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Fluctuations in Responses to Diazepam During the Oestrous Cycle in the Mouse

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CAREY, M. P., A. E. BILLING AND J. P. FRY. *Fluctuations in responses to diazepam during the oestrous cycle in the mouse.* PHARMACOL BIOCHEM BEHAV **41**(4) 719–725, 1992.—Administration of diazepam (0.28 mg/kg, IP; 60 min) to male mice or to female mice at oestrus or dioestrus increased the number of transitions made between the light and dark chambers of a test apparatus, a presumed anxiolytic action. However, the same dose of diazepam had no effect on light/dark transitions at late dioestrus, prooestrus, or metoestrus II. At metoestrus I, this test dose of diazepam induced a decrease in the number of light/dark transitions and significant changes in other test parameters indicative of an increase in fearfulness or light aversion. Concentrations of diazepam in the brain after intraperitoneal injection were not influenced by the stage of the oestrous cycle, suggesting that the observed changes in responses to diazepam reflect changes in sensitivity to this drug rather than alterations in distribution or metabolism. The results indicate a physiological influence of ovarian steroid hormones on sensitivity to the benzodiazepine tranquilisers.

Anxiety Behaviour Benzodiazepines Oestrous Radioimmunoassay

HORMONAL changes that occur during the ovarian cycle in women are known to influence a variety of clinical disorders [see (30,31)]. Disorders of presumed CNS origin include the increased frequency of seizures seen in epileptic patients in the immediate premenstrual and menstrual phase [catamenial epilepsy; (2,19,24,28)]. The premenstrual phase can also be associated with a dysphoria that, if severe, is manifest as the so-called premenstrual syndrome, during which some women are reported to increase their consumption of minor tranquilising drugs, alcohol, or other central depressants (4,39).

A possible site of action for the above effects of ovarian steroids on seizure thresholds and emotionality is provided by the receptor sites for the sedative, anticonvulsant, and anxiolytic 1,4-benzodiazepine minor tranquilising drugs in the CNS. These receptors exist as a macromolecular complex in the neuronal membrane, a complex that also contains recognition sites for the inhibitory transmitter GABA and integral Cl^- ion channel characteristic of the vertebrate $GABA_A$ receptor [see (3,37,38)]. Certain other central depressant drugs, such as the barbiturates, also appear to act through this receptor complex [see (36)].

Some ovarian steroid hormones, in particular the sedative metabolites of progesterone, are known to have direct, barbiturate-like actions on the $GABA_A$/benzodiazepine receptor complex at the level of the neuronal membrane [see (32,43)]. Additional, indirect actions of ovarian hormones on this receptor also appear to occur as revealed by changes in the binding of $GABA_A$/benzodiazepine receptor ligands to membrane preparations or tissue sections from the brains of ovariectomised rats that have been treated with oestrogen and/or progesterone (10,29,41,42). However, since the above studies are based on measures of $GABA_A$ receptor function in vitro and the administration of steroids to ovariectomised animals it is difficult to interpret such results in terms of the interactions that may occur between the ovarian steroids or their metabolites and the $GABA_A$/benzodiazepine receptor complex in the intact animal. In the present study, an attempt was made to investigate the physiological influence of ovarian steroid hormones on $GABA_A$/benzodiazepine receptor function in vivo by employing a simple behavioural test (13) to monitor the fearfulness of mice and their sensitivity to a presumed anxiolytic action of diazepam at different stages of the oestrous cycle. A preliminary report of some of these findings has appeared elsewhere (7).

METHOD

Subjects

Adult C57BL/6J mice weighing 16–30 g (Joint Animal House, U.C.L.) were kept in groups of three or four in standard plastic cages (280 × 158 × 127 mm) with free access to food and water. Animals were housed in the same room used for behavioural observations and allowed to adapt to this room for at least 7 days before the start of an experiment.

