Amino Acids (2001) 21: 119–128

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Regulation of the expression of low affinity $GABA_A$ receptors in rat cerebellar granule cells

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Accepted February 12, 2001

Summary. $GABA_A$ receptors of cerebellar granule cells obtained from neonatal rats and kept in culture were studied by labelled muscimol binding.

The data show that, according to the maturational state of those cells in vivo, one or two binding components appear. The low affinity component seems to be the one appearing later. The expression of this component seems to be regulated by protein tyrosine phosphorylation. In fact, its expression is down regulated by the protein tyrosine kinase (PTK) inhibitor, genistein. Viceversa, its expression is upregulated by insulin like growth factor I (IGF-I), most probably via PTK activation.

A possible interpretation of the data is that in vivo IGF-I is one of the endogenous messages leading to the expression of this component during development. Another endogenous factor involved may be GABA itself.

Low affinity $GABA_A$ receptors appear to be the ones involved in inhibitory synaptic transmission at glomeruli. Whereas the high affinity ones probably correspond to extrasynaptic $GABA_A$ receptors mediating the tonic form of inhibition in cerebellar granules.

Key words: Amino acids – Cerebellar granules – Development – Cells cultures – $GABA_A$ receptors – Muscimol binding – PTK

Introduction

GABA mediated inhibition of granule cells of the cerebellum shapes their electrophysiological activity (Gabbiani et al., 1994). Two types of inhibition have been described for these neurons: a synaptic phasic component in dendrites and a tonic one involving mainly extrasynaptic membrane areas (Brickley et al., 1996; Zhu et al., 1998).

There is also indication that different types of $GABA_A$ receptors with different subunit compositions are involved in these two types of inhibition

(Robello et al., 1999; Cupello and Robello, 2000). In particular, the presence of δ subunit in extrasynaptic dendritic and cell body $GABA_A$ receptors (Nusser et al., 1998) renders them resistent to desensitization (Saxena and Macdonald, 1996), a characteristic quite suitable for receptors involved in tonic inhibition.

From a neurochemical point of view, it has been known for a long time that in cerebellar granules put in culture from 7–8 day old rats, one or two components of GABA or muscimol binding appear according to the culture conditions. In fact, it has been shown that GABA itself may exert a trophic function *in vitro* inducing the expression of a low affinity component in addition to the high affinity one (Meier et al., 1984; Hansen et al., 1987). A likely possibility is that adding GABA to the culture medium mimicks a trophic message *in situ*, which may arise prior to or at the time of synapses formation in the inner granule layer. The message of course may be GABA itself. In addition, other molecules may be involved. In these experiments we show that according to the developmental stage of the donor rats one (only high affinity) or two (high and low affinity) components of muscimol binding appear. In addition, we show that the neurotrophic factor IGF-I is involved in promoting the appearance of the low affinity component. Its action seems to be mediated by activation of protein tyrosine phosphorylation.

This low affinity component may correspond to synaptic δ subunit devoid receptors, whereas the high affinity one would correspond well to δ containing extrasynaptic receptors (Nusser et al., 1998; Saxena and Macdonald, 1996).

Materials and methods

Granule cells cultures

Granule cells cultures were obtained from cerebella of Sprague-Dawley rats whose age ranged from 5 to 9 days, following the procedure of Levi et al. (1984), as described previously (Robello et al., 1993). Briefly, the minced tissue was first suspended in trypsin (0.25 mg/ml, Type III Sigma) for 15 min at 37°C in a shaking water bath, and then in deoxyribonuclease and trypsin inhibitor. Finally, it was dispersed by gently drawing it into a fire polished Pasteur pipette. Cells were resuspended in basal Eagle's medium with Earle's salts supplemented with 10% fetal calf serum (Gibco Bio-Cult Ltd, UK), 25 mM KCl, 2 mM glutamine and 100 μg/ml gentamicine and plated on poly-L-lysine-coated glass coverslips placed in 35 mm plastic dishes at a density of 2.5×10^6 per dish, and kept at 37°C in a humidified 95% air-5% CO_2 atmosphere. 18 hr after plating cytosine arabinoside was added to the cultures to prevent glial cell proliferation.

Binding experiments were performed 7 days after plating.

