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Diazepam fails to potentiate GABA-induced chloride uptake and to produce anxiolytic-like action in aged rats

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Abstract

The pharmacological response to benzodiazepines has been demonstrated to be different in aged individuals in comparison to adults. We studied the age-dependent changes in some of the in vitro and behavioral effects of diazepam in aged (24 months old) rats, comparing them to adults (3 months old). We evaluated the in vitro γ-aminobutyric acid (GABA)-induced $^{36}Cl^-$ uptake and the diazepam potentiation of GABA-stimulated $^{36}Cl^-$ uptake in microsacs from cerebral cortex of both groups of animals. We found no differences in the GABA-stimulated $^{36}Cl^-$ uptake between adult and aged animals, and diazepam failed to potentiate GABA-induced $^{36}Cl^-$ flux in the aged cortical microsacs. We also examined the effect of 0.03–10 mg of diazepam on locomotor activity in an open-field test and the anxiolytic-like action of diazepam in doses ranging from 0.03 to 1 in a dark–light transition test. We observed no anxiolytic-like action of the drug in the dark–light transition test in the aged rats, while there was a shift to the left in the diminution of locomotor activity evaluated by the open-field test. We conclude that the pharmacodynamic changes observed in cortical $GABA_A$ receptors in aged rats could partially explain the lack of anxiolytic-like action but not the oversedation evidenced in this group of animals.

Keywords: Aging; Diazepam; Sedation; Anxiolysis; GABA-induced chloride uptake; Dark–light transition test; Open-field test

1. Introduction

Benzodiazepines constitute a group of drugs that exert, in slightly varying degrees, five major actions: anxiolytic, anticonvulsant, muscular relaxant, hypnotic and amnesic. Since its discovery, and in part due to its safety and efficacy in the treatment of insomnia and anxiety disorders, this group of drugs became one of the most frequently prescribed all over the world. While in many countries the prescription of benzodiazepines decreased (Ashton, 1994), many reports show that elderly population remain disproportionately represented among users (Dunbar et al., 1989; Greenblatt et al., 1975; Koenig et al., 1987; Morgan, 1990; Morgan et al., 1988). These findings may be explained by the fact that insomnia is a frequent complaint among aged people. Up to 40% of individuals over 65 years old report disturbed sleep and this may account for the increased intake of hypnotics in this subpopulation.

Benzodiazepines exert their effect by binding to a specific site in the macromolecular complex of the γ-aminobutyric acid (GABA) receptor. These compounds allosterically potentiate the activity of subsaturating concentrations of GABA and enhance the uptake of chloride into the neuron, the consecutive hyperpolarization and therefore its inhibitory action.

An increased sensitivity to hypnotic doses of benzodiazepines has been evidenced both in aged animals and humans together with higher incidence of confusional states and ataxia, which in turn have been implicated in falls and fractures (Ashton, 1994).

Although aging has been associated with pharmacokinetic changes in the disposition and elimination of benzodiazepines, some authors (Greenblatt and Shader, 1990;

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Greenblatt et al., 1980; Pomara et al., 1989) demonstrated that hypersensitivity would not occur in a fashion entirely dependent on plasma or spinal fluid drug concentration. In contrast, pharmacodynamic changes could be responsible for such different effects.

So, the main goal of our work was to examine the in vitro response to GABA and to diazepam during aging and to correlate it with two parameters of its behavioral effect: the diminution in locomotor activity and the anxiolytic-like action.

2. Materials and methods

We used adult (3 months old, weighting 150–180 g) and aged (24 months old, weighting 400–500 g) male Wistar rats. All the animals were housed in groups of three or four per cage with free access to food and water. Cages were in a room with a 12-h light/dark cycle (lights on 7:00 a.m., off at 7:00 p.m.) with constant temperature.

2.1. Experimental procedures

2.1.1. In vitro effect of diazepam on the potentiation of GABA-stimulated $^{36}Cl^-$ uptake in microsacs of cerebral cortex

GABA-stimulated $^{36}Cl^-$ influx was measured by a radiotracer ion-uptake method (Allan and Harris, 1987).

