# Excitatory and Inhibitory Amino Acid Neurotransmitter Binding Sites in the Cerebellar Cortex of the Pigeon (*Columba livia*)

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## ABSTRACT

We used receptor autoradiography to determine the distribution of excitatory and inhibitory amino acid neurotransmitter binding sites in the cerebellar cortex of the pigeon (*Columba livia*). α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid, kainate and metabotropic binding sites had highest levels in the molecular layer. N-methyl-D-aspartate binding sites, assayed with both [$^3$H]glutamate under selective conditions and with [$^3$H]glycine binding to the associated strychnine-insensitive glycine site, had highest levels in the granule cell layer. There was little specific binding of the non-competitive N-methyl-D-aspartate antagonist, [$^3$H]MK-801. The level of gamma-aminobutyric acid (GABA)-A binding sites was higher than GABA-B binding sites in both molecular and granule cell layers with the highest level of GABA-A sites in the granule cell layer. The highest level of GABA-B binding sites was in the molecular layer. [$^3$H]Flunitrazepam binding levels were approximately the same in both molecular and granule cell layers. With the exception of kainate binding sites, the distribution of binding sites was identical to that seen in the cerebellar cortex of mammals. Our results support the concept that the chemoarchitecture of the cerebellar cortex has been conserved in the course of vertebrate evolution.

KEY WORDS: Glutamate  Gamma-aminobutyric acid  Cerebellum  Avian  Evolution

## INTRODUCTION

Despite significant changes in size and external morphology (Nieuwenhuys, 1967), the neuronal circuitry of the cerebellar cortex has been remarkably conserved in the course of vertebrate evolution (Mugnaini, 1972; see Llinas and Hillman, 1969 for extensive review). This phylogenetic conservation of cerebellar cytoarchitecture and intrinsic connectional anatomy was first noted by Ramon y Cajal, who felt that the uniform organization of the cerebellar cortex across diverse vertebrate phyla amounted to a virtual 'biological law'. The climbing fibre–Purkinje cell and mossy fibre–granule cell–parallel fibre–Purkinje cell circuits appear to exist in almost all vertebrates, and Golgi and ultrastructural studies indicate that the morphology of these synapses is relatively similar across vertebrate phyla (Llinas and Hillman, 1969). The presence of cerebellar cortical interneurons is more variable (Llinas and Hillman, 1969). Amphibians possess a sparse population of stellate cells, while reptiles possess both stellate and Golgi cells. Birds and mammals possess stellate, Golgi and basket cells.

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Electrophysiological studies of a variety of species have shown that the climbing fibre–Purkinje cell and parallel fibre–Purkinje cell synapses are excitatory (Llinas and Hillman, 1969). Similarly, the effect of interneurons is inhibitory in all species examined to date (Llinas and Hillman, 1969). The phylogenetic conservation of cerebellar cortical cytoarchitecture, synaptic morphology and synaptic electrophysiology suggests that the neurotransmitter/receptor chemoarchitecture of the cerebellar cortex may be similarly conserved across vertebrate phyla.

Recent studies of mammals have defined the neurotransmitters of cerebellar cortical neurons and their afferents. The neurotransmitter of mossy fibres, climbing fibres and granule cells is an excitatory amino acid (EAA; Beitz *et al.*, 1986; Garthwaite *et al.*, 1989; Somogyi *et al.*, 1986; Foster and Roberts, 1983; Rea *et al.*, 1983; Toggenburger *et al.*, 1983; Wiklund *et al.*, 1984; Kimura *et al.*, 1985; Young *et al.*, 1974; Hudson *et al.*, 1976; Sandoval and Cotman, 1978; Stone,1979). Stellate, basket, Golgi and Purkinje cells use the inhibitory amino acid, gamma-aminobutyric acid (GABA; Madsen *et al.*, 1985; Ottersen *et al.*, 1988; Wuenschell *et al.*, 1986). Both EAAs and GABA exert their effects through a variety of receptor subtypes. EAAs

