Pharmacology, Biochemistry and Behavior 94 (2010) 510–515

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Adenosinergic system is involved in development of diazepam tolerance in mice

Joanna Listos *, Sylwia Talarek, Sylwia Fidecka

Department of Pharmacology and Pharmacodynamics, Medical University of Lublin, Staszica 4, 20-081 Lublin, Poland

ARTICLE INFO

Article history:
Received 4 May 2009
Received in revised form 28 October 2009
Accepted 16 November 2009
Available online 24 November 2009

Keywords:
Adenosine agonists and antagonists
Benzodiazepine tolerance
Diazepam
Tolerance to motor incoordination effect

ABSTRACT

In the present study the effect of adenosinergic system on the development of diazepam tolerance to motor disturbances in mice was investigated. Diazepam tolerance was obtained by administration of diazepam at a dose of 5.0 mg/kg, s.c. for ten consecutive days. On the 1st and the 10th day of the experiment motor impairments were measured in two behavioural tests: rota-rod and chimney test. We showed that acute diazepam injection produced significant motor impairments in mice and that effect was decreased by repeated diazepam treatment, confirming the development of tolerance to the motor impairing effect of diazepam. We demonstrated that adenosine A_1 and/or A_{2A} receptor agonists: CPA (0.025 and 0.05 mg/kg, i.p.), CGS 21680 (0.1 and 0.2 mg/kg, i.p.), NECA (0.005 and 0.01 mg/kg, i.p.) pretreatment with diazepam were able to attenuate the development of diazepam tolerance and adenosine receptor antagonists: DPCPX (1.0 and 3.0 mg/kg, i.p.), DMPX (3.0 and 6.0 mg/kg, i.p.) and caffeine (10.0 and 20.0 mg/kg, i.p.) induced the opposite effect. The most apparent effects were obtained by non-selective agonist (NECA) and antagonist (caffeine) of adenosine receptors. We conclude that adenosinergic system plays an important role in mechanisms underlying the development of benzodiazepine tolerance.

1. Introduction

Benzodiazepines are the most frequently used psychotropic drugs. They have sedative, anticonvulsant, anxiolytic and myorelaxant properties. At present, it is well known that their repeated exposure may lead to development of tolerance and dependence. In humans, the development of tolerance, i.e. reduced response to benzodiazepine drug, is a major side effect in therapy with these drugs. In animals, behavioural tolerance is often used in experimental studies as one of the parameter of physical dependence. The mechanism underlying benzodiazepine tolerance is not fully understood but, it is well known that it depends on dose level, duration of treatment and elimination half-life of particular benzodiazepine drug. Experimental studies in animals indicate that tolerance to different effects of benzodiazepines is gradually developed in different time period. For example, tolerance to muscle relaxant or ataxic effect develops in a relatively short period while tolerance to anxiolytic effect develops significantly slower [for ref. see Bateson, 2002, Hutchinson et al., 1996). Thus, development of benzodiazepine tolerance is not a fully clarified process leading to limitation of benzodiazepine use in psychiatric diseases.

The activity of benzodiazepines is related to stimulation of γ-aminobutyric acid A ($GABA_A$) receptors by γ-aminobutyric acid (GABA), the most recognized inhibitory neurotransmitter in central nervous system. The $GABA_A$ receptor has a pentameric structure composed of different types of subunits: α, β, γ, δ, ε, θ, π, ρ (Allison and Pratt, 2003, Bateson, 2002, Wafford, 2005). These receptor subtypes are abundantly distributed in brain areas and their stimulation induces inhibitory effect on activity of the central nervous system. Close connections between GABAergic and other neuronal receptors, including dopaminergic (Pérez de la Mora et al., 1997), glutamatergic (MacDermott, 2001) or adenosinergic system (Ferré, 1997) are responsible for a broad spectrum of pharmacological activity of GABAergic drugs in the central nervous system.

Initially, based on the resemblance of benzodiazepine and adenosine activity, such as anxiolytic, sedative or anticonvulsant effects, it was suggested that adenosinergic system was involved in the mechanism of benzodiazepine drugs (Arvidson et al., 1982, Hawkins et al., 1988, Phillis et al., 1980, 1981). Now, it is well known that adenosine is an inhibitory neuromodulator in central nervous system, which acts on four adenosine receptor subtypes, such as A_1, A_{2A}, A_{2B} and A_3. Modulatory properties of adenosine are associated with numerous interactions between adenosine and other ionotropic and metabotropic receptors. Stimulation of adenosine receptors regulates many physiological processes such as seizure susceptibility, neuroprotection, regulation of pain perception or sleep induction (Sichardt and Nieber, 2007). A growing body of evidence is shown

Abbreviation: cAMP, cyclic adenosine monophosphate; CGS 21680, 2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride; CPA, N^6-cyclopentyladenosine; DMPX, 3,7-dimethyl-1-(2-propynyl)-xanthine; DPCPX, 1,3-dipropyl-8- cyklopentylxanthine; GABA, γ-aminobutyric acid; $GABA_A$, γ-aminobutyric acid A receptors; NECA, 5′-N-ethylcarboxamidoadenosine; NMDA receptor, N-methyl-D-aspartate receptor.

