THE DEVELOPMENT OF GABA RECEPTORS IN NORMAL AND MUTANT MOUSE CEREBELLUM

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1. Introduction

The importance of GABA as an inhibitory neurotransmitter in the mammalian cerebellum is well documented [1,2]. The excitatory granule cells, by far the most numerous neuronal type in the cerebellum [3], receive input from GABAergic cells [1]: thus it has been suggested that the majority of GABA receptors are located on granule neurons [4]. The cerebellar mutants, weaver (wv) reeler (rl) and staggerer (sg), present an opportunity to test this hypothesis. A number of workers have examined GABA receptor binding in these mutants [5–7] as well in animals whose cerebella have been depleted of granule cells by viral agents [4]. Although the cerebella of sg, wv and rl are to various extents depleted of granule cells, the primary events which lead to this deficit and the altered cerebellar cytoarchitecture are different for each mutation [8,9]. The sg mutant is also deficient in Purkinje neurons [10]. The hypomyelinating mutant, jimpy (jp), also used in this study, lacks CNS myelin, but cerebellar organization is normal [11,12].

Here we have used the radioligand [^{3}H]muscimol, which binds with high affinity to GABA receptors [13,14], to compare the development of these receptors in normal and mutant cerebella. In addition, we have examined [^{3}H]muscimol binding to isolated cerebellar glomeruli, which contain the post-synaptic specialization of the granule neurons [15]. The results are consistent with the hypothesis that GABA receptors are associated with granule neurons.

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2. Materials and methods

Animals were raised from stock originally obtained from the Jackson Laboratory (Bar Harbor, ME); all were of the B6/CBA strain, except for sg mutants, which were on a C57BL/6 background. Mutants were bred and identified as outlined in [16]. Controls for all the mutants, except sg, were taken from mutant-free B6/CBA stock. Sg controls were normal non-mutant carriers from the sg colony, and identified by their grey coat (marker gene dilute).

2.1. *Preparation of cerebellar glomeruli*

Cerebellar glomeruli were produced from adult CBA mice (obtained from Olac, Bicester) by a modification of the method in [15]. Briefly, this consisted of chopping and homogenizing tissue in ice cold 0.3 M sucrose (this and all subsequently mentioned solutions contained 1 mM $MgSO_4$). The homogenate was filtered through nylon and metal filters and centrifuged. The pellet was resuspended in the sucrose solution and centrifuged and the above procedure repeated. The final pellet was resuspended in 6 ml 0.3 M sucrose and 2 ml aliquots were layered onto a linear gradient generated from 7.5 ml 1.2 M sucrose and 7.5 ml 1.3 M sucrose underlayered with a cushion of 1.4 M sucrose. Tubes were centrifuged (53 000 × *g*) 60 min at 3°C, and the glomeruli collected from the gradient-cushion interface. The glomerular suspension was diluted with distilled water and centrifuged (50 000 × *g*) 30 min at 3°C. The pelleted glomeruli were suspended in 2 ml distilled water and stored at −20°C. This homogenate was treated for analysis as outlined below for the cerebellar homogenates.

2.2. *GABA receptor assay*

[^{3}H]Muscimol binding was determined using a

modification of the method in [14]. Cerebellar tissue (25–55 mg wet wt) was homogenized in 2 ml ice-cold distilled water. Homogenates were centrifuged (50 000 × *g*) 20 min at 3°C. Pellets were resuspended in 2 ml distilled water and stored at −20°C for ≥24 h before analysis. Just prior to analysis, the homogenates were made up to 7 ml with ice cold distilled water and centrifuged as before. Pellets were resuspended in a further 7 ml ice cold water and centrifuged, and the final pellet resuspended in 2.2 ml ice cold water. Duplicate 500 μl aliquots of this suspension were incubated at 0°C for 10 min with 1 ml 60 mM Hepes–Tris buffer (pH 6.8) containing *N*-methylamine-[^{3}H]muscimol ([^{3}H]muscimol, 19 Ci/mmol, Radiochemical Centre, Amersham) in the presence of 100 μM GABA (Sigma). Incubations were terminated by centrifugation (50 000 × *g*, 10 min, 3°C). The supernatants were aspirated, and the pellets were solubilized with 0.1 M NaOH counted by liquid scintillation spectroscopy. The apparent dissociation constant (K_d) for [^{3}H]muscimol binding was determined using a range of [^{3}H]muscimol concentrations from 0.5–33 nM, otherwise the concentration was fixed at 2.8 nM. Specific binding was that displaced by 100 μM GABA. Binding was linear with protein concentration up to 0.55 mg protein/incubation, and was maximally displaced by 100 μM GABA.

