

# Pharmacological analysis of the effects of benzodiazepines on punished schedule-induced polydipsia in rats

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Food-deprived Wistar rats were exposed to a fixed-time 60-s food delivery schedule until they developed schedule-induced polydipsia. Every fifth lick was then followed by an electric shock during two, signalled, 5-min periods, which ran concurrently with the food delivery schedule. Shock intensities were adjusted to reduce licking to 60–70% of the unpunished licking rates. The benzodiazepine full agonists, diazepam (0.3–3.0 mg/kg), chlordiazepoxide (0.3–10.0 mg/kg), oxazepam (0.3–3.0 mg/kg) and the benzodiazepine partial agonist, RU-32698 (3.0–17.0 mg/kg), led to increases in punished responding at intermediate doses and decreases at the highest doses tested. All benzodiazepine agonists brought about dose-dependent decreases in unpunished schedule-induced polydipsia, with doses required to reduce drinking proving higher than doses required to increase punished schedule-induced polydipsia. The antipunishment effect of 0.3 mg/kg of diazepam was dose-dependently antagonized by flumazenil and the benzodiazepine inverse agonist, RU-34000. Flumazenil effects, however, could reflect actions of flumazenil as a partial inverse agonist at GABA<sub>A</sub> receptors. RU-32698 at 10.0 mg/kg further facilitated the

rate-increasing effect of 0.3 mg/kg of diazepam, but at 17.0 mg/kg partially blocked such antipunishment effect. Overall, the present results extend the similarities of the effects of benzodiazepine compounds on adjunctive and operant patterns of behaviour by showing similar interactions within the benzodiazepine receptor complex. *Behavioural Pharmacology* 18:81–87 © 2007 Lippincott Williams & Wilkins.

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## Introduction

Benzodiazepines are highly effective compounds in the treatment of anxiety disorders (see Nutt, 2005). One of the best documented behavioural effects of benzodiazepines is their anxiolytic action in conflict procedures. The operant response rate reduced by the simultaneous occurrence of a schedule of positive reinforcement and a punishment schedule is selectively increased by substances with anxiolytic activity, notably benzodiazepines (see Houser, 1978; Millan, 2003).

The antipunishment effect of benzodiazepines and other anxiolytics depends on the intensity and frequency of the electric shocks that are used as punitive stimuli and determine the punished response rate; low unpunished operant response rates are not increased by anxiolytic drugs (McMillan, 1973; Jeffrey and Barrett, 1979; Dworkin *et al.*, 1989). The degree of the antipunishment effect of benzodiazepines is linked to their clinical efficacy, with positive correlations reported in studies with pigeons (Kleven and Koek, 1999), rats (Cook and Davidson, 1973) and rhesus monkeys (Rowlett *et al.*, 2006).

Behavioural effects of benzodiazepines appear to be mediated by actions at different subtypes of GABA<sub>A</sub>

receptor (see Rudolph *et al.*, 2000). Such mediation can be investigated by blocking GABA<sub>A</sub> receptors with benzodiazepine antagonists, and then testing the absence or diminution of a previously observed effect by the targeted benzodiazepine agonist. In this connection, the antipunishment effect of benzodiazepines and related compounds can be attenuated by previous administration of the benzodiazepine receptor antagonist, flumazenil (e.g. Barrett *et al.*, 1986; Witkin *et al.*, 1997).

Compared with the research undertaken on operant behaviour, very few studies have addressed the effects of drugs on punished adjunctive behaviour. The term 'adjunctive behaviour' refers to any behaviour that occurs during reinforcement schedules without any apparent contingent relationship with the reinforcer, and would correspond to behavioural patterns adjunctive to the operant behaviour needed to obtain the reinforcer. The best studied adjunctive behaviour is schedule-induced polydipsia in rats, that is, when an animal is deprived of food and is subjected to an intermittent reinforcement schedule of food presentation, then, if it is given access to water at the same time, it will drink great amounts of liquid concurrently with its performance under the reinforcement schedule (Falk, 1961). Schedule-induced

polydipsia is adjunctive behaviour because the animals are neither thirsty nor do they have any need to drink to obtain the food reinforcer. Despite formal distinctions between adjunctive and operant behaviour, operant contingencies have been postulated to be involved in the maintenance of schedule-induced polydipsia and adjunctive behaviour in general. Such thinking is derived in part from studies showing similarities in the effects of drugs on adjunctive and operant patterns of behaviour (Pellón and Flores, 2006).