* Corresponding author. Tel.: +48 81 535 73 71; fax: +48 81 528 89 18.
E-mail address: alistos@op.pl (J. Listos).

0091-3057/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2009.11.005

that functional interaction of adenosine receptors with other neuronal receptors may be also useful tool for treatment of some pathological states, e.g. neurological disorders (Chen et al., 2007) or state of dependence (Ferré et al., 2007). Furthermore, adenosine agonists are able to attenuate opioid (Kaplan et al., 1994) and ethanol (Kaplan et al., 1999) withdrawal signs which supports involvement of adenosine receptors in mechanisms underlying addiction. In our previous experiment, we also demonstrated that adenosinergic system was involved in the effect of chronic benzodiazepine treatment. We indicated that adenosine agonists attenuated (Listos et al., 2005) and adenosine antagonists intensified (Listos et al., 2006) diazepam withdrawal signs manifested as increase in a seizure susceptibility. We also demonstrated that adenosine agonists, CGS 21680 and NECA, were able to inhibit development of sensitization to diazepam withdrawal signs (Listos et al., 2008). Thus, the adenosine as an important modulator of the central nervous system is also able to modulate the effects of chronic treatment with abused drugs. In that case links between adenosine and glutamatergic or dopaminergic receptors (Allison and Pratt, 2003, Dunwiddie and Masino, 2001, Tozzi et al., 2007) seem to be the most involved.

Taken together, although benzodiazepine tolerance has been extensively studied, relatively less is known about involvement of adenosinergic mechanisms in that phenomenon. Admittedly there is one report from 1991 (Contreras and Germany, 1991) in which an involvement of adenosinergic system in alprazolam tolerance was investigated, but procedure of that experiment was completely different. In the present study we undertook to investigate the effect of adenosinergic system on development of diazepam tolerance to motor disturbances in two behavioural, generally accepted tests: rota-rod and chimney test. Diazepam was chosen as a representative of clinically available benzodiazepines. In our experiment we used adenosinergic drugs, the selective (A_1 or A_{2A}) and non-selective (A_1 and A_{2A}) adenosine agonists and antagonists. The use of these drugs made possible the assessment of role of particular adenosine receptors in the observed effects. Results were discussed in the context of functional associations of adenosinergic system with other neuronal brain pathways, and with neuroadaptive changes caused by repeated treatment with diazepam. We believe that our study extends the knowledge of benzodiazepine tolerance.

2. Materials and methods

2.1. Animals

The experiments were carried out on male albino Swiss mice (20–30 g). The animals were kept 10 per cage at room temperature of 22.1 °C, on natural day–night cycle (spring). Standard food (Murigran pellets, Bacutil, Motycz) and tap water were freely available. All the experiments were made between 9 a.m. and 2 p.m.

The study was performed according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the European Community Council Directive for Care and Use of Laboratory Animals and it was approved by local ethics committee (The Medical University of Lublin Committee on the Use and Care of Animals).

2.2. Drugs

In the experiments the following drugs were used: diazepam (Relanium, amp., Polfa, Warszawa, Poland), and adenosine receptor ligands: N^6-cyclopentyladenosine (CPA)–the selective adenosine A_1 receptor agonist; 2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680)–the selective adenosine A_{2A} receptor agonist; 5′-N-ethylcarboxamidoadenosine (NECA)–the non-selective adenosine A_1/A_2 receptor agonist; 1,3-dipropyl-8-cyklopentylxanthine (DPCPX)–the selective adenosine A_1 receptor antagonist; 3,7-dimethyl-1-(2-propynyl)-xanthine (DMPX)–the selective adenosine A_{2A} receptor antagonist, (all from Sigma-Aldrich, St. Louis, USA); and caffeine–the non-selective adenosine A_1/A_2 receptor antagonist (Polfa, Poland).

CPA, CGS 21680 and caffeine were dissolved in saline, NECA, DPCPX and DMPX were dissolved in minimal volume of ethanol (5–7 drops) and then, were diluted in saline. Diazepam was diluted to appropriate concentration in saline.

Following doses of drugs were used in the experiments: diazepam (5.0 mg/kg, s.c.), CPA (0.025 and 0.05 mg/kg, i.p.), CGS 21680 (0.1 and 0.2 mg/kg, i.p.), NECA (0.005 and 0.01 mg/kg, i.p.), DPCPX (1.0 and 3.0 mg/kg, i.p.), DMPX (3.0 and 6.0 mg/kg, i.p.) and caffeine (10.0 and 20.0 mg/kg, i.p.).

All drugs were administered in a volume of 10.0 ml/kg. Control animals were injected with appropriate volume of the solvent.

Adenosine ligands were daily injected intraperitoneally (i.p.), 20 min before each subcutaneous (s.c.) injection of diazepam for ten consecutive days. 30 min after diazepam injection (it means, 50 min after injection of adenosine ligands), on the 1st and 10th day of the experiment, two tests were performed.

2.3. Procedure of tolerance development and behavioural tests

To obtain the development of tolerance to motor impairing effect of diazepam, mice were treated with diazepam daily, at a dose of 5.0 mg/kg, s.c., for 10 consecutive days. Instead of diazepam control mice received a respective volume of saline (0.9% NaCl). A motor coordination of mice was measured on the 1st and 10th day of the experiment, using the rota-rod test and the chimney test.