2.1.1.1. Membrane preparation. Tissues from adult and aged animals were always processed in parallel. The animals were sacrificed by decapitation, and the cerebral cortex was dissected out on a Petri dish at 0°C. Briefly, cerebral cortex was homogenized in 40 volumes of buffer (0°C) containing 20 mM of Hepes–Tris, 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (pH 7.4) using a Potter Elvehjem homogenizer with a Teflon pestle (six strokes). The homogenate was centrifuged at $1000 \times g$ for 15 min and after discarding the supernatant, the pellet was resuspended in the same volume of buffer and washed once more. Aliquots of 200 μl of a membrane suspension containing 10 mg/ml of total proteins were preincubated in a shaking water bath for 15 min at 30°C.

2.1.1.2. GABA-induced $^{36}Cl^-$ uptake. Following membrane preincubation, uptake was initiated by the addition and immediate vortexing of 200 μl of solution of GABA in concentrations ranging from 1×10^{-7} to 5×10^{-4} M and 0.5 μCi $^{36}Cl^-$. Chloride uptake was stopped 3 s later with 4 ml of ice-cold buffer containing 0.1 mM picrotoxinin and filtered through Whatman GF/C filters (presoaked with 0.05% polyethylenimine to reduce nonspecific binding). The filters were washed three times with 4 ml of ice-cold buffer and the $^{36}Cl^-$ retained was determined by liquid scintillation spectrometry. The amount of $^{36}Cl^-$ bound to the filters in the absence of GABA (basal chloride uptake) was subtracted from all values to obtain specific chloride uptake induced by GABA. As shown below, as basal and maximum absolute values were not statistically different, results were expressed as percentage of the maximum response.

2.1.1.3. Diazepam potentiation of GABA-stimulated $^{36}Cl^-$ uptake. Membranes from cerebral cortex were prepared and preincubated as described above. Following membrane preincubation, uptake was initiated by the addition and immediate vortexing of 200 μl of a solution containing a fixed concentration of GABA of 5×10^{-6} M (which proved to be equally effective for adult and aged animals in the experiments described in Section 2.1.1.2), 0.5 μCi $^{36}Cl^-$ and diazepam in concentrations ranging from 5×10^{-8} to 2.5×10^{-4} M. All the experiments were performed in triplicates and included three tubes in which the solution of GABA and $^{36}Cl^-$ did not contain diazepam (GABA-stimulated $^{36}Cl^-$ uptake). Results were expressed as percentage of the GABA-stimulated $^{36}Cl^-$ uptake.

2.1.2. In vivo response to diazepam

Behavioral effects of diazepam were assessed 30 min after the injection [intraperitoneal (ip)] of the drug. Control animals received vehicle (polyethyleneglycol/water, 55:45). Previous experiments showed no differences between saline and polyethyleneglycol injections in an open-field test (data not shown). The volume injected was 1 ml/kg. All behavioral tests were performed in the lights-on period, between 10:00 a.m. and 1:00 p.m.

2.1.2.1. Open-field test. The doses tested ranged from 0.03 to 10 mg/kg of diazepam. The open-field apparatus consisted of a wooden cage (1×1 m), with the floor painted in gray and divided by black lines into 10×10 cm squares. Illumination was low in order to avoid novelty-induced stress. At the beginning of the test, the animals were placed in one corner of the cage and the number of lines crossed with all four paws during 5 min was recorded (Introini-Collinson et al., 1987).

2.1.2.2. Dark–light transition test. We used the two-chamber exploratory behavior model (Cancela et al., 1995). The testing apparatus consisted of a square wooden cage ($60 \times 90 \times 90$ cm) divided in the middle to provide both an illuminated and a dark chamber. Its floor was painted in gray. Environmental light from a desk lamp provided the only illumination for the lighted chamber, and a red lamp illuminated the dark enclosure. The rats were always placed in the lit chamber to start the test. A transition was recorded when the animal crossed with all four paws from the dark to the illuminated area. The amount of time spent in the lit chamber was also measured. The total observation time for each animal was 10 min.

