Neuroscience Vol. 35, No. 1, pp. 9–30, 1990
Printed in Great Britain

0306-4522/90 $3.00 + 0.00
Pergamon Press plc

SUBCELLULAR LOCALIZATION OF A PUTATIVE KAINATE RECEPTOR IN BERGMANN GLIAL CELLS USING A MONOCLONAL ANTIBODY IN THE CHICK AND FISH CEREBELLAR CORTEX

PETER SOMOGYI,*† NOMI ESHHAR,*‡ VIVIAN I. TEICHBERG‡ and J. DAVID B. ROBERTS*

*MRC Anatomical Neuropharmacology Unit, South Parks Road, Oxford OX1 3QT, U.K.
‡Department of Neurobiology, The Weizmann Institute of Science, Rehovot 76100, Israel

Abstract—A monoclonal antibody, IX–50, that was raised against a kainate binding protein ($M_r = 49{,}000$) from chicken cerebellum, was used in light and electron microscopic immunocytochemical studies to localize putative kainate receptors. Pre- and postembedding immunoperoxidase and immunogold methods were used in the cerebellar cortices of one to 26-day old chickens and adult rainbow trout.

Immunoreactivity was detected only in association with Golgi epithelial/Bergmann glial cells. Intracellular immunoreactivity was present in the granular and agranular endoplasmic reticulum, Golgi apparatus and in lysosomes, representing the sites of synthesis, glycosylation and degradation of the protein. In the fish the granular endoplasmic reticulum was not immunoreactive. Extracellular immunoreactivity was associated with the plasma membrane. In the fish it was established that the epitope is on the outer surface of the membrane. The protein seems to be uniformly distributed along the membrane including the somata, the radial stem processes and the leafy lamellae surrounding Purkinje cell dendrites. Areas of the glial membrane in contact with other glial cells were also immunopositive.

High-resolution light microscopy demonstrated all the Bergmann glial plasma membrane in the cortex, providing a "negative" image of Purkinje cell dendrites. It is apparent that Bergmann glial processes selectively outline the dendrites of the Purkinje cells by surrounding the parallel fibre terminal/Purkinje cell spine synaptic complexes. The parallel fiber terminals were highly immunoreactive for glutamate, as shown by an immunogold procedure.

The association of Bergmann glial processes, carrying the $M_r = 49{,}000$ kainate binding protein, with the Purkinje cell dendrites and spine synapses could provide a basis for neuronal signalling to the Bergmann glia, possibly by glutamate.

Acidic amino acids such as glutamate, aspartate and homocysteate are released in the central nervous system and act as mediators of cellular interactions. The actions of these and similar compounds, collectively called excitatory amino acids, are mediated by several classes of receptor, which regulate cationic channels in the membrane (for review see Refs 52, 54, 83, 84). Kainic acid, isolated from the marine alga *Digenea simplex*, is a rigid cyclic analogue of glutamate. It activates receptors which are thought to form one class of excitatory amino acid receptor, the so called kainate receptors. Kainate activates a conductance that is not voltage dependent, and its current reverses polarity close to 0 mV membrane potential,[52] indicating that the receptor controls the membrane permeability for both Na^+ and K^+. Furthermore, kainate receptors are more sensitive to the antagonists gamma-D-glutamylaminomethylsulphonate[39] and quinoxalinediones[35] than is another class of excitatory amino acid receptor, the *N*-methyl-D-aspartate receptor. Using physiological and pharmacological characteristics, kainate receptors have been found on both neurons (for review see Refs 52, 54, 83) and glia.[3,7,76,81]

Kainate also binds selectively to isolated membranes and to tissue sections. Radiolabelled kainic acid is displaced from these sites by low concentrations of domoate and glutamate, while other acidic amino acids are less potent.[52,54,83,84] Although ligand binding studies have provided a great deal of information on the regional distribution of kainate binding sites (for review see Refs 10, 31, 47), little is known about their cellular localization and subcellular distribution. In addition, the biochemical identity of the binding sites and the physiologically defined kainate receptors remains to be established.

Kainate binding sites are particularly abundant in the brains of lower vertebrates,[15,27,29,48] which have recently been used for the isolation of kainate binding proteins. Thus proteins composed of a single polypeptide have been purified from frog,[23,24] pigeon[45] and chicken[19] brain. Of all brain areas, the molecular layer of the cerebellar cortex contains the highest density of kainate binding sites in these species.[15,19,23,27,28] The selective localization of kainate binding sites to the molecular layer has also been confirmed using

†To whom correspondence should be addressed.