Flores and Pellón (1998) showed that adjunctive drinking reduced by contingent presentation of electric shocks could be increased after administration of the benzodiazepine, diazepam, but not after administration of buspirone or D-amphetamine. Moreover, this effect depended on the intensity of shock used, and was not seen in animals that received noncontingent electric shocks upon licking. In a later study, Flores and Pellón (2000) observed that the more adjunctive drinking was reduced by the use of electric shocks of different intensity, the greater was the antipunishment effect of diazepam. The procedure used by Flores and Pellón (2000) consisted of inducing adjunctive drinking in rats by means of a schedule that dispensed a food pellet every 60 s regardless of the animals' behaviour [a fixed-time (FT) schedule], and alternating this schedule with another which, in addition to food, dispensed electric shocks contingent on every fifth lick. Indeed, this procedure, based on the presentation of alternate punishment and nonpunishment components, was the one followed in the present experimental series.

Unlike the punishment studies outlined above, moderate doses of benzodiazepine increase unpunished adjunctive drinking, though mainly in terms of water intake rather than licking (Bacotti and Barrett, 1976; Sanger and Blackman, 1976; Pellón *et al.*, 1992), a finding that suggests that benzodiazepines might alter the topography of drinking.

This study sought to investigate the pharmacological mechanisms of action of benzodiazepines on punished schedule-induced polydipsia, by the comparative study of different benzodiazepine agonists and the possible antagonism through the blockade of benzodiazepine receptors with flumazenil. To this end, the high rates of licking a water spout by rats, induced by an FT 60-s schedule of food delivery, were punished by the presentation of lick-contingent shocks during signalled 5-min periods. First, the full benzodiazepine agonists, diazepam, chlordiazepoxide, oxazepam and the partial benzodiazepine agonist, RU-32698 (Tully *et al.*, 1991), were tested on punished and nonpunished schedule-induced drinking. Thereafter, diazepam was combined with doses of flumazenil and RU-32698, as well as with

doses of the inverse benzodiazepine agonist, RU-34000 (Gardner and Budhrum, 1991). The study of the pharmacological mediation of actions of benzodiazepines on punished schedule-induced polydipsia should provide further comparisons of adjunctive and operant behaviour to advance the understanding of the mechanisms involved in the development and maintenance of adjunctive behaviour.

## Methods

### Subjects

Six experimentally naive, male Wistar rats, obtained from IFFA-CREDO (Lyon, France), were used. They were approximately 90 days old and had a mean free-feeding weight of 414 g (range: 380–432 g) at the start of the experiment. The rats were individually housed and placed in a room with controlled environmental conditions (ambient temperature 22°C, 60% relative humidity and an 08.00/20.00 h light/dark cycle). After a 10-day period of habituation to the living conditions, and before training, the rats were gradually reduced to and maintained at 80% of their ideal free-feeding weight through food restriction. Clean, fresh water was freely available in all animals' home cages. These procedures were in accordance with European Union Directive 2003/65/EC and Spanish Royal Decree 1201/2005 governing the protection of animals used for experimental purposes.

### Apparatus

The experiment was conducted in six, identical 29 × 24.7 × 35.5 cm, LI-836 rodent conditioning chambers (Leticia Instruments, Barcelona, Spain) with grid floors. The chambers were enclosed in a soundproofed housing. The front panel of each chamber was made of aluminium, the right-hand wall of black Plexiglas, and the remaining sides and roof of transparent Plexiglas. Scrambled electric shocks were administered via each chamber's grid floor, by six independent LI 200–20 generators, (Leticia Instruments, Barcelona, Spain). A calibrated water bottle was attached to the external side of the right wall of each chamber, with its spout accessible to the rat through a 3.2 × 3.9 cm aperture, located 20 cm from the front wall and 7 cm above the floor. The spout was positioned 2 cm behind the aperture, in such a way that the rat could lick, but not maintain permanent contact with it. Each lick completed a circuit between the bottle spout and the grid floor, but the shocks were not administered through the spout, nor was the recording of the licks interrupted by administration of the shocks. The conditioning chambers were illuminated by two internal 3-W bulbs during the experimental sessions. The ambient noise produced by the ventilation was 60 dB, which served to mask any other possible external sounds. With the aid of a Leticia Instruments dispenser fitted to the outside of the front panel, standard 45-mg rodent food pellets (Bio-Serv,

Frenchtown, New Jersey, USA) were delivered into a receptacle situated in the centre of the front wall, at a height of 3.7 cm above the grid floor. Experimental events were scheduled and recorded using a BBC microcomputer (Acorn Computers, Cambridge, UK), programmed in SPIDER.