3. Results

[^{3}H]Muscimol was bound with high affinity to a single population of receptors with K_d = 13 ± 1 nM and B_{max} = 1270 ± 100 pmol/g protein (fig.1, [17]). The K_d values at 10 and 20 days of age were not significantly different (fig.1).

Although significant levels of binding were found at all ages examined (0.028 pmol/cerebellum at 5 days), development of [^{3}H]muscimol binding sites occurred mainly postnatally (fig.2). The maximum increase in binding occured at 15–20 days and increased up to 30 days of age.

With the exception of jp, [^{3}H]muscimol binding

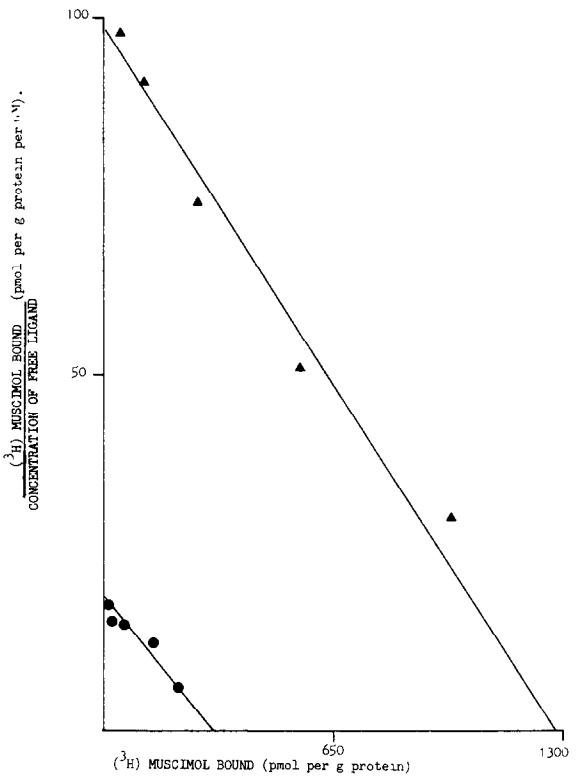

Fig.1. Scatchard plot of [^{3}H]muscimol binding to the total particulate fraction of 10 day (•) and 20 day (▲) mouse cerebellum. Each point is the mean of 2 expt (range <15%). The binding parameters, calculated from this data by linear regression (Edwards, 1967) [30] were as follows: 10 days, K_d = 16.4 ± 3.4 nM, B_{max} = 310 ± 48 pmol/g protein; 20 days, K_d = 13.0 ± 1.4 nM, B_{max} = 1282 ± 105 pmol/g protein. The regression coefficients for the linear regressions were 0.86 and 0.95 for 10 and 20 days, respectively. The difference between the K_d values was not statistically significant (t = 0.51, p < 0.50, 2-tailed Student's t-test).

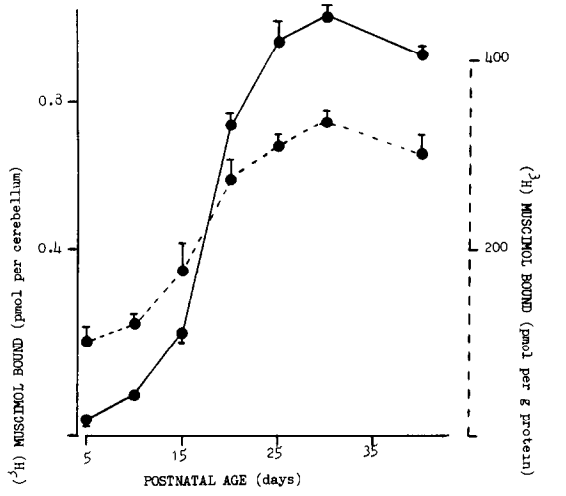

Fig.2. The development of [^{3}H]muscimol binding in normal mouse cerebellum. Results plotted as mean ± SEM, n = 5. (——) Data expressed as pmol/cerebellum; (– –) data as pmol/g protein.