Labelled muscimol binding

The binding experiments were performed at room temperature with (^{3}H)-muscimol. [Methylamine-^{3}H]Muscimol, specific activity 607 Gbq/mmol (Amersham pharmacia biotech, Bristol, UK) was used. For the binding experiments, the culture medium was removed, the cells washed twice with 1 ml of Locke's buffer (NaCl 154 mM, KCl 5.6 mM, $NaHCO_3$ 3.6 mM, $CaCl_2$ 2.3 mM, $MgCl_2$ 1 mM, D-glucose 5.6 mM, Hepes 5 mM; pH 7.4) and then incubated 15 min with 1 ml of Locke's buffer containing various concentrations

of labelled muscimol in order to determine the saturation curves. In particular, the labelled muscimol concentrations used were: 10, 20, 40, 200, 800 and 1,000 nM. Parallelly to the experimental samples, always run in duplicate, blanks were performed, also in duplicate, where, in addition to labelled muscimol, unlabelled 1 mM GABA was present. At the end of the binding incubations, the medium was rapidly removed and the cells washed three times with 2 ml of buffer. After washing, the cells were treated with 1 ml of NaOH 0.1 M for 2 hours at 4°C. Then, 0.8 ml were taken, neutralized and counted with 10 ml Readygel (Beckman Instruments, CA), 0.150 ml aliquots were used in order to determine protein by the Lowry method. At the various labelled muscimol concentrations, specific binding (SB) was obtained subtracting from total bindings the unspecific ones, after having corrected for the protein contents. Saturation curves were built plotting specific binding (fmol/mg prot) vs. muscimol concentration (nM) and fitted to a Michaelis-Menten type equation according to a non linear regression analysis program. When binding was performed at a single muscimol concentration (1,000 nM), the cell dishes for the control and the various pretreatments (see below) in duplicate were parallel to a pair of dishes where excess cold GABA (1 mM) was added (blanks). Obviously, the overall binding procedure was the same as that followed in the experiments for the saturation curves. Analogous was also the procedure for evaluating the specific binding.

Cells pretreatments with drugs

In the single concentration binding experiments, before performing the actual binding experiments granule cells were pretreated for 20 min at room temperature with:

a) Dimethylsulphoxide (DMSO), 0.2% (control)
b) DMSO, 0.2% + IGF-I, 20 ng/ml
c) Genistein, either 100 or 200 μM (vehicle DMSO final conc. 0.2%)

After these preincubations, the media were removed and the cells washed once with 1.5 ml of buffer. Then, the cell dishes were used for binding with (^{3}H) muscimol, 1,000 nM.

Materials

Unlabelled muscimol, GABA, IGF-I and genistein were from Sigma, St. Louis, MO.

Results

Components of (^{3}H)-muscimol binding to granule cells from 7–8 day old rats: saturation curves

In preliminary experiments we could verify that binding of labelled muscimol reached a steady state within 10 min (data not shown here). We decided to run all our binding experiments with 15 min incubations.

The muscimol binding experiments gave different results in different granule cells cultures, where the cells were obtained from 7–8 day old animals and were then kept 7 days in vitro (DIV). In particular, in 2 out of 7 cases only a high affinity component appeared, in the remaining 5 there was both a high and a low affinity component. The data of the first two cases and those of the other ones are reported together in, respectively, Fig. 1A and B. In Fig. 1B it is evident that in addition to the high affinity component there is also a low affinity one. The latter could be fitted with the following parameters: K_D = 1,885 nM and B_{max} = 4,160 fmol/mg prot.