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The room was maintained at 20–22°C on a 12 L/12 D cycle with lights on between 0600 and 1800 h or 0900 and 2100 h and providing a maximum background illumination of 280 lux. Cages containing male or female mice were alternated in the cage racks to encourage regular oestrous cycles. Females were not used for experiments until they had shown at least two regular oestrous cycles, each of 4–5 days duration. Stages of the oestrous cycle were monitored by taking daily vaginal smears (1) and corroborated on the day of the experiment by taking a final vaginal smear immediately after the behavioural test. In one experiment, the handling female mice experienced from the taking of vaginal smears was mimicked in males over a period of 14 days by daily removing them from their home cages for a period of up to 10 s and then returning them.

Apparatus

The light/dark exploratory apparatus was constructed from black perspex and consisted of two chambers connected by a tunnel (101 × 30 × 50 mm). The light chamber (295 × 210 × 210 mm) was open-topped and evenly illuminated by a fluorescent lamp (2 × 15 W) situated 335 mm above the floor of the box. The other chamber (148 × 210 × 210 mm) was darkened by a lid. Both chambers and tunnel had a clean sheet of matt black paper as a floor, which was replaced after each test. Photocells were mounted 3 mm above the floor of the box, immediately opposite a small infrared light source, and set to detect interruptions of the light beam. There were two such photocells in the light chamber, four in the tunnel, and one in the dark chamber and they were connected to a recording system monitoring both the overall locomotor activity of the mouse and the number of transitions between the two chambers. In addition, this system was also set to monitor the amount of time the animal spent in each chamber.

Test Procedure

Subjects were naive to the light/dark box apparatus and tested individually for a period of 15 min. Preliminary experiments had shown that this test duration produced the optimal difference in the number of light/dark tunnel transitions between mice treated with diazepam and those treated with the drug vehicle alone. Each test was initiated by placing the mouse in the dark chamber. For each experiment, the test parameters measured were as follows: the number of light/dark transitions, the time spent in the light and dark chambers, the latency to first emerge from the dark chamber, and the number of rearings made in the light chamber. Testing took place between 1300 and 1800 h.

Drug

Diazepam (a gift from Roche Products Ltd., Welwyn Garden City) was dissolved initially in a propylene glycol/ethanol mixture (2 : 1 v/v) that was then diluted to 4% (v/v) with phosphate buffered saline (PBS; 100 mM NaCl, 50 mM Na_2HPO_4/NaH_2PO_4; pH 7.4). This drug solution was injected at 10 ml/kg, intraperitoneally, 60 min before the test. The various treatments to be given (untreated, sham or saline injection, injection of drug vehicle alone, or injection of diazepam) were assigned at random to experimental animals to be used on each day.

Measurement of Whole Brain Concentrations of Diazepam

To measure the brain concentration of diazepam after intraperitoneal injection, mice were treated with the drug as described above and then killed 60 min later by cervical dislocation prior to removal of the whole brain for storage (3–11 wk) at −70°C. Brains were homogenised in 5 vol ice-cold 0.1 M HCl. Concentrated perchloric acid (72% w/w) was then added to a concentration of 0.5 M and the homogenates left on ice for 10 min to allow precipitation of proteins. Samples were centrifuged (28,000 *g*; 10 min; 4°C) and the clear supernatant decanted into a fresh tube before addition of 2.5 vol 1.0 M $KHCO_3$ to neutralise the extract and precipitate the perchlorate. After a further 20 min on ice, extracts were centrifuged again and the supernatants decanted for storage overnight at −20°C.

Concentrations of diazepam (or its active metabolites) in the brain extracts were measured by inhibition of [^{3}H]flunitrazepam binding to immunoglobulin G (IgG) class antibodies specific for the intact, pharmacologically active benzodiazepine nucleus (17). Dilutions of brain extracts were incubated overnight at 4°C, in triplicate, with antiserum at a dilution of 1 : 7500 in PBS containing thimerosal (0.01% w/v) and bovine serum albumin (0.1% w/v), in a final total volume of 0.5 ml. The [^{3}H]flunitrazepam (Amersham; 2.92 TBq/mmol) was then added to a concentration of 0.2 nM, followed by a further incubation for at least 2 h at room temperature. Benzodiazepine binding IgG's could then be adsorbed onto *Staphylococcus aureus* cells (50 μl of a well-washed suspension at 0.1% v/v in PBS) and isolated by filtration through glass-fibre paper under vacuum. The *S. aureus* cells had been prepared beforehand as a heat-killed, formalin-fixed suspension (27) and were a gift from M. Ginsburg, I.C.R.F. Laboratories, Clare Hall, UK. Radioactivity bound to the antibodies and trapped on the filters by the *S. aureus* cells was measured by scintillation counting. Tubes containing a range of known diazepam concentrations were included in each assay for the construction of standard curves. Known amounts of diazepam (60–120 pmol) were also added to brain homogenates to estimate the recovery of this drug through the extraction procedure. Results have not been corrected for this recovery, which was found to be 107 ± 14% (mean ± SEM; n = 8). Amounts of diazepam extracted from different brain samples have been expressed as pmol/g wet tissue weight.