Table 1. GABA and EAA binding site assays

| Binding Site | Ligand | Conc (nM) | Buffer | Blockers | Blank | Reference |
|---|---|---|---|---|---|---|
| BDZ | [³H]Flunitrazepam | 5 | 50 mM-Tris–acetate, pH 7.4 | | 5 μM-Clonezepam | Albin and Gilman (1990) |
| GABA-A | [³H]GABA | 20 | 50 mM-Tris–Cl + 2.5 mM-CaCl₂ | 100 μM-Baclofen | 100 μM-Isoguvacine | Chu *et al.* (1990) |
| GABA-B | [³H]GABA | 20 | 50 mM-Tris–Cl + 2.5 mM-CaCl₂ | 10 μM-Isoguvacine | 100 μM-Baclofen | Chu *et al.* (1990) |
| NMDA | [³H]Glutamate | 65 | 50 mM-Tris–acetate, pH 7.4 | 2.5 μM-Quisqualate 100 μM-Kainate | 1 μM-NMDA or 100 μM-CPP | Makowiec *et al.* (1991) |
| Glycine | [³H]Glycine | 100 | 50 mM-Tris–acetate, pH 7.4 | 1 μM-Strychnine | 1 μM-Glycine | McDonald *et al.* (1990) |
| MK-801 | [³H]MK-801 | 5 | 50 mM-Tris–acetate, pH 7.4 | | 5 μM-MK-801 | Sakurai *et al.* (1991) |
| AMPA | [³H]AMPA | 35 | 50 mM-Tris–Cl + 2.5 mM-CaCl₂ + 30 mM-KSCN | | 1 μM-Glutamate | Nielsen *et al.* (1988) |
| MET | [³H]Glutamate | 100 | Tris–Cl + 2.5 mM-CaCl₂ + 30 mM-KSCN | 100 μM-AMPA | 2.5 μM-Quisqualate | Cha *et al.* (1990) |
| Kainate | [³H]Kainate | 60 | 50 mM-Tris–acetate, pH 7.2 | | 100 μM-Kainate | Wamsley *et al.* (1982) |

act through four generally recognized types of receptors, named after their selective agonists (Young and Fagg, 1990; Monaghan *et al.*, 1989). α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors activate ion channels while the metabotropic (MET) receptor is coupled to inositol phospholipid metabolism. The NMDA receptor possesses a strychnine-insensitive binding site for glycine, and another binding site for the non-competitive antagonists MK-801 and phencyclidine. Gamma-aminobutyric acid receptors are divided into GABA-A receptors, linked to an inhibitory chloride channel and modulated by an associated benzodiazepine (BDZ) binding site (Enna, 1988), and GABA-B receptors, a G-protein-coupled receptor that modulates potassium and calcium channels (Bowery, 1989). To test the hypothesis that the neurotransmitter architecture of the cerebellar cortex has been conserved in the course of vertebrate evolution, we used receptor autoradiography to determine the distribution of EAA and GABA binding site subtypes in the cerebellar cortex of pigeons

(*Columba livia*). We compare our findings with previously published information on mammalian cerebellar cortex. The last common ancestor of avians and mammals is thought to have lived 320 million years ago (Carroll, 1988). Consequently, comparison of these two phyla provides a reasonable view of a large segment of vertebrate evolution. A preliminary account of this work has appeared (Albin *et al.*, 1989).

## MATERIALS AND METHODS

White Carneaux pigeons were purchased from Palmetto Farms (South Carolina). [³H]Glutamate, [³H]kainate, [³H]glycine, [³H]GABA and [³H] flunitrazepam were purchased from Amersham (Arlington Heights, ILL). [³H]AMPA and [³H]MK-801 were purchased from DuPont-NEN (Boston, MA). Non-radioactive MK-801 was a gift of Dr L. L. Iversen (Merck, Sharp & Dohme). Baclofen was a gift of Dr Peter Sorter (Ciba-Geigy). Non-radioactive AMPA, kainate, NMDA, quisqualate,

3-(2-carboxypiperazin-4-yl)-propyl-1-phosphoric acid (CPP) and isoguvacine were purchased from CRB (Wilmington, DE). The remaining reagents were purchased from Sigma (St Louis, MO) and were of the highest possible purity.

