Agmatine Inhibit The Tolerance To The Anxiolytic Effect Of Diazepam In Rats

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Abstract

Long-term treatment leads to tolerance to and dependence on benzodiazepines. Benzodiazepines dependence and tolerance are produced by the glutamate antagonist. The present study investigating the role of agmatine on tolerance to the anxiolytic effect of diazepam in rats using social interaction test. Rats tested after an acute dose of diazepam (2 mg/kg, i.p.) showed an anxiolytic effect, measured by an increase in the time spent in social interaction, whereas repeated administration of diazepam (2 mg/kg, i.p.) for 21 days produced tolerance to anxiolytic effect that was inhibited by the pretreatment with agmatine (80 mg/kg, i.p.) and its modulators known to increase endogenous agmatine levels in brain viz., L-arginine, an agmatine biosynthetic precursor (100 µg/rat, i.c.v.), ornithine decarboxylase inhibitor, DFMO (125 µg/rat, i.c.v.), diamine oxidase inhibitor, aminoaguanidine (65 µg/rat, i.c.v.) and agmatinase inhibitor, arcaine (50 µg/rat, i.c.v.).

1. Introduction

Tolerance is decreased effect of a drug with repeated administration. Dependence is also a state produced by repeated administration of a drug, but is only expressed after administration is terminated. Tolerance and dependence are thought to result from neuronal adaptations produced by repeated drug exposure (Trujillo and Akil, 1991). Increasing evidence suggests that N-methyl-D-aspartate (NMDA) receptors may have an important role in mediating the neural and behavioural plasticity changes produced by administration of a variety of psychoactive drugs. (Plaznik et al., 1996). The involvement of NMDA receptors in the development of tolerance to ethanol has been suggested by the results of different studies (Khanna et al., 1995). Trujillo (1991) reported that NMDA receptors play an important in the development of opiate tolerance and dependence. In particular, the non-competitive NMDA receptor antagonist, dizocilpine (MK-801), has been shown to block the development of tolerance to a range of drugs and behavioural responses. For example, it blocks the development of tolerance to the effects of cocaine on locomotor activity (De Montis et al., 1992), to the analgesic effects of morphine (Trujillo and Akil, 1991), to the aversive stimulus effects of nicotine (Shoaib, and Stolerman, 1996) and to the motor impairing effects of ethanol (File and Fernandes, 1999; Wu et al, 1993). Agmatine acts as an endogenous neurotransmitter and/or neuromodulator. It is bioactive, releasing several neurotransmitters and hormones including catecholamines from adrenomedullary chromaffin cells, luteinizing hormones from the pituitary and insulin from pancreatic islet cells (Reis and Reganathan, 2000). Recently, it was reported that exogenous agmatine has a neuroprotective effect (Feng et al., 2002); it also enhances opioid analgesia (Su et al., 2003) and improves neuropathic pain (Fairbanks et al., 2000). Pharmacological studies show that agmatine activates imidazoline receptors and α2 adrenoceptor (Li et al., 1994), and inhibits nitric oxide synthase (NOS) (Feng et al., 2002) and N-methyl-D-aspartate (NMDA) receptor channels (Yang and Reis, 1999; Yamakura et al., 2005).

Agmatine has a weak analgesic effect in tail flick test and enhances morphine-induced antinociception (Kolesnikov et al., 1996; Yesilyurt and Uzbay, 2001). Furthermore, it reduces tolerance to morphine (Fairbanks and Wilcox, 1997; Li et al., 2002) and attenuates behavioral signs of morphine abstinence syndrome in vitro and in vivo (Arcioglu-Kartal and Uzbay, 1997; Li et al., 1998; Arcioglu et al., 2003a, b). The beneficial effects of agmatine in potentiating...
morphine analgesia and at the same time reducing the withdrawal symptoms have been clearly documented (Arıcıoğlu-Kartal and Uzbay, 1997; Li et al., 2002; Li et al., 1998). In previous studies, agmatine was injected at the time of inducing withdrawal with naloxone and it has been proposed that agmatine acts by inhibiting N-methyl D-aspartate receptors to reduce withdrawal symptoms (Arıcıoğlu-Kartal and Uzbay, 1997; Li et al., 2002; Regunathan et al., 2004).