Statistical Analysis

A Kruskal–Wallis one-way analysis of variance (K–W ANOVA) was used to test for any significant changes in the number of light/dark transitions made by untreated female mice during the oestrous cycle. Statistical significance of differences in test parameters between different groups was determined using the Mann–Whitney U-test (two-tailed). A K–W ANOVA was also used to test for any significant change in diazepam concentrations in female mouse brain during the oestrous cycle. Diazepam concentrations from male and female mouse brain were compared using the Student's t-test (two-tailed).

RESULTS

Effect of Diazepam on the Number of Light/Dark Transitions Made by Male Mice

In male mice, injection of diazepam (0.2–2.0 mg/kg, IP, 60 min) caused a dose-dependent increase in the number of transitions between the light and dark chambers of the exploratory apparatus (Fig. 1). Injection of the drug vehicle alone caused a significant ($p < 0.05$) decrease in the number of light/dark transitions in comparison to untreated mice. Simi-

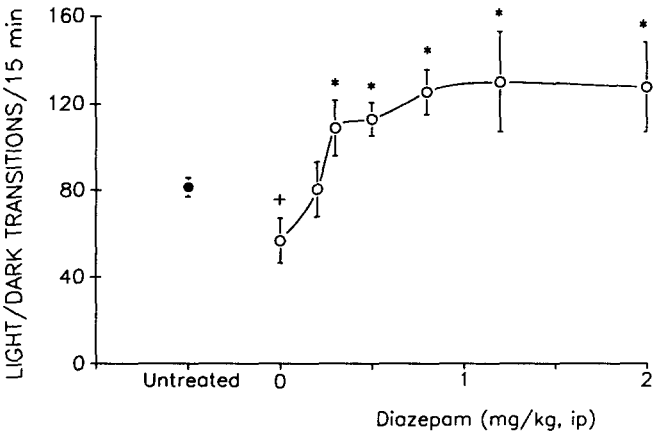

FIG. 1. Effect of diazepam (0.2–2.0 mg/kg, IP) on the number of transitions made by male mice between the light and dark chambers of an exploratory apparatus. Mice were injected with diazepam or the drug vehicle alone 60 min before the test, which lasted for 15 min and was initiated by placing the mouse in the dark chamber. Each mouse was tested only once and experiments included untreated mice for comparison. Points are mean ± SEM ($n \geq 6$). *$p < 0.05$ vs. vehicle alone, $^{+}p < 0.05$ vs. untreated; Mann–Whitney U-test.

lar decreases in light/dark transitions were also seen in male mice given a sham injection or an injection of PBS (Fig. 2a), but were not seen if the vehicle-treated animals had been handled daily for 14 days before the test (Fig. 2b), a degree of handling comparable to that experienced by female mice in the sampling of vaginal smears.

Behaviour of Untreated and Diazepam-Treated Female Mice at Different Stages of the Oestrous Cycle Tested in the Light/Dark Box

From the above observations in male mice, a dose of diazepam (0.28 mg/kg, IP) that caused an increase in light/dark transitions to about 50% of maximum was chosen to test the sensitivity of female mice to the drug at different stages of the oestrous cycle. The results of this study are shown in Fig. 3. During the cycle, no significant fluctuations were found in the number of light/dark transitions made by untreated female mice ($p > 0.05$). Injection of the drug vehicle alone induced a (nonsignificant) decrease in the number of transitions at late dioestrus and prooestrus but no apparent effect of injection with the drug vehicle alone was observed at any other stage of the cycle. Diazepam at 0.28 mg/kg increased the number of transitions when given to female mice at oestrus and dioestrus ($p < 0.05$) but had no significant effect at late dioestrus, prooestrus, or metoestrus II. At metoestrus I, this test dose of diazepam significantly ($p < 0.05$) decreased the number of light/dark transitions.