The rota-rod test (Dunhann and Miya, 1957) assesses the ability of animals to maintain a balance on rotating rod (20 mm diameter) during 60 s. We measured time which mice spent on rotating rod. The rod revolves with constant speed 18 rpm. The chimney test (Boisser et al., 1960) evaluates the ability of mice to go backwards, vertically from the tube. We measured time which animals spent in the tube (maximum 60 s). The tube is made from Plexiglas with rough surface (30 mm diameter and 25 cm length).

Before the experiments began all animals had been trained: each mouse was placed on the rod and in the tube for 3 min. The number of trials for each mouse was unlimited. In experiment we approved all animals which were able to stay on rotating rod for 60 s or which were able with easy (up to 15 s) to go backwards, vertically from the tube.

2.4. Statistical analysis

The obtained data, presented in the figures as mean ± S.E.M, were statistically calculated using the one-way analysis of variance (one-way ANOVA). Post hoc comparisons were carried out by Tukey–Kramer test. Chronically diazepam-treated mice were compared vs. animals injected with acute dose of diazepam. Animals treated with adenosine ligands were compared with chronically diazepam-treated mice. A probability (P) value of 0.05 or less was considered as statistically significant. Each group of animals consisted of 10 mice.

3. Results

As it is shown in Fig. 1, animals injected with adenosine ligands alone did not show motor coordination impairments in both rota-rod test (CPA: $F_{2,\ 27}=1.326$, $P=0.28$; CGS 21680: $F_{2,\ 27}=0.92$, $P=0.407$; NECA: $F_{2,\ 27}=1.076$, $P=0.355$; DPCPX: $F_{2,\ 27}=0.22$, $P=0.803$; DMPX: $F_{2,\ 27}=1.27$, $P=0.297$; caffeine: $F_{2,\ 27}=0.286$, $P=0.753$) and chimney test (CPA: $F_{2,\ 27}=0.885$, $P=0.424$; CGS 21680: $F_{2,\ 27}=3.074$, $P=0.627$; NECA: $F_{2,\ 27}=0.678$, $P=0.516$; DPCPX: $F_{2,\ 27}=3.44$, $P=0.066$; DMPX: $F_{2,\ 27}=3.645$, $P=0.069$; caffeine: $F_{2,\ 27}=0.825$, $P=0.44$). In contrast, animals that received acute dose of diazepam (1st day of the experiments) produced considerable motor coordination impairments in comparison with control mice ($P<0.001$) and this effect was significantly ($P<0.001$)

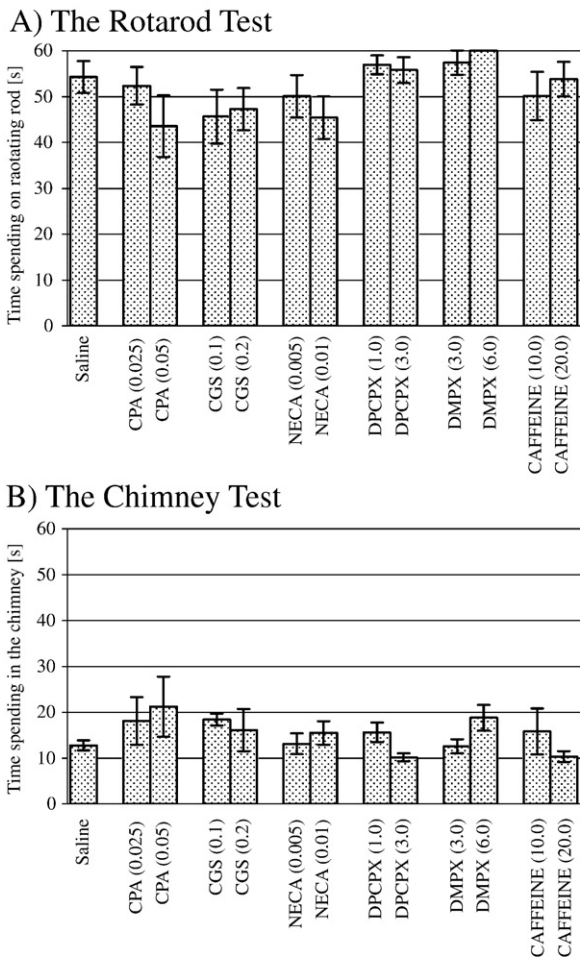

Fig. 1. Effects of adenosine agonists and antagonists on motor coordination of mice in rota-rod test (A) and the chimney test (B). Adenosine ligands were injected 20 min before the tests. Results are expressed as mean ± S.E.M. ($n = 10$ mice/group).

