Coll. Antropol. **27** Suppl. 1 (2003) 175–182
UDC 591.1:577.175.8
Original scientific paper

Effect of Ergot Alkaloids on ^{3}H-Flunitrazepam Binding to Mouse Brain $GABA_A$ Receptors

Ante Tvrdeić[1] and Danka Peričić[2]

[1] Department of Pharmacology, School of Medicine, University of J. J. Strossmayer Osijek, Osijek, Croatia

[2] Laboratory for Molecular Neuropharmacology, »Ruđer Bošković« Institute, Zagreb, Croatia

A B S T R A C T

In vitro effects of dihydroergotoxine, dihydroergosine, dihydroergotamine, α-dihydroergocriptine (ergot alkaloids), diazepam, methyl-β-Carboline-3-carboxilate (β-CCM), flumazenil (benzodiazepines), γ-amino butyric acid (GABA) and thiopental (barbiturate) were studied on mouse brain (cerebrum minus cerebral cortex) benzodiazepine binding sites labeled with ^{3}H-flunitrazepam. Specific, high affinity (affinity constant, K_d = 57.7 8.6 nM) binding sites for ^{3}H-flunitrazepam on mouse brain membranes were identified. All benzodiazepine drugs inhibited ^{3}H-flunitrazepam binding with nanomolar potencies. In contrast to benzodiazepines, all ergot drugs, GABA and thiopental produced an enhancement of ^{3}H-flunitrazepam binding to its binding site at the $GABA_A$ receptor of the mouse brain. The rank order of potency was: neurotransmitter (GABA) > dihydroergotoxine > thiopental > α-dihydroergocriptine > dihydroergosine > dihydroergotamine. The results suggest that dihydrogenated ergot derivatives do not bind to the brain benzodiazepine binding sites labeled with ^{3}H-flunitrazepam. However, an enhancement of ^{3}H-flunitrazepam binding by all ergot drugs tested, clearly identifies an allosteric interaction with the benzodiazepine binding sites of $GABA_A$ receptors.

Key words: *ergot drugs, ^{3}H-flunitrazepam, $GABA_A$, mouse.*

Introduction

The $GABA_A$ receptor is a ligand gated chloride channel, an ionothropic receptor that is opened after release of γ-amino butyric acid (GABA) from presynaptic neuron and binding of GABA to neurotransmitter recognition site[1]. It contains neurotransmitter binding site, the benzodiazepine modulatory center with binding sites for anxiolytic and anxiogenic compounds, the picrotoxin/convulsant bind-

Received for publication January 7, 2003

ing site, the barbiturate binding site and the steroid binding site that seems to mediate rapid, nongenomic effects of neuroactive steroid hormones in the brain[2,3]. The recognition sites for other classes of compounds on $GABA_A$ receptors were also hypotesized[2]. Ergot drugs have been used clinically in many settings: as diagnostics, cognition enhancers and in the management of orthostatic hypotension. The primary uses of ergot alkaloids today are limited to treatment of postpartum hemorrhage and migraine. To a varying degree these drugs act at peripheral or brain α-adrenergic, dopaminergic and serotonergic receptors[4]. The results of behavioral experiments indicate that these drugs might be also active at brain $GABA_A$ receptors. Dihydroergotoxine and dihydroergosine, for example, affect the occurrence and latency of convulsions produced by antagonists of $GABA_A$ receptors[5,6]. Besides, these ergot compounds prolonged pentobarbital induced sleeping time in mice and produced anticonflict effect in rats[7,8]. A more direct interaction of dihydrogenated ergot derivatives with brain $GABA_A$ receptors was suggested by receptor binding experiments. Ergot alkaloids non-competitively displaced the binding of ^{3}Ht-butyl-bicycloorthobenzoate (TBOB), a compound that labels picrotroxin/convulsant binding site of $GABA_A$ receptors, with IC_{50} values comparable or even lower (2.5 times, dihydroergotoxine) than that of GABA[7,9]. Moreover, GABA enhanced the affinity of dihydroergotoxine for ^{3}H- TBOB binding to mouse brain $GABA_A$ receptors by two orders of potency and this effect was completely abolished by $GABA_A$ receptor competitive antagonist bicuculline. The authors suggested that dihydroergotoxine binds to an unidentified recognition site of the brain $GABA_A$ receptor complex, other than that labeled with ^{3}H-TBOB, to produce its aforementioned behavioral actions[7]. To test the hypothesis whether benzodiazepine binding site is target for ergot derivatives at brain $GABA_A$ receptors, we studied the effects of dihydroergotoxine, α-dihydroergocriptine, dihydroergosine and dihydroergotamine, benzodiazepine receptor ligands (diazepam, β-CCM, flumazenil), GABA and barbiturate (thiopental) on ^{3}H-flunitrazepam binding to mouse brain (cerebrum minus cerebral cortex) membranes. The brain region was chosen since our results using ^{3}H-TBOB as a ligand have shown the greatest binding affinity of dihydroergotoxine (the most potent inhibitor of ^{3}H-TBOB binding) in this brain region[6].