Further experiments were performed to investigate the responses of female mice to diazepam at metoestrus I. Figure 4 compares the effect of diazepam at 0.28 and 1.0 mg/kg on four test parameters in these mice. At metoestrus I, the lower dose of 0.28 mg/kg diazepam not only decreased the number of light/dark transitions but also caused a significant ($p < 0.02$) decrease in the time spent in the light chamber and in the number of rearings made in this chamber, while the latency to emerge from dark to light was significantly ($p < 0.05$) increased. None of these changes was seen upon administration of the higher dose of 1.0 mg/kg diazepam to mice at metoestrus I. Indeed, this higher dose of diazepam caused a significant ($p < 0.05$) increase in light/dark transitions (Fig. 4a) comparable to that seen upon administration of the lower dose of the drug to male or female mice at oestrus or dioestrus.

Measurements of Whole Brain Concentrations of Diazepam in Male and Female Mice at Different Stages of the Oestrous Cycle

The whole brain concentrations of diazepam (or its active metabolites) 60 min after the intraperitoneal injection of this drug (at 0.28 mg/kg) to male or female mice at different stages of the oestrous cycle are shown in Table 1. Concentrations of benzodiazepine were significantly lower in female than in male mouse brain ($p < 0.02$) but no significant fluctuations were seen during the oestrous cycle ($p > 0.05$).

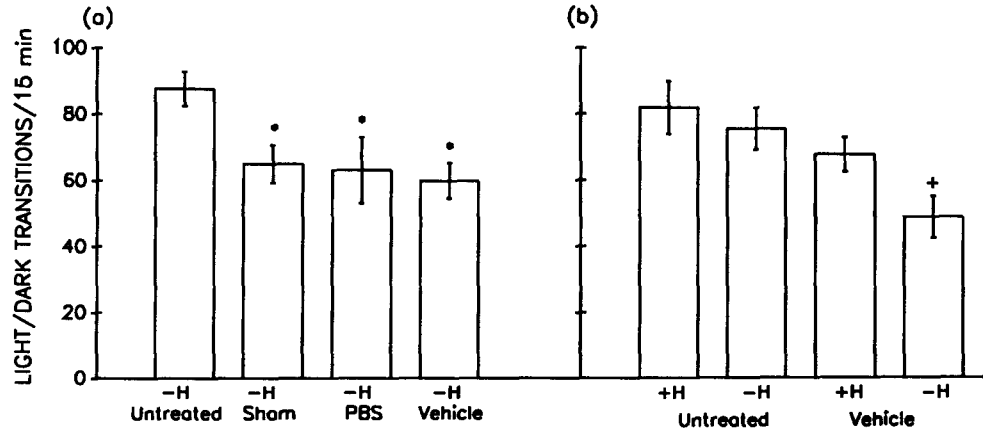

FIG. 2. Effect of a sham intraperitoneal injection, an injection of PBS, or an injection of the diazepam vehicle (see the Methods Section) on the number of light/dark transitions made in the exploratory apparatus by male mice that were a) naive (−H) and b) naive (−H) or handled (+H) daily for 14 days before the test. Values are mean ± SEM ($n \geq 9$). *$p < 0.05$ vs. untreated, $^{+}p < 0.05$ vs. handled and vehicle treated; Mann–Whitney U-test.