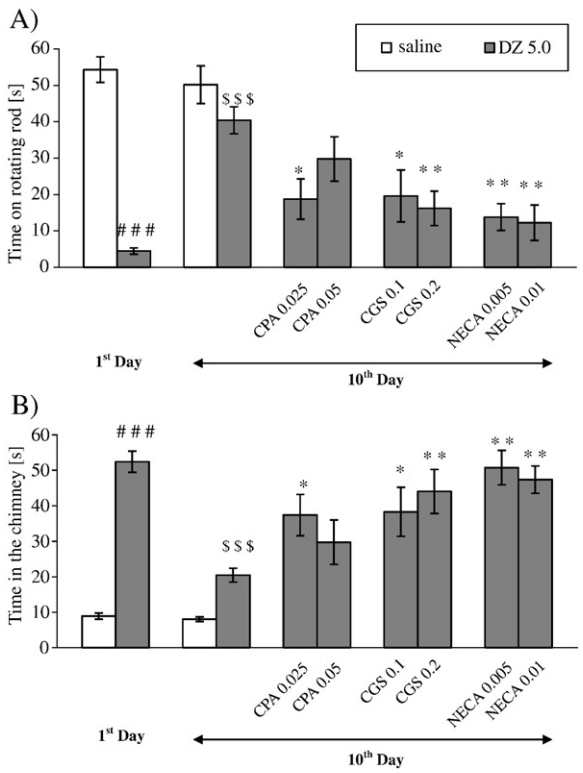

Fig. 2. Effects of adenosine agonists: CPA (0.025 and 0.05 mg/kg, i.p.), CGS 21680 ("CGS" 0.1 and 0.2 mg/kg, i.p.) and NECA (0.005 and 0.01 mg/kg, i.p.) on the development of tolerance to the motor impairing effect of diazepam ("DZ" 5.0 mg/kg, s.c.), measured in rota-rod (A) and chimney (B) tests. Adenosine ligands were injected 20 min before each application of diazepam for ten consecutive days. 30 min after diazepam injection (it means, 50 min after injection of adenosine ligands), on the 1th and 10th day of the experiment, two tests were performed. Results are expressed as mean ± S.E.M. ($n = 10$ mice/group). $^{\#\#\#}P<0.001$ vs saline treated mice; $^{\$\$\$}P<0.001$ vs. acute diazepam-treated mice; $^{*}P<0.05$, $^{**}P<0.01$ vs. diazepam chronic treated mice (Tukey–Kramer's test).

attenuated on 10th day of the study in both used tests (Figs. 2 and 3). That result demonstrated development of behavioural tolerance to diazepam-induced motor incoordination.

Fig. 2 shows that all used adenosine receptor agonists inhibit diazepam tolerance in the rota-rod (CPA: $F_{5,\ 54} = 18.088$, $P<0.0001$; CGS 21680: $F_{5,\ 54} = 19.458$, $P<0.0001$; NECA: $F_{5,\ 54} = 30.897$, $P<0.0001$) and in the chimney (CPA: $F_{5,\ 54} = 8.57$, $P = 0.0002$; CGS 21680: $F_{5,\ 54} = 7.422$, $P = 0.0005$; NECA: $F_{5,\ 54} = 16.687$, $P<0.0001$) tests. Significant effects were produced in both tests by low dose of CPA (0.025 mg/kg) $P<0.05$, and both doses of CGS 21680 (0.1 mg/kg, $P<0.05$ and 0.2 mg/kg, $P<0.01$) and NECA (0.005 and 0.01 mg/kg, $P<0.01$).

Effects of adenosine receptor antagonists on development of tolerance to diazepam-induced motor incoordination in rota-rod (DPCPX: $F_{5,\ 54} = 3.404$, $P = 0.048$; DMPX: $F_{5,\ 54} = 0.115$, $P = 0.89$; caffeine: $F_{5,\ 54} = 23.557$, $P<0.0001$) and in chimney test (DPCPX: $F_{5,\ 54} = 18.121$, $P<0.0001$; DMPX: $F_{5,\ 54} = 0.809$, $P = 0.45$; caffeine: $F_{5,\ 54} = 32.488$, $P<0.0001$) are illustrated in Fig. 3. In the rota-rod test, significant intensification of diazepam tolerance was produced by low dose (1.0 mg/kg) of DPCPX ($P<0.01$) and both doses of caffeine (10.0 and 20.0 mg/kg, $P<0.05$). In the chimney test only caffeine (10.0 and 20.0 mg/kg) significantly ($P<0.05$ and $P<0.01$, respectively) intensified development of tolerance to diazepam-induced motor incoordination.

4. Discussion

In the present experiments we used rota-rod and chimney tests to study development of tolerance to diazepam-induced motor disturbances and to assess an involvement of adenosinergic mechanisms in that effect. The present results showed that acute diazepam administration produced significant motor impairments in mice and that effect was decreased by repeated administration of diazepam, indicating that diazepam tolerance to motor disturbances had been developed. Our result supported the literature data in which diazepam tolerance to muscle relaxant effect developed during a relatively short time, it means, within 5–14 days of benzodiazepine treatment (Hutchinson et al., 1996, Licata and Rowlett, 2008). The main finding of the present study was that adenosine receptor agonists co-administered with diazepam were able to attenuate development of tolerance while adenosine receptor antagonists intensified that effect. All adenosine ligands, however, did not alter normal motor coordination of mice in the absence of diazepam (Fig. 1) and had no effect on motor impairments induced by acute dose of diazepam (data were not placed in the figure). The strongest changes we observed after administration of non-selective adenosine A_1/A_{2A} receptor agonist and antagonists, NECA and caffeine, because both doses of these drugs induced clear and significant effects in each test. Thus, simultaneous stimulation or blockade of both adenosine A_1 and A_{2A} receptor resulted in the most intensive effects on development of diazepam tolerance. Our results are not in agreement with the experiment described by Contreras and Germany (1991) in which both, adenosine agonists and antagonists attenuated the alprazolam tolerance. We suggest that difference between each other experiment is associated with completely different experimental procedure e.g. ineffective doses of diazepam have been applied for ten consecutive days in our experiment while alprazolam has been given twice at a