Material and Methods

Animals

Female CBA/HZgr mice from »Ruđer Bošković« Institute, Zagreb, Croatia, weighing 20–25 g were used. They were housed at a constant temperature (22 °C) and under a light cycle of 11h light/13 h darkness (lights on at 7:00 a.m.). Food and water were freely available.

Drugs

Dihydroergotoxine methane sulfonate, dihydroergosine methane sulfonate, α-dihydroergocriptine methane sulfonate and dihydroergotamine methane sulfonate, all from Lek, Ljubljana, Slovenia, were used. GABA and diazepam were from Sigma, St. Louis, MO. Flunitrazepam, flumazenil and β-CCM were from Hoffman – La Roche, Basel. Thiopental sodium was from Byk Gulden, Konstantz. ^{3}H-flunitrazepam (specific activity 85 Ci/ mmol) was purchased from Amersham.

Preparation of the membranes

Synaptic membranes were prepared from the mouse brain according to a method previously described[10]. Briefly, the brains (cerebrum minus cortex) from four mice were pooled and homogenized in 20 volumes of ice – cold 50mM Tris citrate

buffer, pH = 7.4. After centrifugation at 10,000 ms^{-2} for 10 min, the pellet was discarded and the supernatant centrifuged again at 120,000 ms^{-2} for 20 min. The second pellet was resuspended and centrifuged under the same conditions two more times. The resultant pellet was resuspended and suspension frozen at –20 °C for 24 hours. After 24 hours suspension was thawed at room temperature and centrifuged again as above. Freeze – thaw – centrifugation cycle was repeated to remove endogenous GABA. The final pellet was obtained by centrifugation at 170,000 ms^{-2} for 20 min and resuspended in 40 volumes of 50 mM Tris citrate buffer containing 250 mM NaCl (pH = 7.4 at 37 °C) to give a protein concentration of ~0.7 mg/mL. Protein concentration was determined as described by Lowry et al[11].