Abbreviations: BSA, bovine serum albumin; HRP, horseradish peroxidase; NGS, normal goat serum; PB, phosphate buffer; PBS, phosphate-buffered saline; TPBS, Tris–phosphate-buffered saline.

kainate conjugated to bovine serum albumin (BSA), a high affinity ligand for the kainate binding sites,[15] or using monoclonal antibodies raised to the isolated kainate binding protein from chick[19] and pigeon[45] cerebellum. The high density of kainate binding sites in the molecular layer of the cerebellum of lower vertebrates raises the question of their precise localization as well as that of their physiological role. Since the molecular layer has several cellular components which are unique to this layer, the present high-resolution immunocytochemical study was carried out in the cerebellar cortex in order to establish the cellular and subcellular distribution of the putative kainate receptor. A preliminary report of these results has been published elsewhere.[16]

EXPERIMENTAL PROCEDURES

Antibodies

A mouse monoclonal antibody, IX-50, produced to sucrose gradient purified kainate binding protein from chicken cerebellum, was used. The antibody was purified from ammonium sulphate precipitated ascites proteins, on a QAE-Sephadex A-50 column. In Western blots, the antibody recognizes two polypeptides with $M_r = 49{,}000$ and 93,000 in the chicken cerebellum, and a polypeptide with $M_r = 49{,}000$ in the fish cerebellum. The $M_r = 49{,}000$ polypeptide binds kainate, as described earlier.[19]

A mouse monoclonal antibody, bd-24, specific to the α-subunit of the $GABA_A$/benzodiazepine receptor/chloride channel complex was used as one of the controls for method specificity. The purification of the receptor complex and the preparation of the antibody were described earlier.[25,68] In the present experiment, tissue culture supernatant, containing 10% calf serum, was used for immunocytochemistry. The antibody was donated by Dr J. G. Richards.

A rabbit polyclonal antiserum, raised against glutamate conjugated to keyhole limpet hemocyanin with glutaraldehyde,[30] was used for the localization of glutamate immunoreactivity. This antiserum was characterized earlier and has been found to give only weak cross-reactivity with conjugated aspartate and carboxyl terminal peptidyl glutamate. The antiserum was donated by Dr P. Petrusz.

Animals

White Cobb chickens (*Gallus domesticus*, one to 26 days old, Hooks Hatcheries, Coate, U.K.) and young adult (300–400 g) rainbow trout (*Salmo gairdneri*), obtained from a local farm, were used. The chickens were anaesthetized with chloral hydrate (BDH, 500 mg/kg, i.p.); the fish were anaesthetized with MS 222 (Sandoz, 100 ppm) mixed in their water.

Fixation

The animals were perfused initially with saline for approximately 0.5–1 min, followed by fixative for approximately 30 min, at room temperature for the chickens, and chilled for the fish. The latter were kept on ice during perfusion. Two fixatives were used: the first[74] contained paraformaldehyde (4%), glutaraldehyde (0.025–0.05%) and picric acid (approximately 0.2%), made up in 0.1 M phosphate buffer (PB, pH 7.2–7.4); the second fixative contained all these components, but glutaraldehyde was at a higher concentration (1.25%). The brains were dissected after perfusion and the cerebellum was sliced. Tissue blocks were washed free of aldehydes in 0.1 M PB.

Tissue processing for standard electron microscopy

Blocks from animals fixed with fixative No. 2 were washed free of aldehydes in PB, and postfixed in 1% OsO_4 (dissolved in PB pH, 7.2) for 1 h. They were then dehydrated in alcohol and embedded in epoxy resin (Durcupan, ACM, Fluka).

Tissue processing for pre-embedding immunocytochemistry

Blocks from animals fixed with fixative No. 1 were placed sequentially in 0.1 M PB containing 10 and 20% sucrose until they sank. To facilitate the penetration of reagents, the blocks were frozen in liquid N_2 then thawed in 0.1 M PB as described earlier.[74] Sections were cut on a Vibratome at a thickness of 50 μm, and were treated with 1% sodium borohydride[67] dissolved in phosphate-buffered saline (PBS, pH 7.4).