### Behavioural procedure

After being stabilized at 80% of their free-feeding weight, all rats underwent a water-intake test for two consecutive days. Fifty-five 45-mg food pellets were placed on a saucer in the home cages, and the total amount of water consumed by each rat in 55 min was duly recorded. This procedure furnished a baseline rate against which to compare the level of schedule-induced polydipsia subsequently observed in the experiment, during which each animal individually received the 55 food pellets in 55-min experimental sessions.

The following day, the rats were placed in the conditioning chambers for the first time, kept there for a period of 55 min and allowed to eat 20 food pellets previously deposited in the respective food hoppers. During this session, no water bottles were installed.

After this adaptation session, the rats were exposed to a FT 60-s schedule of one food-pellet presentation over 14, 55-min experimental sessions. The food pellets were dispensed at regular 1-minute intervals, regardless of the animal's behaviour. Immediately before each experimental session, bottles were filled with 100 ml of fresh tap water and installed in each conditioning chamber as described before. General illumination lights were switched on at the beginning of each session. The sessions were held 5 days per week. The following measures were taken per rat per session: (a) total number of licks, which enabled the number of licks per minute to be calculated and (b) total volume of water consumed (in ml).

Food continued to be administered at regular 60-s intervals in experimental sessions of 55 min each. Now, however, two 5-min periods signalled by a tone (70 dB, 40 Hz) were superimposed, and began 15 and 35 min, respectively, after the start of each session. During these periods, every fifth lick was followed by a 0.3-s electric shock delivered through the grid floor (a FR-5 schedule). The intensity of the electric shock was adjusted for each rat so as to obtain a 60–70% suppression in the lick rate vis-à-vis that registered during the nonsignalled periods (shock range: 0.10–0.12 mA). Licks were separately registered for each rat in both shock and no-shock components, thereby making it possible for the number of licks per minute of each component in the multiple schedule to be calculated. Although water consumption continued to be measured, these data were not reported

because of the impossibility of differentiating between the respective intakes in each component. This experimental phase lasted 28 sessions.

### Pharmacological procedure

All animals received intraperitoneal administrations of benzodiazepine compounds at a volume of 1 ml/kg, 10 min before the experimental session. Initially, benzodiazepine-full agonists were administered: firstly diazepam (0.1, 0.3, 1.0 and 3.0 mg/kg), then chlordiazepoxide (0.3, 1.0, 3.0 and 10.0 mg/kg) and finally oxazepam (0.3, 1.0 and 3.0 mg/kg). Thereafter, the benzodiazepine partial agonist, RU-32698, was administered at 3.0, 10.0 and 17.0 mg/kg. Diazepam was suspended in a solution of distilled water and three drops of Tween 80 (Sigma-Química, Madrid, Spain), chlordiazepoxide and RU-32698 were dissolved in 0.9% saline solution, and oxazepam was suspended in 50% propylene glycol and saline serum. Drug doses were administered following a random order, but all rats received the same dose on the same day. Drug administration sessions were on Tuesdays and Fridays. On Thursdays, the animals received intraperitoneal injections of vehicle at a volume of 1 ml/kg, 10 min before the session. Monday sessions served as control condition without injection.

Once the dose–response functions of the benzodiazepine agonists had been established, we proceeded to evaluate the combination of 0.3 mg/kg of diazepam (as it brought about the greatest antipunishment effect: see the Results section): first with saline serum; then at doses of 3.0 and 10.0 mg/kg of the benzodiazepine antagonist, flumazenil (Ro 15–1788); later, at doses of 10.0 and 17.0 mg/kg of the benzodiazepine partial agonist, RU-32698; and lastly, with doses of 1.0 and 3.0 mg/kg of the inverse benzodiazepine agonist, RU-34000. Flumazenil was suspended in a solution of distilled water and three drops of Tween 80, and RU-34000 was dissolved in a 0.9% saline solution. Ten minutes before each experimental session, the animals received the two corresponding injections. Both the randomization procedure and administration pattern were the same as those described above.

The pharmacological study lasted 52 sessions, during which lick rates continued to be measured separately for the punished and unpunished components. The percentage change generated by each pharmacological administration with respect to control condition without injection was calculated, by dividing licks per minute in the presence of any given drug by the control-session lick rate, and multiplying this by 100.

