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Facilitation of Lordosis Behaviour is Differentially Mediated by 5-HT_2 Receptor Agents in Diazepam-Tolerant Female Rats

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Abstract.- Diazepam-tolerant but ovariectomized rats were primed with 50 µg oestradiol benzoate and 500 µg progesterone to induce a lordosis quotient of moderate intensity. Enhancing of lordosis was induced by treating with 5-$HT_{2A/2C}$ receptor agonist, DOI. The effect of the 5-HT_{2C} receptor antagonist, ketanserin and the 5-HT_{2A} receptor antagonist, ritanserin, on lordosis behaviour were investigated in ovariectomized but steroids-primed diazepam-tolerant rats. Both ketanserin and ritanserin inhibited lordosis behaviour. A potent protective effect of DOI was noted following antagonists ketanserin and ritanserin treatment. DOI attenuated the lordosis inhibiting effect of ritanserin-injected to diazepam-tolerant rats. These data show that diazepam-tolerance as such did not cause significant change in the lordosis profile. Behavioural profile as a consequence of ritanserin and DOI treatment further reinforce the prior inference that facilitation of female lordosis behaviour is differentially mediated by 5-HT_2 receptor agents and tolerance to anxiolytic does not alter the lordosis profile, significantly.

Keywords: Lordosis, lordotic quotient, serotonin, diazepam-tolerance, open field, elevated plus-maze test

INTRODUCTION

Lordosis is an essential reflex for reproduction in female rats and many other mammals. It is produced as a response to copulatory stimulation by the male (Pfaff and Modianos, 1985). The reflex is regulated via gonadal hormone-dependent modulation of serotoninergic (5-HT) system (Ahlenius *et al*., 1986; Mendelson, 1992). Serotoninergic modulation of female rate lordosis behaviour is believed to include both facilitatory and inhibitory components located in the ventromedial hypothalamus (Aiello-Zaldivar *et al*., 1992; Uphouse *et al*., 1992; Maswood *et al*., 1997).

Beside reproductive functions, the serotoninergic system in the CNS has been strongly implicated in the neural regulation of mood and anxiety state. Accordingly, many commonly used antidepressant and antianxiety medications target this system. Several lines of evidence suggest that after diazepam administration, a decrease in 5-HT levels in the hippocampus (Wright *et al*., 1992) or dorsal raphe nuclei (Soubrie *et al*., 1983; Thiebot *et al*., 1984) is associated with the observed anxiolytic profile and is probably mediated via 5-HT_{1A} receptors (Glennon and Dukat, 1995). Zimmerberg and Farley (1993) reported that the sex difference in the therapeutic effect of benzodiazepines and other non-benzodiazepine anxiolytics depend upon the endocrine basis of the ovarian cycle. In the 5-HT_{1A} receptors knock-out mice diazepam administration appeared insensitive to the anxiolytic effects of the drug (Sibille *et al*., 2000). These findings mechanistically links together the 5-HT and GABAergic system, both of which have been implicated in the complex process of anxiety.

Several studies indicate that benzodiazepine administration modulate the sexual behaviour of both male and female rats. For example, diazepam administration in rats was reported to enhance sexual performance in males and conversely decrease receptivity in females (McDonell *et al*., 1986, 1987). It has also been reported that drugs which induce an anxiolytic effect also raise plasma corticosterone level (Pellow and File, 1985; File *et al*., 1988). Moreover, experimental evidence has shown that estradiol and progesterone exert anxiolytic effects in paradigms that are typically used to assess the anxiolytic potency of drugs (Nomikas and Spiraki, 1988; Rodriguez-Sierra *et al*., 1986; Mora, 1996). The elevated plus-maze test used in the present study has been behaviourally,

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physiologically and pharmacologically validated as an animal model of anxiety in rats (Pellow and File, 1985; Reibaud and Bohme, 1993).

To-date there is no consensus regarding the effect on lordosis behaviour after tolerance development to benzodiazepine. Introduction of serotoninergic anxiolytic drugs in recent years has added a new dimension as the serotonin is regarded as a known mediator for its involvement in the regulation of gonadotrophin release and lordosis behaviour (James *et al*., 1989; Siddiqui *et al*., 2000). Evidence suggests that lordosis reflex involves activation of 5-HT $_{2A/2C}$ receptors agonist which is reported to facilitate the reflex in non-sexually receptive females (Wolf *et al*., 1998) whereas in sexually receptive rats 5-$HT_{2A/2C}$ receptor antagonists inhibit lordosis behaviour (Uphouse *et al*., 1992). The present study was designed to investigate into the effects of serotoninergic agents on lordosis behaviour in diazepam-tolerant rats. Intact cycling female rats or ovariectomized rats were used to develop tolerance to diazepam and either primed with estrogen and progesterone or not primed. Diazepam-tolerant rats were then treated with ketanserin, a selective 5-HT_{2C} antagonist and ritanserin, a selective 5-HT_{2A} antagonist followed by DOI, selective 5-$HT_{2A/2C}$ agonist to assess lordosis facilitating effects of DOI in moderately receptive female rats.