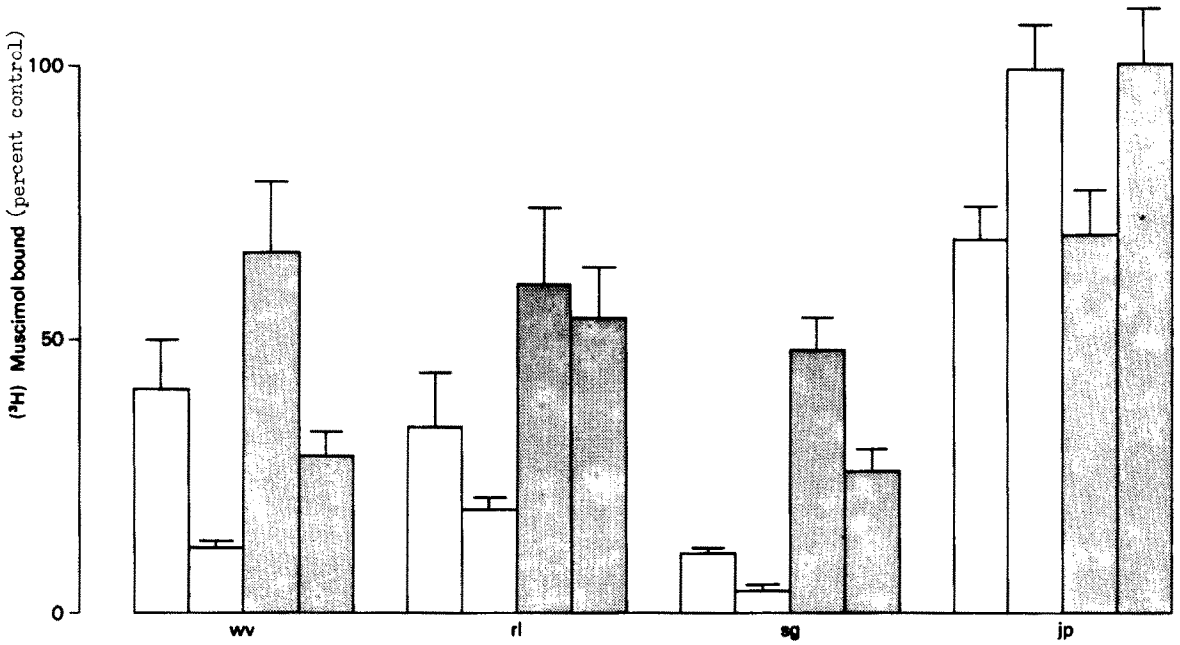

Fig.3. [^{3}H]Muscimol binding expressed as mean percentage of control ± SEM (n = 5) at age 10 and 20 days, respectively: binding/cerebellum (□); binding/unit protein (▨). The bsolute control values at age 10 days were as follows: wv, rl and jp controls, 0.080 ± 0.012 pmol/cerebellum, 99 ± 10 pmol/g protein; sg controls, 0.070 ± 0.11 pmol/cerebellum, 67 ± 7 pmol/g protein. At 20 days the corresponding values were: wv, rl and jp controls, 0.533 ± 0.025 pmol/cerebellum, 273 ± 14 pmol/g protein; sg controls, 0.344 ± 0.021 pmol/cerebellum, 152 ± 11 pmol/g protein.

Table 1
Summary of analysis of variance performed on [^{3}H]muscimol binding (per cerebellum) in mutants and their controls

Mutant	Age (10 *vs* 20 days)	Mutant *vs* control
wv	28.2	33.7
sg	17.7	48.5
rl	32.5	24.4
jp	381	0.03[a]

[a] $p > 0.25$ (not significant)