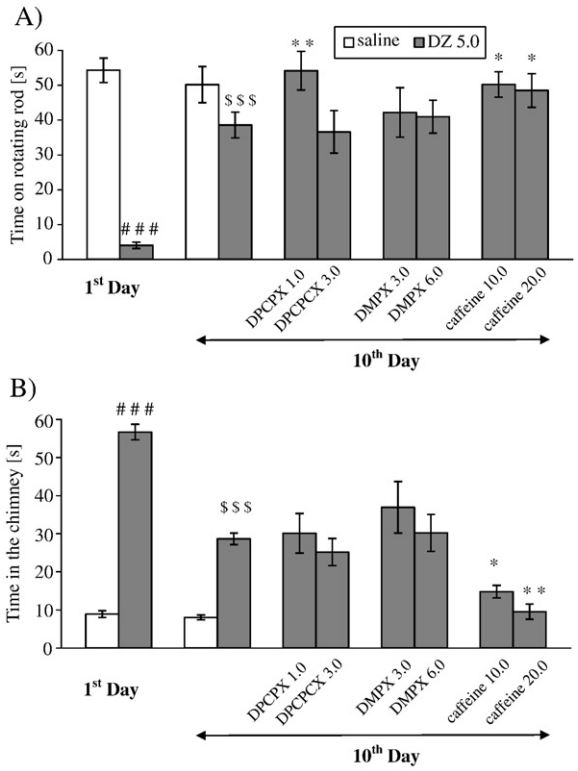

Fig. 3. Effects of adenosine antagonists: DPCPX (1.0 and 3.0 mg/kg, i.p.), DMPX (3.0 and 6.0 mg/kg, i.p.) and caffeine (10.0 and 20.0 mg/kg, i.p.) on the development of tolerance to the motor impairing effect of diazepam ("DZ" 5.0 mg/kg, s.c.), measured in rota-rod (A) and chimney (B) tests. Adenosine ligands were injected 20 min before each application of diazepam for ten consecutive days. 30 min after diazepam injection (it means, 50 min after injection of adenosine ligands), on the 1th and 10th day of the experiment, two tests were performed. Results are expressed as mean ± S.E.M. ($n = 10$ mice/group). $^{\#\#\#}P<0.001$ vs saline treated mice; $^{\$\$\$}P<0.001$ vs. acute diazepam-treated mice; $^{*}P<0.05$, $^{**}P<0.01$ vs diazepam chronic treated mice (Tukey–Kramer's test).

large dose (100.0 mg/kg). Furthermore, the pharmacological profile of both used benzodiazepines is also heterogenous: diazepam posses all properties which are specify for $GABA_A$ receptor agonists, like anxiolytic, sedative, anticonvulsant or miorelaxant activity, while alprazolam induces predominantly anxiolytic effect. That difference is caused by various abilities to stimulation of particular $GABA_A$ receptor subunits. Thus, we suppose that all these factors affect the divergent results in each experiment.

Drug tolerance is defined as the necessity to increase dose of the drug to obtain the same behavioural effect. It can be due to the metabolic changes (the pharmacokinetic tolerance) or adaptive changes within the central nervous system (the pharmacodynamic tolerance). A large body of evidence from animal studies suggests that tolerance to benzodiazepines has pharmacodynamic nature because development of tolerance does not correlate with changes in benzodiazepine levels in blood plasma, cerebrospinal fluid or in brain tissue [for ref. see Hutchinson et al., 1996). Therefore, we suppose that relationship between adenosine ligands and benzodiazepines observed in our study is also due to pharmacodynamic, not pharmacokinetic, interactions in the central nervous system.