It is reported that diazepam and nicotine increase social interaction in gerbils and shown the anxiolytic action (File et al., 2001). Plaznik (1996) reported the effect of repeated administration of diazepam, MK-801, CGP 37849 on rat behavior in two models of anxiety. Dizocilpine prevent the development of tolerance to the sedative effects but does not prevent to the anxiolytic effect of diazepam in rats (File and Fernandies, 1999). Diazepam dependence prevented by glutamate antagonists (Steppuhn, 1993). Thus it can be intrigued whether agmatine and its interaction with NMDA receptor system endogenously play an active role to reverse the tolerance to the anxiolytic effect of diazepam by using social interaction test.

2. Materials and Methods

2.1. Animals

Adult healthy Sprague Dawley rats (230–250 g) were housed four per cage (640 × 410 × 250 mm height) or individually after intracerebroventricular (i.c.v.) cannulation, under controlled conditions (25 ± 2 °C and 12 h light/dark cycle, light on at 07:00 am) with free access to food and water. All experimental procedures were approved by the Institutional Animal Ethical Committee and executed in strict accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Govt. of India.

2.2. Drugs

Diazepam (Roche Products, Welwyn Garden City, Hertfordshire, UK.) was suspended with a drop of Tween 20 in 20 ml of distilled water and left in an ultrasonic water bath for 20 min before injection. Agmatine sulfate, DL-α-difluoromethyl ornithine hydrochloride (DFMO), aminoguanidine hemisulfate, aracaine sulfate, L-arginine mono-hydrochloride, D-arginine, D-cycloserine (Sigma-Aldrich Co., USA.), (+) MK-801 (RBI, Natick, U.S.A.), neramexane hydrochloride (MRZ 2/579; 1-aminoo-1, 3, 3, 5, 5-pentamethyl-cyclohexan hydrochloride (Merz Pharmaceuticals GmbH, Frankfurt am Main, Germany). Drugs like agmatine and D-cycloserine were dissolved in saline (0.9%), MK-801 and neramexane dissolved in distilled water and administered by intraperitoneal (i.p.) route. Drugs like L-arginine, DFMO, aracaine and aminoguanidine, D-arginine were injected by intracerebroventricular (i.c.v., 5 µl/rat) route to alter the levels of brain agmatine and avoid peripheral effects. For intracerebroventricular (i.c.v.) administration of drugs, dilutions were made with artificial cerebrospinal fluid (aCSF) of following composition 0.2 M NaCl, 0.02 M NaH2CO3, 2 mM KCl, 0.5 mM KH2PO4, 1.2 mM CaCl2, 1.8 mM MgCl2, 0.5 mM Na2SO4, and 5.8 mM D-glucose. Doses and timing of the drug injections with respect to the behavioral testing employed in the protocols were selected on the basis of previous experiments in our lab and available literature (B.G. Taksande et al., 2009, File and Fernandies, 1999, Sukhotina et al., 2004).

2.3. Surgery

Rats were anesthetized with thioptenone sodium (60 mg/kg, i.p.) (Abbott Pharmaceuticals Ltd., Mumbai) and a 22-gauge stainless steel guide cannula (C3113G/Spc, plastic UK) was stereotaxically (David Kopf Instruments, CA, USA) implanted (Kokare et al., 2006) into the right lateral ventricle. The surgical coordinates−0.8 mm, posterior, +1.2 mm lateral to midsline and −3.5 mm ventral to bregma were used for i.c.v. cannulation (Paxinos andWatson, 1998). The guide cannula were then fixed to the skull with dental cement (DPI-RR cold cure, acrylic powder, Dental Product of India, Mumbai) and secured in two stainless steel screws. A 28-gauge stainless steel dummy cannula was used to occlude the guide cannula when not in use. Following surgery, the rats were placed individually in cage and allowed to recover at least for 7 days before being tested in elevated plus maze (EPM). Rats were then randomly assigned to different groups (n=8) and habituated to the testing environment by transferring to experimental room and twice daily handling for 1 week. Drugs were injected (5 µl/rat) into the right ventricle over a one min period with a microliter syringe (Hamilton, Reno, NV, USA) connected by PE-10 polyethylene tubing to a 28-gauge internal cannula (C3131/Spc, plastic one, internal diameter 0.18 mm, outer diameter 0.20 mm) that extended 0.5 mm beyond the guide cannula. The internal cannula was held in a position for another 1 min after each injection to promote diffusion of drugs before being slowly withdrawn to prevent backflow.