A standard avidin–biotin–peroxidase complex method was used for immunocytochemistry. Briefly, free-floating sections were incubated first with 10% normal horse serum for 1 h to block non-specific binding of the antibodies, then either overnight or for two days at 4°C, with antibodies to either the kainate binding protein (IX-50, diluted 1:100–200) or to the GABA receptor complex (diluted 1:2). This was followed by incubation with biotinylated horse anti-mouse IgG (dil. 1:50, Vector) overnight at 4°C. The sections were then washed in PBS containing 1% normal horse serum, followed by incubation in avidin–biotin–horseradish peroxidase (HRP) complex (diluted 1:100, Vector) for 4 h.

In some cases specimens were prepared for light microscopy only and Triton X-100 (0.05–0.2%) was added to the blocking and to the primary antibody solutions to increase penetration of antibodies and to reveal possible masked immunoreactive sites.

As controls for method specificity, some sections were incubated in tissue culture medium, and other sections were incubated with omission of the primary antibody step from the sequence.

The peroxidase enzyme reaction was carried out in the dark by preincubating the sections for 30 min in 0.05% diaminobenzidine tetrahydrochloride (Sigma), dissolved in 50 mM Tris buffer (pH 7.4), followed by incubation in the same solution containing 0.01% H_2O_2 for 3–10 min. After washing in PB, the sections for combined light and electron microscopy were treated with OsO_4 (1% in PB) for 30 min, dehydrated in ethanol and embedded flat on glass slides in epoxy resin. To increase contrast for electron microscopy the sections were treated with 1% uranyl acetate in 70% ethanol for 40 min during dehydration. Lead staining was not used. Ultrathin sections were cut from the surface layers of the thick Vibratome sections because the immunoreactivity was usually limited to the superficial 10–15 μM of the sections. At least two areas from each animal were cut for electron microscopy.

Tissue processing for postembedding immunocytochemistry on semithin (0.5 to 1 μm-thick) sections

Perfusion-fixed brain slices from animals fixed with either fixative 1 or 2 were washed free of aldehydes in PB. They were dehydrated and embedded in epoxy resin in capsules. Sections were mounted on albumin-coated slides and reacted as described earlier.[70] Briefly, the resin was etched with ethanolic sodium hydroxide. The slides were washed and incubated at room temperature, sequentially with 10% normal rabbit serum, with antibody IX-50 (diluted 1:100–200), and then with rabbit anti mouse IgG conjugated to HRP (Dako, dil. 1:50). The slides were washed and reacted to reveal HRP enzyme activity as described above. The peroxidase reaction end-product was enhanced with a mild OsO_4 treatment. Then, following dehydration, the sections were covered in synthetic mounting medium.

Controls for method specificity included replacement of antibody IX-50 with antibody bd-24, or omission of the primary antibody from the solution.

Tissue processing for postembedding immunogold reaction with antibody IX-50 on ultrathin sections

(1) Blocks from brains fixed either with fixative 1 or 2 were washed free of aldehydes.

(2) Sections (100-μm-thick) were cut on a Vibratome and washed further in 0.1 M PB, followed by washing for 3 × 30 min in veronal/acetate buffer,[66] pH 7.0, containing 10% sucrose.

(3) Sections were stained with uranyl acetate (filtered), 1% in veronal/acetate buffer plus 10% sucrose pH 5.0 in the dark with agitation for 2 h.

(4) Sections were washed in veronal/acetate buffer for 2 × 15 min.

(5) Sections were dehydrated through 30, 50, 70, 90, 95, 2 × 100% alcohols for 5–10 min each followed by 2 × 10 min propylene oxide and then they were placed into Durcupan resin overnight at room temperature prior to embedding in capsules.

This procedure produced good structural preservation without OsO_4 treatment and was carried out in order to produce contrast for electron microscopy, and to stabilize membranes.

Immunostaining procedure

(1) Serial ultrathin sections were cut and picked up onto formvar-coated 300 mesh nickel grids (TAAB). Alternate grids were used for immunostaining, the remaining grids were contrasted with lead citrate for electron microscopy.

(2) The following steps were carried out in small glass dishes:

(a) resin removal by submerging grids in mixture of sodium ethanolate (one part) absolute alcohol (two parts) for 30 s to 2 min;[50,72]

(b) grids were transferred through 2 × 100% ethanol, 90 and 70% ethanol and then into distilled water.