Although the mechanisms underlying diazepam tolerance have already been described (Licata and Rowlett, 2008), they are far from being completely understood. The experimental data demonstrated, that neuroadaptive changes in GABAergic system underlay benzodiazepine tolerance. In vitro, Biggio et al. (2003) indicated that chronic treatment with diazepam induced alterations in the amount of $GABA_A$ receptor subunits. They observed significant decrease in the amount of α_1, γ_2, but not α_4 subunit mRNA, and observed changes were associated with a reduction of diazepam ability to potentiate GABA-evoked chloride conductance in $GABA_A$ receptor. Earlier study also showed a reduction in amount of α_1 subunit and upregulation of α_3, α_5, β_2, β_3 and γ_2 subunits in $GABA_A$ receptor following two weeks of exposure to diazepam (Pesold et al., 1997), confirming that GABAergic mechanism was important component of benzodiazepine tolerance. On the other hand, a large body of evidence (Steppuhn and Turski, 1993, Tsuda et al., 1999) demonstrated that balance between inhibitory and excitatory mechanisms was disturbed after chronic treatment with benzodiazepines. An excitatory mechanism, such as glutamatergic system, became more sensitive after repeated exposure to benzodiazepines, as compensatory effects. It suggests that changes in glutamatergic mechanisms may also be important factor of benzodiazepine tolerance (Allison and Pratt, 2003). For example, experimentally data have shown that NMDA receptor antagonists, like CPP and MK-801 are able to prevent the development of diazepam tolerance to sedative effect in animals (File and Fernandes, 1994, Steppuhn and Turski, 1993). Furthermore, some modifications, like alterations in N-methyl-D-aspartate (NMDA) receptor subunit expression have been shown during the development of benzodiazepine tolerance (Izzo et al., 2001). Thus, all above mentioned data show that mechanisms underlying benzodiazepine tolerance are extremely complicated and various neurotransmitter systems, including GABAergic or glutamatergic pathways are possible to be involved in that phenomenon.

From all adenosine receptor, A_1 and A_{2A} are the most abundant in the central nervous system, and their role is the most recognized. Stimulation of A_1 receptor produces a decrease in different neurotransmitter release, such as dopamine, GABA, acetylcholine, noradrenaline and induces a decrease in locomotor activity, sedation or anticonvulsant effect (Dhalla et al., 2003). We suppose that attenuating effect of CPA observed in our study could be caused by reduction in GABA release in brain areas. Furthermore, it is documented that upon chronic activation of A_1 receptors by agonists (i.g. CPA) A_1 receptors undergo desensitization (Green, 1987, Parsons and Stiles, 1987) and that mechanism might be responsible for weak effect of CPA ($P<0.05$) in our experiment.

On the other hand, we suggest that involvement of CGS 21680 in diazepam-induced tolerance may be mediated by interaction of A_{2A} receptors with NMDA receptors. A glutamatergic hypothesis of benzodiazepine tolerance is strongly supported in experimental studies (Izzo et al., 2001, Tsuda et al., 1999). In striatum, the glutamatergic system is under tight control of adenosine A_{2A} receptors and their stimulation facilitates the glutamate release in that area (Popoli et al., 1995). Moreover, the glutamatergic pathways of cerebellar cortex are also involved in motor impairments induced by ethanol (Al-Rejaie and Dar, 2006) or cannabinoids (Dar, 2002) which supports glutamatergic hypothesis of involvement of adenosine A_{2A} agonist in our study.

Interestingly, although effect of stimulation of adenosine A_1 and A_{2A} receptors on adenylyl cyclase is completely different, we observed that A_1 and A_{2A} ligands produced the same, compatible effect on diazepam tolerance. According to literature data, the major brain areas involved in ethanol-induced motor incoordination, are cerebellum (Dar, 2006), striatum (Meng and Dar, 1994) and motor cortex (Barwick and Dar, 1998). Meng et al. (1998) showed that acute dose of ethanol significantly decreased in cyclic adenosine monophosphate (cAMP) levels in striatum, which functionally correlated with motor disturbances of ethanol. Similarly, we suggest that motor impairments observed in our study could be also related to reduction in cAMP level in motor brain areas: CPA and acute dose of diazepam directly produced a reduction in cAMP level. While CGS 21680, as adenosine A_{2A} receptor agonist, by interaction with other G-coupled receptor system, could indirectly diminish cAMP level. Thus, the common denominator, which underlies the effects of adenosinergic drugs in diazepam tolerance may be a reduction in cAMP level.

Moreover, we hypothesize that the strongest effects of non-selective compounds, NECA and caffeine, may be associated with

additive effect of these drugs on A_1 and A_{2A} adenosine receptors, and with limitation the activity of A_1 by A_{2A} receptors which results in an increase in glutamate release in central nervous system. In brain, A_1 receptors are co-localized with A_{2A} receptors in the same striatal glutamatergic terminals (Schiffmann et al, 2007) which suggest that interactions between these receptors may mediated effects shown in our study. It is known that in normal circumstances adenosine has affinity mainly to A_1 receptors inducing a decrease in glutamate release and higher concentration of adenosine is able to stimulate A_{2A} receptors and to produce an increase in glutamate release. However, in some circumstances, stimulation of A_{2A} receptors "switch off" the activity of A_1 receptors on glutamatergic system and promotes glutamate release (Ciruela et al., 2006a,b). In that way, A_{2A} receptors are able to limit the activity of A_1 receptors. We suggest that this interaction may be involved in the effects of NECA and caffeine in our experiments.

From among all used adenosine receptor antagonists, both doses of caffeine, the non-selective A_1/A_{2A} receptor antagonist, significantly reduced motor impairments in chronically diazepam-treated mice. Our result is in accordance with other study, in which motor incoordination induced by acute dose of ethanol was attenuated by caffeine and DPCPX (Connole et al., 2004). We suggest that activity of caffeine was mainly associated with blockade of A_1 receptor, all the more that higher dose of DPCPX, selective adenosine A_1 receptor antagonist, evoked similar to caffeine effect. DMPX had no effect on diazepam tolerance. Thus, caffeine, generally known as a drug with ability for reverse intoxicating effects of ethanol both in humans and rodents, via blockade of A_1 receptor was able to attenuate motor impairments in chronically diazepam-treated mice. On the other hand, Batista et al. (2005) demonstrated that blockade of adenosine A_1 receptors by selective (DPCPX) or non-selective (caffeine) drugs inhibited development of ethanol tolerance in mice. It should be mentioned, however, that ethanol has been applied for only two days in that experiment.