^{3}H-flunitrazepam binding assay

^{3}H-flunitrazepam binding assay was performed according to the method of Zarkovsky[12]. To determine the concentration of drug required to displace 50% of ^{3}H-flunitrazepam from receptor sites, $IC_{50,}$ a single concentration of ^{3}H-flunitrazepam (0.05 mL, 1 nM final concentration), the various concentrations of unlabeled drugs (0.05 mL), 100 μM diazepam (0.05 mL) to define non-specific binding and 0.05 mL of assay buffer (50 mM Tris citrate + 250 mM NaCl, pH = 7.4 at 37 °C) or drug solvent were incubated with 0.3 mL of the synaptosomal membrane suspension for 30 minutes at 37 °C. To determine the number of ^{3}H-flunitrazepam binding sites, B_{max}, and the ligand dissociation constant, K_d, hot-cold dilution binding assays were performed – the same compound, flunitrazepam, was used as labeled and unlabeled ligand. Assay conditions were, in general, the same as aforementioned, except the concentrations of flunitrazepam ranged from 0.1–1,000 nM. In any case, incubation was stopped by filtration of 0.5 mL (final volume) incubation mixture through Whatman GF/C filters. The filters were rapidly rinsed with 10 mL of assay buffer, transferred to counting vials and dried. After addition of scintillation cocktail (toluene, PPO, POPOP), the radioactivity retained in the filters was counted by liquid scintillation counter at 40–45% efficiency. Specific ^{3}H-flunitrazepam binding was defined as the difference between binding in the absence and presence of diazepam and was 75–85% of the total binding. Binding data were analyzed using a computer-based equilibrium binding data analysis (EBDA) program[13]. EBDA calculates K_d, B_{max} and IC_{50} values from binding data. In the case of enhancement of ^{3}H-flunitrazepam binding, it is not possible to use EBDA program. Therefore, we used another computer-based program[14] to calculate the EC_{50} values (the concentration of drug required for the half of the maximum enhancement) from the linear portion of the enhancement curve. E_{max} is the maximum enhancement of radioligand binding observed in the presence of drug over the control value (100% specifically bound 1 nM ^{3}H-flunitrazepam without drug). The data, expressed as the mean standard error of the mean (SEM), were subjected to two way analyses of variance (ANOVA) followed, if significant, with Newman – Kuels multiple comparison procedure. P values of less than 0.05 were considered significant.

Results

^{3}H-flunitrazepam binding affinity, density and pharmacological specificity

Analysis of hot-cold dilution binding data revealed a mean dissociation constant (K_d) of 57.7 ± 8.6 nM and a mean maximum receptor density (B_{max}) of 0.485 ± 0.130 pmol/mg protein for ^{3}H-flunitrazepam binding sites at mouse brain $GABA_A$ receptors (Table 1). All benzodiazepine ligands displaced ^{3}H-flunitra-

TABLE 1
DISSOCIATION CONSTANT, K_D, AND A MAXIMUM RECEPTOR DENSITY, B_{MAX}, FOR ^{3}H-FLUNITRAZEPAM BINDING SITES AT MOUSE BRAIN $GABA_A$ RECEPTORS

	K_d (nM) X ± SEM	B_{max} (pmol/mg protein) X ± SEM	Number of experiments*
^{3}H-flunitrazepam	57.7 ± 8.6 nM	0.485 ± 0.130	3

* The brains (cerebrum minus cortex) from four mice were pooled and used in each separate experiment

zepam binding in a concentration dependent manner and with nanomolar potency (Table 2). Benzodiazepine receptor antagonist flumazenil was the most potent occupying agent (IC_{50} = 6.3 ± 2.5 nM), followed by full agonist diazepam (IC_{50} = 28.6 ± 9.1 nM) and inverse agonist β-CCM (IC_{50} = 32.8 ± 11.6 nM).

TABLE 2
DISPLACEMENT POTENCIES OF BENZODIAZEPINE RECEPTOR LIGANDS ON ^{3}H-FLUNITRAZEPAM BINDING TO MOUSE BRAIN $GABA_A$ RECEPTORS

	IC_{50} (nM) X ± SEM	Number of experiments*
Flumazenil	6.3 ± 2.5	3
Diazepam	28.6 ± 9.1	3
β-CCM	32.8 ± 11.6	3

* The brains (cerebrum minus cortex) from four mice were pooled and used in each separate experiment. IC_{50} is the molar concentration of drug required to displace 50% of ^{3}H-flunitrazepam from specific binding sites.