2.1.3. Statistical calculations

Results were expressed as mean ± S.E.M. Comparisons between groups were assessed by a two-way ANOVA followed by Tukey test when $P < .05$.

2.1.4. Drugs

Diazepam was kindly provided by Roche Laboratories (Argentina). ^{36}Chloride (specific activity 14.58 mCi/g) was from New England Nuclear. GABA, picrotoxinin and HEPES [*N*-2-hydroxiethyl piperazine-*N*′-(2-ethanesulfonic acid)] were from Sigma (St. Louis, MO).

3. Results

3.1. In vitro effect of diazepam on the potentiation of GABA-stimulated $^{36}Cl^-$ uptake in microsacs of cerebral cortex

3.1.1. GABA-induced $^{36}Cl^-$ uptake

Mean values ± S.E.M. of basal uptake (without GABA) were not different between groups: 74 ± 7 and 52 ± 3 pmol/mg of protein for adults and aged rats, respectively (Student's *t* test, $P = .06$). The effect of increasing concentrations of GABA on $^{36}Cl^-$ uptake was calculated as the difference between the uptake for each concentration and the basal values of each experiment. Maximum response to GABA was not different between groups either (mean ± S.E.M., 156 ± 27 vs. 107.9 ± 6 pmol/mg of protein for adult and aged rats, respectively; Student's *t* test, $P = .9$). The results in the concentration–response curve (Fig. 1) are expressed as percentage of the maximum response for each group. There were no age-attributable differences in the curves, assessed by a two-way ANOVA ($P = .6945$). EC_{50} were 0.9×10^{-5} and 1×10^{-5} M for the adult and aged groups, respectively. A concentration of 5×10^{-6} M of GABA was chosen for the diazepam potentiation experiments.

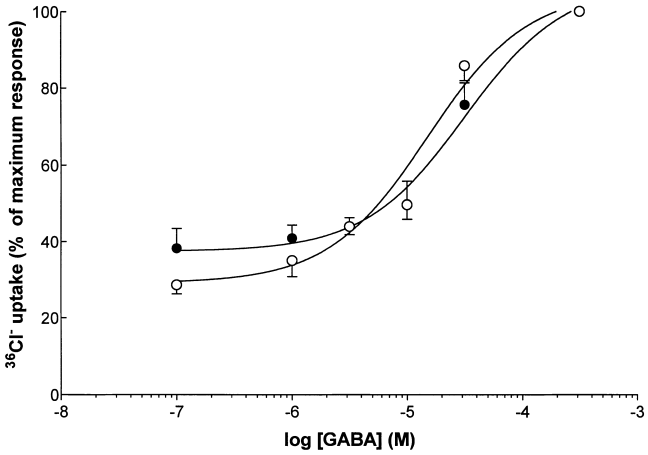

Fig. 1. $^{36}Cl^-$ uptake in microsacs of cerebral cortex: Dose–response curve to GABA in concentrations ranging from 1×10^{-7} to 5×10^{-4} M. Membrane preparations from aged (●) and adult (○) rats were tested. Results are expressed as mean ± S.E.M. of percentage of maximum response ($n = 5–7$ animals per group). Comparisons were performed using a two-way ANOVA test. No significant differences were found between groups.