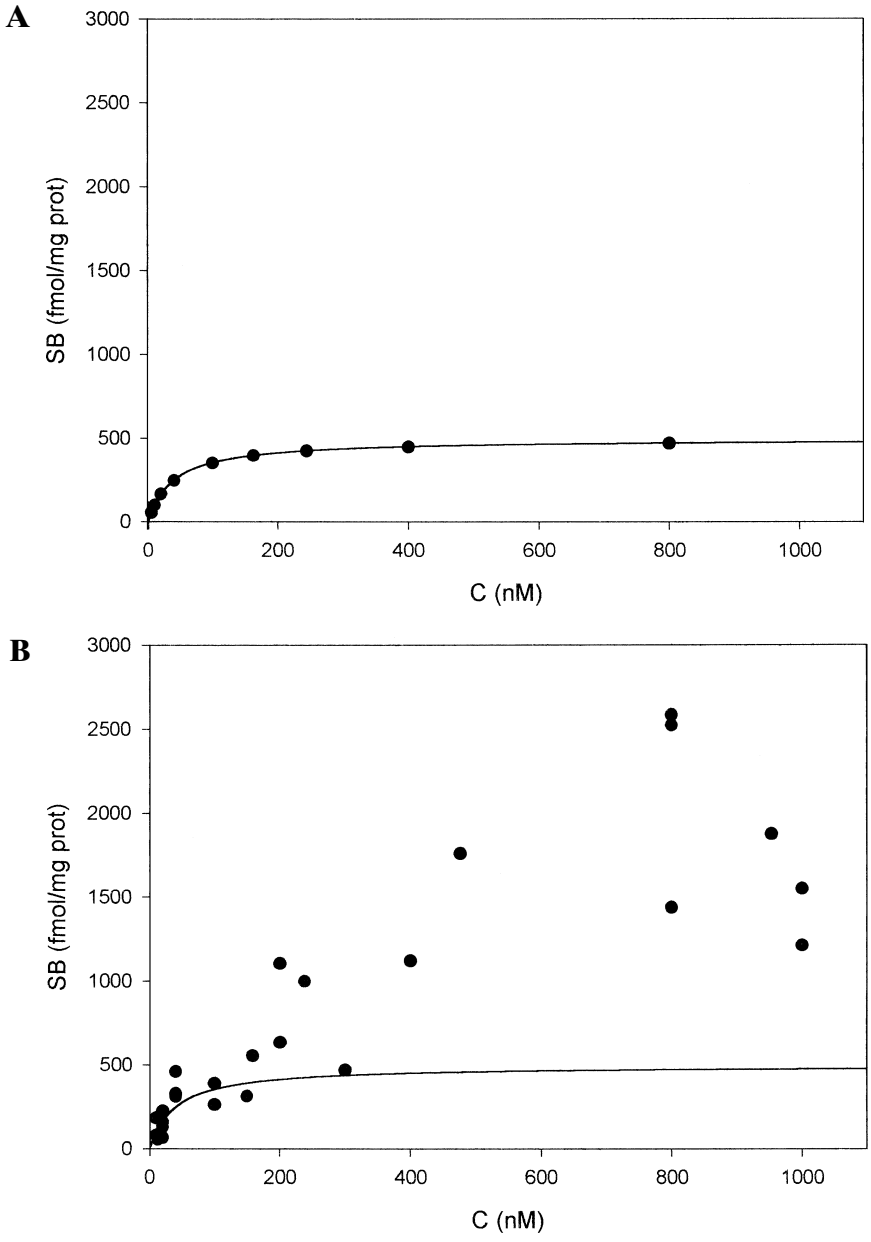

Fig. 1. Saturation curves for the specific binding (*SB*) of labelled muscimol to rat cerebellar granule cells in culture. **A** Collective data from two cases showing only a high affinity component. All the points are the means of two plates. Best fit gave: $K_D = 40 \pm 35$ (SD) nM; $B_{max} = 494 \pm 147$ (SD) fmol/mg prot. **B** Collective data of 5 cases showing two components. Also in this case all the single points derive from two plates. The trace represents the saturation curve for the high affinity according to the parameters of Fig. 1A. A second component (low affinity) is evident ($K_D = 1{,}885 \pm 1{,}734$ nM; $B_{max} = 4{,}160 \pm 1{,}082$ fmol/mg prot)

Components of (^{3}H)-muscimol binding to granule cells from 7–8 day old rats: single muscimol concentration point

We then chose to work at a labelled muscimol concentration able to put into evidence mainly the low affinity binding: 1,000 nM. At this (^{3}H)-muscimol concentration, according to the parameters found in the experiments of Fig. 1, the high affinity component (K_D = 40 nM) is saturated with a specific binding corresponding to its B_{max}(494 fmol/mg prot).