MATERIALS AND METHODS

The 5-$HT_{2A/2c}$ receptor agonist, 1-(2,5-dimethroxy-4-iodophenyl) -2- aminopropane hydrochloride (DOI) and a selective 5-HT_{2c} antagonist, 3-[2-[4-4-flourobenzoyl)-1- piperdinyl] ethyl] -2, 4(1H,3 H)-quinazolin edionetartarate (Ketanserin tartrate) and ritanserin, a selective 5-HT_{2A} antagonist were purchased from Research Biochemicals Inc., Natick, MA, USA. Oestradiol benzoate (OB) and progesterone (P) were purchased from Sigma, Dorset, U.K.

Animals, housing conditions and surgical procedures

Female Wistar rats (n = 72) from litters born and raised in the animal housing facility at Aga Khan University were weaned at 25 days of age. At 75-80 days of age, half of the females were anaesthetized with diethyl ether and bilaterally ovariectomized via bilateral lumbar incision. Immediately post-surgery, these females were housed in groups of five in shoe-box cages in a room maintained at 24^{o}C under a reversed 12 hour light/dark cycle (lights off at 0600 hrs). Rat chow and water were available ad lib.

Steroids-priming and lordosis testing procedures

Following recovery, groups of rats were primed either with 50µg OB subcutaneously (s.c.) 50 h before the experiment 48 h following OB treatment animals were injected with 0.5 mg P, s.c. so as to potentiate the sensitizing effect of OB. Administration of OB alone exerts a negative feedback effect on LH release and induces relatively low levels of plasma LH. 48 h after the OB and 2 h before the experiment, this treatment enchances sexual receptivity associated with the release of an LH surge with a peak concentration about 2-3.5 h after the P.

Sexual behaviour was recorded during the dark portion of the light dark cycle and was initiated within 3-5 h after lights were off. Then, 2 to 3 h after P administration, the rats were pretested for sexual receptivity with a sexually active male.

Lordosis testing involved presentation of a test female to a sexually active male rat in a cylindrical pyrex arena measuring 50cm in height and 30cm in diameter. Males were given brief access to fully receptive females (each given 50 µg OB 48 hours followed by 0.5mg P, s.c., 2-3 h before exposure) immediately prior to sessions with test females. Sessions were conducted 4-5 h after commencement of the dark cycle. The female was allowed to adjust to the chamber for 5-10 min before the male was placed with the female. For experiments with moderately receptive females *i.e.* females with lordotic quotient (LQ) ranging from 35% - 55%, pretesting took place in the male's home cage. The test female was then injected with 5HT agents. 20 min after the injection and was again placed into the male's home cage. If a male would not mount, the female was placed in a different arena containing another male. A female's response to a mount was considered a lordosis response if some degree of concavity of the back

was observed. A test was completed either at completion of 10 mounts or 5 min. with sexually active male rat, which ever occurred first.

Experimental protocol

A group of 36 ovariectomized female rats and equal number of intact female rats were used to analyze whether the long term exposure to diazepam produces any alteration in the sensitivity of 5-HT_{1A} receptor or not. Rats from the two groups were randomly assigned to two subgroups (n = 18 each) that received either diazepam (5mg/kg) or saline (1ml/kg) intra-peritoneally (i.p.) twice daily with an interval of 10-12 hours till development of tolerance to diazepam. The animals were subjected to open-field and elevated plus-maze tests subsequently to measure the anxiety level after 15 and 30 min of diazepam treatments, respectively. Each animal was tested at 5-7 days intervals till the development of tolerance or at the maximum 22 days of treatment. Following the treatment period, whether tolerance to diazepam developed or not, diazepam-treatment was continued and experiments with 5-HT agents and ovariectomized rats were commenced as described below:

A) a group of ovariectomized and diazepam-tolerant rats (n=6) were primed with OB+P was injected with 5-$HT_{2A/2C}$ agonist, DOI (0.25 mg/kg; i.p.) and tested for percentage LQ against sexually active male rats.

B) a group of ovariectomized and diazepam-tolerant rats (n = 5) injected with DOI (0.25 mg/kg; i.p.) without been primed with OB+P and tested for percentage LQ against sexually active male rat.