After pigeons were decapitated, their brains were extracted rapidly from the cranial vault, covered in Lipshaw embedding matrix, and frozen in crushed dry ice. Brains were stored at −70°C until the time of sectioning. After equilibration at −20°C, brains were sectioned in a coronal plane at 20 microns on a Lipshaw cryostat. Sections were thaw mounted onto gelatin-coated slides, air dried and stored at −20°C until time of assay. All assays were performed within 3 days of sectioning.

EAA and GABA binding site subtypes were assayed according to standard techniques (Table 1). In all assays, non-specific binding is less than 10%, except for MET binding, where non-specific binding approaches 50%. GABA-A, GABA-B, NMDA, AMPA, MET, kainate and glycine binding sites were all assayed in a similar manner. Sections were prewashed in buffer at 4°C for 30 min, dried under a stream of cool air and immersed in cytomailers containing buffer at 4°C with radioactive ligand and the required selective blocking agents. After a 45-min incubation period (except for [$^3$H]glycine, with an incubation period of 35 min), the sections received three (GABA assays) or four (remaining assays) rapid 4-ml rinses of 4°C buffer followed by one (GABA assays) or two (remaining assays) rapid 3-ml rinses of 2.5% glutaraldehyde in acetone at 4°C, and dried under a stream of hot air.

MK-801 binding was assessed with the method of Sakurai et al. (1991, Table 1). Sections were prewashed in buffer for 30 min, dried under a stream of cool air, and immersed in ligand solution containing 5 nM [$^3$H]MK-801. Following incubation for 2 h at room temperature, sections were rinsed in buffer at 4°C for 80 min, and dried under a stream of hot air. Sections were assayed simultaneously with sections of pigeon forebrain at levels where we have documented abundant [$^3$H]MK-801 binding (Sakurai et al., 1990).

To measure BDZ binding site density, sections received 3 × 10-min washes in 4°C buffer, were dried under a stream of cool air, and immersed in 4°C ligand solution for 30 min. Following incubation with ligand, sections received one quick dip in buffer at 4°C followed by 2 × 5-min rinses in buffer at 4°C, and were dried under a stream of hot air.

Three pigeon brains were used for assay of GABA-A, GABA-B, BDZ and kainate binding, and three other brains were used for assay of MK-801, NMDA, glycine, AMPA and MET binding sites. All assays were run in duplicate or triplicate. Following conclusion of the assays, the slides were apposed to tritium-sensitive film and exposed along with known radioactive standards for 10 days to 6 weeks (Table 1).

Table 2. GABA and EAA binding sites in pigeon cerebellar cortex. N of three animals for each binding assay. All values in fmol/mg protein (SEM)

| Receptor | Molecular layer | Granule cell layer |
|---|---|---|
| BDZ | 331 (27) | 440 (6) |
| GABA-A | 695 (32) | 2473 (232) |
| GABA-B | 154 (7) | 65 (7) |
| Kainate | 23598 (1200) | N.S. |
| AMPA | 1660 (32) | 345 (13) |
| MET | 673 (166) | 198 (56) |
| NMDA | 185 (60) | 540 (50) |
| MK-801 | 70 (10) | 80 (10) |
| GLY | 370 (30) | 1500 (90) |

Films were developed in Kodak D-19 and binding site density in the molecular and granule cell layers was quantified with computer-assisted densitometry using the MCID system (Imaging Research, St Catherines, Ont.). It was impossible to determine reliably the extent of the Purkinje cell layer, and ligand binding in this layer was not measured. At least 50 readings were taken in each layer with a variable size cursor. Film density was converted to bound radioactivity with a polynomial regression curve derived from the co-exposed standards.