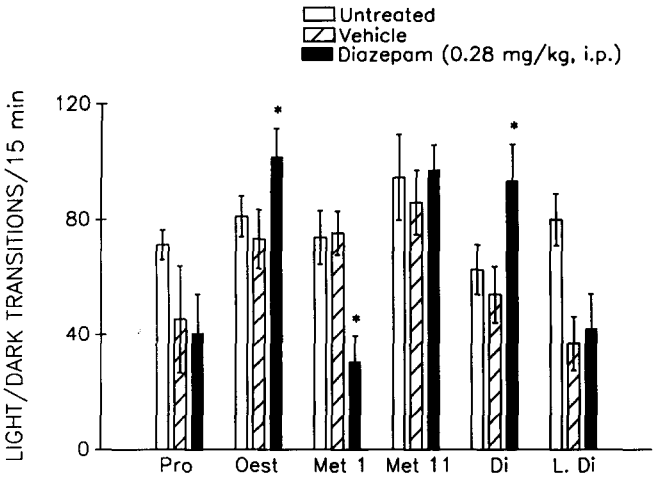

FIG. 3. Transitions between light and dark compartments of the exploratory apparatus made by female mice at different stages of the oestrous cycle: prooestrus (Pro), oestrus (Oest), metoestrus 1 (Met 1), metoestrus II (Met II), dioestrus (Di), and late dioestrus (L. Di). Mice were tested 60 min after injection of diazepam (0.28 mg/kg, IP), after injection of the drug vehicle alone, or without any prior treatment. The values shown are mean ± SEM ($n \geq 6$). *$p < 0.05$ vs. drug vehicle alone; Mann-Whitney U-test.

DISCUSSION

As described previously (12), intraperitoneal injection of diazepam caused a dose-dependent increase in the number of transitions made by male mice between the light and dark chambers of a novel environment. In our hands, injection of the drug vehicle alone to naive male mice caused a significant decrease in the number of light/dark transitions. Similar decreases were seen in mice injected with the same volume of PBS or given a sham injection, but no such effect of the intraperitoneal injection procedure was seen in male mice that had been handled daily for the preceding 14 days. We subjected male mice to this period of daily handling in an attempt to mimic the handling experienced by female mice in the sampling of vaginal smears. If injected with the drug vehicle alone, these female mice only made fewer light/dark transitions than untreated mice when tested at late dioestrus or prooestrus. Thus, our results suggest that daily handling of male or female mice renders them less susceptible to the acute stressful effects of an intraperitoneal injection but that at late dioestrus or prooestrus in female mice other mechanisms intervene that influence this adaptation to stress. One such mechanism might be the release of adrenocorticotropic hormone (ACTH). Both corticotropin-releasing factor (CRF) and ACTH have apparent anxiogenic effects in the rat (14,16) and the release of ACTH appears to be responsible for the decreased exploratory behaviour of mice following acute restraint stress (6,21). Release of ACTH is known to fluctuate during the oestrous cycle, with peak plasma concentrations at prooestrus (8). An increase in CRF and ACTH release from late dioestrus to prooestrus could have rendered the female mice at these stages in our experiments more susceptible to the acute stressful effects of the intraperitoneal injection procedure.

A dose of diazepam (0.28 mg/kg, IP) that caused approximately 50% of the maximum increase in light/dark transitions in male mice was used to test the effects of this drug in females at different stages of the oestrous cycle. While this dose of diazepam increased the number of light/dark transitions at oestrus and dioestrus, it decreased the number of transitions at metoestrus I and had no significant effect at late dioestrus, prooestrus, or metoestrus II. These changes in responses to diazepam during the oestrous cycle do not appear to be due to variations in the amount of the drug, or of its active metabolites, reaching the specific neuronal benzodiazepine receptors in the brain because no significant differences were found in the whole brain concentrations of benzodiazepine-like immunoreactivity extracted from the brains of mice injected with diazepam at different stages of the cycle. In addition to diazepam, our immunoassays would also detect the three major active metabolites of diazepam found in brain: *N*-desmethyl diazepam, oxazepam, and 3-hydroxydiazepam (17). Although concentrations of benzodiazepines extracted from the female mouse brain after intraperitoneal injection of diazepam did not fluctuate during the oestrous cycle, they were significantly lower than those recovered from male mice. In this respect, the mouse may be similar to man and have a faster metabolic clearance of diazepam in the female (20).