C) a group of ovariectomized and diazepam-tolerant rats (n = 6) was administered with corn oil, s.c., as vehicle followed by an injection of 0.9% saline i.p. and tested for percentage LQ against a sexually active male rat.

D) three groups of ovariectomized and diazepam-tolerant rats (n = 6 each) were administered with OB+P and injected with $5HT_{2C}$ antagonist, ketanserin (3 mg/kg), 5-HT_{2A} antagonist, ritanserin (0.25 mg/kg) or 0.9% saline and tested to evaluate change in LQ against sexually active male rats.

After a gap of two weeks the same three groups of ovariectomized and diazepam-tolerant rats administered with OB+P were injected with $5HT_{2C}$ antagonist, ketanserin, (3 mg/kg) 5-HT_{2A} antagonist, ritanserin (0.25 mg/kg) or 0.9% saline i.p. injected with DOI (0.25 mg/kg) and within 20 min tested to evaluate percentage LQ against sexually active male rats.

To minimize the desensitizing effects of the 5-HT agents used, all treatments and observations were carried out twice with a 2 weeks gap between the two observations.

RESULTS

Intact and ovariectomized female rats were used to develop tolerance to diazepam and examine the effect of 5-HT_{1A} receptor sensitivity in the brain and the effects on lordosis behaviour in response to mounting attempts by sexually active male rats. Amongst the group of intact female rats (n=5) that remained unprimed, another group of females (n=6) that was ovariectomized and was administered corn oil, as vehicle only and finally another group that was ovariectomized but primed with OB+P (n=6) were treated with diazepam (5mg/kg), twice a day till the development of tolerance to diazepam. On an average, a 15-22 days treatment with diazepam produced tolerance towards the sedative and anxiolytic actions of diazepam as determined by using open field tests and elevated plus-maze tests.

Exploratory activity during the open field test

The results of the open field test in intact ovariectomized and ovariectomized but OB+P-primed female rats are shown in Figure 1 as observations on day 1, 5, 10, 15 and 22 of diazepam treatment. Exploratory activity was monitored as number of squares crossed per 5 min.

Exploratory activity in an open field arena in ovariectomized but unprimed female rats show that diazepam treatment caused initial decline but showed a rise by day 10 of the diazepam treatment and then it declined through to day 22 and the number of squares crossings was comparable to the control group (Fig. 1). In the group that was ovariectomized but primed with standardized doses of OB + P showed a clear evidence for

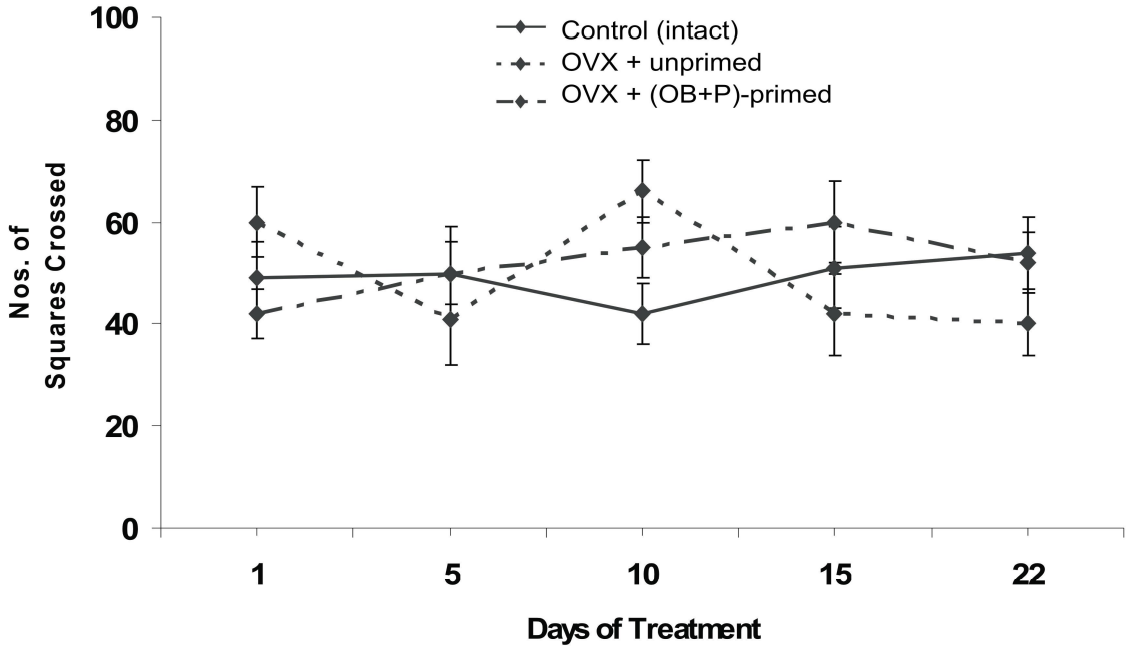

Fig. 1. Time course of development of tolerance to the anxiolytic effect of diazepam in intact, ovariectomized (OVX) but unprimed and OVX but OB+P primed female rats demonstrated in open field test. The data represent mean±S.D. in control and treated groups (n = 18).