2.4. Social Interaction Test

The social interaction test arena was a wooden box 60 cm² × 35 cm deep. Infrared photocells were
mounted in the walls of the box, 4.5 and 12.5 cm from the floor and the interruption of these beams provided automated measures of locomotor activity and rearing, respectively. A closed circuit camera was mounted vertically above the arena and the rats were observed from a monitor in an adjacent room. Photocell output and the scores of the observer, who was blind to the drug treatment, were entered into a computer. The light level in the test arena was 33 scotopic lux (File and Fernandies, 1999). Two days prior to testing, animals were allocated to test partners within their drug group, on the basis of weight; such that members of a pair did not differ by more than 10 g. (Both members of a test pair received the same chronic treatment and the same drug treatment on the test day.) Animals were given a 4.5 min social interaction test in a low-light, unfamiliar test arena on the test day (day 21), and 30 min after a diazepam or vehicle injection and 60 min after receiving different drugs or vehicle. (Low light, unfamiliar conditions of the social interaction test were selected as these conditions is more sensitive to anxiolytic drug effects).

At the beginning of each social interaction trial, members of a test pair were placed at opposite corners of the arena, facing each other. An observer blind to the drug treatment of the rat pairs recorded the time each pair spent in active social interaction. The following social interaction behaviors were scored and expressed in seconds for each rat pair: sniffing, following, rooming, crawling under or over a partner, boxing, wrestling, kicking, biting, mounting and horizontal submission. (There were no incidences of passive interaction, i.e., when the rats were sitting or lying with their bodies in contact but not interacting with each other, and therefore, this was not scored). At the end of each social interaction test, the arena was wiped with a damp cloth and faecal pellets removed. All testing took place under quiet conditions, in an order randomized for drug treatment, between 08:00 and 12:30 h.

2.5. Treatments

Rats were randomly allocated among the different treatment groups listed in Table 1. All animals received 21 days of i.p. injections of Diazepam (2 mg/kg, i.p.) or vehicle (distilled water). On day 22, acute administration of different doses of drugs administered 30 min before the 21 days of diazepam (2 mg/kg) or vehicle (water/Tween 20) as appropriate and were subjected to Social interaction test.

### Table No-1 List of Treatments

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments (days 1-21)</th>
<th>Test (day 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Veh + 21 days Veh</td>
<td>V + V</td>
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<tr>
<td>Diaz (Acute)</td>
<td>Veh + 21 days Veh</td>
<td>V + Diaz</td>
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<tr>
<td>21 days Diaz</td>
<td>Veh + 21 days Veh</td>
<td>V + 21 Diaz</td>
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<tr>
<td>Agmatine (Ac)</td>
<td>Agmatine + 21 days Veh</td>
<td>21 days Diaz</td>
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<td>Agmatine + 21 days Veh</td>
<td>Agmatine + 21 days Veh</td>
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<tr>
<td>Arginase - 21 days</td>
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<td>Arginase - 21 days</td>
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<td>DMFO + 21 days</td>
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<td>L-Arginine + 21 days Veh</td>
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<td>D-cycloserine + 21 Veh</td>
<td>D-cycloserine + 21 Diaz</td>
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Doses: Diazepam: 2 mg/kg, i.p., Agmatine (acute study): 10-80 mg/kg, i.p.), [Agmatine (80 mg/kg, i.p.) + 21 days Diaz], MK-801 (1 mg/kg, i.p.), Neraexane (1 mg/kg, i.p.), D-cycloserine (3 mg/kg, i.p.) or saline, L-Arginine (100 µg/rat, i.c.v.), DMFO (125 µg/rat, i.c.v.), Arginine (50 µg/rat, i.c.v.) and D-Arginine (100 µg/rat, i.c.v.) or acSF (5 µr/l, i.c.v.) respectively.