The diazepam-induced decrease in transitions at metoestrus I could not have been due to these animals being supersensitive to the drug and displaying the sedative actions normally seen at higher doses (12) because an increase of the dose given, from 0.28 to 1.0 mg/kg, caused an increased number of transitions. Rather, our results suggest that female mice tested after treatment with the lower dose of 0.28 mg/kg diazepam at metoestrus I display an increased fearfulness and/or photophobia as reflected by an increased latency to enter the light chamber and a reduction of rearing activity and time spent in the light chamber. Such a decrease in light/dark transitions, with a corresponding decrease in the time spent in the light chamber of a similar exploratory apparatus, has been reported following the administration of anxiogenic β-carboline derivatives to male mice (5,11). Other authors (15) reported that the sensitivity of female rats to an apparent anxiolytic action of diazepam is lower at metoestrus than at prooestrus. In contrast to the present findings, their study did not reveal an anxiogenic action of low doses of diazepam at metoestrus.

What physiological mechanisms could underlie the ob-

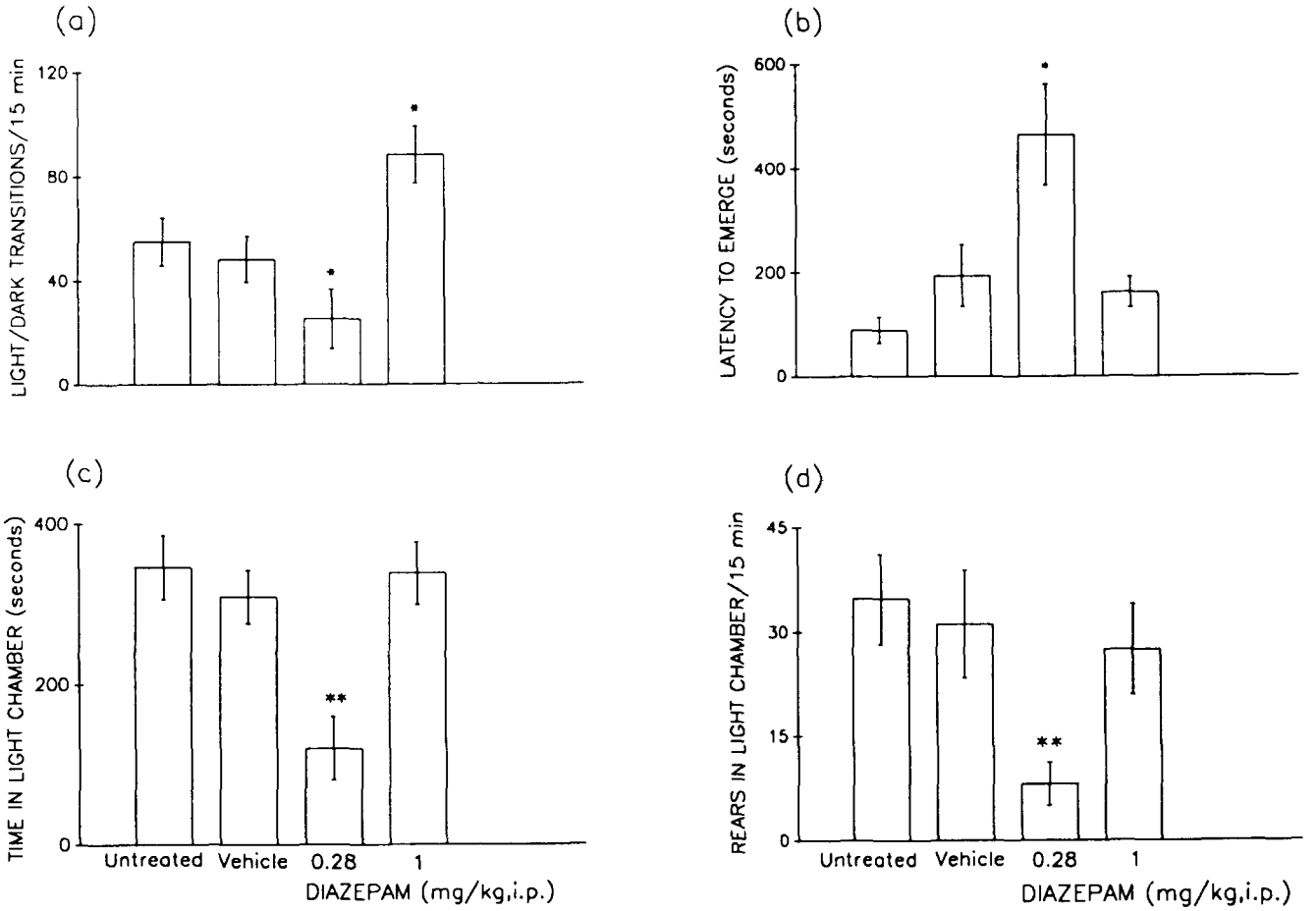

FIG. 4. Comparison of the effect of diazepam at 0.28 and 1.0 mg/kg IP on four parameters of the behaviour of female mice tested in the exploratory apparatus at metoestrus I: a) number of light/dark transitions, b) latency to emerge from dark chamber, c) time spent in light chamber, and d) number of rears made in light chamber. All values mean ± SEM ($n \geq 10$). *$p < 0.05$, **$p < 0.02$ vs. drug vehicle alone; Mann-Whitney U-test.

TABLE 1

WHOLE BRAIN CONCENTRATIONS OF DIAZEPAM AFTER IP INJECTION IN MALE MICE AND IN FEMALE MICE AT DIFFERENT STAGES OF THE OESTROUS CYCLE

Sex	Whole Brain Diazepam Equivalents (pmol/g wet wt)
Male	106.4 ± 9.9*
Female	
Prooestrus	82.2 ± 9.4
Oestrus	67.9 ± 10.5
Metoestrus I	88.3 ± 8.2
Metoestrus II	83.1 ± 8.3
Dioestrus	87.6 ± 10.9

Whole brain concentrations of diazepam and its pharmacologically active metabolites were determined 60 min after injection of the drug at 0.28 mg/kg IP into male and female mice at different stages of the oestrous cycle. All values mean ± SEM ($n \geq 8$).

*$p < 0.02$ vs. females; Student's t-test.