development of tolerance as the numbers of squares crossed during open field activity test on day 22 were almost similar (Fig. 1). This observation in contrast to intact females and ovariectomized but unprimed (with steroids) females where the exploratory activity kept fluctuating during the whole observation period.

Exploratory activity during elevated plus-maze test

The results of the plus-maze activity tested in intact female rats for 5-HT_{1A} receptor sensitivity also did not show much change as a result of diazepam treatment. Exploratory activity in ovariectomized but unprimed rats monitored as the time spent in open arms for 5 min. remained more or less steady uptil day 15 of treatment (Fig. 2). In contrast, the group that was ovariectomized but primed with OB + P, where there was an initial increase in the time spent in the open arm that later showed a decline by day 22 and was comparable to control group thus indicating a development of tolerance to diazepam (Fig. 2).

Effect on lordosis behaviour

Two groups of diazepam-tolerant and control female rats primed with 50 µg OB plus 0.5 mg P exhibiting a moderate range of LQ of 35%-55% were injected with $5HT_{2A/2C}$ agonist, DOI and tested against sexually active male rats for effects on sexual receptivity. The mean results show that treatment with DOI enhanced the lordotic response and mean LQ was risen to 78% on the first test whereas in the subsequent test it was reduced to 66% (Fig. 3). In other experiment, treatment to ovariectomized and OB + P-primed but diazepam-tolerant rats with $5HT_{2C}$ antagonist, ketanserin and 5-HT_{2A} antagonist, ritanserin caused a significant decrease in the LQ as compared to the control group ($p < 0.05$). The decline in LQ caused by ritanserin was more intense ($p<0.01$) compared to ketanserin and persisted in the subsequent test as well (Fig. 4).