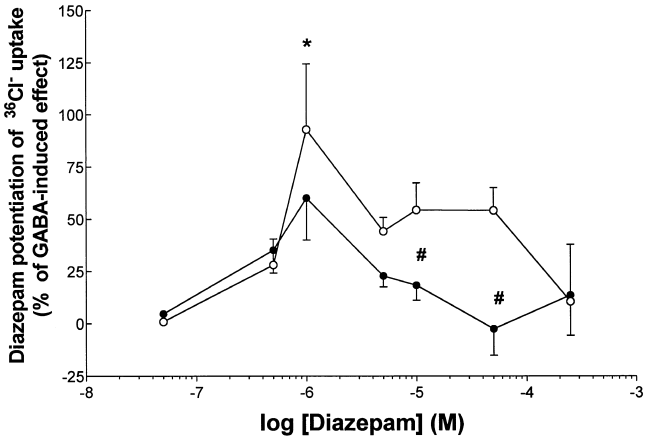

Fig. 2. Diazepam potentiation of GABA-stimulated $^{36}Cl^-$ uptake: Effect of different concentrations of diazepam ranging from 5×10^{-8} to 2.5×10^{-4} M on the $^{36}Cl^-$ uptake induced by 5×10^{-6} M of GABA. Membrane preparations from aged (●) and adult (○) rats were tested. Results are expressed as mean ± S.E.M. of percentage of potentiation of GABA-stimulated $^{36}Cl^-$ uptake ($n = 4–7$ animals per group). Comparisons were performed using a two-way ANOVA test followed by the Tukey test. $^*P < .05$ vs. GABA-induced effect in adult animals, $^{\#}P < .05$ vs. the same concentration of diazepam in adults.

3.1.2. Diazepam potentiation of GABA-stimulated $^{36}Cl^-$ uptake

Fig. 2 shows the dose–response curves obtained with different concentrations (5×10^{-8}–2.5×10^{-4} M) of diazepam on $^{36}Cl^-$ uptake induced by a fixed concentration of 5×10^{-6} M of GABA. Results are expressed as percentage

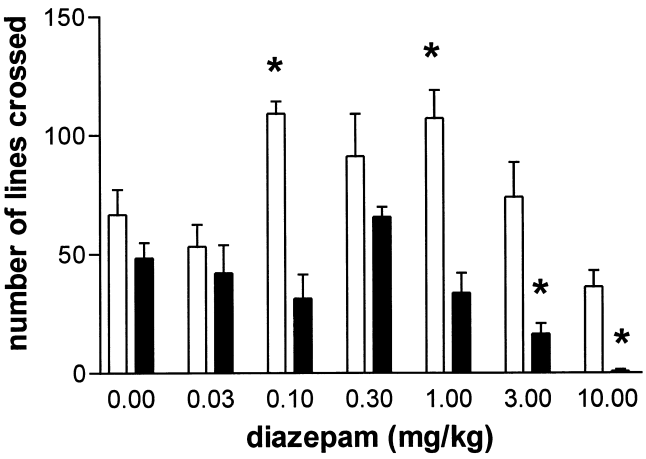

Fig. 3. Open-field test: 30 min before the test, animals were injected (intraperitoneal) with different doses of diazepam ranging from 0.03 to 10 mg/kg, and the effect on the locomotor activity was recorded on aged (filled bars) and adult (open bars) rats. Results are expressed as mean ± S.E.M. of the number of lines crossed. Comparisons among groups were performed by a two-way ANOVA followed by Tukey test. $^*P < .05$ compared with vehicle-treated animals from each group ($n = 3–9$ animals per group).

of GABA-induced $^{36}Cl^-$ uptake. Age- ($P<.05$) and concentration- ($P<.05$) dependent differences were found using a two-way ANOVA. In adult animals, a 92% increase in $^{36}Cl^-$ uptake was evidenced with 1×10^{-6} M of diazepam (Tukey test, $P<.05$ compared with GABA-induced effect in the same group). On the contrary, no significant potentiation was found in aged rats with the same diazepam concentration. Moreover, the results obtained with higher concentrations of diazepam in aged rats were significantly lower than those seen in adult ones (21.4% and 56.5% less with 5×10^{-6} and 5×10^{-5} M diazepam, respectively; $P<.05$).