## RESULTS

### GABA binding sites

The overall level of GABA-A binding sites was higher than GABA-B binding sites in both granule cell and molecular layers (Table 2, Figure 1). There was a higher level of GABA-A binding sites in the granule cell layer than in the molecular layer. GABA-B binding sites had a higher level in the molecular layer than in the granule cell layer.

### BDZ binding sites

BDZ binding sites had similar levels in both layers (Table 2, Figure 1).

### NMDA receptors

When assayed with NMDA or CPP competition of [$^3$H]glutamate binding, more NMDA binding sites were found within the granule cell layer than the molecular layer (Table 2, Figure 2). After NMDA or CPP competitive displacement of [$^3$H]glutamate binding there was a significant amount of residual [$^3$H]glutamate binding, particularly in the molecular layer (Figure 2). The amount of specific binding was identical regardless of whether NMDA or CPP were used to define non-specific binding. The pattern of [$^3$H]glycine binding was identical to that of NMDA/CPP competitive displacement of [$^3$H]glutamate binding (Table 2, Figure 2), with more binding in the granule cell layer than the molecular layer. Little

Fig 1. GABA and BDZ binding site distribution in pigeon cerebellar cortex. (A) BDZ binding sites; ligand binding levels are approximately equal in both molecular and granule cell layers. (B) GABA-A binding sites; ligand binding is highest in the granule cell layer. (C) GABA-B binding sites; ligand binding is highest in the molecular layer. ML = molecular layer, GL = granule cell layer, scale bar = 0.88 mm.

specific [³H]MK-801 binding was found in either layer of the cerebellar cortex (Table 2, Figure 2) while abundant specific [³H]MK-801 binding was found in sections of simultaneously assayed pigeon forebrain (data not shown).

### Non-NMDA EAA receptors

AMPA, kainate and MET binding sites were found at highest level in the molecular layer (Table 2, Figure 3). Kainate binding sites had an exceptionally high level in the molecular layer with virtually no binding in the granule cell layer under these conditions.

### DISCUSSION

#### Methodological considerations

Comparison of our present results with data obtained in mammals assumes that the binding site assays we employed identify identical sites in birds and mammals. Rigorous verification of this assumption would require an extensive series of pharmacological and kinetic experiments beyond the scope of this paper. Available evidence indicates that GABA-A, BDZ, AMPA and NMDA binding sites in avian brain are identical to those of mammals. Several groups have investigated the pharmacology, regulatory properties and kinetics of GABA-A and BDZ receptors in a broad variety of species (Dietl *et al.*, 1988; Nielsen *et al.*, 1978; Tehrani and Barnes,1986; Maggi *et al.*, 1980). These properties have been found to be conserved across a broad

range of species. Similarly, biochemical studies have shown that BDZ binding proteins in avians have physical properties similar to those of mammals (Hebebrand *et al.*, 1986, 1987; Friedl *et al.*, 1988). Henley and Barnard (1989) have found the pharmacology and kinetics of AMPA binding sites in chick brain homogenates to be similar to those described in mammals. Our own studies of NMDA receptor pharmacology, kinetics and regulation in pigeon brain (Sakurai *et al.*, 1990) and those of McCabe and Horn (1988) in chick brain have shown that NMDA binding sites in avian brain have properties similar to those of mammals.

