Brain Research, 595 (1992) 164–166

BRES 25424

$GABA_A$, $GABA_B$, and benzodiazepine binding sites in the cerebellar cortex of the red-eared turtle (*Pseudemys scripta*)

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(Accepted 4 August 1992)

Key words: Stellate cell; Purkinje cell; Granule cell; Golgi cell; Molecular layer; Evolution; Benzodiazepine

We used receptor autoradiography to ascertain the distribution of $GABA_A$ and $GABA_B$ binding sites in the cerebellar cortex of the red-eared turtle (*Pseudemys scripta*). $GABA_A$ binding sites were found in both molecular and granule cell layers with highest levels in the granule cell layer. $GABA_B$ binding sites were found at highest level in the molecular layer. Benzodiazepine binding sites were found in approximately equal abundance in both layers. Little binding of any ligand was seen in the Purkinje cell layer. Our results are similar to those found in mammals and other non-mammalian vertebrates and indicate that the organization of inhibitory pathways of the cerebellar cortex has been conserved in the course of vertebrate evolution.

The cytoarchitecture and synaptic organization of the cerebellar cortex has been remarkably conserved in the course of vertebrate evolution (see review by Llínas and Hillman[9]). This is particularly true of excitatory pathways. The mossy fiber–granule cell–parallel fiber–Purkinje cell and the climbing fiber–Purkinje cell circuits are present in virtually all vertebrates with Golgi and ultrastructural studies indicating that the morphology of these synapses is relatively conserved across vertebrate phyla[9]. The presence of cerebellar GABAergic interneurons is more variable. Amphibians possess a sparse population of stellate cells only, reptiles possess both stellate and Golgi cells, while mammals and avians possess stellate, Golgi and basket cells[9]. To investigate further the evolution of inhibitory pathways within the cerebellar cortex, we used receptor autoradiography to determine the distribution of $GABA_A$, $GABA_B$, and benzodiazepine (BDZ) binding sites in the cerebellar cortex of red-eared turtles (*Pseudemys scripta*).

Four turtles were decapitated, the brains extracted rapidly from the calvarium, frozen in crushed dry ice, and stored at −70°C until the time of assay. Twenty-micron frozen sections of the cerebellum were cut on a Lipshaw cryostat, thaw-mounted onto gelatin coated glass slides, and stored at −20°C until time of assay. All assays were done within 3 days of sectioning and triplicate sections of each animal were used in each assay. $GABA_A$ and $GABA_B$ binding sites were assayed with [^{3}H]GABA (Amersham, 100 Ci/mmol) under selective conditions according to the method of Chu et al.[5]. Briefly, sections were prewashed in 50 mM Tris-HCl buffer containing 2.5 mM $CaCl_2$ (pH 7.4 at 4°C) for 30 min and dried under a stream of cool air. Sections were then incubated in the same buffer containing 20 nM [^{3}H]GABA and selective blocking agents for 45 min. For $GABA_A$ binding, 100 μM baclofen was added; for $GABA_B$ binding, 10 μM isoguvacine was added. Non-specific binding was assessed with the addition of 100 μM baclofen and 100 μM isoguvacine. Following incubation in ligand solution, slides received 3 × 3 ml rapid rinses with the same buffer followed by 1 × 2 ml rapid rinse with 2.5% glutaraldehyde in acetone. To measure BDZ binding sites, slides were prewashed in 50 mM Tris-citrate buffer (pH 7.4 at 4°C), dried under a stream of cool air, and incubated in the same buffer containing 5 nM [^{3}H]flunitrazepam (Amersham, 82 Ci/mmol) for 30 min. Following incu-

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TABLE I

Distribution of $GABA_A$, $GABA_B$, and benzodiazepine (BDZ) binding sites in the molecular and granule cell layers of turtle cerebellar cortex

All values in fmol/mg protein (±S.E.M.)

	Molecular layer	*Granule cell layer*
BDZ	162 (17)	132 (13)
$GABA_A$	1483 (123)	3444 (179)
$GABA_B$	441 (56)	211 (39)

bation with ligand, sections received one quick dip in the same buffer followed by 2 × 5 min washes in the same buffer, and were dried under a stream of hot air.

After completion of the assays, slides were apposed to tritium-sensitive film (Hyperfilm, Amersham) along with known radioactive standards and exposed for 10 days to 3 weeks. Films were developed in Kodak D-19, and ligand binding levels were quantitated with computer assisted densitometry using the MCID system (Imaging Research, St. Catherines, Ont.). Multiple readings were taken of the molecular and granule cell layers using a variable size cursor. Film optical density was converted to bound radioactivity with a polynomial regression curve derived from the co-exposed standards.

High levels of $GABA_A$ binding were found in both molecular and granule cell layers with a higher level of binding in the granule cell layer (Table I, Fig. 1). For $GABA_B$ binding, the pattern was reversed with highest levels of ligand binding in the molecular layer (Table I, Fig. 1). BDZ binding levels were approximately the same in both molecular and granule cell layers (Table I, Fig. 1). Little binding of any ligand was seen in the Purkinje cell layer (Fig. 1), though the actual levels of ligand binding could not be quantitated because the narrow width of the Purkinje cell layer precluded accurate measurement.