The experiments were designed for 2 × 2 factorial analysis of variance. However, a significant difference in cell variances was observed in all comparisons ($p < 0.05$, from variance ratio distribution statistic). [Homogeneity of variance is a prerequisite for analysis of variance (Edwards, 1967) [30]]. Thus the simple effects of age and mutation were computed using a modification of analysis of variance, which takes into account the heterogeneity of variance, outlined in Winer (1971) [31]. The F ratios are given above for the simple effects of age and mutation (n = 5, 1 and 19 degrees of freedom). All F ratios are highly significant ($p < 0.001$) unless indicated otherwise

was reduced in all mutant cerebella (fig.3, also see table 1 for a summary of analysis of variance of these data). Binding/cerebellum was most reduced in sg to 11% and 4% of control values at 10 and 20 days, respectively. The corresponding values for wv were 41% and 12% and for rl, 34% and 19%. Although binding in all cerebellar mutants decreased over this period relative to controls, absolute values increased as did the controls. The concentration of [^{3}H]muscimol binding sites in preparations enriched in cerebellar glomeruli was ~3-fold greater than that of the total particulate fraction (table 2).

Table 2
The binding of [^{3}H]muscimol and [^{3}H]QNB (pmol/g protein) to fractions enriched in cerebellar glomeruli

Ligand	Total homogenate	Glomerular fraction
[^{3}H]Muscimol	431 ± 159	1076 ± 295[a]
[^{3}H]QNB	152 ± 30	142 ± 10

[a] Significantly different from total homogenate value t = 3.363, $P < 0.05$ (2-tailed Student's t-test)

Values expressed as mean ± SEM (n = 3)

4. Discussion

Since the cerebellum is undergoing rapid development over the period studied [21] and because the cerebellar weights of mutant animals are much reduced with respect to their controls, data concerning changes in the concentration of binding sites can be misleading [18]. For this reason the results are discussed in terms of binding cerebellum unless stated otherwise.

The age of maximal cerebellar binding (1.3 pmol/mg protein, fig.1) was similar to that observed by other workers for rat and mouse cerebral cortex [12, 14,19]. The K_d for muscimol binding in mouse cerebellar tissue (13 nM at 20 days, fig.1) was similar to that reported for high affinity binding to mouse cerebral cortex [19], but higher than those in [13,14]. The biphasic nature of binding reported in [13,19] was not observed in this study. At the concentration of muscimol used here, binding was limited to a single population of high affinity sites. The K_d-values for binding to cerebellar tissue in 10 and 20 day animals did not change. Thus the increases seen during development were due to an increase in the number of sites and not a change in receptor affinity. The K_d of muscimol binding in mutant cerebella could not be determined due to the unavailability of the large amounts of tissue necessary. It was thus assumed that any differences observed in muscimol binding to mutant cerebellar tissue were due to changes in the number of receptors.

The developmental profile for muscimol binding (fig.2) followed that reported for sodium-independent GABA binding to rat cerebellar material [20]. The major increase in muscimol binding occurred relatively late during development (15–20 days, fig.2), coinciding with the later stages of granule cell migration [21]. These data are consistent with the majority of GABA receptors being localized on granule neurons. This conclusion was further supported by the finding that muscimol binding levels were elevated in cerebellar glomeruli, which contain golgi axon terminals and granule cell dendrites. Receptor formation may preceed synaptogenesis [22,23]. The significant levels of muscimol binding observed in 5 day mouse cerebellum suggests that this may be true for GABA receptors in the cerebellum, since according to [21], earliest GABAergic synapses appear around day 7.

Although the most marked feature of the cerebellar mutants is the granule cell deficit, other anomalies occur [24–29]. However, the finding that muscimol binding is severely reduced in the cerebella of these animals (fig.3) strongly suggests that a large proportion of the muscimol binding defecit is due to the loss of granule cells. The binding deficit, while similar for wv and rl, is more marked for sg (fig.3). Whether the increased deficit in sg is the result of known Purkinje cell loss is unclear. However, the reduced levels of the GABA synthetic enzyme, glutamate decarboxylase in sg compared with wv [18], suggest less GABAergic neuron activity in sg.

In all cerebellar mutants the deficit in muscimol binding relative to controls became larger over 10–20 days (fig.3), indicating that perhaps the responsible processes continue to operate over this period. These may include death and/or shortfall in maturing granule cells which occur maximally at 10–21 days [28]. The relatively normal levels of muscimol binding seen in jp mutant is consistent with the apparent normal neuronal development of the cerebellum of these animals [11–13].

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