(3) The following steps were carried out in a moist chamber with grids floating on drops placed on parafilm, section down, slightly modified from that described earlier.[70] Briefly:

(a) wash in Tris–phosphate-buffered saline (TPBS);

(b) blocking non-specific protein binding with 1% ovalbumin (Sigma) in TPBS for 30 min, followed by washing;

(c) primary antibody IX-50 diluted 1:100 with TPBS plus 1% normal goat serum (NGS) for 1–3 h;

(d) wash in 0.05 M Tris–HCl buffer, pH 7.2, plus 0.5 mg/ml polyethylene glycol (mol. wt 20,000, BDH);

(e) goat anti-mouse IgG coupled to 15-nm gold particles (Bioclin, Cardiff, U.K.) diluted 1:40 in the solution described in (d);

(f) wash in 0.05 M Tris buffer 5 min; wash in 0.1 M PB 2 × 5 min;

(g) fix in 2% glutaraldehyde in 0.1 M PB for 10 min, and wash in PB;

(h) wash in distilled water.

(4) The remaining steps were carried out in glass dishes as described earlier.[50] The grids were dehydrated through 70, 90, 2 × 100% ethanol, 2–3 min each and into a solution of 2% Durcupan resin in 100% ethanol prepared fresh and allowed to stand for 30 min. Grids were removed from the resin and were carefully blotted between two sheets of type 54 filter paper (Whatman) and placed vertically in slots cut in a silicon rubber flat embedding mould. The grids were then placed in an oven at 56°C for 24–48 h.

(5) When the resin had cured the sections were contrasted by placing them on drops of saturated aqueous uranyl acetate for 20 min, washed, and then onto drops of lead citrate for 1 min, washed and dried.

Controls for method specificity included replacement of antibody IX-50 by antibody bd-24, or the omission of the antibody from the solution.

Golgi impregnation

In order to correlate the immunostaining with the pattern of cellular processes in the cerebellar cortex, rapid Golgi impregnation followed by gold toning was carried out as described earlier.[73] Slices of chicken cerebellum fixed with fixative 1 (but with 0.1% glutaraldehyde) were washed, and then sequentially treated with OsO_4, $K_2Cr_2O_7$ and $AgNO_3$, followed by sectioning, illumination and gold toning. Eventually the sections were embedded in epoxy resin in the same way as immunoreacted sections. Ultrathin sections were cut from selected areas containing Bergmann glial cells and contrasted with lead citrate.

RESULTS

Controls

Immunoreactivity was absent in the cerebellum when the primary antibody IX-50 was omitted from the incubation. In the pre-embedding incubations, peroxidase reaction end-product was only associated with red blood cells, and it was attributed to the peroxidase activity of haemoglobin. In postembedding incubations, no peroxidase reactivity or immunogold deposition was observed without using the primary antibody. When antibody IX-50 was replaced with antibody bd-24 for immunostaining of the chicken cerebellum, the molecular layer was only very weakly reactive in a pattern similar to that described earlier for other species.[75] In contrast, the granular cell layer was strongly immunoreactive with antibody bd-24 specific for the $GABA_A$ receptor complex. From these results, we concluded that the reaction that was obtained with antibody IX-50 was due to immunoreactive sites recognized by this antibody.

Light microscopic distribution of immunoreactivity detected by antibody IX-50

Both in the fish and in the chicken, strong reactions were obtained in the molecular layer of the cerebellar cortex (Figs 1–4). In the other layers, glial cells showed weak staining, but this was inconsistent from experiment to experiment and was only observed in the pre-embedding condition. The two species differed in that in the chicken the Purkinje cell layer was also immunoreactive, as seen in thick sections (cf. Figs 1B and 4A). Immunoreactivity was localized to the space between Purkinje cells, which themselves were outlined by immunoreactivity around their somata in the chicken. In the latter species immunoreactivity was associated with Golgi epithelial/Bergmann glial cells (Bergmann glial cells below) which were distributed both below and between the Purkinje cells, forming a thick layer and vastly outnumbering Purkinje cells (Fig. 5). In the fish Bergmann glial cells were less conspicuous, and they did not seem to form an obvious layer (Fig. 4). However, when they were seen, it was clear that the cells were immunoreactive (Fig. 4B). It was not possible to establish with the light microscope where this immunoreactivity was located. The reaction seemed to form a shell around the cells and their proximal processes (Fig. 4B and C).

In the molecular layer, both the main dendrites and the spiny branchlets of Purkinje cells were outlined by immunoreactivity (Figs 1B, 4A and B). In the fish, branching filopodia with fine protrusions were seen entering the top of the granule cell layer from the molecular layer and arborizing amongst granule and Purkinje cells (Fig. 4). However, in spite of the strong immunoreactivity it was impossible to establish which cells carry the immunoreactive sites. Semithin sections provided some clues.

