Amino Acids (1994) 6: 155–163

Amino
Acids

$GABA_A$ receptors on rat cerebellar granule cells are potently activated by muscimol but only slightly modulated by the benzodiazepine agonist flunitrazepam

P. Baldelli[1], **A. Cupello**[2], and **M. Robello**[1]

[1] Dipartimento di Fisica dell'Università, Genova, Italy
[2] Centro di Neurofisiologia Cerebrale, C.N.R. Genova, Italy

Accepted September 3, 1993

Summary. $GABA_A$ receptors present on rat cerebellar granule cells in culture were studied by the whole cell patch clamp technique. Muscimol appeared to be more potent than GABA itself in activating Cl^- currents. A benzodiazepine, flunitrazepam, only slightly (10%) potentiated the GABA action.

These results support the previous suggestion that $GABA_A$ receptors containing the δ subunit, such as those in the cerebellum granule cells, are potently activated by muscimol. The present results also bear out the concept that GABA action on receptors containing the δ subunit is not potentiated by benzodiazepines.

Keywords: Amino acids – Granule cells – $GABA_A$ receptors – Muscimol – Flunitrazepam

Introduction

The $GABA_A$ receptor with its intrinsic Cl^- channel is made up of 5 polypeptides arranged in a pentameric supramolecular complex (Olsen and Tobin, 1990; De Lorey and Olsen, 1992). The two main subunits α and β have been the first ones to be cloned and sequenced (Schofield et al., 1987). Since then, other subunit classes and variants within the classes have been discovered (De Lorey and Olsen, 1992).

Among these are six α, four β, three γ, δ and ρ members. The γ_2 subunit appears to be of particular importance for the pharmacological properties of the receptor. In fact, the presence of such a polypeptide in the receptor oligomer gives it the ability to be modulated by benzodiazepines (Pritchett et al., 1989). It has been observed that the γ_2 and δ subunit mRNA's are present in different neuronal subpopulations (Shivers et al., 1989).

A clear cut example is represented by the granule cells in the rat cerebellum, which are devoid of the mRNA for the γ_2 subunit but which contain a high amount of the δ subunit mRNA (Shivers et al., 1989).

We now report experiments aimed to characterize, by patch clamp analysis, the $GABA_A$ receptors of the rat cerebellum granule cells in terms of their activation by GABA and muscimol and their modulation by a benzodiazepine, flunitrazepam.

Materials and methods

Granule cells were prepared from 8-day-old Wistar rat cerebella following the procedure of Levi et al., 1984 and suspended in Basal Eagle's medium with Earle's salts supplemented with 10% fetal calf serum (Gibco Bio-Cult Ltd, U.K.), 25 mM KCl, 2 mM glutamine and 100 μg/ml gentamicine. They were plated on poly-L-lysine-coated glass coverslips placed in 35 mm plastic dishes at a density of 1.8×10^6 per dish, and kept at 37°C in a humidified 95% air/5% CO_2 atmosphere. Experiments were performed between days 5 and 12 after plating.

Membrane currents were measured with the standard whole-cell patch-clamp technique. Patch electrodes were manufactured from borosilicate glass capillaries (Type 1406129 Hilgenberg, Malsfeld, Germany) with a Sachs and Flaming puller (model PC-84). The currents were amplified by an EPC-7 (List-Electronic, Darmstadt, Germany). Both stimulation and data acquisition were performed with a Labmaster board driven by pCLAMP software (Axon Instrument, Burlingame, CA). Data were fitted to models using the software Asystant (Asyst Software Technologies, Rochester, NY). All experiments were performed at room temperature.

In all experiments the external solution contained (mM): 135 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 10 Glucose. The pH was adjusted to 7.4 with NaOH.The pipette filling solution contained (mM); 142 KCl, 4 $MgCl_2$, 10 HEPES, 2 ATP. The pH was adjusted to 7.3 with Trizma base.

GABA and Muscimol were dissolved directly in the external solution at the final desired concentration just before experiments were conducted. Flunitrazepam was dissolved in Dimethyl Sulfoxide (DMSO) at a concentration of 10^{-2} M; and, when needed, was diluted with the external solution to a final concentration of 10^{-7} M. Drugs were applied in the external solution, to the cell bath (volume about 0.15 ml) by steady perfusion (3 ml/min gravity flow). Control experiments established that the final DMSO concentration used (0.001%) had no effect on Cl^- activated currents. All chemicals were purchased from Sigma Chemical Co. St. Louis, MO.