A different situation exists with respect to kainate binding sites. Our work confirms previous reports of a high density of kainate binding sites in the molecular layer of the avian cerebellum (Henke *et al.*, 1981). In mammalian cerebellar cortex, [³H]kainate binding is found mainly in the granule cell layer (Monaghan and Cotman, 1982; Unnerstall and Wamsley, 1983). In both mammals and birds, kainate binding sites exist in high and low affinity forms (Henke *et al.*, 1981; Voukelatou *et al.*, 1986; London *et al.*, 1980). Henke *et al.* (1981) have documented that pigeon cerebellar cortical kainate binding is almost exclusively to the low affinity site. This binding protein has been purified and a monoclonal antibody directed against the kainate binding protein developed (Klein *et al.*, 1988). Somogyi *et al.* (1990) have shown that this monoclonal antibody binds to cerebellar cortical glia. Cloning of the gene for the avian cerebellar kainate binding protein has also been accomplished and its sequence has relatively little resemblance to the rodent kainate receptor (Gregor *et al.*, 1989). It seems unlikely that
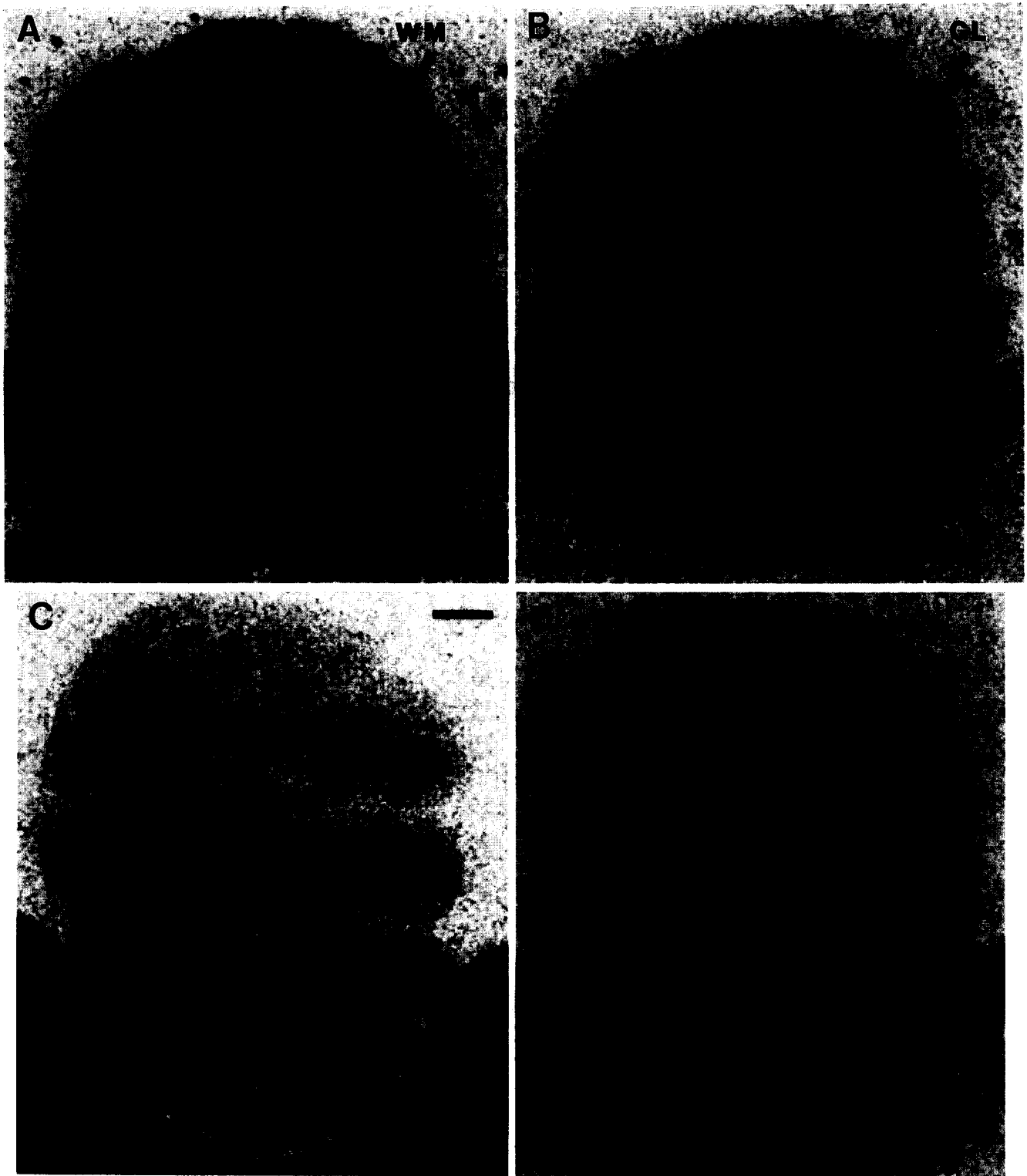
Fig 2. NMDA binding site distribution in pigeon cerebellar cortex. (A) [³H]Glutamate binding in the absence of NMDA or CPP. (B) [³H]Glutamate binding in the presence of NMDA. Note the decrease in binding in the granule cell layer and the persistence of binding in the molecular layer. (C) Strychnine-insensitive glycine binding sites; ligand binding is highest in the granule cell layer, paralleling the distribution of [³H]glutamate binding that is competitively displaced by NMDA or CPP. (D) [³H]MK-801 binding; little binding is seen. Abbreviations and scale as in Fig. 1.