The effect of ergot drugs on ^{3}H-flunitrazepam binding

In contrast to benzodiazepines, dihydroergotoxine (1 nM–90 μM; ANOVA: F (9.45) = 17.01; p<0.01), α-dihydroergocriptine (10 nM–500 μM; ANOVA: F (6,10) = 8.91; p<0.01), dihydroergosine (100 nM–1 mM; ANOVA: F (7,14) = 8.88; p<0.01) and dihydroergotamine (100 nM–900 μM; ANOVA: F (5,13) = 30.43; p< 0.01), all produced an concentration dependent enhancement of ^{3}H-flunitrazepam binding to its binding site at the $GABA_A$ receptor of the mouse brain (Table 3). The rank order of potency for ^{3}H-flunitrazepam binding enhancement was: dihydroergotoxine > α-dihydroergocriptine > dihydroergosine > dihydroergotamine. The most effective enhancer of ^{3}H-flunitrazepam binding, as judged by E_{max} values listed in Table 3, was dihydroergotamine (E_{max} = 338 ± 32 % over control value), followed by dihydroergotoxine (E_{max} = 241 ± 11 %), dihydroergosine (E_{max} = 81 ± 20 %) and α-dihydroergocriptine (E_{max} = 66 ± 5 %).

The effect of GABA and thiopental on ^{3}H-flunitrazepam binding

100 nM–1mM concentrations of GABA (ANOVA: F (8,15) = 28.19; p<0.01) and 100 nM–1 mM concentrations of thiopental (ANOVA: F (4,12) = 7.88; p<0.01) enhanced ^{3}H-flunitrazepam binding to its binding site at the $GABA_A$ receptor of the mouse brain. GABA was the most potent enhancer of ^{3}H-flunitrazepam binding (EC_{50} = 4.3 ± 1.5 μM, Table 3) among all drugs used, with EC_{50} value about 8 times lower than that of the most potent ergot drug dihydroergotoxine and about 30 times lower than that of thiopental (EC_{50} = 117.5 ± 19.4 μM, Table 3). E_{max} values of GABA and thiopental listed in Table 3 were lower than that of dihydroergotamine and dihydroergotoxine.

TABLE 3
THE ENHANCEMENT POTENCIES AND EFFICACIES OF ERGOT DRUGS, GABA AND THIOPENTAL ON ^{3}H-FLUNITRAZEPAM BINDING TO MOUSE BRAIN $GABA_A$ RECEPTORS

	EC_{50} (μM) X ± SEM	E_{max} (%) X ± SEM	Number of experiments*
GABA	4.3 ± 1.5	127 ± 3	3
Dihydroergotoxine	32.4 ± 2.6**	241 ± 11††	6
Thiopental	117.5 ± 19.4	135 ± 19	3
α-dihydroergocriptine	174.1 ± 43.9**	66 ± 5	3
Dihydroergosine	340.4 ± 64.4	81 ± 20	3
Dihydroergotamine	388.5 ± 19.1	338 ± 32††	2

* The brains (cerebrum minus cortex) from four mice were pooled and used in each separate experiment.

EC_{50} = molar concentration of drug required for 50% of the maximum observed enhancement; E_{max}) = ^{3}H-flunitrazepam binding over the control value (100% specifically bound 1 nM ^{3}H-flunitrazepam without drug, about 0.040 pmol/mg protein).

** $p < 0.01$ for dihydroergotoxine and α-dihydroergocriptine EC_{50} values against dihydroergosine and dihydroergotamine EC_{50} values, Newman Kuels test (ANOVA: F (3,10) = 26.47).

†† $p<0.01$ for dihydroergotoxine and dihydroergotamine E_{max} values against dihydroergosine and α-dihydroergocriptine E_{max} values, Newman Kuels test (ANOVA: F (3,10) = 59.31).