In summary, it has already been described that chronic treatment with diazepam produces tolerance to different effects. The present findings, which are related to chronic diazepam treatment and developing of diazepam tolerance to motor disturbances, show analogies with repeated treatment with benzodiazepines in human and support the addictive role of these drugs during therapy with benzodiazepines. We showed in behavioural experiments that repeated treatment with diazepam, for ten consecutive days, produced development of tolerance to motor impairing effects. The co-administration of diazepam with selective and non-selective adenosine receptor agonists inhibited development of tolerance while adenosine receptor antagonists produced the opposite effects. The strongest effects were obtained by administration of non-selective agonist (NECA) and antagonist (caffeine) of adenosine receptors. It is reasonable to conclude that adenosinergic system may play an important role in mechanisms underlying development of benzodiazepine tolerance and physical dependence. These findings strongly support further investigation of adenosinergic system in dependence mechanism.

References

Allison C, Pratt JA. Neuroadaptive processes in GABAergic and glutamatergic systems in benzodiazepine dependence. Pharmacol Ther 2003;98:171–95.

Al-Rejaie S, Dar MS. Behavioral interaction between nicotine and ethanol: possible modulation by mouse cerebellar glutamate. Alcohol Clin Exp Res 2006;30:1223–33.

Arvidson SB, Ekstrom-Jodal B, Martinell SAG, Niemand D. Aminophilline antagonizes diazepam sedation. Lancet 1982;11:1467.

Barwick VS, Dar MS. Adenosinergic modulation of ethanol-induced motor incoordination in the rat motor cortex. Prog Neuropsychopharmacol Biol Psychiatry 1998;22:587–607.

Bateson AN. Basic pharmacologic mechanisms involved in benzodiazepine tolerance and withdrawal. Curr Pharm Des 2002;8:5-21.

Batista LC, Prediger RD, Morato GS, Takahashi RN. Blockade of adenosine and dopamine receptors inhibits the development of rapid tolerance to ethanol in mice. Psychopharmacology 2005;181:714–21.

Biggio G, Dazzi L, Biggio F, Mancuso L, Talani G, Busonero F, et al. Molecular mechanisms of tolerance to and withdrawal of GABA(A) receptor modulators. Eur Neuropsychopharmacol 2003;13:411–23.

Boisser JR, Tardy J, Diverres JC. Une nouvelle simple methode pour explorer l'action "tranquilisante": le test de la chemnée. Med Exp 1960;3:81–4.

Chen JF, Sonsalla PK, Pedata F, Melani A, Domenici MR, Popoli P, et al. Adenosine A_{2A} receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and "fine tuning" modulation. Prog Neurobiol 2007;83:310–31.

Ciruela F, Casadó V, Rodrigues RJ, Luján R, Burgueño J, Canals M, et al. Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. J Neurosci 2006a;26:2080–7.

Ciruela F, Ferré S, Casadó V, Cortés A, Cunha RA, Lluis C, et al. Heterodimeric adenosine receptors: a device to regulate neurotransmitter release. Cell Mol Life Sci 2006b;63:2427–31.

Connole L, Harkin A, Maginn M. Adenosine A_1 receptor blockade mimics caffeine's attenuation of ethanol-induced motor incoordination. Basic Clin Pharmacol Toxicol 2004;95:299–304.

Contreras E, Germany A. Adenosine analogs attenuate tolerance-dependence on alprazolam. Gen Pharmacol 1991;22:637–41.

Dar MS. Mouse cerebellar adenosine-glutamate interactions and modulation of ethanol-induced motor incoordination. Alcohol Clin Exp Res 2002;26:1395–403.

Dar MS. Co-modulation of acute ethanol-induced motor impairment by mouse cerebellar adenosinergic A_1 and $GABA_A$ receptor systems. Brain Res Bull 2006;71:287–95.

Dhalla AK, Shryock JC, Shreeniwas R, Belardinelli L. Pharmacology and therapeutic applications of A_1 adenosine receptor ligands. Curr Top Med Chem 2003;3:369–85.

Dunhann NW, Miya TS. A note on a simple approach for determinig neurological deficits in rats and mice. J Amer Pharmacol Assoc 1957;46:208–9.

Dunwiddie TV, Masino SA. The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci 2001;24:31–55.

Ferré S. Adenosine-dopamine interactions in the ventral striatum. Implications for the treatment of schizophrenia. Psychopharmacology 1997;133:107–20.

Ferré S, Diamond I, Goldberg SR, Yao L, Hourani SM, Huang ZL, et al. Adenosine A_{2A} receptors in ventral striatum, hypothalamus and nociceptive circuitry implications for drug addiction, sleep and pain. Prog Neurobiol 2007;83:332–47.