3.2. In vivo response to diazepam

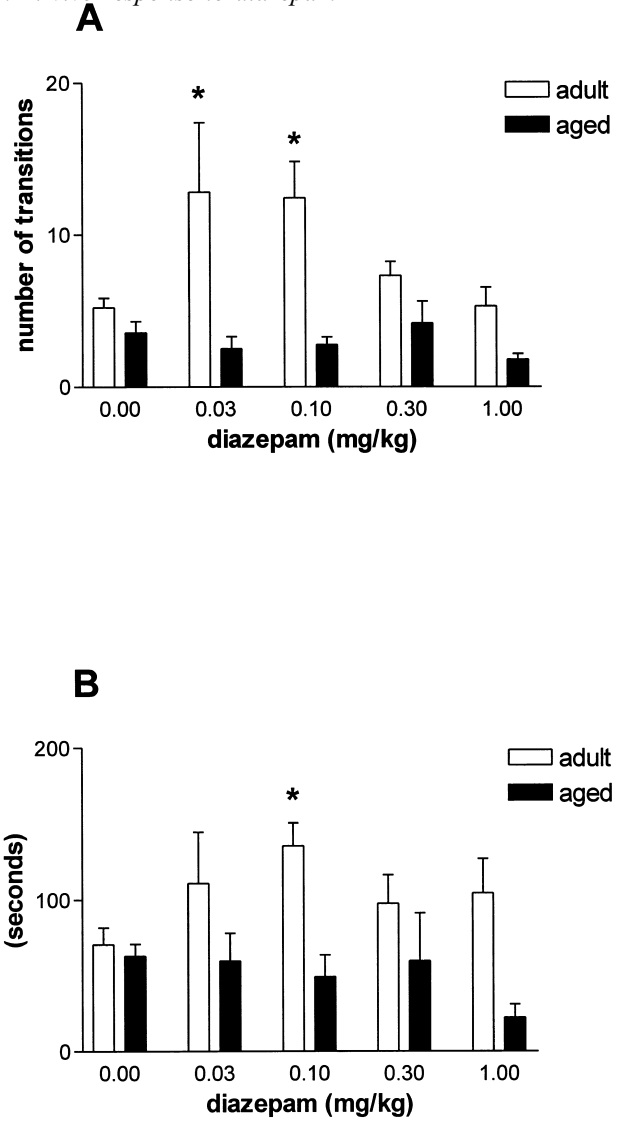

Fig. 4. Dark–light transition test: 30 min before the test, animals were injected (intraperitoneal) with different doses of diazepam ranging from 0.03 to 1 mg/kg. Panel A represents the number of transitions from the dark to the illuminated area and Panel B, the time spent in the light. Aged rats (filled bars), adult rats (open bars). Results are expressed as mean ± S.E.M. Comparisons among groups were performed by a two-way ANOVA followed by Tukey test. * $P<.05$ compared with vehicle-treated animals from each group ($n=4-15$ animals per group).

3.2.1. Open-field test

Results of this test are shown in Fig. 3. Results obtained with vehicle-treated rats were not different between groups. Diazepam (0.1 and 1 mg/kg) induced a significant increase in the locomotor activity of adult rats (63%, $P<.05$ and 60%, $P<.05$, respectively, compared to vehicle), while no changes were found in aged animals with these doses. The tendency to a diminution in the locomotor activity observed with 10 mg/kg in the adult group did not reach statistical significance. On the contrary, 3 and 10 mg/kg proved to induce a significant decrease in this parameter in the aged animals (35%, $P<.05$ and 98%, $P<.001$ with 3 and 10 mg/kg, respectively, compared to vehicle).

3.2.2. Dark–light transition test

Fig. 4 shows the results obtained for both adult and aged rats in the dark–light transition test. The test was performed with doses ranging from 0.03 to 1 mg/kg, as higher doses had induced a significant diminution in the locomotor activity in the aged rats. Results obtained after vehicle injection were similar for both adult and aged rats. In the adult group, 0.03 and 0.1 mg/kg of diazepam increased the number of transitions (Fig. 4A; 145%, $P<.05$ and 137%, $P<.05$, respectively, compared to adult control rats). Similarly, 0.1 mg/kg increased the time spent in the illuminated area (Fig. 4B; 92%, $P<.05$, compared to adult control rats). However, none of the doses tested produced any effect in the aged animals.