Discussion

^{3}H-flunitrazepam binding affinity, density and pharmacological specificity

In this kind of experiments is crucial to prove that radioligand, in our case ^{3}H-flunitrazepam, has identified the correct binding site, in our case benzodiazepine binding site at the brain $GABA_A$ receptor. Dissociation constant (K_d) and a maximum receptor density (B_{max}) values for ^{3}H-flunitrazepam listed in Table 1, are in agreement with the data[15,16] obtained under similar conditions (physiological or near physiological incubation temperature, presence of NaCl in incubation mixture, absence of detergents during membrane preparation). To further validate ^{3}H-flunitrazepam binding assay, benzodiazepine drugs with well-known potencies for benzodiazepine binding sites, GABA and thiopental were used in competitive binding experiments. Again, there is a good match between IC_{50} values for flumazenil, diazepam and β-CCM reported here (Table 2) and IC_{50} values reported elsewhere using the same drugs under similar ^{3}H-flunitrazepam binding conditions[17,18]. One of the most consistent findings on the pharmacology of $GABA_A$ receptor is the existence of several binding sites on these receptors, all of which exhibit multiple allosteric binding interactions with each other[2,19]. Since benzodiazepine/GABA/barbiturate binding sites allosteric interactions remain intact in the absence of detergents during membrane preparation[20], GABA and thiopental enhanced ^{3}H-flunitrazepam binding to mouse brain membranes (Table 3). It has been already reported that binding of benzodiazepines to the brain membranes which are not subjected to the treatment with detergents is stimulated by GABA, by depressant barbiturates and by anxiolytic, anticonvulsant and hypnotic steroids[2,21–23]. EC_{50} and E_{max} values for GABA and thiopental presented here are in rea-

sonable agreement with literature data[16,24,25]. Taking together, above mentioned results clearly suggest that ^{3}H-flunitrazepam labels high affinity benzodiazepine binding sites, the same site at the brain $GABA_A$ receptor complex by which the benzodiazepines exert their clinically important actions[1].

The effect of ergot drugs on ^{3}H-flunitrazepam binding

As shown in Results, all ergot drugs produced an enhancement of ^{3}H-flunitrazepam binding to its binding site at the $GABA_A$ receptor of the mouse brain. To our knowledge, this is the first demonstration that ergot compounds affect brenzodiazepine binding sites labeled with ^{3}H-flunitrazepam. Moreover, E_{max} values for ergot drugs listed in Table 3 are comparable or even higher (2-3 times, dihydroergotoxine and dihydroergotamine) than that for GABA or thiopental. Sometimes is difficult or even impossible to unmistakably conclude whether drug interacts directly or allosterically with binding sites of $GABA_A$ receptor complex, especially in the case of inhibition of radioactive ligand binding. On the contrary, an enhancement of binding of radioactive ligand by the compound to be investigated in any case identifies an allosteric interaction with the respective binding site[2]. Thus, the fact that all ergot compounds used in our study stimulate rather than inhibit ^{3}H-flunitrazepam binding clearly indicate an allosteric inter- action of these drugs with benzodiazepine binding site at the brain $GABA_A$ receptor. Because ergot compounds allosterically modulate ^{3}H-flunitrazepam binding to benzodiazepine binding site, we can presume that ergot drugs do not bind to the benzodiazepine recognition site at the brain $GABA_A$ receptor. The mechanisms responsible for the enhancing effect of ergot drugs on ^{3}H-flunitrazepam binding to the brain $GABA_A$ receptor in vitro could be a few. Although ergot drugs are known to have a high, nanomolar potency for brain amine receptors[26], the enhancement effect of ergot drugs on ^{3}H-flunitrazepam binding in vitro could not be explained on the basis of a non- $GABA_A$ receptor mechanism, since ^{3}H-flunitrazepam exclusively labels high affinity benzodiazepine binding sites in our experimental system. Regarding $GABA_A$ re- ceptor related mechanisms, the neurotransmitter site could also be excluded as a possible explanation of the results presented here. Namely, Hruska and Silbergerd reported that ergot alkaloids do not affect ^{3}H-GABA binding to brain $GABA_A$ receptors[26]. These results are in accordance with our unpublished data, showing very low, nearly milimolar potency of ergot drugs for ^{3}H-muscimol (GABA analogue) binding. It has been already reported that dihydrogenated ergot drugs bind with high affinity to $GABA_A$ receptor associated chloride ionophore labeled with ^{3}H-TBOB[9]. The potency for inhibition of 3H-TBOB binding sites was 2-6 times higher than that for enhancement of ^{3}H-flunitrazepam binding sites listed in Table 3. Moreover, in the presence of physiological concentration of GABA, at least one of these drugs, dihydroergotoxine, non-competitively displaced ^{3}H-TBOB binding with potency (IC_{50} = 46 nM) comparable to that reported for brain amine receptors[8]. Besides, the rank order of potency for ergot drugs on ^{3}H-TBOB inhibition[8] was the same as that for enhancement of ^{3}H-flunitrazepam binding reported here: dihydroergotoxine > α-dihydroergocriptine > dihydroergosine > dihydroergotamine (Table 3). Therefore, we can presume that binding site for ergot drugs at the brain $GABA_A$ receptor responsible for the enhancing effect of ergot drugs on ^{3}H-flunitrazepam binding is located near to picrotoxin/convulsant site. Finally, intri- guing possibility of direct interaction between ergot drugs and steroid binding site of the brain $GABA_A$ re-

