic studies of audible "distress vocalizations" in other species have implicated an endogenous opiate system in the mediation of these calls. Exogenous opiates administered peripherally [34] or centrally [33] appear to decrease while naloxone appears to increase [34] the number of audible "distress vocalizations." Recent studies using electrophysiologic stimulation of opiate-rich areas of brain [18] have begun to suggest a neuroanatomic map for the opiate mediation of isolation calls in the guinea pig. The guinea pig is an excellent species for such studies as its isolation calls are audible and the call-reducing effects of low doses of exogenous opiates can be dissociated (to some extent) from the non-specific effects of these drugs on locomotor activity [19]. In preliminary studies however, we were unable to dissociate morphine's effects on ultrasonic isolation calls in rat pups from the drug's sedative properties (author's unpublished data). As clinical studies have recently linked a history of "separation anxiety" in childhood to anxiety disorders in adulthood [16], we hypothesized that clinically potent anxiolytics, such as the benzodiazepines, might more selectively decrease rodent isolation calls.

In addition to their clinical potency, benzodiazepines are of interest because their anxiolytic effects appear to be mediated by a specific, membrane-bound receptor (functionally linked to the GABA-A receptor and a chloride ion channel) [46,47] with a well-defined neuroanatomic distribution [48,49]. New ligands for the benzodiazepine receptor permit the pharmacologic dissection of receptor mediated effects. For instance, the imidazobenzodiazepine, RO 15-1788, binds with high affinity to the receptor and antagonizes the behavioral effects of diazepam, but in most paradigms has little intrinsic anticonflict or anxiolytic activity on its own [10, 11, 20, 30]. Other synthetic compounds, such as certain substituted beta-carboline esters and pentylenetetrazol, appear to have anxiogenic effects mediated through the benzodiazepine-GABA-chloride channel complex [12, 22, 26, 45]. In this study, we report that these various anxiolytic and anxiogenic compounds affect rat pup ultrasonic distress vocalizations, and we present evidence implicating the benzodiazepine-GABA receptor-chloride channel complex in the physiologic mediation of these calls.

METHOD

Sprague-Dawley rat pups, 6–10 days old, were used. Pups were housed with both parents in a cage that was undisturbed from the time of conception. Litters of less than 6 or more than 12 pups were rejected. All testing was done in the