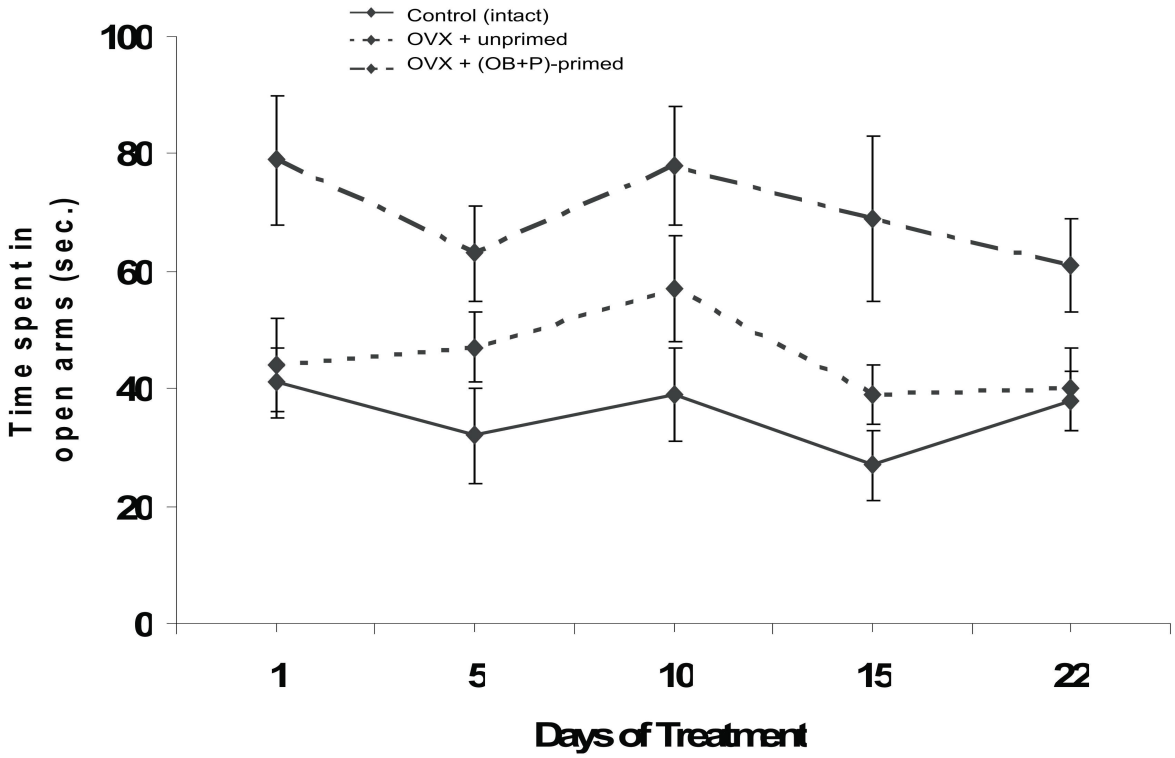

Fig. 2. Time course of development of tolerance in intact, OVX but unprimed and OVX but OB+P primed female rats to the anxiolytic effect of diazepam demonstrated in elevated plus-maze test. The data represent Mean±S.D. in control and treated group (n=18).

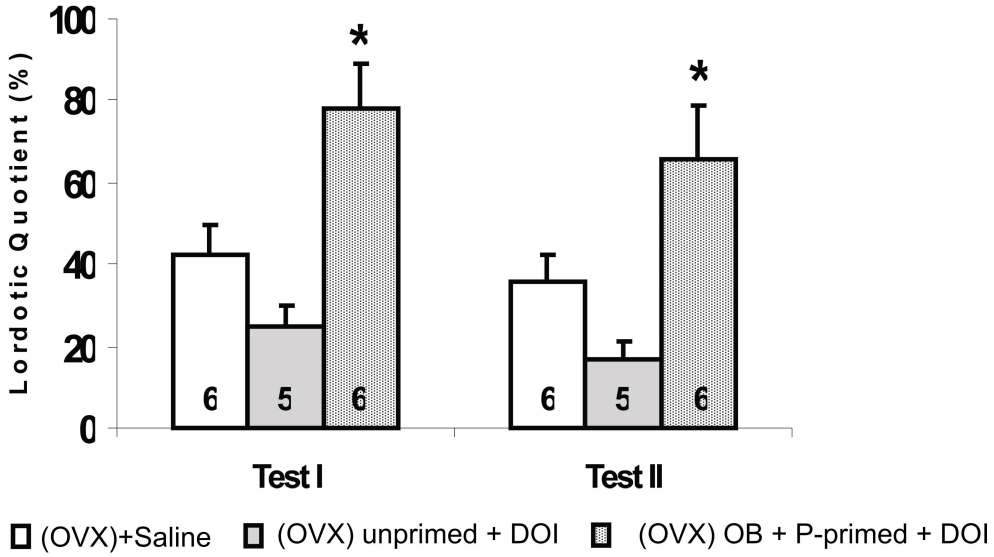

Fig. 3. Effect of DOI 5 $HT_{2A/2C}$ agonist on lordotic quotient measured in OVX controls; OVX but unprimed and OVX (OB+P)-primed diazepam-tolerant female rats. Measurement was made for two tests with a gap of two weeks. * $p<0.01$ vs control (OVX+saline) and unprimed (OVX only).