ceptor could not be excluded, since binding of benzodiazepines is stimulated also by anxoiolytic, anticonvulsant and hypnotic steroids[23].

In conclusion, the results of present study suggest that dihydrogenated ergot derivatives do not bind directly to the brain benzodiazepine binding sites labeled with ^{3}H-flunitrazepam. However, these findings indicate that ergot compounds have an appreciable modulation activity at the benzodiazepine binding site labeled with ^{3}H-flunitrazepam, and affect the mouse brain $GABA_A$ receptor complex in a manner which is typical for drugs acting on this allosteric receptor complex. Therefore, the results presented here further support the hypothesis that ergot drugs interact with the brain $GABA_A$ receptor complex.

REFERENCES

1. CHARNEY, D. S., J. MIHIC, R. A. HARRIS, Hypnotics and sedatives. In: HARDMAN, J. G., L. L. LIMBIRD, A. GOODMAN GILMAN (Eds.): Goodman & Gilman's the pharmacological basis of therapeutics. (McGraw Hill, New York, 2001). — 2. SIEGHART, W., Pharmacol. Rev., 47 (1995) 181. — 3. FALKENSTEIN, E., H. C. TILLMAN, M. CHRIST, M. FEURING, M. WEHLING, Pharmacol. Rev., 52 (2000) 513. — 4. HOFMANN, B. B., Catecholamines, sympathomimetic drugs and adrenergic receptor antagonists. In: HARDMAN, J. G., L. L. LIMBIRD, A. GOODMAN GILMAN (Eds.): Goodman & Gilman's the pharmacological basis of therapeutics. (McGraw Hill, New York, 2001). — 5. PERIČIĆ, D., H. MANEV, Psychopharmacology, 80 (1983) 171. — 6. PERIČIĆ, D., H. MANEV, J. Neural. Transm., 79 (1990) 125. — 7. TVRDEIĆ, A., D. PERIČIĆ, Eur. J. Pharmacol., 221 (1992) 139. — 8. PERIČIĆ, D., A. TVRDEIĆ, Eur. J. Pharmacol., 235 (1993) 267. — 9. TVRDEIĆ, A., D. PERIČIĆ, Eur. J. Pharmacol., 202 (1991) 109. — 10. GORDON – WEEKS, P. R., Isolation of synaptosomes, growth cones and their subcellular components. In: TURNER, A. J., H. S. BACHELARD (Eds.): Neurochemistry: A practical approach. (IRL Press, Oxford, 1987). — 11. LOWERY, O. H., N. J. ROSEBROUGH, A. L. FARR, R. J. RANDALL, J. Biol. Chem., 193 (1951), 265. — 12. ZARKOVSKY, A. M., Neuropharmacology, 26 (1987) 1653. — 13. McPHERSON, G. A., Comput. Prog. Biomed., 17 (1983) 107. — 14. TALLARIDA, R. J., R. B. MURAY: Manual of pharmacologic calculationas with computer programs. (Springer Verlag, New York, 1986). — 15. WONG, P. T. H., Can. J. Physiol. Pharmacol., 