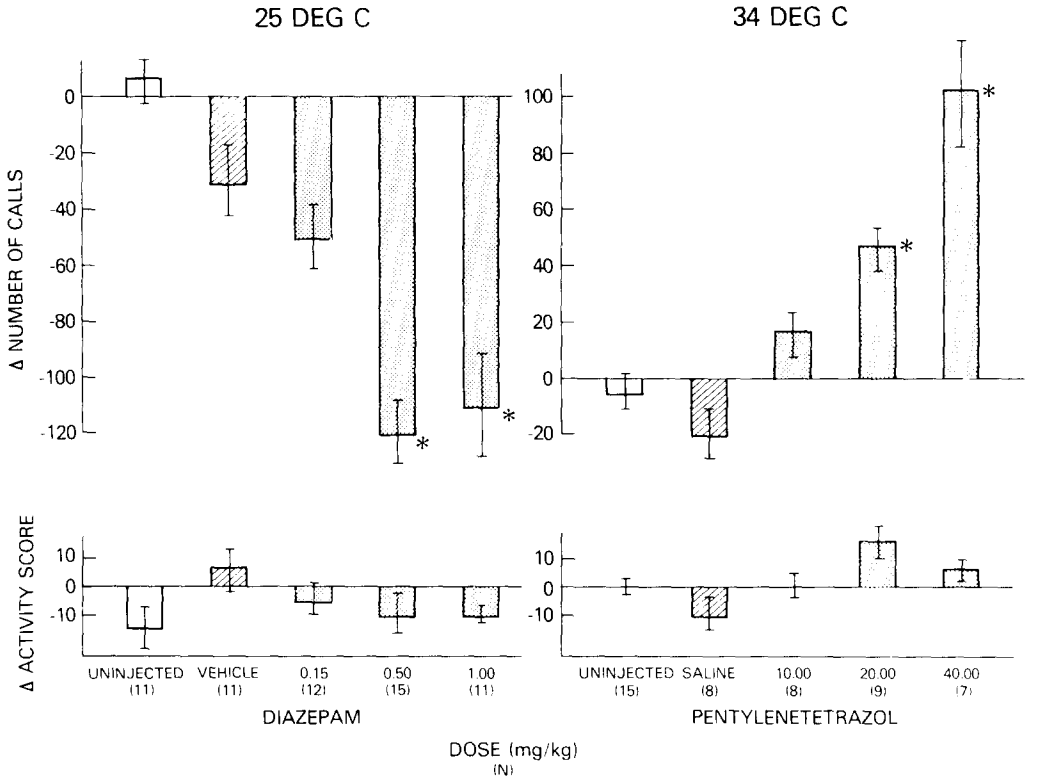

FIG. 1. Values represent means (±SEM) of absolute changes from baseline scores 30 minutes following diazepam, pentylenetetrazol, vehicle, or saline injection. Uninjected littermates were used to identify effects of saline or vehicle alone. No significant differences were evident at baseline across treatment groups at either temperature. The * signifies a significant difference (i.e., $p<0.05$) from vehicle or saline injections on post hoc Student-Newman-Keuls test. Neither vehicle nor saline differs significantly from uninjected controls. Changes in locomotor activity were not significant for diazepam, $F(4,58)=2.26$, $p>0.05$, but due to a prominent decrease after saline, were significantly different across the pentylenetetrazol condition, $F(4,42)=6.14$, $p=0.0006$.

TABLE 1

CHANGES IN PHYSICAL CHARACTERISTICS OF ISOLATION CALLS FOLLOWING DRUG ADMINISTRATION

	Number of Calls	Mean Peak Frequency	Mean Total Power
Baseline (24°C)	131.7 ± 15.0	38.8 ± 0.6	459.3 ± 33.3
Diazepam (0.5 mg/kg)	12.5 ± 5.0*	36.7 ± 0.5*	198.9 ± 30.4*
Baseline (34°C)	28.3 ± 7.7	38.0 ± 0.7	269.9 ± 23.7
PTZ (20 mg/kg)	77.8 ± 10.3*	39.0 ± 0.6	385.3 ± 46.2†

*$p<0.01$; †$p<0.05$; two tailed paired t-tests comparing post-drug values to baseline values.

last 6 hours of the light cycle (14 hours light, 10 hours dark). Each pup was used only one time. Weight and sex were determined and then pups were placed in a 41×24 cm Plexiglas recording chamber for testing. Vocalization was recorded during 2 minutes of separation with a microphone (Bruel and Kjaer Model 4385, Copenhagen) suspended within a parabolic reflector approximately 10 cm above the floor of the chamber. Following a baseline trial, 0.1 ml of drug or vehicle was administered subcutaneously at the nape of the neck. Injected pups or non-injected untreated controls were then returned to the litter for 30 minutes after which they were retested for 2 minutes for the drug trial. Vocalization signals were transformed by a digital sound spectrum analysis system providing on-line the number of calls in each two-minute session [8]. In addition, records were stored on a magnetic disk to permit subsequent analysis of peak frequency (kHz) and relative power for each call [8]. Locomotor activity was recorded by visual scoring of crossovers on a 2″×2″ grid on the floor of the recording chamber. Each drug condition included pups of at least two different litters with littermates used as vehicle controls. Experimental temperature was controlled by a water bath beneath the isolation chamber.