In the cases of granule cells cultures with only the high affinity component the overall specific binding should be around this level. Conversely, if the low affinity component is also present, the specific binding would be significantly higher.

We found in 3 out of 11 cases a specific binding at (^{3}H)-muscimol 1,000 nM which was $\leq$639 fmol/mg prot (within one SD in the respect of the best fit B_{max} for the high affinity, 494 $\pm$ 147 fmol/mg prot). In the other 8 cases the specific binding was $\geq$1,300 fmol/mg prot, thus these specific binding values were more than three SD higher than the B_{max} for the high affinity. In other words, in the first case (n = 3) the specific binding can be accounted for solely by the high affinity component. In the second case (n = 8), one must admit the presence also of the low affinity component. This corresponds nicely to the incidence of saturation curves with one or two components (around 30% showing only the high affinity, see above).

Binding of 1,000 nM (^{3}H)-muscimol to granule cells from rats of 5–9 day of age

Then we studied the specific binding of 1,000 (^{3}H)-muscimol to granule cells obtained from rats of 5, 6, 7, 8 and 9 days of postnatal (PN) age. The cells were kept in culture for 7 days. The data in Fig. 2 show that starting from 970 fmol/mg prot at 5 day of age the

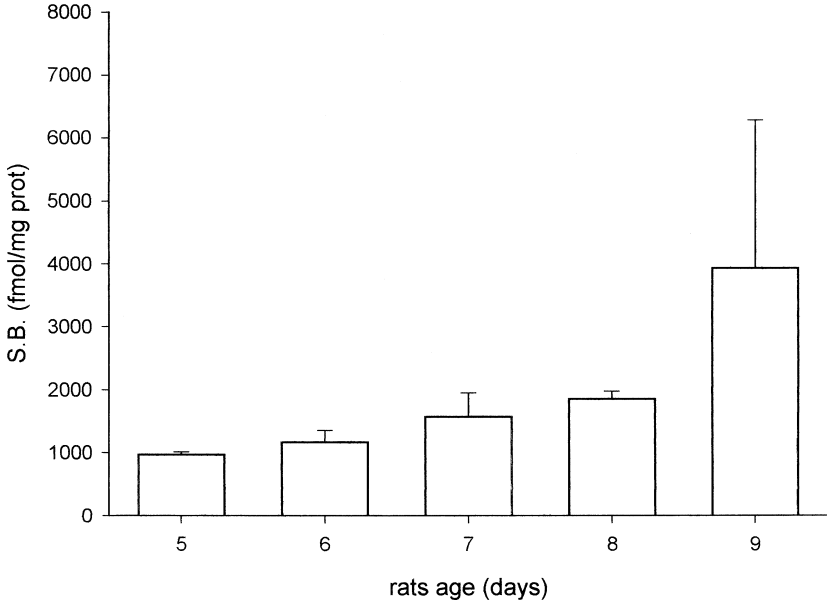

Fig. 2. Specific binding of labelled muscimol (1,000 nM) to cerebellar granule cells cultures from rats of different PN ages. The columns represent average SB's and the bars represent SEM. In all cases, but the 5 days one, the results are the average of 3 different cultures. In the case of 5 days, the average was between 2 different cultures. From 8 days the SB is statistically different from the one at 5 days ($p < 0.01$)

average specific binding of muscimol gradually increases to around 4,000 fmol/mg prot at 9 days PN. Whereas the average specific binding at 5 days is at the limit of the B_{max} of the HA component +3SD (494 + 3 × 147 fmol/mg prot), this does not happen from the 6th day PN on.

Thus, we conclude that a second, low affinity, component is in the process of being expressed from the 5th PN day on.

Effects of IGF-I and genistein on low affinity binding

Granule cells cultures from 7–8 day old rats were treated for 20 min at room temperature with IGF-I (20 ng/ml) in Locke's buffer before the binding experiments at 1,000 nM (^{3}H)-muscimol. Muscimol specific binding was compared with that to cells treated just with buffer for the same time period (controls). When in controls there was no low affinity binding (3 cases, see above), IGF-I induced a significant increase of the binding (Fig. 3, left column). Viceversa, when in the controls there was also the low affinity component (8 cases, see above), IGF-I caused a reduction of the binding (Fig. 3, right column).