Results

The data in Fig. 1 represent the Cl^- currents activated by, respectively, 1 μM muscimol and 1 μM GABA. The curves show that muscimol, at equal concentration, activates more potently the $GABA_A$ receptor. The dose-response curves for the two substances (in terms of peak current, I_p) are reported in Fig. 2 (top). The experimental points can be fitted by Hill equation:

$$I = I_{max} C^n / C^n + K^n \quad (1)$$

where I_{max} is the saturation current, C is the GABA or muscimol concentration, n is the Hill number and K the concentration where $I = I_{max}/2$. The values of the parameters fitting the data to this equation are reported in Table 1. The two substances give the same maximal peak current, implying the ability for

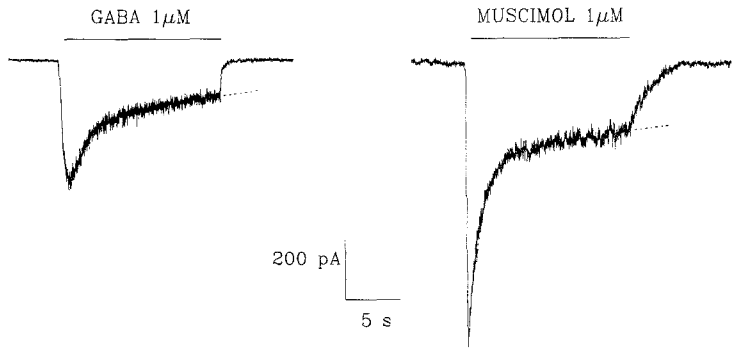

Fig. 1. Current activated by 1 μM GABA (left) and 1 μM muscimol (right) in a granule cell voltage-clamped at −80 mV. GABA, muscimol and external solution were applied by perfusion. Time-course of desensitization is fitted by Eq. 2 (dashed lines) using the following parameter values: for muscimol $I_1 = 583$ pA, $I_2 = 368$ pA, $\tau_1 = 2.3$ s, $\tau_2 = 50$ s and for GABA $I_1 = 238$ pA, $I_2 = 200$ pA, $\tau_1 = 3$ s, $\tau_2 = 58$ s

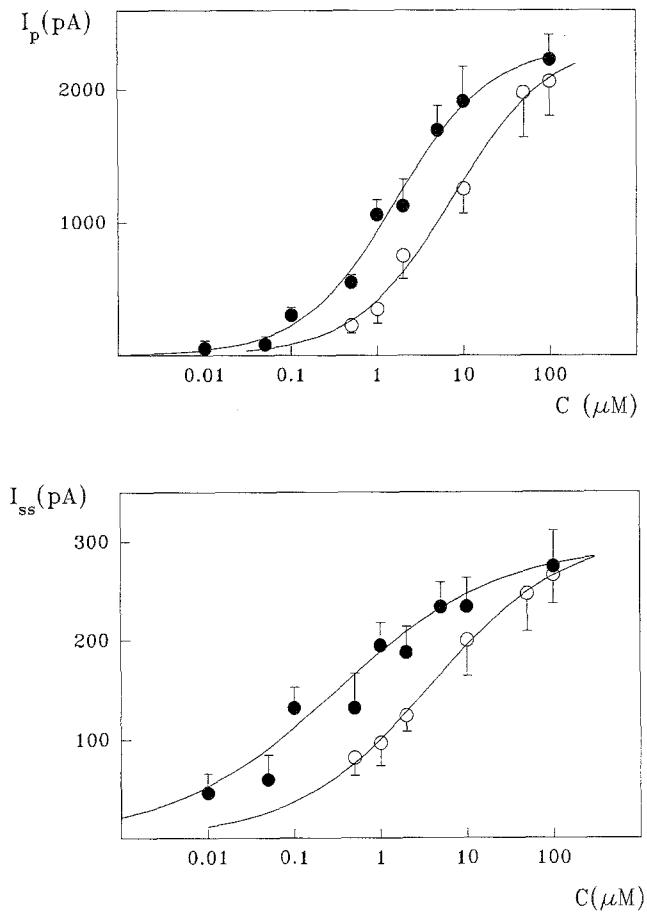

Fig. 2. Dose response curve. Semilogarithmic plot of the peak (top) and of the steady-state (bottom) current amplitude as a function of GABA (o) and muscimol (•) concentration. Theoretical fitting is obtained using the equation (1). The value of the parameters for GABA and muscimol are reported in Table 1. Each cell was voltage clamped at −80 mV. Each point is the average of 3 ÷ 20 cells ± standard deviation