Diazepam (Hoffmann-La Roche) was used as a prototype benzodiazepine receptor agonist. The standard formulation for parenteral diazepam was diluted in saline to a concentration giving the requisite dose in 0.1 ml. Pentylenetetrazol (Summit Hill Laboratory), which appears to directly affect the chloride channel [22,45] was used as a standard "anxiogenic." The benzodiazepine receptor antagonist RO 15-1788 (generously supplied by F. Hoffmann-La Roche) and the benzodiazepine receptor inverse agonists FG 7142 (generously supplied by Leif Jensen, Ferrosan Inc.,

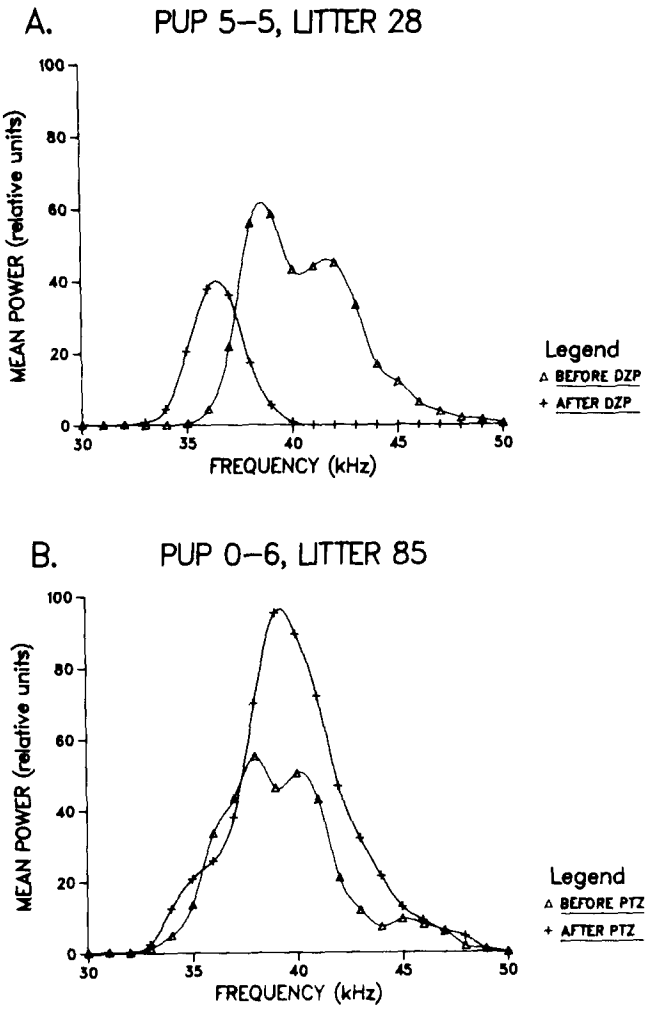

FIG. 2. Representative records from individual pups (A) before and after diazepam (0.5 mg/kg) and (B) before and after PTZ (20 mg/kg). Y-axis represents mean power output at each frequency across the two-minute recording session.

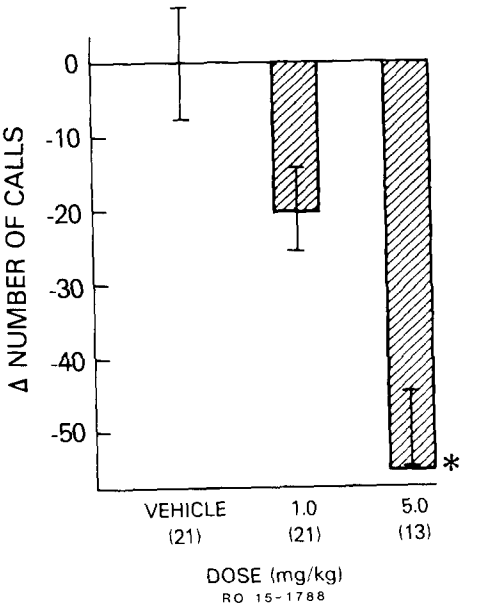

FIG. 3. Change from number of calls at baseline following vehicle or RO 15-1788 injection. Analysis, as in Fig. 1, yields a significant decrease (* signifies $p<0.05$) in number of calls only at higher dose of RO 15-1788. No significant change in activity was noted at either dose.

Copenhagen) and β-CCE (Research Biochemicals) were suspended in a vehicle of 40% propylene glycol, 10% ethanol, and 50% saline and then the stock solution was further diluted in saline to the required concentration for injection.