In Fig. 4, we report the change of (^{3}H)-muscimol specific binding at 1,000 nM in IGF-I treated vs. control granule cells. The data show that IGF-I had a definite stimulation effect on binding in the first group of 3 cases, data to the left of the vertical line in the figure. For the other 8 cases, where the low affinity component was present according to the criterion discussed above, data to the right of the vertical line, the IGF-I effect disappeared. In these cases instead, if anything, IGF-I had a negative effect. Overall, the data show a significant ($r = 0.82$; $p < 0.01$) negative correlation between basal specific muscimol binding at 1,000 nM and the effect (increase or decrease of specific binding) by IGF-I.

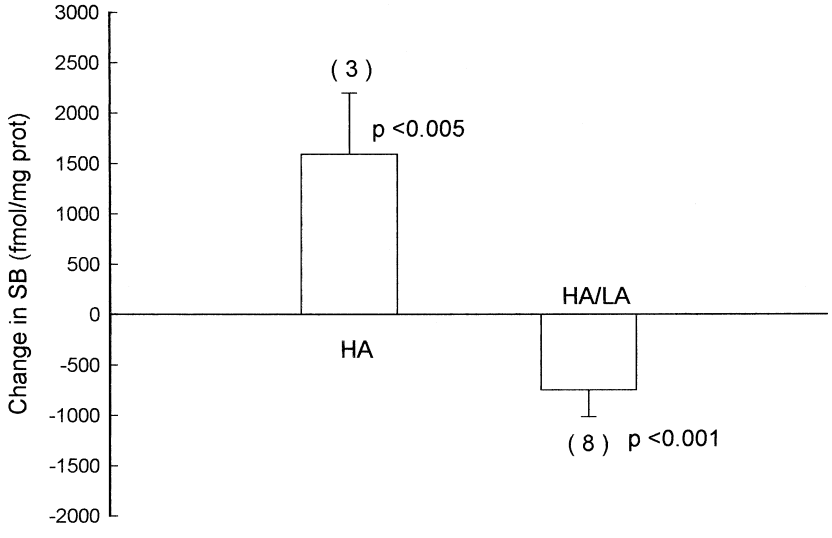

Fig. 3. Different effect of IGF-I on labelled muscimol specific binding, at a [muscimol] = 1,000 nM, whether (*HA/LA*) or not (*HA*) a low affinity component is present. The columns represent the change in muscimol specific binding. The change was calculated as the difference of SB of the treatment case (in the presence of IGF-I) *vs* the relevant control (in the absence of IGF-I). This change is positive (an increase) in the case of absence of the low affinity component (column to the left), it is negative (a decrease) when such a component is present (column to the right). In parentheses the numbers of different cultures. p refers to the comparison, Student's t test, with the untreated control