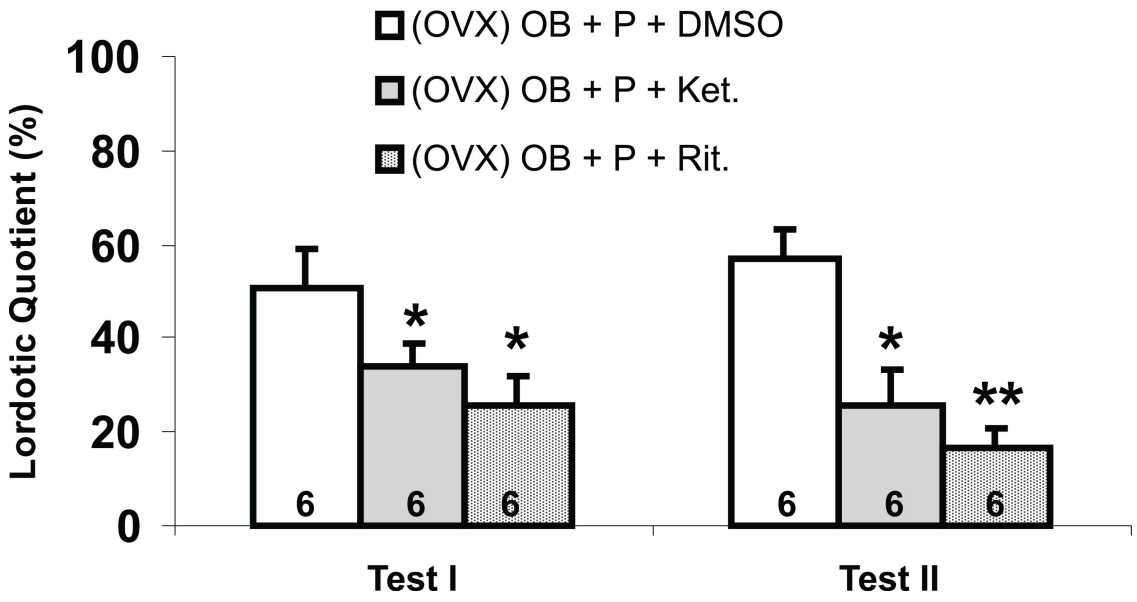

Fig. 4. Effect of ketanserin (5-HT_{2C} antagonist) and ritanserin (5-HT_{2A} angatonist) treatment to OVX, diazepam-tolerant but OB+P-primed rats on lordosis behaviour. * $p<0.05$; ** $p<0.01$ vs OVX controls.

In order to investigate whether $5HT_{2A/2C}$, agonist facilitates lordosis in those ovariectomized but steroids primed diazepam-tolerant rats that are already under inhibitory influence of $5HT_{2C}$ antagonist, ketanserin or $5HT_{2A}$ antagonist, ritanserin it was noted that inhibitory influence was released significantly in both ketanserin and ritanserin treated female rats (Fig. 5). This protective effect, however, was more significant in ritanserin, $5HT_{2A}$ antagonist treated rats ($p<0.05$) as compared to $5HT_{2C}$ antagonist, ketanserin treated female rats. The LQ values as a result of treatment by ritanserin was close to the LQ in control group.

DISCUSSION

A considerable volume of both behavioural and biochemical data suggested involvement of serotonin in the physiology of anxiety and moreover, that the sexual behavioural effects of benzodiazepines might be due to actions on this neurotransmitter system. The use of benzodiazepines has been limited by concern regarding dependence, withdrawal and abuse (Rickels *et al*., 1999). In the CNS, benzodiazepines like diazepam and lorazepam are known to exert their effects via GABA receptor / benzodiazepine receptor / chloride ionophore complex (Schoch *et al*., 1985).

Anxiolytic (fear-reducing) drugs are expected to increase the number of square crossings in the open field activity test and number of entries and time spent in the open arms seems to reveal exploratory activity in rats during elevated plus-maze test (Pellow *et al*., 1985) although these parameters cannot be considered independent of the anxiety state. It has been shown that diazepam anxiolytic-like effects vary depending upon oestrous cycle phase (Fernandez-Guasti and Picazo, 1990). Female rats that were intact, ovariectomized but steroids unprimed and ovariectomized and OB + P-primed, were used as to find out whether gonadal steroids modulate development of tolerance to diazepam or not. In contrast to intact, ovariectomized but not primed with steroids (as controls) steady increase in the number of squares crossings during open field test (Fig. 1) and in the time spent during elevated plus maze test (Fig. 2) was observed in ovariectomized and OB+P-primed rats is in agreement with previous findings showing that the gender, oestrous cycle and ovarian hormones modify exploration of the elevated plus-