Temperature and gender effects were evaluated by a Student's t-test of baseline values. Drug effects were analyzed with a linear models ANOVA (Statistical Analysis System) with post hoc Student-Newman-Keuls tests applied to individual pairs within groups where significant overall effects were present.

RESULTS

Pups tested at room temperature (24–25°C) gave a mean (±SEM) of 98.5±7.1 calls during the two minute baseline trial. By contrast, pups tested at 33–36°C, the temperature at the core of a huddled litter, gave a mean (±SEM) of 27.8±8.2 calls, $t(110)=42.37$, $p<0.0001$. To avoid ceiling effects in the pharmacologic studies, 25°C was used to test anxiolytics; the 33–36°C condition was used to test anxiogenics. Gender did not have a significant effect on baseline calling rate.

Administration of the benzodiazepine, diazepam, in doses ranging from 0.15 to 1.0 mg/kg, but not vehicle (50% saline, 40% propylene glycol, 10% ethanol) alone, significantly reduced the number of calls compared to untreated controls, $F(4,58)=14.63$, $p<0.0001$ (Fig. 1). Following diazepam administration, locomotor activity was not significantly decreased. Analysis of individual vocalization records revealed a significant decrease in the mean peak frequency and in the total power following 0.5 mg/kg diazepam (Table 1 and Fig. 2A). Pretreatment with 5.0 mg/kg of RO 15-1788 significantly attenuated the decrease in isolation calls induced by 0.5 mg/kg diazepam ($n=10$, mean±SEM change from baseline $=-2.1\pm13.3$ calls) demonstrating that the decrease following diazepam alone was mediated by the central benzodiazepine receptor.

Pentylenetetrazol (PTZ) administration was followed by increased distress vocalizations at 20 and 40 mg/kg, $F(4,42)=20.82$, $p<0.0001$ (Fig. 1). Mean peak frequency did not change following administration of 20 mg/kg PTZ, although the mean power of the calls increased (Table 1 and Fig. 2B). PTZ did not cause seizures at any of these doses, although subsequent convulsant testing with pups at this age revealed a very steep dose response curve with a CD_{50} (i.e., dose at which 50% of animals seized) of 50 mg/kg.

To further investigate if the convulsant properties of PTZ were critical to the drug induced increase in isolation calls, we administered the benzodiazepine inverse receptor agonist, FG 7142, which has been shown to be anxiogenic, but non-convulsant in man and non-human primates [12,21]. Following FG 7142 (25 mg/kg), the mean (±SEM) number of calls increased from 14.9 (±6.9) to 30.7 (±15.0), a nearly significant difference, $t(8)=2.06$, $p<0.06$, when compared to

changes after vehicle administration. Administration of the convulsant benzodiazepine receptor inverse agonist, β-carboline-3-carboxylic acid ester (β-CCE) at a dose of 1.5 mg/kg was associated with an inconsistent increase in the number of calls. Several pups in this sample (7 out of 28) had exceedingly high baseline calling rates and actually showed a decrease in the number of calls following β-CCE administration. For those pups with less than 100 calls in the baseline period (n=21), the mean (±SEM) number of calls increased from 29.8 (±5.3) to 45.8 (±8.8) following β-CCE, $t=2.04$, $p=0.05$.

The benzodiazepine receptor antagonist, RO 15-1788, was given alone to determine if some endogenous anxiogenic or anxiolytic receptor ligand might affect the isolation call. Given by itself, at 24°C, RO 15-1788 significantly reduced isolation calls at 5.0 mg/kg (107.2±11.2 baseline to 53.1±9.6 post-drug) but not at 1.0 mg/kg (96.5±10.0 baseline to 71.4±11.9 post-drug), $F(2,52)=10.84$, $p<0.0001$ (Fig. 3). RO 15-1788 did not affect locomotor behavior, $F(2,52)=1.84$, NS.