served changes in the apparent sensitivity of the CNS to diazepam during the oestrous cycle? Certain ring-A 5α-reduced progesterone metabolites are known to increase the affinity of specific benzodiazepine receptor binding in rat brain membranes and to enhance the actions of GABA in vitro (22, 23,33). A role for these metabolites of ovarian origin in producing the observations of the present study seems unlikely, however, because their secretion is highest from late prooestrus to metoestrus, falling to low levels by late metoestrus (25,26). In contrast, the highest sensitivities to diazepam were seen in the present study at oestrus and dioestrus and not in the intervening metoestrus stages.

Other possible actions of ovarian steroid hormones that could account for the behavioural fluctuations in sensitivity to diazepam during the oestrous cycle might be mediated by oestrogen or progesterone receptors regulating gene expression in the CNS. Pretreatment for 24 h with low doses of oestradiol has been reported to attenuate diazepam sensitivity in the ovariectomised rat (35). Although this action of oestradiol might explain the present observations of decreased diazepam sensitivity at late dioestrus and prooestrus, when plasma oestrogens are at their peak (9,34), it is unlikely to explain the decreased sensitivity to diazepam seen at metoestrus. A more

plausible explanation would be the modulation of CNS benzodiazepine sensitivity by progesterone receptors operating through the genome. We put forward this explanation because the two ovarian cycle stages, oestrus and dioestrus, at which the highest sensitivity to diazepam was observed are the only stages preceded by an increased production of progesterone (9,34). Progesterone actions at oestrogen-induced receptors in the brain are well known to induce the expression of genes facilitating and terminating sexual receptivity and oestrous behaviour in rodents [see (18)] and to reduce conditioned avoidance behaviour in the rat (40).

In conclusion, our results indicate a physiological influence of ovarian steroid hormones on the behavioural responses of mice to diazepam. The neurochemical alterations responsible for these apparent changes in diazepam sensitivity could underlie the changes in epileptic seizure frequency, mood, and consumption of central depressant drugs that sometimes occur during the ovarian cycle.

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