Table 1. Calculated parameters fitting to "Eq (1)" the data of the dose response curves for GABA and muscimol

		I_{max} (pA)	K (μM)	n
GABA	I_p	2370	7.5	0.8
	I_{ss}	310	3.7	0.5
Muscimol	I_p	2320	1.6	0.8
	I_{ss}	300	0.4	0.5

muscimol to fully activate the available receptors on the granule cells. Likewise, the n values are the same and somewhat lower than unity. Muscimol however displays a higher affinity than GABA itself for the receptors with a K of 1.65 vs. 7.5 μM.

We next studied the concentration dependence of the steady state current (I_{ss}) for the two substances (Fig. 2 (bottom) and Table 1).

Overall, the data show that the I_{pmax}/I_{ssmax} ratio is the same for the two agonists. Likewise the n value for the steady-state component is the same (around 0.5) in the two cases. The K value is lower for muscimol than for GABA also for the steady state component. In any case, both for GABA and muscimol the K is lower for the I_{ss} component than for the I_p one.

When expressing the current data for both I_p and I_{ss} in the form of Scatchard plots for both GABA and Muscimol (Fig. 5), two components were found. The relevant parameter values are reported on Table 3.

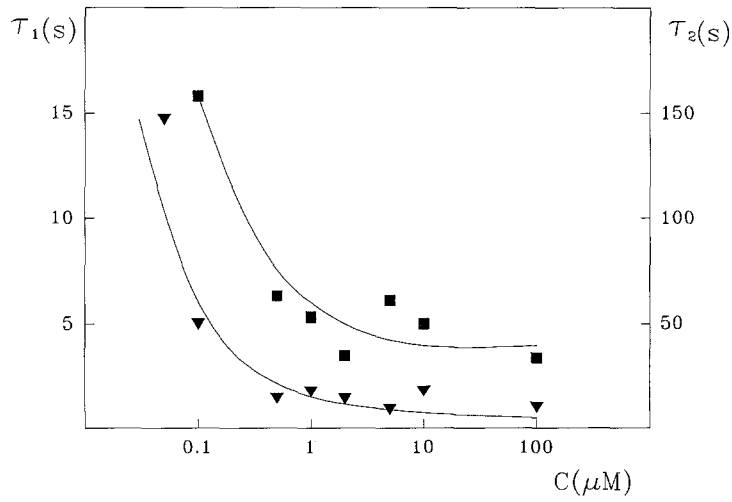

Fig. 3. τ_1 (▼) and τ_2 (■) desensitization time constant obtained using "Eq. (2)" plotted as a function of muscimol concentration. Points are the mean value of 3 experiments. The continous lines have been drawn as a visual guide

As in the case of GABA (Robello et al., 1993) the decay of Cl^- current activated by muscimol is not adequately fitted by a single exponential function, but by the sum of two exponential:

$$I = I_1 e^{-t/\tau_1} + I_2 e^{-t/\tau_2} \quad (2)$$

where I_1 and I_2 are the amplitudes and τ_1 and τ_2 the time constants of the fast and the slow component respectively. As an example, from the currents in Fig. 1, using equation (2) one calculates: $I_1 = 583$ pA, $I_2 = 368$ pA, $\tau_1 = 2.3$ s, $\tau_2 = 50$ s. As a comparison, the same concentration (1 μM) of GABA gives: $I_1 = 238$ pA, $I_2 = 200$ pA, $\tau_1 = 3$ s, $\tau_2 = 58$ s. Figure 3 shows the behaviour of τ_1 and τ_2 as a function of the muscimol concentration. Here one can see that after an initial decrease, the two parameters are stable in spite of the increase of the agonist concentration.