The main dendrites as well as small processes of the Purkinje cells were completely revealed in semithin sections, because in the postembedding method penetration barriers to the antibodies are removed (Figs 2 and 3). The Purkinje cell geometry was demonstrated remarkably by the immunoreactivity. The most striking feature in the fish was that the Purkinje cell dendrites originating from different cells were lined up close to each other along the longitudinal axis of the parallel fibre bundles (Fig. 3C). In the chick, the Purkinje cell dendrites were followed by spine-like dots (Fig. 2), while in the fish the staining formed continuous rings along the dendrites (Fig. 3). The semithin sections clearly showed that the Bergmann glial cells were immunoreactive. It could not be established whether the immunoreactivity was extracellular or intracellular in the chick, because of the thin rim of cytoplasm around the nucleus (Fig. 2). However, in the fish, the cells are larger and have a higher cytoplasm to nucleus ratio, thus the intracellular and plasma membrane associated immunoreactivity could be resolved (Fig. 4C). The intracellular reaction was restricted to small short perinuclear dots and lines. The reactivity outlining the Bergmann cells was much thinner than the reactivity outlining the Purkinje cell dendrites. This suggested that the plasma membrane of Bergmann glial cells was reacting, but the question regarding the location of the protein(s) on the Purkinje cell dendrites was not resolved.

Composition of the molecular layer as revealed by Golgi impregnation

Since the light microscopy showed that at least some of the immunoreactivity was associated with glial cells, we studied the distribution of glial cell processes in the chick by Golgi impregnation. As also reported in earlier studies,[13,56,58,63] it was found that the glial cells around the Purkinje cells were Bergmann glial cells as identified by their radial processes (Fig. 5A and B). The radial fibres have thin stalks which emit elaborate leafy lamellae protruding up to 5 μm from the radial fibre. The radial fibres terminate with end-feet on the pia. In the chick there were immature granule cells in the outer granular layer, and this lamina was penetrated by the Bergmann glia as straight fibres without protrusions (Fig. 5A).

The Bergmann glial processes run radially and do not follow the obliquely branching Purkinje cell dendrites. This means that the dendritic arbour of a Purkinje cell is passed by the processes of a large number of Bergmann glial cells. This was confirmed by electron microscopic examination of single impregnated Bergmann processes. Only short segments of a dendrite were covered by the leafy lamellae originating from individual Bergmann glial cells. One such segment is shown in Fig. 5D. The lamellae followed the dendritic shafts and also surrounded the spine parallel fibre complexes. However, the parallel fibre bundles were devoid of Bergmann glial processes. The pattern described above corresponded

Fig. 1. Chicken cerebellum, 17 days old, 50-μm-thick sections. (A) Immunoreactivity detected by antibody IX-50 is localized to the molecular (ml) and Purkinje cell layers. Dots in the granular layer, in the white matter and in the deep cerebellar nuclei are not immunoreactive sites. They represent red blood cells demonstrating peroxidase enzyme activity of a haemoglobin. (B) At higher magnification immunoreactivity outlines Purkinje cells (Pc), their dendrites, basket cells (Bc), and also seems to be associated with Bergmann glial cells (Bg). Scale bars: A = 500 μm; B = 50 μm.

Fig. 2. Chicken cerebellum, 1 day old, 1-μm-thick sections. Postembedding immunocytochemistry with antibody IX-50 shows immunoreactivity outlining Purkinje cells (Pc), their main ascending dendrites (d) as well as their spiny branchlets. Immunoreactivity is associated with Bergmann glial cells (Bg) which lie beneath and amongst the Purkinje cells. Note that at high magnification occasional straight fibres (small arrows) are also immunopositive. Some of them (double arrows) seem to be continuous with the subpial immunopositive lamella. Scale bars: A = 50 μm; B and C = 20 μm.

Fig. 3. Fish cerebellum, adult, 1-μm-thick sections. (A and B) Postembedding immunocytochemistry with antibody IX-50 shows immunoreactivity localized to the molecular layer (ml). The reaction outlines the dendrites (d) of Purkinje cells, delineating dendritic geometry. Long arrows (pf) indicate approximate direction of parallel fibres. In the area between triangles the parallel fibres run roughly perpendicular to the plane of the paper. Purkinje cell bodies (Pc) are not outlined as in the chick. Bergmann glial cells (Bg) are not conspicuous. Framed area is shown at higher magnification in Fig. 4C. (C) Section cut parallel with the pia. gcl, granule cell layer. Scale bars: A = 100 μm; B = 50 μm; C = 20 μm.