kainate binding in the pigeon cerebellar cortex identifies a homologue of the mammalian neuronal kainate receptor.

Interpretation of receptor autoradiography with tritiated ligands is complicated by the existence of the so-called 'quenching phenomenon'. Tritium
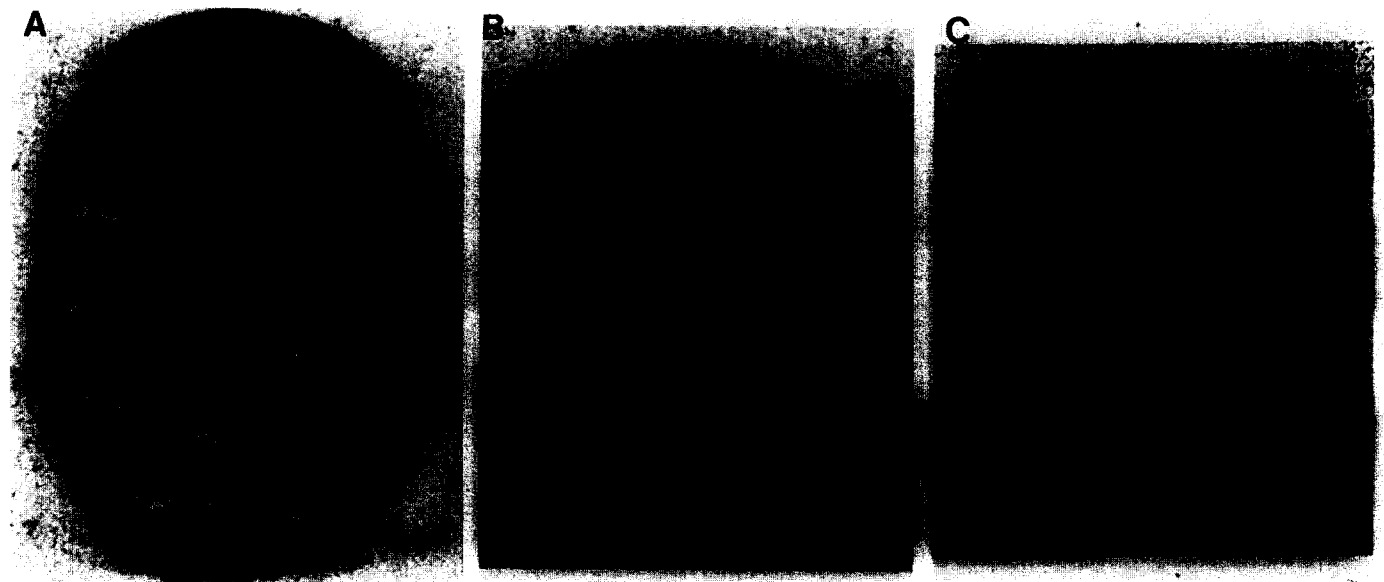
Fig 3. (A) Kainate binding sites; there is very high [³H]kainate binding in the molecular layer. (B) AMPA binding sites; ligand binding is highest in the molecular layer. (C) MET binding sites; ligand binding is highest in the molecular layer. Image made by digital subtraction of non-specific binding from total binding because of the relatively high non-specific binding in the assay. Abbreviations and scale identical to those of Fig. 1.