Finally, the effect of the benzodiazepine agonist flunitrazepam (100 nM) on the Cl^- current activated by 10 μM GABA was studied (Fig. 4 and Table 2). The data show that there is only a limited effect (around +10%) by the benzodiazepine on both I_1 and I_2, the rapidly and the slowly desensitizing

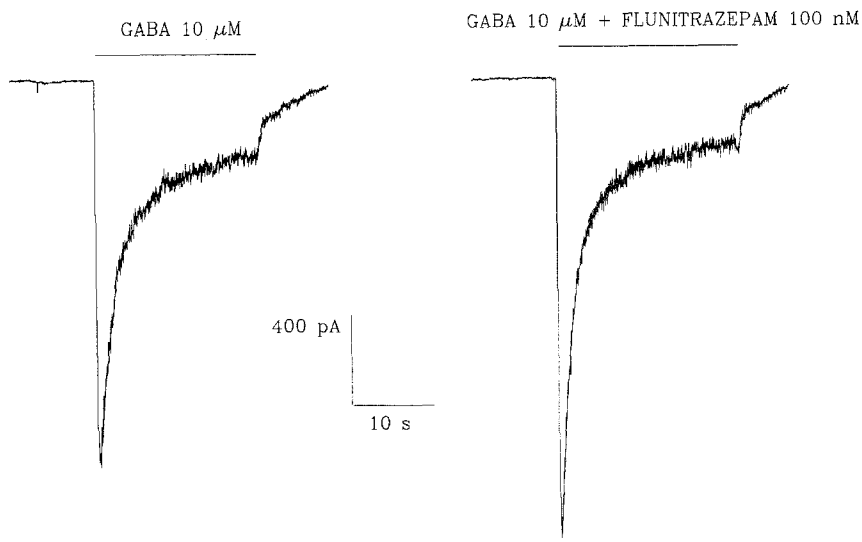

Fig. 4. Current activated by 10 μM GABA (left) and 10 μM GABA + 100 nM flunitrazepam (right) in a granule cell clamped at −80 mV

Table 2. Calculated parameters fitting to "Eq (2)" the current desensitization for GABA and GABA + flunitrazepam

	I_1 (pA)	I_2 (pA)	τ_1 (s)	τ_2 (s)
GABA (10 μM)	1080	500	2.5	47
GABA (10 μM) + Flunitrazepam (100 nM)	1200	530	1.7	32