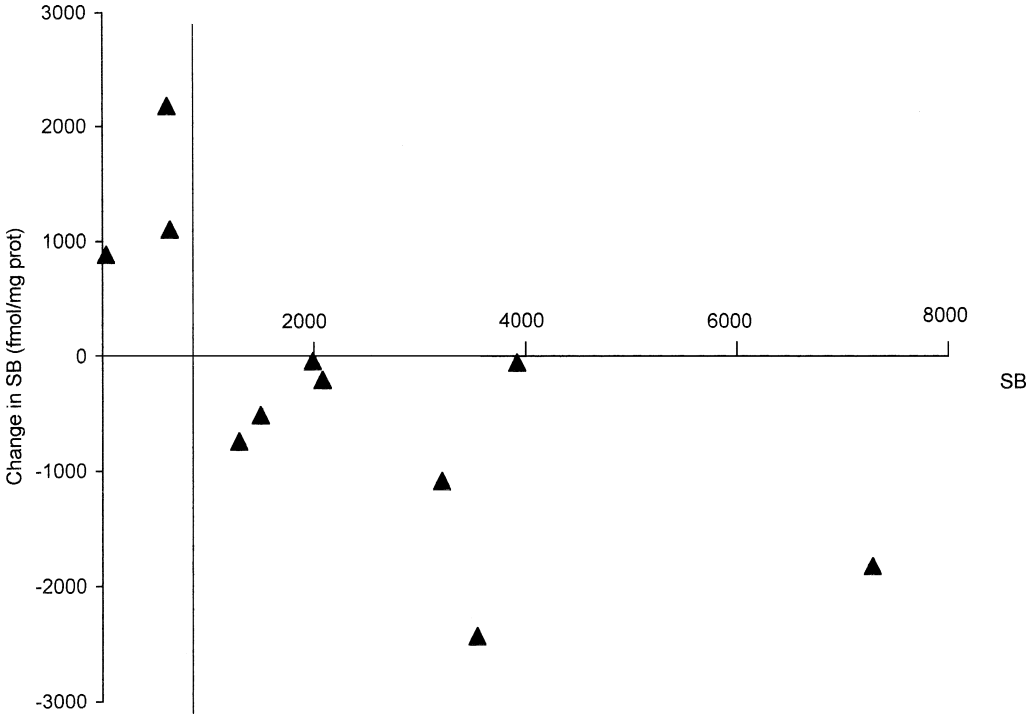

Fig. 4. Effect of IGF-I on muscimol specific binding (fmol/mg prot), at [muscimol] 1,000 nM, as a function of specific binding in the control (not treated with IGF-I). The vertical line within the plot underlines 935 fmol/mg prot specific binding, corresponding to the B_{max} for the high affinity plus 3 × SD. The change in muscimol SB by IGF-I treatment (defined as in Fig. 3) is significantly correlated to basal SB, in the relevant controls ($r = 0.82$; $p < 0.01$)

The protein tyrosine kinase inhibitor genistein at 100–200 μM caused a somewhat different result in comparison with IGF-I. Namely, when the low affinity component was present and the specific binding at labelled muscimol 1,000 nM was ≥1,300 fmol/mg prot, genistein caused a decrease of it which became statistically significant at a genistein concentration of 200 μM (Fig. 5).

Discussion

The data we present in this report show that when rat cerebellum granule cells were put in culture from 7–8 day old rats, only a high affinity (≈30% of the cases) or both a high and a low affinity component of muscimol binding appear (Fig. 1). This result is reminiscent to us of previous data (Meier et al., 1984) by another group showing that in such neuronal cultures only the high affinity component was present unless the cultures were treated with exogenous GABA. Our interpretation is that in our experiments the age of the animals was in the range of a switch *in vivo* from an immature condition in which only high affinity $GABA_A$ receptors are expressed in granule cells to a more mature one in which both components are expressed. The data of the binding at 5–9 days PN (Fig. 2) support this idea. In fact, the average specific

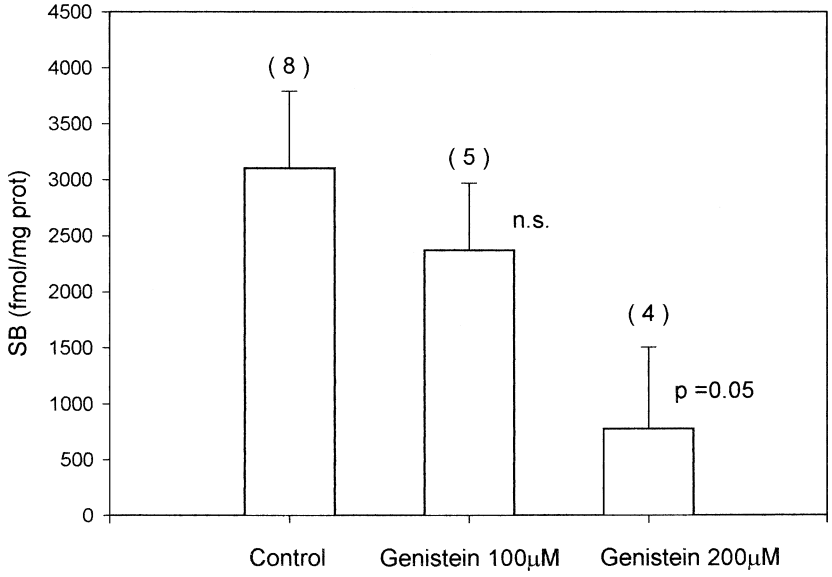

Fig. 5. Effect of genistein on muscimol 1,000 nM SB in granule cells cultures expressing the LA, according to the criterion expressed in the text. The columns represent the averages of SB, the bars represent SEM and the numbers in parentheses are the numbers of determinations. The p values is in reference to the control and it was evaluated by the Student's t test