The distribution of $GABA_A$, $GABA_B$, and BDZ binding sites in the cerebellar cortex of red-eared turtles is similar to that of mammals and those non-mammalian vertebrates that have been examined to date. Abundant $GABA_A$ binding has been found in the cerebellar cortex of mammals with highest levels of binding sites in the granule cell layer[2,12,13]. An identical pattern of $GABA_A$ binding site distribution has been found in the cerebellar cortex of sharks, trout, toads, lizards, chickens, and pigeons[3,6,15]. The distribution of $GABA_B$ binding sites has been studied in a smaller range of species, but the predominance of $GABA_B$ binding in the molecular layer has been observed in human, rodent, and pigeon cerebellar cortex[2,3,5]. We have, however, found that $GABA_B$ binding in the vermis of rat cerebellum is characterized by alternating high and low density parasagittal zones[1], a pattern reminescent of the parasagittal distribution of olivocerebellar fibers and other markers of parasagittal zonation within the cerebellar cortex. Kunzle has demonstrated that olivocerebellar terminals exhibit parasagittal zonation in the molecular layer of turtle cerebellar cortex[8]. We have also been unable to find parasagittal zonation of $GABA_B$ binding sites in pigeon cerebellar cortex, suggesting that this phenomenon may be confined to mammals[3]. Finally, studies of BDZ binding in a variety of vertebrate species have shown that BDZ binding sites are found in vari-

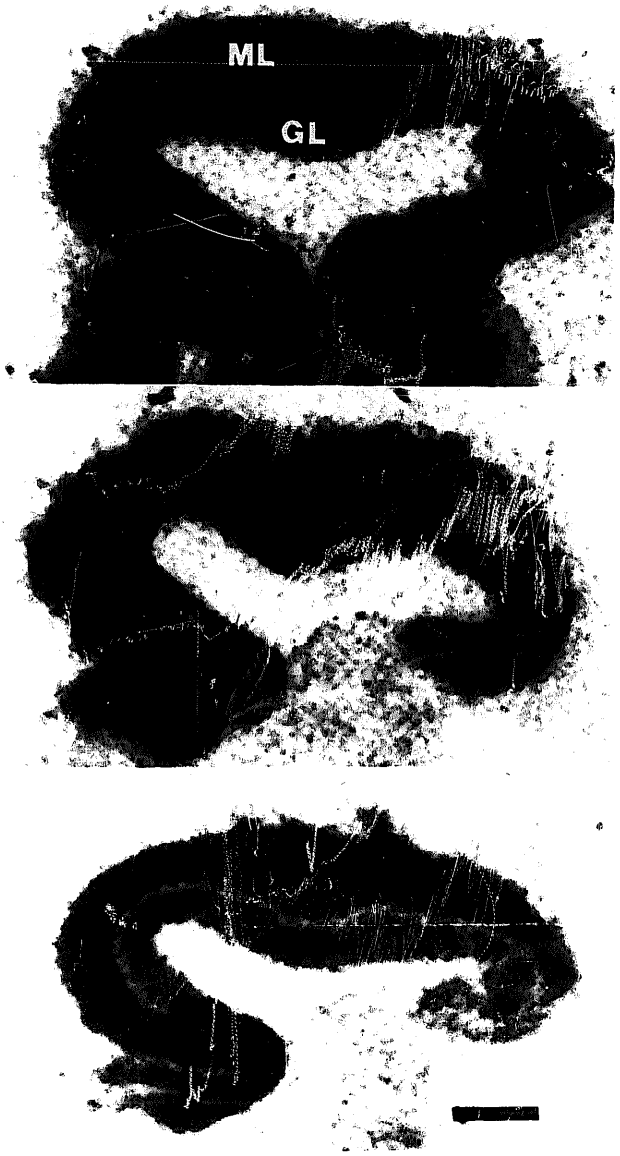

Fig. 1. Distribution of benzodiazepine (A), $GABA_A$ (B), and $GABA_B$ (C) binding sites in turtle cerebellar cortex. Benzodiazepine binding sites are found in equal abundance in both molecular layer (ML) and granule cell layer (GL). $GABA_A$ binding site levels are higher in GL than ML and $GABA_B$ binding site levels are higher in ML than GL. Less binding is seen in the Purkinje cell layer, the relatively pale band between ML and GL, though Purkinje cell layer binding could not be quantitated accurately. Bar = 1 mm.

able proportions in the molecular and granule cell layers[6,15]. Schmitz et al. have obtained identical results in immunohistochemical studies of cerebellar cortex using antibodies directed against the BDZ binding site[15].

Our conclusions are based on the assumption that the assays we employ identify identical binding sites in mammals and non-mammalian species. This assumption has not been verified for $GABA_B$ receptors, but prior studies indicate that the pharmacology, regulatory properties, and kinetic properties of BDZ and $GABA_A$ binding sites have been conserved across a broad range of species[6,7,10,11,16], indicating that these assays can be legitimately applied to non-mammalian species.

The phylogeny of turtles has been controversial[4], however, recent paleontologic analysis indicates that turtles are a distinct lineage sharing a remote common ancestor with other modern amniotic vertebrates[14]. Modern turtles consequently serve as a good outgroup for comparisons with other amniote lineages. Our data and prior studies show that the regional distribution of $GABA_A$, $GABA_B$, and BDZ binding sites in cerebellar cortex has been conserved in the course of vertebrate evolution. Studies of rodents and humans with toxic or genetic lesions of cerebellar cortical neuron subpopulations indicate that most $GABA_A$ and BDZ binding sites are localized on Purkinje and granule cells while most $GABA_B$ binding sites are located on Purkinje cells (reviewed in Albin and Gilman[2]). The phylogenetic conservation of regional GABA and BDZ binding site distribution suggests that the cellular localization of these binding sites is also conserved. If so, the evolution of the cerebellar cortex in amniotic vertebrates appears to be characterized by the addition of and functional specialization of populations of GABAergic interneurons rather than by qualitative changes in the distribution of GABAergic synapses within the cerebellar cortex.

We thank Dr. Anton Reiner for the donation of turtle brains. Supported by NS 01300 and NS 19613.

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