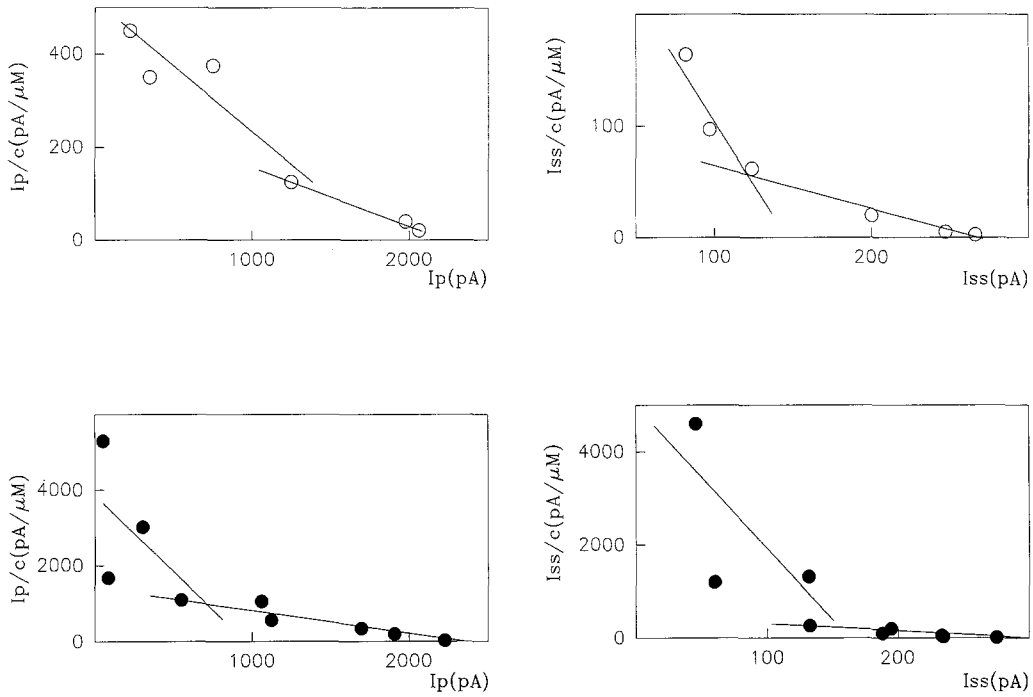

Fig. 5. Scatchard plots of the data reported in Fig. 2 for GABA (o) and muscimol (•) for the peak (left) and steady-state current (right). The plots are non linear. The solid lines represent linear least-squares fits to the experimental data

components, respectively. The same slight increase was found with a higher (10^{-5} M) concentration of flunitrazepam.

Discussion

Dissociated cerebellar granule cultures from 8-day postnatal rat contain more than 90% granule cells (Levi et al., 1984) and cells of different types are easily identified by their morphology.

The electrical properties of these cells have been investigated in different cerebellar preparations and the patch-clamp technique has made possible the recording of voltage and neurotransmitter-activated ionic current.

In previous experiments we have studied the Cl^- currents which can be activated by GABA in rat cerebellum granule cells in culture (Robello et al., 1993). Such currents are mediated by $GABA_A$ receptors since they are antagonized by picrotoxin. Such experiments showed that $GABA_A$ receptor function is modulated by at least two different kinase activities.

The present results demonstrate that muscimol is more potent than GABA itself in eliciting Cl^- currents on such neurons. Furthermore, this agonist is able to activate all $GABA_A$ receptors present on the granule cells. In fact, both muscimol and GABA display the same I_{pmax} and I_{ssmax}. Moreover, muscimol induces Cl^- currents which decline in two phases, a rapid and a slow one (Fig. 1). The two phases may correspond to two populations of $GABA_A$ receptors with different desensitization kinetics. This possibility is supported by the Hill

Table 3. Parameters derived from the Scatchard plots of the currents activated by muscimol and GABA

	I_p	I_{ss}
Muscimol	$K_1 = 0.2\ \mu M$ $K_2 = 1.6\ \mu M$	$K_1 = 0.033\ \mu M$ $K_2 = 0.5\ \mu M$
	$Vmax_1 = 805$ pA $Vmax_2 = 2037$ pA	$Vmax_1 = 145$ pA $Vmax_2 = 265$ pA
GABA	$K_1 = 3.7\ \mu M$ $K_2 = 8.3\ \mu M$	$K_1 = 0.43\ \mu M$ $K_2 = 2.3\ \mu M$
	$Vmax_1 = 1855$ pA $Vmax_2 = 2342$ pA	$Vmax_1 = 148$ pA $Vmax_2 = 264$ pA

coefficients of less than unity for both I_p and I_{ss} for both GABA and Muscimol (Table 1). Such a result is expected in the case of site heterogeneity (Dahlquist, 1979).

Taking those two components (see Table 3) as a basis for evaluating n for the case of site heterogeneity, as discussed by Dahlquist (1979), we could calculate theoretical values quite close to the experimental ones for both I_p and I_{ss}. Moreover, in the case of GABA, such an agreement was closer admitting 1 binding site ($n = 0.79$ for I_p and $n = 0.44$ for I_{ss}) than admitting two binding sites on the receptors ($n = 1.0$ for I_p and $n = 0.34$ for I_{ss}). A close look at the data referring to the two components in the Scatchard plots for both GABA and muscimol shows that the K values referring to steady state I_{ss}, most probably representing desensitized states, are lower than the I_p counterparts. Moreover, for both I_p and I_{ss} the K values are lower for muscimol indicating a higher affinity of this agonist for the two populations of $GABA_A$ receptors in both the active and desensitized states.

On the basis of equation (2) the τ parameters describe the speed at which the GABA current signal decreases with time during desensitization. Since desensitization is more evident with increasing concentrations of the agonist (this report and Robello et al., 1993), it is logical that the τ's decrease at higher GABA concentration. Similar results, at least for the slow component of $GABA_A$ receptor desensitization, have been already described in the literature (Akaike et al., 1986).