File SE, Fernandes C. Dizocilpine prevents the development of tolerance to the sedative effects of diazepam in rats. Pharmacol Biochem Behav 1994;47:823–6.

Green A. Adenosine receptor down-regulation and insulin resistance following prolonged incubation of adipocytes with an A_1 adenosine receptor agonist. J Biol Chem 1987;262:15702–7.

Hawkins M, Pravica M, Radulovacki M. Chronic administration of diazepam downregulates adenosine receptors in the rat brain. Pharmacol Biochem Behav 1988;30:303–8.

Hutchinson MA, Smith PF, Darlington CL. The behavioural and neuronal effects of the chronic administration of benzodiazepine anxiolytic and hypnotic drugs. Prog Neurobiol 1996;49:73–97.

Izzo E, Auta J, Impagnatiello F, Pesold C, Guidotti A, Costa E. Glutamic acid decarboxylase and glutamate receptor changes during tolerance and dependence to benzodiazepines. Proc Natl Acad Sci USA 2001;98:3483–8.

Kaplan GB, Leite-Morris KA, Sears MT. Alterations of adenosine A_1 receptors in morphine dependence. Brain Res 1994;657:347–50.

Kaplan GB, Bharmal NH, Leite-Morris KA, Adams WR. Role of adenosine A_1 and A_{2A} receptors in the alcohol withdrawal syndrom. Alcohol 1999;19:157–62.

Licata SC, Rowlett JK. Abuse and dependence liability of benzodiazepine-type drugs: GABA (A) receptor modulation and beyond. Pharmacol Biochem Behav 2008;90:74–89.

Listos J, Malec D, Fidecka S. Influence of adenosine receptor agonists on benzodiazepine withdrawal signs in mice. Eur J Pharmacol 2005;523:71–8.

Listos J, Malec D, Fidecka S. Adenosine receptor antagonists intensify the benzodiazepine withdrawal signs in mice. Pharmacol Rep 2006;58:643–51.

Listos J, Talarek S, Fidecka S. Adenosine receptor agonists attenuate the development of diazepam withdrawal-induced sensitization in mice. Eur J Pharmacol 2008;588:72–7.

MacDermott AB. Glutamate and GABA: a painful combination. Neuron 2001;32:376–8.

Meng ZH, Dar MS. Intrastriatal Ro15-4513 functionally antagonizes ethanol-induced motor incoordination and striatal adenosinergic modulation of ethanol-induced motor incoordination in rats. J Pharmacol Exp Ther 1994;271:524–34.

Meng ZH, Pennington SN, Dar MS. Rat striatal adenosinergic modulation of ethanol-induced motor impairment: possible role of striatal cyclic AMP. Neuroscience 1998;85:919–30.

Parsons WJ, Stiles GL. Heterologous desensitization of the inhibitory A_1 adenosine receptor-adenylate cyclase system in rat adipocytes. Regulation of both N_s and N_i. J Biol Chem 1987;262:841–7.

Pérez de la Mora M, Ferré S, Fuxe K. GABA-dopamine receptor–receptor interactions in neostriatal membranes of the rat. Neurochem Res 1997;22:1051–4.

Pesold C, Caruncho HJ, Impagnatiello F, Berg MJ, Fritschy JM, Guidotti A, et al. Tolerance to diazepam and changes in GABA(A) receptor subunit expression in rat neocortical areas. Neuroscience 1997;79:477–87.

Phillis JW, Siemens RK, Wu PH. Effects of diazepam on adenosine and acetylocholine release from rat cerebral cortex: further evidence for a purinergic mechanism in action of diazepam. Br J Pharmacol 1980;70:341–8.

Phillis JW, Wu PH, Bender AS. Inhibition of adenosine uptake into rat brain synaptosomes by the benzodiazepines. Gen Pharmacol 1981;12:67–70.

Popoli P, Betto P, Reggio R, Ricciarello G. Adenosine A_{2A} receptor stimulation enhances striatal extracellular glutamate levels in rats. Eur J Pharmacol 1995;287:215–7.

Schiffmann SN, Fisone G, Moresco R, Cunha RA, Ferré S. Adenosine A_{2A} receptors and basal ganglia physiology. Prog Neurobiol 2007;83:277–92.

Sichardt K, Nieber K. Adenosine A(1) receptor: functional receptor–receptor interactions in the brain. Purinergic Signal 2007;3:285–98.

Steppuhn KG, Turski L. Diazepam dependence prevented by glutamate antagonists. Proc Natl Acad Sci USA 1993;90:6889–93.
Tozzi A, Tscherter A, Belcastro V, Tantucci M, Costa C, Picconi B, et al. Interaction of A_{2A} adenosine and D_2 dopamine receptors modulates corticostriatal glutamatergic transmission. Neuropharmacology 2007;53:783–9.
Tsuda M, Shimizu N, Suzuki T. Contribution of glutamate receptors to benzodiazepine withdrawal signs. Jpn J Pharmacol 1999;81:1–6.
Wafford KA. $GABA_A$ receptor subtypes: any clues to the mechanism of benzodiazepine dependence? Curr Opin Pharmacol 2005;5:47–52.