Fig. 4. Fish cerebellum (A and B) 50-μm-thick (C) 1-μm-thick sections. (A and B) Immunoreactivity outlines the dendrites (d) of Purkinje cells, the surface of Bergmann glial cells (Bg) and is also associated with filopodia (f) intruding into the granule cell layer (gcl). One of the Bergmann glial cells emits smooth lightly immunopositive processes (arrows). Purkinje cells (Pc) are not outlined. (C) Postembedding reaction showing outlined immunopositive Bergmann glial cells (Bg), one of them also containing intracellular reaction (arrow). gc, granule cells. Scale bars: A = 50 μm; B and C = 20 μm.

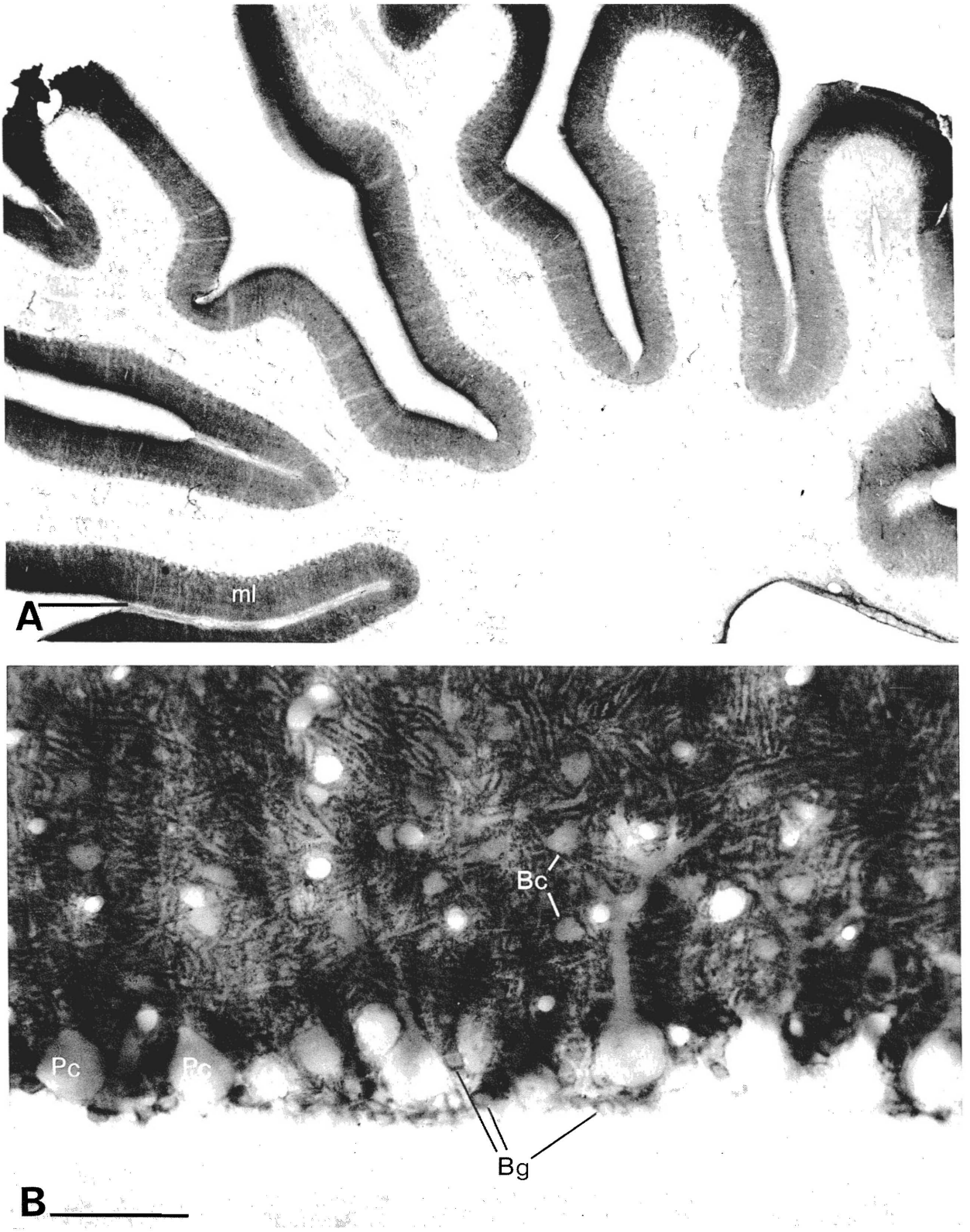


Fig. 1.