#### 2.6. Data analysis

At the end of the experiment, dilute India ink was injected by i.c.v. route and the animals were euthanized by an overdose of pentobarbital sodium (80 mg/kg, i.p.). Immediately, the brain of ratwas dissected out and cannula placement was verified histologically for distribution of ink in the ventricles. The guide cannulae were found to be incorrectly placed in some animals (20%) and these were excluded from the observations. Data from only those animals with uniform distribution of ink in the ventricles were considered for statistical analysis. The results are presented as mean ± S.E.M. The effects of different acute drug treatments were statistically analyzed by one way analysis of variance (ANOVA). A value of P < 0.05 was considered significant.

### 3. Results and Discussion

#### 3.1. Anxiolytic effect of agmatine

Acute administration of agmatine (80 mg/kg, i.p.) significantly increased [F (4, 29) = 5.178, P < 0.001] the time spent in social interaction test than the animals receiving vehicle. There was significant changes in the locomotor activity (P < 0.001) but did not change in the number of rears as compared to the vehicle. These results are depicted in Fig. 1.

#### 3.2. Agmatine inhibited the tolerance to the anxiolytic effect of diazepam.

In the social interaction test, acute administration of diazepam significantly increased the time spent (P < 0.05), decreased locomotor activity (P < 0.05), but did not change the number of rears as compared to the vehicle. The post hoc test indicated that prior administration of agmatine (80 mg/kg, i.p.) significantly increased the time spent in social interaction test [F (3, 23) = 7.182, P < 0.001] with no change in locomotor activity and number of rears. Agmatine reversed the decreased time spent of 21 days diazepam treated animals in social interaction test. These results are depicted in Fig. 2.
3.3. Enhanced brain agmatine content attenuated the tolerance to anxiolytic effect of diazepam.

As depicted in Fig. 3, pretreatment with the agmatine modulators viz., L-arginine (100 µg/rat, i.c.v.), DFMO (125 µg/rat, i.c.v.), aminoguanidine (65 µg/rat, i.c.v.) and arcaine (50 µg/rat, i.c.v.) showed significant increase in the time spent in social interaction test [F (5, 35) = 5.25, P < 0.001]. Post hoc Newman–Kuels comparisons showed significant inhibition of tolerance by L-arginine (P < 0.001), DFMO (P < 0.05), aminoguanidine (P < 0.001) and arcaine (P < 0.05). However, treatments of arcaine (P < 0.05) and L-arginine (P < 0.05) had shown significant change in locomotor activity but not in number of rearing compared to control or 21 days diazepam treated group.

3.4. Agmatine synthesis inhibitor, D-arginine failed to inhibition of tolerance to anxiolytic effect of diazepam.

As depicted in Fig. 4, the effect of arginine decarboxylase inhibitor, D-arginine on tolerance to anxiolytic effect of diazepam. Pretreatment with D-arginine (100 µg/rat, i.c.v.) elevated the anxiogenic effect in Social interaction test by decreasing time spent. No changes in locomotor activity and rearing number.

3.5. NMDA antagonist inhibited and its agonist potentiated the tolerance to the diazepam anxiolytic effect in SCT.

Prior administration of NMDA receptor antagonists MK-801 (1 mg/kg, i.p) (P < 0.001) or Neramexane (1 mg/kg, i.p) (P < 0.001) significantly inhibited the tolerance [F (3, 23) = 7.454, P < 0.001] by increasing the time spent of 21 days diazepam treated animals in SCT. MK-801 and Neramexane decreased the locomotor activity but not significantly. Neramexane shown the change in the number of rearing (P < 0.05) compared to respective control. These results are shown in Fig. 5.