emits relatively weak beta particles and some of these particles are absorbed by surrounding tissue, especially white matter (Geary and Wooten, 1985; Geary *et al.*, 1985; Rainbow *et al.*, 1984). In the cerebellar cortex, this phenomenon may lead to an underestimate of the amount of ligand binding in the molecular layer relative to the granule cell layer. However, studies of [¹⁴C]glutamate binding in the rat pallidum and hippocampus show that white matter quenching does not cause a significant change in the pattern of binding site distribution (Greenamyre *et al.*, 1987; McDonald *et al.*, 1990), indicating that white matter quenching is not a major determinant of the qualitative distribution of binding sites. Finally, our experiments were all performed with single concentrations of ligands. We cannot, consequently, compare the absolute densities ($B_{max}$) of binding sites for different ligands. We can, however, determine the relative amounts of binding for a single ligand in the different layers of the cerebellar cortex. This analysis assumes only that the affinity ($K_d$) of binding sites for a given ligand is the same in both the granule cell and molecular layer.

### Comparison with mammals and other phyla

In general, our results show that the distribution of GABA and EAA binding sites in pigeon cerebellar cortex is identical to that seen in mammals and those non-mammalian species that have been examined.

GABA-A binding site distribution has been well characterized in mammalian cerebellar cortex and in some non-mammalian species and found to be similar across a wide variety of species. In mammals, GABA-A binding sites are found in both molecular

and granule cell layers with a significantly higher level of GABA-A binding sites in the granule cell layer (Palacios *et al.*, 1980, 1981; Albin and Gilman, 1990). Schmitz *et al.* (1988), using [³H]muscimol as a GABA-A ligand, have obtained similar results in a variety of non-mammalian species including trout, toads and chickens. We have found also that turtle cerebellar cortex displays a similar distribution of GABA-A binding sites (Albin *et al.*, 1989). Similarly, cerebellar cortical BDZ binding site distribution has also been well characterized in mammals and some non-mammalian species. In rodents, BDZ binding sites are found at highest level in the molecular layer while primates have a high level of BDZ binding sites in both molecular and granule cell layers (Young and Kuhar, 1979; Palacios and Kuhar, 1982; Albin and Gilman, 1990). Dietl *et al.* (1988) and Schmitz *et al.* (1988) have both found that avians and some other non-mammalian species have roughly equal levels of BDZ binding sites in the molecular and granule cell layers. Our present data in pigeons, obtained with a different GABA-A ligand and an identical BDZ binding site assay, confirm previous results.

No prior study has reported the distribution of GABA-B binding sites in a non-mammalian species. In both rodent and human cerebellar cortex, GABA-B binding sites are found predominantly in the molecular layer (Chu *et al.*, 1990; Bowery *et al.*, 1987; Albin and Gilman, 1990). Our data in pigeons reveal an identical pattern of GABA-B binding site distribution and density relative to GABA-A binding sites. There may be one important difference in GABA-B binding site distribution between mammals and birds. In the vermis of the rat cerebellar cortex, we have shown that GABA-B binding

sites are arranged in alternating high and low density parasagittal zones (Albin and Gilman, 1989), a pattern similar to the well-characterized parasagittal distribution of olivocerebellar fibres and other markers of parasagittal zonation in the cerebellar cortex. Freedman *et al.* (1977) have shown that the olivocerebellar system in pigeons has a parasagittal termination pattern similar to that seen in mammals. We found, however, no evidence of parasagittal zonation of GABA-B binding sites in the pigeon cerebellar cortex. Study of more mammals and non-mammalian species will be necessary to determine if the absence of parasagittal zonation of GABA-B binding sites in pigeon cerebellum is a significant inter-phyletic difference.