69 (1991) 109. — 16. DE LOREY, T. M., G. B. BROWN, J. Neurochem., 58 (1992) 2162. — 17. MAGUIRE, P. A., H. O. VILLAR, M. F. DAVIES, G. H. LOEW, Eur. J. Pharmacol., 226 (1992) 233. — 18. BROWN, C. L., I. L. MARTIN, Eur. J. Pharmacol., 106 (1985) 167. — 19. COSTA, E., Neuropsychopharmacology, 2 (1989) 167. — 20. ENNA, S. J., S. H. SNYDER, Mol. Pharmacol., 13 (1977) 442. — 21. KAROBATH, M., G. SPERK, Proc. Natl. Acad. Sci., 76 (1979) 1004. — 22. THYAGARAJAN, R., R. RAMNJANEYULU, M. K. TICKU, J. Neurochem., 41 (1983) 578. — 23. GEE, K. W., Mol. Neurobiol., 2 (1988) 291. — 24. BUREAU, M. H., R. W. OLSEN, J. Neurochem., 61 (1993) 1479. — 25. PRICE, R. J., M. A. SIMMONDS, Neurosci. Lett., 135 (1992) 273. — 26. HRUSKA, R. E., E. K. SILBERGELD, J. Neurosci., 6 (1981) 1.

A. Tvrdeić

Department of Pharmacology, School of Medicine, University of J. J. Strossmayer Osijek, J. Huttlera 4, 31000 Osijek, Croatia

UČINAK ^{3}H-FLUNITRAZEPAMA ZA GABA$_A$ RECEPTORE IZ MOZGA MIŠA

S A Ž E T A K

U in vitro uvjetima istraživani su učinci dihidroergotoksina, dihidroergozina, dihidroergotamina, α-dihidroergokriptina (ergot alkaloidi), diazepama, metil-β-karbolin-3-karboksilata (β-CCM), flumazenila (benzodiazepini), γ-amino maslačne kiseline (GABA) i tiopentala (barbiturat) na vezno mjesto za benzodiazepine obilježeno ^{3}H-flunitrazepamom iz mozga miša (veliki mozak bez kore). Na membranama pripremljenim iz mozga miša identificirano je specifično vezno mjesto, visokog afiniteta (konstanta afiniteta $K_d = 57.7 \pm 8.6$ nM) za ^{3}H-flunitrazepam. Svi benzodiazepini su inhibirali vezanje ^{3}H-flunitrazepama sa nanomolarnom potencijom. Za razliku od njih, svi ergot alkaloidi, GABA i tiopental su povećavali vezanje ^{3}H-flunitrazepama za njegovo vezno mjesto na GABA$_A$ receptorima iz mozga miša. Redoslijed potencije za taj učinak je bio: neurotransmiter (GABA) > dihidroergotoksin > tiopental > α-dihidroergokriptin > dihidroergozin > dihidroergotamin. Spomenuti rezultati sugeriraju da se ergot alakloidi ne vezuju za vezno mjesto za benzodiazepine obilježeno ^{3}H-flunitrazepamom iz mozga miša. Međutim, povećanje vezanja ^{3}H-flunitrazepama sa svim ergot alkaloidima upotrebljenim u ovom istraživanju jasno ukazuje na alosteričku interakciju ergot alkaloida sa veznim mjestom za benzodiazepine na GABA$_A$ receptorima.