binding of muscimol to granule cells from rats of 5 days is 970 fmol/mg prot whereas that to granule cells from animals of 9 days is around 4,000 fmol/mg prot. This change (around 3,000 fmol/mg prot) cannot be accounted for by the HA component (B_{max} = 494 fmol/mg prot), it can only be accounted for by the LA one (B_{max} = 4,160 fmol/mg prot). The basis for this change may be the gradual increase of the granules reaching the internal granule layer (Altman, 1972). In this process *in vivo*, factors may induce the expression of the low affinity component. One of those factors of course may be GABA itself which could begin to increase in the internal granule layer due to incoming immature (but glutamic acid decarboxylase positive) axons of the Golgi cells (Mc Laughlin et al., 1975).

In addition, our present experiments suggest that an additional factor may be insulin-like growth factor-I. This factor has been shown to prevent apoptosis in cerebellar granule cells maintained in low K^+ (D'Mello et al., 1997). In addition, it has been shown, in the same system, to be able to induce glutamate sensitivity (Calissano et al., 1993). The actions of IGF-I are mediated by activation of the relevant receptors and protein tyrosine kinase activity. Via a similar mechanism insulin promotes expression on the cell membrane of functional $GABA_A$ receptors in hippocampal neurons. This effect is blocked by the protein tyrosine kinase inhibitor genistein (Wan et al., 1997).

In addition, in previous experiments (Amico et al., 1998) we showed that the protein tyrosine kinase inhibitors genistein and lavendustin A induce a run-down of cerebellar granules $GABA_A$ receptor activity, as studied by the

whole cell patch-clamp technique. More recent unpublished results from our group show the same effect by genistein on rat brain $GABA_A$ receptors expressed in the heterologous system of Xenopus oocytes, after rat brain mRNA injections.

Also the effects we describe here are probably mediated by tyrosine kinase activity stimulation, as borne out by the data about the effects of IGF-I itself and of genistein. The almost paradoxical fact that, under conditions of full expression of the low affinity component, IGF-I and presumably protein tyrosine kinase activity reduce its muscimol binding (Fig. 4) may be explained by the presence of a set-point for tyrosine phosphorylation either of the $GABA_A$ receptor itself or of a closely related protein. In other words, over-phosphorylation of tyrosines might result in a less efficient expression of the receptor's activity.

The low affinity component of muscimol binding appears to be related to a $GABA_A$ receptor population involved in the peak chloride current evoked by GABA and muscimol in granule cells in culture (Baldelli et al., 1994). This component is probably related to synaptic dendritic $GABA_A$ receptors (Robello et al., 1999; Cupello and Robello, 2000). These receptors may appear later in cerebellar granule cells development.

Thus, in summary, we propose that in the postnatal development of cerebellar granules the most precocious type of $GABA_A$ receptors are the high affinity ones. Later on, under the influence of trophic factors, such as GABA itself and IGF-I, other $GABA_A$ receptors types are expressed. These present a lower affinity for GABA but they mediate from then on phasic, rapid inhibitory messages at synapses in glomeruli. *In situ*, GABA may be provided as a trophic factor for differentiation of granule cells in terms of $GABA_A$ receptors by GABAergic fibers incoming to the internal granule layer (Mc Laughlin et al., 1975).

In addition, our present results suggest that at around P7, in addition to GABA, there may be another trophic factor inducing the expression of low affinity synaptic $GABA_A$ receptors, IGF-I. IGF-I has been demonstrated to be present in the rat cerebellum only in its early development and particularly present in neuroglial cells surrounding granule cells, in addition to being present in granule cells themselves, whether migrating through the molecular layer or in the internal granule layer (Andersson et al., 1988). This factor could act *in vivo* on granule cells in a limited developmental time window, being released extracellularly either by neuroglial cells or, in an autocrine fashion as suggested by Andersson et al., by granule cells themselves.

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Received December 12, 2000