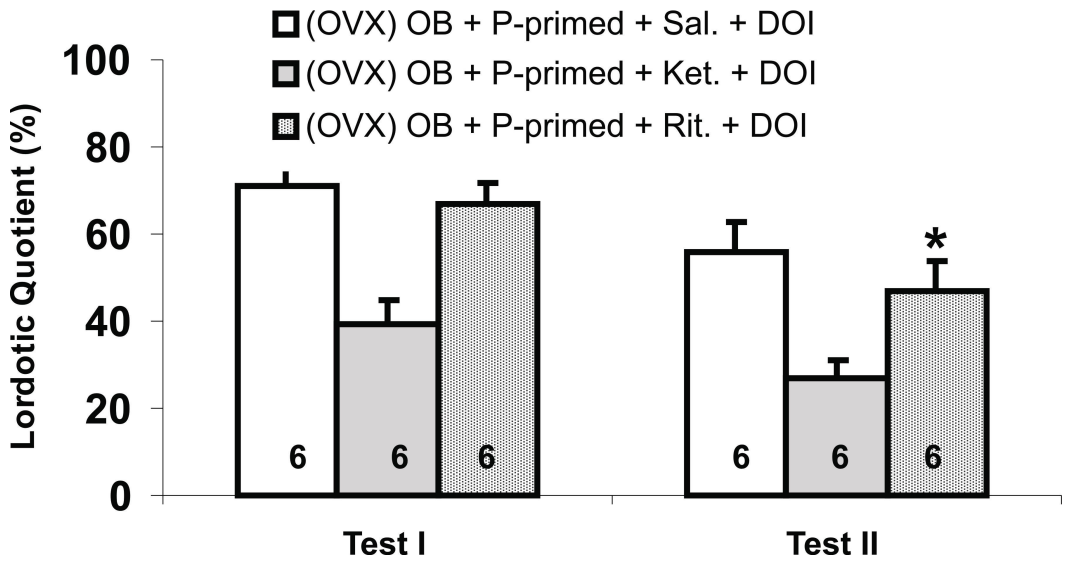

Fig. 5. Effect of ketanserin (5-HT $_{2C}$ antagonist) and ritanserin (5HT$_{2A}$ antagonist) treatment to OVX, diazepam-tolerant but OB+P-primed rats on lordosis behaviour. * $p<0.01$ vs OVX (OB+P) + ket. + DOI treated rats.

maze (Mora, 1996). A variable dose of OB has always been used to induce lordosis in various studies (McCarthy and Becker, 2002). Use of 50 µg OB in our study was considered as a relative high dose which may be attributed to over sensitivity to steroids in rats used in our laboratory. Furthermore, P as a component of the steroids priming regimen, in addition to potentiating the lordosis-inducing effect of OB, also elicit anxiolytic properties (Bitran *et al*., 1991; Weiland *et al*., 1991). The anti-anxiety action has been proposed to occur through its ability to stimulate the GABA-benzodiazepine receptor complex (Picazo and Fernandez-Guasti, 1995). In the context of LH release P-dependent increase in GABA activity in the OB-primed rats has also been reported to contribute to termination of LH surge (Grattan *et al*., 1993). On these basis it may be proposed that benzodiazepines' actions may be influenced by changes in steroid hormones.

An interesting observation of the present investigation is that in the elevated plus maze, ovariectomized but OB+P primed rats showed a decreased anxiety-like behaviour (spending more time in the open arms / entering more frequently into these arms), in comparison with the ovariectomized control group *i.e.* not primed with steroids during the observations made in the initial days of experimentation. Fluctuations in ovarian hormones over the oestrous cycle or following ovariectomy, with or without estrogen replacement are associated with major perturbations in the lordosis. Previously it has been reported that this behavioural activity is facilitated at diestrous day of oestrous cycle but it is diminished at oestrous and metestrous days (Nomikos and Spiraki, 1988; Cabrera *et al*., 1993; Mora, 1996). We reasoned that females closer to the threshold of intense display of the behaviour might be more vulnerable to events that were disruptive to the behaviour. To test this hypothesis, ovariectomized rats were treated with a dose of OB (50 µg) that, when followed by 500 µg P, elicited lordosis behaviour in response to male's mounting behaviour by postural adjustments which culminate in the lordosis reflex (Pfaff and Modianos, 1985). However, only in a subset of the rats in response to this dose regimen while a considerable number remained moderately receptive with an LQ range of 35%-55%. With this priming sequence used in Wistar rats in this study, approximately 60% of the rats show an LQ of 35%-55%.

The high level of anxiogenic-like behaviour observed generally by all female rats in present study could be attributed to diestrous day of the

ovarian cycle (though ovarian cyclicity was not monitored). However, following a treatment day of 15 to 22, anxiolytic effect of diazepam was discernable, possibly because of desensitization of 5HT receptors involved. Both 5-HT and GABA are well documented to influence lordosis (McCarthy *et al*., 1991; Uphouse *et al*., 1992; Farmer *et al*., 1996; Gonzalez *et al*., 1997). Since 5-HT would be expected to activate multiple receptors families, the behavioural effect of the neurotransmitter probably reflects 5-HT's activation of multiple 5-HT's activations of multiple 5-HT receptors. Activation of 5-HT_{1A} within VMN is established to inhibit lordosis (Uphouse *et al*., 1992). The inhibitory effect of 5-HT_{1A} receptor agonists on lordosis behaviour has been thoroughly described and the ventromedial nuclueus (VMN) is at least one brain area where such inhibition is mediated (McCarthy and Becker, 2002). The 5-HT_{1A} receptors belong to the super family of G-protein-coupled receptors and activation of 5-HT_{1A} receptors is generally associated with inhibition of adenyl cyclase or opening of a K^+ channel (Andrade *et al.,* 1986; Newberry, 1992). However, 5-HT_{1A} receptors have been suggested to couple to multiple second messenger systems (Zifa and Fillion, 1992), so it remains unclear exactly what effects of 5-HT_{1A} receptors is responsible for the decline in female rat lordosis behaviour. The possible importance of 5-HT_{1A} receptors in anxiety was raised by evidence that the clinically effective anxiolytic, buspirone, had 5-HT_{1A} receptor agonist/partial agonist properties (Fernandez-Guasti and Picazo, 1990). 5-HT_{1A} receptors occur both pre-synaptically in their projection areas.