### Results

All rats developed schedule-induced polydipsia after being exposed to the FT 60-s food delivery schedule. During the last 5 days of exposure to the schedule, the rats drank an

average of approximately 28 ml of water (range: 19–42 ml), which was four times as much as they had consumed during the water-intake test in their home cages (mean 6.5 ml, range: 5.0–8.5 ml). With the introduction of the punishment procedure, the lick rates in the punished component were reduced by 60–70% with respect to the lick rates in the unpunished component. Specifically, during the last five sessions of the multiple schedule, the mean rate of licks per minute in the punished component was  $25.33 \pm 3.72$  and that of the unpunished component,  $75.83 \pm 17.48$ . Throughout the study, animals collected and ate all food pellets delivered in any experimental session; therefore, food intake was not substantially altered with the introduction of the punishment procedure or the administration of drugs (see below).

### Benzodiazepine agonists

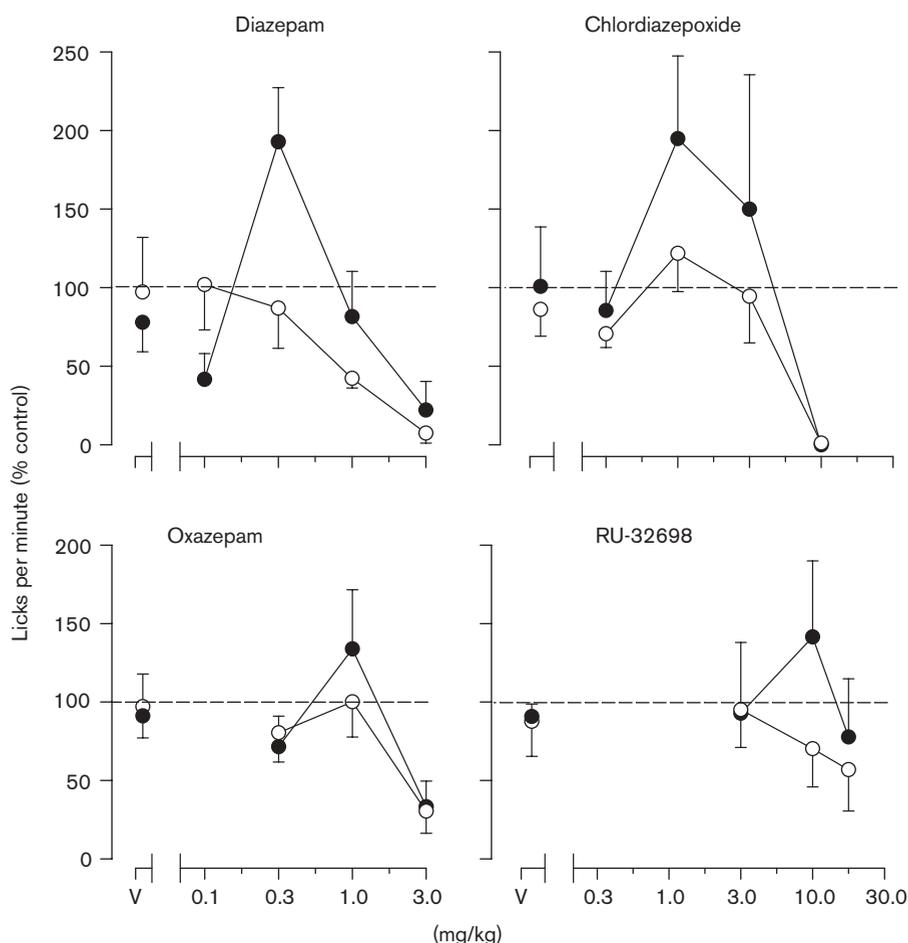
Figure 1 depicts the effects of the different doses of diazepam, chlordiazepoxide, oxazepam and RU-32698 on the licking rates in the punished (black circles) and

unpunished components (white circles). Furthermore, shown are the means and standard errors of the licks per minute in the sessions in which the vehicle (V) corresponding to each drug was administered. The data represent the percentage change with respect to the mean licking rate in control condition during which the animals were not injected. In no case did administration of vehicle result in noteworthy changes in the punished and unpunished licking rates.

Insofar as the unpunished component was concerned, none of the drugs led to increases in licks per minute. For diazepam and RU-32698, dose-dependent decreases were observed; for chlordiazepoxide and oxazepam, low or intermediate doses were observed to have no effect on unpunished licks per minute, whereas the highest doses of both drugs substantially reduced polydipsic drinking.