The distribution of NMDA binding sites in pigeon cerebellar cortex appears also to be identical to that of mammals. When assayed with either [$^3$H]glutamate under selective conditions or with [$^3$H]glycine, the highest density of NMDA binding sites is found in the granule cell layer in both mammals and pigeons (Olson *et al.*, 1987; Greenamyre *et al.*, 1985; Monaghan and Cotman,1985; McDonald *et al.*, 1990; Makowiec *et al.*, 1991). Our data confirm previous [$^3$H]glutamate binding data obtained in chick brain by Mitsacos *et al.* (1990), who used less selective assays. As previously documented by Henley and Barnard (1989) in chick brain, the density of [$^3$H]MK-801 binding was relatively low, despite the presence of a moderate density of NMDA binding sites. This phenomenon is also seen in mammals, where the binding site for MK-801 and related ligands is found in the cerebellar cortex but in a low affinity state (Vignon *et al.*, 1986; Sakurai *et al.*, 1991). Our [$^3$H]glutamate/NMDA assay reveals also a high level of [$^3$H]glutamate binding in the molecular layer that is not competitively displaced by NMDA or CPP. Since this assay is done in the presence of high concentrations of kainate and quisqualate, this residual binding is insensitive to kainate or quisqualate competition. Preliminary experiments (Albin *et al.*, unpublished data) have shown that this residual binding is competitively displaced by glutamate. This non-NMDA, non-kainate, non-quisqualate glutamate binding site exists also in human and rat molecular layer and appears to be located on neurons (Albin and Gilman, 1990; Higgins *et al.*, 1989; Greenamyre *et al.*, 1990).

Binding to non-NMDA EAA binding sites in pigeon cerebellar cortex is also largely similar to that seen in mammals, where AMPA and MET binding occur at highest levels in the molecular layer (Makowiec *et al.*, 1990, 1991; Cha *et al.*, 1990; Nielsen *et al.*, 1988). As mentioned above, [$^3$H] kainate binding in avian cerebellar cortex has a different distribution from that seen in mammals and available evidence indicates that this binding protein is not homologous to the kainate receptor of mammals. It is intriguing that the only binding assay that gives a pattern of binding different from that seen in mammals is also the only one where the avail-able evidence indicates lack of homology between mammalian and avian binding proteins. This counter-example supports the idea, originally put forward by Deitl and Palacios (1988), that use of binding site assays developed in mammals provides a legitimate basis for comparison of neurotransmitter chemoarchitecture when similar binding site distributions are found in homologous structures.

Finally, experiments in rodents using toxic, viral or genetic lesions of cerebellar cortical neuron subpopulations have allowed assignment of specific binding site types to cerebellar cortical neurons (reviewed in Albin and Gilman, 1990). GABA-A, BDZ and NMDA binding sites are found on granule cells, while AMPA, MET, GABA-B and BDZ binding sites are found on Purkinje cell dendrites. The identity of distribution of GABA and EAA binding sites between mammals and pigeons suggests that the cellular localization of these binding sites is also phylogenetically conserved. There is no direct evidence in avians to confirm this hypothesis, but it is consistent with electrophysiological data showing that climbing and parallel fibre activation in both birds, reptiles and mammals produces very similar patterns of post-synaptic potentials in Purkinje cells (Llinas and Hillman, 1969).

In summary, our data support the conclusion that the chemoarchitecture of the cerebellar cortex is conserved in the course of vertebrate evolution.

## ACKNOWLEDGEMENTS

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