The present studies were also designed a) to test the hypothesis that whether like in the intact female rats, in ovariectomized steroids-primed diazepam-tolerant rats activation of 5-$HT_{2A/2C}$ receptors can facilitate lordosis and b) to determine whether 5-$HT_{2A/2C}$ receptors are sole candidate in mediating the lordosis.

Serotoninergic modulation of female rats lordosis behaviour is believed to include both facilitatory (via 5-$HT_{2A/2C}$ receptors) (Wilson and Hunter, 1985; Mendelson and Gorzalka, 1986; Mendelson, 1992; Maswood *et al*., 1997) and inhibitory (via 5-HT_{1A} receptors) (Aiello-Zaldivar *et al*., 1992; Mendelson, 1992; Mendelson and Gorzalka, 1986).

In the moderately receptive rats used in this study DOI also facilitated the lordosis behaviour in response to male's mounts. In agreement with previous studies activation of 5-$HT_{2A/2C}$ receptors may produce facilitation of lordosis behaviour as a consequence of their ability to depolarize neurones in the VMN involving decrease in K^+ conductance (Sheldon and Aghajanian, 1991; Zifa and Fillion, 1992). In fact, Wolf *et al*. (1998) reported that the 5-$HT_{2A/2C}$ receptors agonist, DOI requires 15-20 min to facilitate lordosis behaviour which is consistent with the slow depolarizing action of 5-$HT_{2A/2C}$ receptors. Lordosis behaviour was also examined following i. p. administration of 5-HT_{2C} receptors antagonist, ketanserin and 5-HT_{2A} receptors antagonist, ritanserin in separate groups. More significant inhibition as seen induced by ritanserin rather than ketanserin, thus clearly depicting a stronger repolarising effect mediated by 5-HT_{2A} receptors.

Ketanserin was noted not very effective in preventing DOI from increasing lordosis behaviour of moderately receptive rats. Yet, the antagonist effectively attenuated DOI-facilitated lordosis behaviour, once it had occurred. Like other G-protein coupled receptors, 5-$HT_{2A/2C}$ receptors may exist in both active and inactive states (Westphal and Sanders-Bush, 1994) so that the effect of a ligand, at a given point in time, depends on the ligand's relative preference for the two receptor states. If a larger portion of the receptors are in an inactive state, kitanserin might be expected to be more effective while the agonist action of DOI would be accentuated when more receptors are in the active state. It should be noted that ketanserin is not specific of 5-HT receptors (Hoyer *et al*., 1987) so we cannot eliminate the possibility that ketanserin-induced inhibition involves mechanisms other than 5-$HT_{2A/2C}$ receptors.

Although the present studies have raised several interesting issues regarding the mechanism responsible for the effects 5-$HT_{2A/2C}$ receptor compounds on lordosis behaviour, they are significant in providing direct evidence that 5-$HT_{2A/2C}$ receptors in the VMN contribute to the regulation of lordosis behaviour. Whether 5-HT_{2A} or

5-HT_{2C} receptors, or both, are responsible for modulation of the behaviour is not known. DOI has roughly comparable affinity for both receptor subtypes. Ketanserin is 10-100 fold more potent at 5-HT_{2A} than at 5-HT_{2C} receptors (Hoyer and Schoeffer, 1991; Pierce *et al*., 1992).

Collectively, the present findings support the earlier suggestion that ovarian hormones modulate anxiety level. Tolerance to anxiolytic action of diazepam as such did not cause a significant deviation in the lordosis profile. Behavioural profile as a result of ritanserin and DOI treatment to diazepam-tolerant rats reinforce prior inference that activation of 5-$HT_{2A/2C}$ receptors can facilitate lordosis behaviour. However, lordotic response as result of ritanserin and DOI treatment indicate differential involvement of some other 5-HT receptors subtypes as on LH release, 5-HT_{2A} receptor antagonist, ritanserin antagonize the inhibitory effect of both 5-HT and 8-OH DPAT (Siddiqui *et al*, 2000). Further studies with more potent compounds are required to delineate the specific role of specific 5-HT receptor subtypes involved in modulation of lordosis behaviour.

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