In the case of the punished component, dose-dependent effects were observed for all drugs according to the

Fig. 1



Effects of diazepam, chlordiazepoxide, oxazepam and RU-32698 on the licking rates of punished (filled circles) and unpunished (open circles) components, represented as the mean  $\pm$  SEMs ( $n=6$ ) of the percentage change with respect to average licks per minute in control sessions without injection. Data above V correspond to administration of vehicle.

following general pattern, whereas the lowest doses failed to produce effects on the punished licking rate (except for 0.1 mg/kg of diazepam which reduced the behaviour), increases were observed at intermediate doses and decreases at the highest doses (except for 17.0 mg/kg of RU-32698). The punished licking rate increased to approximately double after doses of 0.3 mg/kg of diazepam and 1.0 mg/kg of chlordiazepoxide, as it did, albeit to a lesser extent, after 1.0 mg/kg of oxazepam and 10.0 mg/kg of RU-32698 (and 3.0 mg/kg of chlordiazepoxide). The punished licking rate decreased after doses of 3.0 mg/kg of diazepam, 10.0 mg/kg of chlordiazepoxide and 3.0 mg/kg of oxazepam.

### Benzodiazepine antagonists

Figure 2 shows the effects of the combination of different doses of flumazenil, RU-32698 and RU-34000 with 0.3 mg/kg of diazepam on licks per minute in the punished component, represented as the mean and standard error of the percentage change with respect to the control rate without injection. It also shows the effect of administering 0.3 mg/kg of diazepam preceded by vehicle (black circles). In this instance, it is seen that the drug increased the punished licking rate by an order of magnitude similar to that observed in the previous figure, when the same dose of diazepam was administered alone.

The combination of 0.3 mg/kg of diazepam with flumazenil (white circles) resulted in antagonism of the

antipunishment effect of diazepam, which was dependent on the flumazenil dose, that is, with the dose of 3.0 mg/kg, no appreciable effects were generated, but with 10.0 mg/kg, licks per minute were reduced even below the baseline levels of punished behaviour. Similar results were observed after combining 0.3 mg/kg of diazepam with the inverse benzodiazepine agonist, RU-34000 (white squares), that is, with 1.0 mg/kg no effect whatsoever was in evidence, but with 3.0 mg/kg the antipunishment effect of diazepam was completely blocked (doses of 10.0 mg/kg of flumazenil and 3.0 mg/kg of RU-34000 alone led to changes of 87.59 and 84.89%, respectively, vis-à-vis the licking rate in control condition without injection, with no appreciable effects *per se* and, thus, an absence of anxiogenic effects). Lastly, joint administration of 0.3 mg/kg of diazepam with the benzodiazepine partial agonist, RU-32698 (white triangles), resulted in an even greater increase (in the order of 50–75%) in punished drinking, when combined with a dose of 10.0 mg/kg, and an absence of effect or a slight decline in the licking rate, when combined with a dose of 17.0 mg/kg.

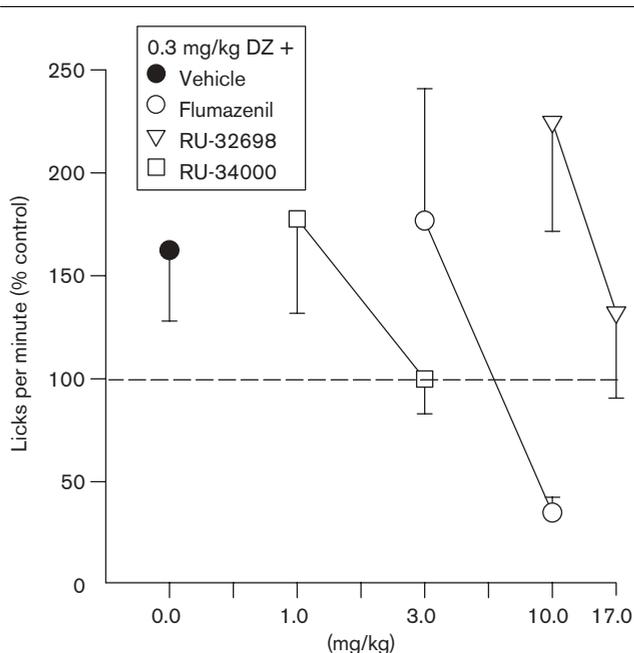
### Discussion

Results from the present studies indicate, first, that unpunished schedule-induced polydipsia was dose-dependently reduced by acute administration of several benzodiazepines, thus in keeping with previously published reports on diazepam (Sanger and Blackman, 1976; Mittleman *et al.*, 1988) and chlordiazepoxide (Bacotti and Barrett, 1976), and extending such findings to oxazepam, another full benzodiazepine agonist, and to the partial benzodiazepine agonist, RU-32698.