DISCUSSION

These results following diazepam and PTZ administration suggest that the isolation call is quite sensitive to drugs which affect the benzodiazepine-GABA receptor-chloride channel complex. The reduction in isolation calls following diazepam could be secondary to sedative effects of the drug, but this seems unlikely as we noted no decrease in locomotor behavior. Previous studies have reported activating rather than sedating effects of diazepam administration to infant rats [35]. In one previous report in young dogs, diazepam had non-specific effects on separation distress, but this investigator used doses ranging up to 8 mg/kg [43]. The only previously published study of diazepam effects on rodent isolation calls also noted a decrease in calls in the absence of locomotor effects [15]. Our results confirm this report and provide further data demonstrating decreases in frequency and power of isolation calls following administration of clinically anxiolytic doses of diazepam.

Although the anxiogenic drugs FG 7142 and β-CCE appear much weaker than PTZ, this difference may be largely pharmacodynamic as both of these drugs are esters with very brief plasma half-lives (less than one minute for β-CCE) in the rat, in contrast to their much slower metabolism in primates [21]. Such species differences in plasma esterase activity point out the difficulty in extrapolating from drug effects in the rodent to effects in man. A further problem with the interpretation of our data with the inverse agonists is the considerable variability in baseline values. The explanation for this variability is not clear, but several possibilities such as individual differences in temperature, hunger, or sleep-wake cycles seem likely. The isolation call, although ostensibly a "simple" behavior, is probably affected by an array of internal and external factors, only a few of which can be fully assessed.

As temperature is such a critical variable for eliciting ultrasonic isolation calls, one explanation for our results with diazepam and PTZ could be a direct effect on body temperature. To test this possibility, rectal and surface temperatures were measured in 12 pups, 30 minutes after drug administration. Mean temperatures following DZP (0.5 mg/kg) were 32.6°C rectal, 33.6°C surface; following PTZ (20 mg/kg) were 32.4°C rectal, 32.8°C surface; differences that were neither consistent nor significant.

It appears then that the anxiolytic diazepam and the anxiogenic PTZ can, respectively, decrease and increase isolation calls in the absence of sedative, convulsant, or thermoregulatory effects. Furthermore, the diazepam effects appear to be benzodiazepine receptor mediated as they are entirely blocked by RO 15-1788. Our finding that RO 15-1788 by itself has intrinsic properties is somewhat more surprising, although others [9,40] have also reported diazepam-like effects of RO 15-1788 in stressful behavioral paradigms. One interpretation of this agonist-like activity of RO 15-1788 is that in situations of distress, such as isolation of an infant, an endogenous "anxiogenic" ligand occupies the receptor. Indeed, an endogenous ligand which appears to be anxiogenic has recently been described [17]. Since RO 15-1788 also blocks the effects of diazepam, this "receptor antagonist" appears to have contrasting effects that are determined by the mileu of the receptor [4,36].

Taken together, these data following agonist, antagonist, and inverse agonist administration would point to a role for the benzodiazepine-GABA receptor-chloride channel complex in rodent attachment behavior. Two other kinds of evidence would support such a role. First, we have found that the pups of Maudsley reactive rats emit five fold more isolation calls than their non-reactive congeners (author's unpublished data). The Maudsley reactive strain was originally selected on the basis of increased defecation in the open field [6] and was subsequently demonstrated to show increased emotional reactivity on an extensive repertoire of behavioral tests [3,7]. This strain has also been reported to have a decreased number of brain BZD receptors [39]. Although this strain difference in the number of isolation calls is consistent with the hypothesis that BZD receptors have a physiologic role in the mediation of these cries, a definitive statement must await demonstration of differences in receptor number in the Maudsley reactive pups.

A second kind of evidence, albeit indirect, arises from the paradox that the BZD receptor is phylogenetically recent [25], emerging roughly with the evolutionary appearance of such social behaviors as parenting and attachment [23], yet is distinguished by a very early appearance in ontogeny [5, 24, 32, 37]. In the rat, for instance, the concentration of BZD receptors increases from 35% to nearly 100% of adult levels within the first postnatal week [24]. The relatively late phylogenetic emergence and early ontogenetic appearance of this receptor would be consistent with our pharmacologic data suggesting a role in attachment behavior. Ultimately, however, the isolation of endogenous ligands for this receptor will be essential before more conclusively defining a physiologic role for this anatomically discrete complex in the brain.

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