4. Discussion

In our work, we investigated the in vitro effect on $^{36}Cl^-$ uptake of diazepam and two behavioral responses to the drug such as the diminution of the locomotor activity and the anxiolytic-like action in aged rats.

Although we found no statistical differences in the cortical $^{36}Cl^-$ uptake in response to increasing concentrations of GABA alone (Fig. 1), a tendency towards an age-dependent decrease was observed in both the basal and the maximum values of the concentration–response curve. Basal and GABA-induced $^{36}Cl^-$ uptake in cortical membranes of aged rodents was previously reported with contradictory results. Some authors found an increase in basal (i.e. nonstimulated) values but a shift to the right in the GABA curve (Concas et al., 1988). Nevertheless, other investigators observed, in agreement with our findings, no changes in the response to muscimol, a specific $GABA_A$ agonist (Barnhill et al., 1990).

On the other hand, a different stimulation pattern was observed in aged rats compared to adult controls when the diazepam potentiation of GABA-induced $^{36}Cl^-$ uptake was assessed (Fig. 2). In adult rats, there was a significant potentiation of the $^{36}Cl^-$ flux with 1×10^{-6} M of diazepam, and higher concentrations maintained this tendency in spite of not reaching statistical significance. This potentia-

tion is in accordance to that shown in a similar work (Yu et al., 1988), in which 1×10^{-6} M diazepam exerted an enhancement of 75% in the chloride influx compared to that obtained with GABA alone. The biphasic shape of the curve evidenced in our results was similar to what has been previously reported with pentobarbital and flunitrazepam (Ikeda et al., 1989; Schwartz et al., 1986) and is in agreement with other works, which demonstrated that high concentrations of some benzodiazepines decreased GABA-induced hyperpolarization. This bell-shaped curve has been attributed to an heterologous desensitization between muscimol and pentobarbital (Nistri and Berti, 1984). On the other hand, diazepam failed to potentiate the GABA-induced $^{36}Cl^-$ flux in aged rats. Moreover, with the concentrations of 5×10^{-6} and 5×10^{-5} M diazepam, the potentiation obtained in aged rats was significantly lower than that seen in adult rats. As far as we know, there are no previous studies on the in vitro effect of benzodiazepines on the GABA-induced chloride uptake in microsacs from aged rats, but other authors observed a decrease in pentobarbital potentiation of $^{36}Cl^-$ uptake in cortical microsacs of aged animals (Concas et al., 1988). At least two phenomena could explain these results. On the one hand, our results may reflect that diazepam, acting at its binding site in the $GABA_A$ receptor, exerts a lower effect in the aged cerebral cortex than that seen in the adult brain. On the other hand, as experiments were performed at a fixed 3-s point, it cannot be ruled out if a more rapid desensitization occurs in $GABA_A$ receptor's response to diazepam potentiation. Changes in the benzodiazepines in vitro effect such as these could be caused by modifications in the number or affinity of benzodiazepines binding sites. While many authors failed to find age-associated changes in these parameters (Pedigo et al., 1981; Ruano et al., 1996; Sieghart et al., 1983; Tsang et al., 1982), some other investigators reported an age-dependent diminution in the affinity of diazepam binding sites in cortical membranes (Barnhill et al., 1990). Molecular modifications in the $GABA_A$ receptor could be associated with these neurochemical changes.

$GABA_A$ receptors show different multisubunit composition. In recombinant receptors, it has been observed that the best response to benzodiazepines was obtained when GABA receptor possessed α, β and γ subunits. Particularly, α_1 or α_3 subunits conferred the maximum response to benzodiazepines (Huh et al., 1995, 1996), while the presence of α_4 subunits rendered benzodiazepine-insensitive $GABA_A$ receptors (Huh et al., 1996; Knoflach et al., 1996). Age-dependent changes in the $GABA_A$ receptor function and in its α-subunit isoform expression have been reported (Gunnersen et al., 1996; Gutiérrez et al., 1996). However, the functional consequences of these changes remain controversial. While some authors failed to find changes in the allosteric interaction between GABA and benzodiazepines (Ruano et al., 1996), other works demonstrated that a diminution in α_2-subunit mRNA but not in α_1 mRNA underlies a decrease in the binding of *t*-butyl-bicyclophosphonothionate (a specific ligand for Cl^- channels) to $GABA_A$ receptors in aged cerebellum (Mhatre and Ticku, 1998). Our results may suggest that a shift from a benzodiazepine-sensitive to a benzodiazepine-insensitive $GABA_A$ receptor could take place during aging in cerebral cortex, and that this may influence both the in vitro and in vivo pharmacological responses.