On the other hand, NMDA receptor agonist, D-cycloserine (3mg/kg, i.p.) significantly [F (2, 17) = 8.368, P < 0.001] potentiated the anxiogenic activity of 21 days diazepam treated group by decreasing in time spent, significantly elevated the locomotor activity (P < 0.05) but no change in number of rearing as compared to 21 days diazepam treated group alone. These results are depicted in fig. 6.
compared with 21 days diazepam alone. (One way repeat measure ANOVA followed by Newman-Keuls post hoc test)

Fig. 4. Mean (± S.E.M.) time (s) spent in social interaction (top panel) and number of rears (bottom panel) made by rats treated with acute treatment of the D-arginine (100 µg/rat, i.c.v.) (n = 6) 30 min before dose of 21 days of diazepam (2 mg/kg, i.p.) (n = 6) or control or 21 days diazepam (n = 6) respectively. Animals were subjected in the social interaction arena 30 min after receiving their appropriate drug treatment. (One way repeat measure ANOVA followed by Newman-Keuls post hoc test).

Fig. 5. Mean (± S.E.M.) time (s) spent in social interaction (top panel) and number of rears (bottom panel) made by rats treated with acute treatment of MK-801 (1 mg/kg, i.p.) (n = 6), Neramexane (1 mg/kg, i.p.) (n = 6) 30 min before dose of 21 days of diazepam (2 mg/kg, i.p.) (n = 6) or control or 21 days diazepam (n = 6) respectively. Animals were subjected in the social interaction arena 30 min after receiving their appropriate drug treatment. (One way repeat measure ANOVA followed by Newman-Keuls post hoc test).

Table 2. Shown data as mean ± SEM Locomotor Activity (Beam Breaks) and Number of Rearing made by vehicle and different drugs treated groups (n = 6-8). *P < 0.05, **P < 0.001 compared with control, *P < 0.05 compared with 21 days diazepam respectively.

4. Conclusion
Consistent with the previous reports (File et al., 2001), the present experiments demonstrated that acute administration of diazepam shown an anxiolytic effect in social interaction test by increased time spent in the central area. Diazepam significantly decreased the locomotor activity but no change in the number of rearing when compared with control. As reported earlier (Netto et al., 2003) agmatine (80 mg/kg, i.p.) exerted anxiolytic action in rats by increasing time spent in the arena of social interaction test with significant change in locomotor activity but no change in the number of rearing. The present study demonstrated the effects of acute dose agmatine on tolerance to the anxiolytic effect of diazepam in rats using social interaction test. The present work shows for the first time that agmatine (80 mg/kg, i.p.) significantly attenuated the tolerance to the anxiolytic effect of diazepam by increasing the time spent in the arena without any change in the locomotor activity and rearing number in the social interaction test.