Referring to the particular type of $GABA_A$ receptors present on the granule cells, we recall that it has been previously shown by autoradiography that these cells are rich in muscimol binding sites (Palacios et al., 1980, 1981; Olsen et al., 1990). More recently, it has been shown that they are enriched in the δ subunit mRNA (Shivers et al., 1989). For this reason it has been suggested that the presence of the δ subunit gives to the $GABA_A$ receptor high affinity for muscimol (Olsen and Tobin, 1990).

The present results give physiological evidence for the fact that neurons abundantly expressing δ subunit mRNA, the rat cerebellum granule cells, are potently activated by muscimol. In addition, this study shows that a powerful

benzodiazepine agonist, flunitrazepam (Farrant et al., 1990), only slightly potentiates GABA activation of Cl^- channels in these neurons. This result is in line with the absence of benzodiazepine sites in the cerebellar granule cell layer in the rat as demonstrated autoradiographically (Unnerstall et al., 1981). The lack of benzodiazepine effect may be explained by the absence of the γ_2 subunit mRNA in rat cerebellar granule cells (Shivers et al., 1989). Alternatively, these neurons express γ_2 subunit mRNA in combination with the α_6 subunit that are present in $GABA_A$ receptors which have no affinity for β-carbolines or benzodiazepines other than RO 15-4513 (Lüddens et al., 1990).

Acknowledgements

This work has been supported by the C.N.R. Target Project "Biotechnology and Bioinstrumentation".

References

Akaike N, Inoue M, Krishtal OA (1986) Concentration clamp study of GABA-induced chloride current kinetics in frog sensory neurons. J Physiol 379: 171–195

Dahlquist FW (1979) The meaning of Scatchard and Hill plots. In: Hirs CHW, Timasheff SN (eds) Methods in enzymology, vol. 48. Academic Press, New York, pp 270–299

De Lorey T, Olsen RW (1992) γ-Aminobutyric $acid_A$ receptor structure and function. J Biol Chem 267: 16747–16750

Farrant M, Gibbs TT, Farb OH (1990) Molecular and cellular mechanisms of GABA/Benzodiazepine-receptor regulation: electrophysiological and biochemical studies. Neurochem Res 15: 175–191

Levi G, Aloisi F, Ciotti M, Gallo V (1984) Autoradiographic localization and depolarization induced release of amino acids in differentiating granule cell cultures. Brain Res 290: 77–86

Lüddens H, Pritchett DB, Köhler M, Killisch I, Keinänen K, Monyer H, Sprengel R, Seeburg PH (1990) Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist. Nature 346: 648–651

Olsen RW, Mc Cabe RT, Wamsley JK (1990) $GABA_A$ Receptor subtypes: autoradiographic comparison of GABA, benzodiazepine and convulsant binding sites in the rat CNS. J Chem Neuroanat 3: 59–76

Olsen RW, Tobin AJ (1990) Molecular biology of $GABA_A$ receptors. FASEB J 4: 1469–1480

Palacios JM, Scott-Young W, Kuhar MJ (1980) Autoradiographic localization of GABA receptors in the rat cerebellum. Proc Natl Acad Sci USA 77: 670–674

Palacios JM, Wamsley JK, Kuhar MJ (1981) High affinity GABA receptors-autoradiographic localization. Brain Res 222: 285–307

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, Seeburg PA (1989) Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. Nature 338: 582–585

Robello M, Amico C, Cupello A (1993) Regulation of $GABA_A$ receptor in cerebellar granule cells in culture: differential involvement of Kinase activities. Neuroscience 53: 131–138

Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA, Seeburg PH, Barnard FA (1987) Sequence and functional expression of the $GABA_A$ receptor shows a ligand gated receptor superfamily. Nature 328: 221–227

Shivers BD, Killisch I, Sprengel R, Sontheimer H, Köhler M, Schofield PR, Seeburg PA (1989) Two novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations. Neuron 3: 327–337

Unnerstall JR, Kuhar MJ, Niehoff DL, Palacios JM (1981) Benzodiazepine receptors are coupled to a subpopulations of GABA receptors: evidence from a quantitative autoradiographic study. J Pharmacol Exp Therap 218: 797–804

Authors' address: Dr. M. Robello, Dipartimento di Fisica, Università di Genova, Via Dodecaneso 33, I-16146 Genova, Italy.

Received May 17, 1993