In fact, when we investigated the in vivo response to the drug, we observed an absence of anxiolytic-like action in the dark–light transition test in the aged animals (Fig. 4), an effect that has not been previously observed. This test is suitable for the study of the anxiolytic action of a drug on a naturally occurring behavior. Animals with nocturnal habits prefer the dark area of the apparatus but, under the effect of diazepam or other anxiolytic compounds, they exhibit a two to threefold increase in the number of transitions from the dark to the illuminated area and spend more time in the light (Chaouloff et al., 1997; Costall et al., 1992; Crawley, 1981). Lack of the anxiolytic activity of diazepam in the aged animals with doses ranging from 0.03 to 1 mg/kg of diazepam seems not to be due to the sedative action of the drug, as we demonstrated in the open-field test that aged rats were sensitive to the sedative action of diazepam in doses of 3 mg/kg or higher (Fig. 3).

It is tempting to suggest that there could be a relationship between the absence of anxiolytic-like effect of diazepam and the diminution of the in vitro diazepam potentiation of GABA-stimulated $^{36}Cl^-$ influx in aged rats, caused in turn by cortical changes in $GABA_A$ receptor subunit expression.

On the other hand, when the open-field test was performed in aged rats, we observed a shift to the left in the dose–effect curve of diazepam-induced diminution of locomotor activity, compared to adults (Fig. 3). An exacerbation of benzodiazepine-induced sedation in the aged population has been previously reported (Ashton, 1994; Barnhill et al., 1990; Dunbar et al., 1989; Greenblatt et al., 1980). This increased in vivo response could be related to pharmacokinetic or to pharmacodynamic modifications in aged subjects. It is well known that aging is associated with an increase in plasma and brain elimination half-life (Barnhill et al., 1990; Greenblatt and Shader, 1990; Greenblatt et al., 1975). However, peak plasma and brain concentrations, as observed 30 min after intraperitoneal injection, are not modified by aging (Barnhill et al., 1990). So, it seems likely that pharmacodynamic rather than pharmacokinetic modifications should explain the oversedating effect of one dose of diazepam tested 30 min after administration.

If our previous speculation is correct, this excessive sedation may correlate with changes in $GABA_A$ receptor subunits, which cause an increased sensitivity to diazepam. As we have found a decreased response to diazepam in cerebral cortex, we suggest that other areas involved in the sedative action of benzodiazepines (e.g., locus coeruleus and raphe nuclei) may be expressing supersensitive $GABA_A$ receptors. This hypothesis should be experimentally evaluated. Moreover, two recent works demonstrated that the

mutation of the α_1 subunit of the $GABA_A$ receptor has a different impact on the anxiolytic and sedative effects of diazepam. Particularly, it was observed that while diazepam continues to exert its anxiolytic-like action, the sedative effect was not preserved (McKernan et al., 2000; Uwe et al., 1999). So, it could be hypothesized that age-dependent changes in $GABA_A$ receptor composition could affect sedation and anxiolysis in a different way.

In summary, we have demonstrated that the behavioral effect of diazepam in aged animals shows a predominantly sedative profile, without anxiolytic action evaluated in a dark–light transition test. The latter could be correlated with the lack of diazepam-induced potentiation of GABA-stimulated $^{36}Cl^-$ uptake, evidenced in cerebral cortex of aged rats. On the contrary, we could not correlate the age-dependent oversedation observed in vivo with in vitro cortical changes; this should be further investigated in other areas of the central nervous system.

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