We have also investigated the attenuation of tolerance due to 21 days administration of diazepam in the presence of agents like L-arginine, DFMO, aminoguanidine and arcaine modulating endogenous brain agmatine levels. The greater agmatine levels in brain may be accomplished by increased biosynthesis or decreased degradation of this substance. Biosynthesis of agmatine by L-arginine decarboxylase (L-ADC) depends upon the availability of L-arginine...
(Su et al., 2003). L-arginine is also converted into ornithine and nitric oxide (NO) by an enzyme arginase and nitric oxide synthase, respectively (Reis and Regunathan, 2000). Ornithine subsequently converted into putrescine by L-ornithine decarboxylase. Inhibition of either metabolic pathway is reported to increase activity in other metabolic pathways. Administration of DFMO, which inhibits both L-ornithine decarboxylase and arginase enzyme (Slokin et al., 1982) and subsequently stimulates L-ADC (Hernandez and Schwarz de Tarlovsky, 1999), might increase the availability of agmatine in brain (Lu et al., 2003). Agmatine is metabolized to putrescine and guanidobutanoic acid by enzyme agmatinase and diamine oxidase respectively (Reis and Regunathan, 2000) and inhibition of these enzymes results in augmentation of endogenous agmatine (Regunathan, 2006; Lu et al., 2003; Huang et al., 2003). In the present study we used diamine oxidase (DAO) inhibitor, aminoguanidine (Lu et al., 2003) and agmatinase inhibitor, aracine (Huang et al., 2003; Regunathan, 2006). In fact, results of our previous study clearly indicate that all these drugs substantially increase the levels of agmatine in rat brain (Taksande et al., 2009). All these manipulations attenuate the anxiogenic effect of diazepam due to tolerance in SCT. Conversely, L-ADC inhibitor, D-arginine potentiated an anxiogenic effect of diazepam due to tolerance. D-arginine (isomer of L-arginine) is known to inhibit ADC in plants and bacteria (Rosenfeld and Roberts, 1976; Hao et al., 2005) and thereby blocks conversion of L-arginine to agmatine. Moreover, substantial agmatine immunoreactivity has been detected in areas like VTA, NAC, amygdala, etc that are involved in regulation of anxiety (Otake et al., 1998; Davis, 1997; Koob, 2003). The central effects of agmatine are diverse and have been commonly linked to its ability to bind NMDA and imidazoline receptors. In agreement with previous reports, Anxiolytic activity resulting from NMDA receptor antagonism was reported as early as 1986 when Stephens and colleagues (Stephens et al., 1986) found that high doses of 2-amino-7-phosphohenotanoic acid (APH, 160 mg/kg) increased locomotion in mice in the four plate test, and Bennett and Amrick (1986) showed anxiolytic effect in the conflict test in rats. Later, anxiolytic activity of uncompetitive and competitive NMDA receptor antagonists has been shown in the conflict test (Corbett and Dunn, 1993; Plaznik et al., 1994), the social interaction test (Dunn et al., 1989; Corbett and Dunn, 1993), the elevated plus-maze (Dunn et al., 1989; Corbett and Dunn, 1993). NMDA receptor antagonist, MK-801 and Neramexane in their effective doses inhibited the tolerance to the anxiolytic effect of diazepam. Thus, the possibility that the reversal of anxiogenic effect may be related to blocking of excitatory NMDA receptor function by these agents. In contrast, NMDA receptor agonist, D-cycloserine (3 mg/kg, i.p.) respectively failed to inhibit the tolerance to the anxiolytic effect of 21 days diazepam treated group. Steppuhn and Turski (11) and Dunworth and Stephens (23) reported that in mice they could either prevent the expression or block the manifestation of diazepam withdrawal symptoms by injecting AMPA and NMDA receptor antagonists. They hypothesized that a long-term treatment with BZ-RS ligand, may cause a maximal amplification of GABA-gated Cl− current intensities, leading to a compensatory enhancement of glutamate receptor function, which they argue tends to minimize the consequences of an up-regulation of GABAergic tone elicited by BZ-RS ligands. Pharmacological experiments with agonists or antagonists of specific glutamate receptor subtypes suggested that whereas N-methyl-D-aspartate (NMDA)-dependent mechanisms may underlie the expression of signs of dependence, AMPA receptor activation may be essential for the induction of the withdrawal syndrome following a protracted diazepam administration. Taken together, these data suggest that: (i) the tolerance to the actions of BZ-RS ligands acting as FAMs may be associated with changes in GABAergic transmission; (ii) the dependence elicited by these drugs may be associated with an enhanced glutamatergic transmission; and (iii) an enhanced glutamatergic transmission may be a common mechanism underlying the abrupt discontinuation of protracted treatment with GABAergic transmission enhancers. Glutamic acid decarboxylase and glutamate receptor changes during tolerance and dependence to benzodiazepines. In conclusion, this study demonstrated that agmatine inhibited the tolerance to anxiolytic effect of diazepam. We also showed similar influence of NMDA receptor antagonists or drugs augmenting endogenous agmatine levels on above tolerated rats. Anxiogenic effect of diazepam due to tolerance was potentiated by NMDA agonist. During tolerance and dependence to benzodiazepines, the glutamate receptors changed and might be unregulated. Taken together it was reasonable to conclude that tolerance to anxiolytic effect of diazepam might be linked to NMDA receptor. Agmatine act as NMDA antagonist significantly inhibited the tolerance to the anxiolytic effect of diazepam by NMDA receptor antagonism. Additional studies are necessary to better define the role of agmatine in the dependence and development of tolerance to the anxiolytic effect of benzodiazepines.
REFERENCES