Second, all benzodiazepine agonists showed dose-related effects on punished schedule-induced polydipsia. Low doses generally had no effect, intermediate doses increased suppressed drinking and high doses normally further suppressed punished schedule-induced polydipsia. These results confirm previous findings with diazepam (Flores and Pellón, 1998, 2000) and extend the antipunishment effect to other benzodiazepines, including the partial agonist, RU-32698. Chlordiazepoxide increased punished adjunctive drinking to the same degree as did diazepam, whereas oxazepam and RU-32698 only led to increases amounting to half of those registered by diazepam and chlordiazepoxide.

Benzodiazepine-induced increases in the rates of punished schedule-induced polydipsia took place at doses lower than those required to decrease rates of unpunished schedule-induced polydipsia, thus showing a great specificity in the effects. These results are in line with previous papers on operant conditioning using rats and other animals (Cook and Davidson, 1973; Kleven and Koek, 1999; Rowlett *et al.*, 2006). Indeed, the relative

Fig. 2



Effects on punished licking rate of the combination of different doses of flumazenil, RU-32698 and RU-34000 with 0.3 mg/kg of diazepam, represented as the mean  $\pm$  SEMs ( $n=6$ ) of the percentage change with respect to average licks per minute in control sessions without injection.

potency of drug effects on schedule-induced polydipsia is comparable to findings on punished operant behaviour (cf. Houser, 1978). The ranking of potencies for the rate-increasing effects on punished schedule-induced polydipsia was diazepam > chlordiazepoxide = oxazepam > RU-32698, exactly the same as that obtained for the rate-decreasing effects on unpunished schedule-induced polydipsia (see Fig. 1). In a recent report on operant behaviour in rhesus monkeys, the antipunishment effect of benzodiazepines correlated positively with the therapeutic use of benzodiazepines in humans, for example, with diazepam proving as efficacious as, yet three times more potent than, chlordiazepoxide (Rowlett *et al.*, 2006), just as in this study.

The diazepam antipunishment effect was dose-dependently antagonized by the benzodiazepine antagonist, flumazenil, and by the benzodiazepine inverse agonist, RU-34000. In light of some recent evidence, however, flumazenil interactions with diazepam could be more complex than a mere antagonistic effect, and might reflect actions of flumazenil as a GABA<sub>A</sub> partial inverse agonist (Rowlett *et al.*, 2006). The finding that 10.0 mg/kg of flumazenil not only antagonized the antipunishment effect of 0.3 mg/kg of diazepam, but also suppressed licking, might reflect an anxiogenic effect typical of benzodiazepine inverse agonists. Interaction of flumazenil with diazepam was similar to interaction of RU-34000 with diazepam, thus suggesting that flumazenil may have an action similar to that of an inverse agonist.

Depending on the dose, the partial agonist, RU-32698, facilitated or antagonized the rate-increasing effect of 0.3 mg/kg of diazepam on suppressed schedule-induced polydipsia. Partial benzodiazepine agonists have also been shown to have mixed effects in other measures of anxiolytic activity (Jones *et al.*, 1994). When partial agonists have no effect (such as 17.0 mg/kg of RU-32698; see Fig. 1), they have been reported to act as antagonists of the effects of more efficacious benzodiazepine agonists (Lelas *et al.*, 2001). When partial agonists have an effect (such as 10.0 mg/kg of RU-32698; see Fig. 1), this effect can enhance that of a full agonist. Such dependency on the type of RU-32698 effect could, in theory, explain the dose–response curve that resulted from combinations of diazepam with different doses of RU-32698. Alternatively, the decrease observed in responding when 0.3 mg/kg of diazepam was combined with 17.0 mg/kg of RU-32698 could simply reflect dose additivity.

Benzodiazepines have been repeatedly shown to exert similar effects on schedule-induced polydipsia and operant behaviour, in studies on both punished and unpunished behaviour. Similarities between drug effects on adjunctive and operant patterns of behaviour have

been documented for several classes of drugs (see Pellón and Flores, 2006). The present results extend these similarities by showing that the antipunishment effect of diazepam on punished schedule-induced polydipsia is mediated by the benzodiazepine receptor complex, much in the same way as the antipunishment effects of benzodiazepines on punished operant responding are mediated by benzodiazepine receptors.

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