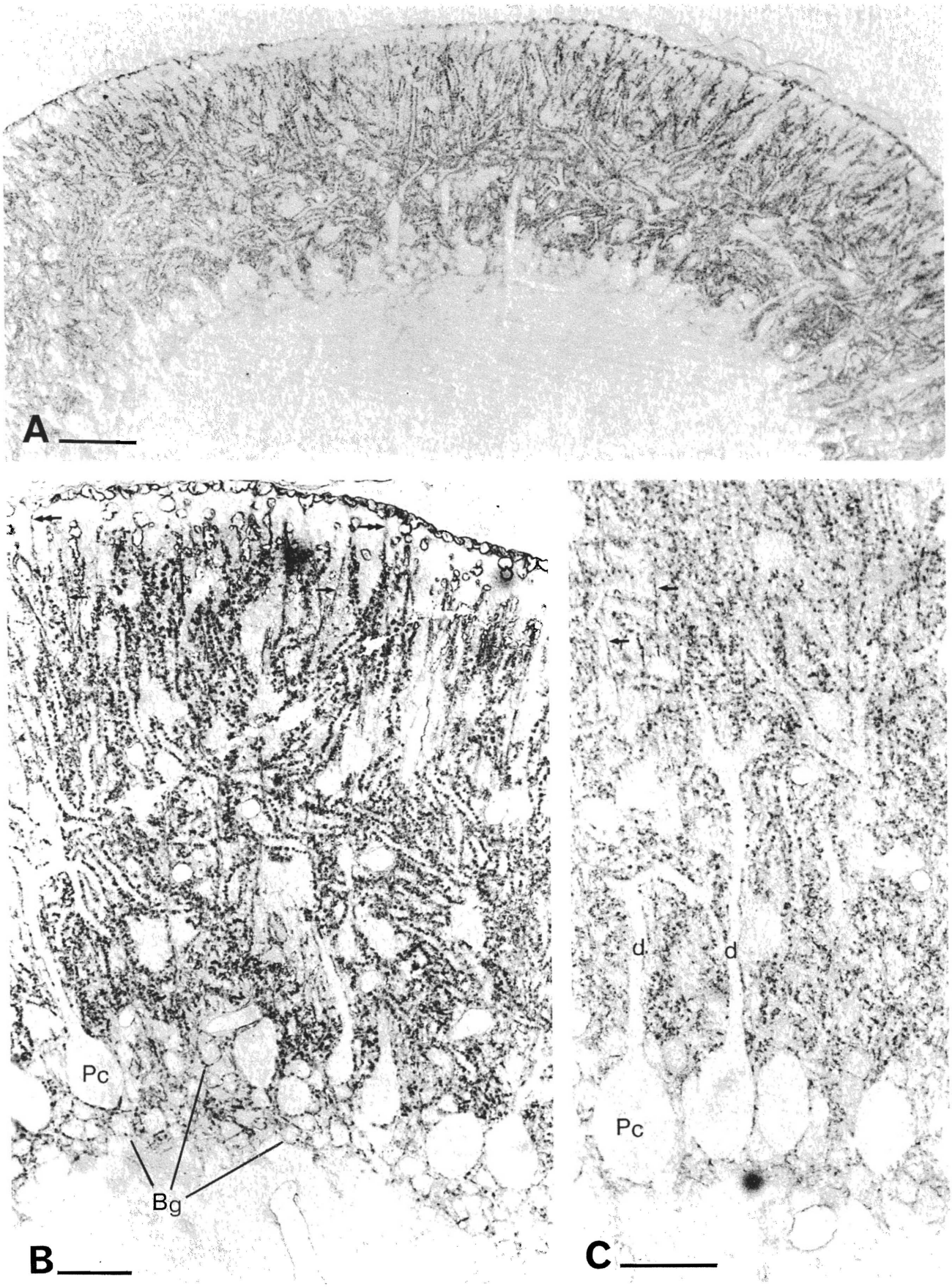


Fig. 2.

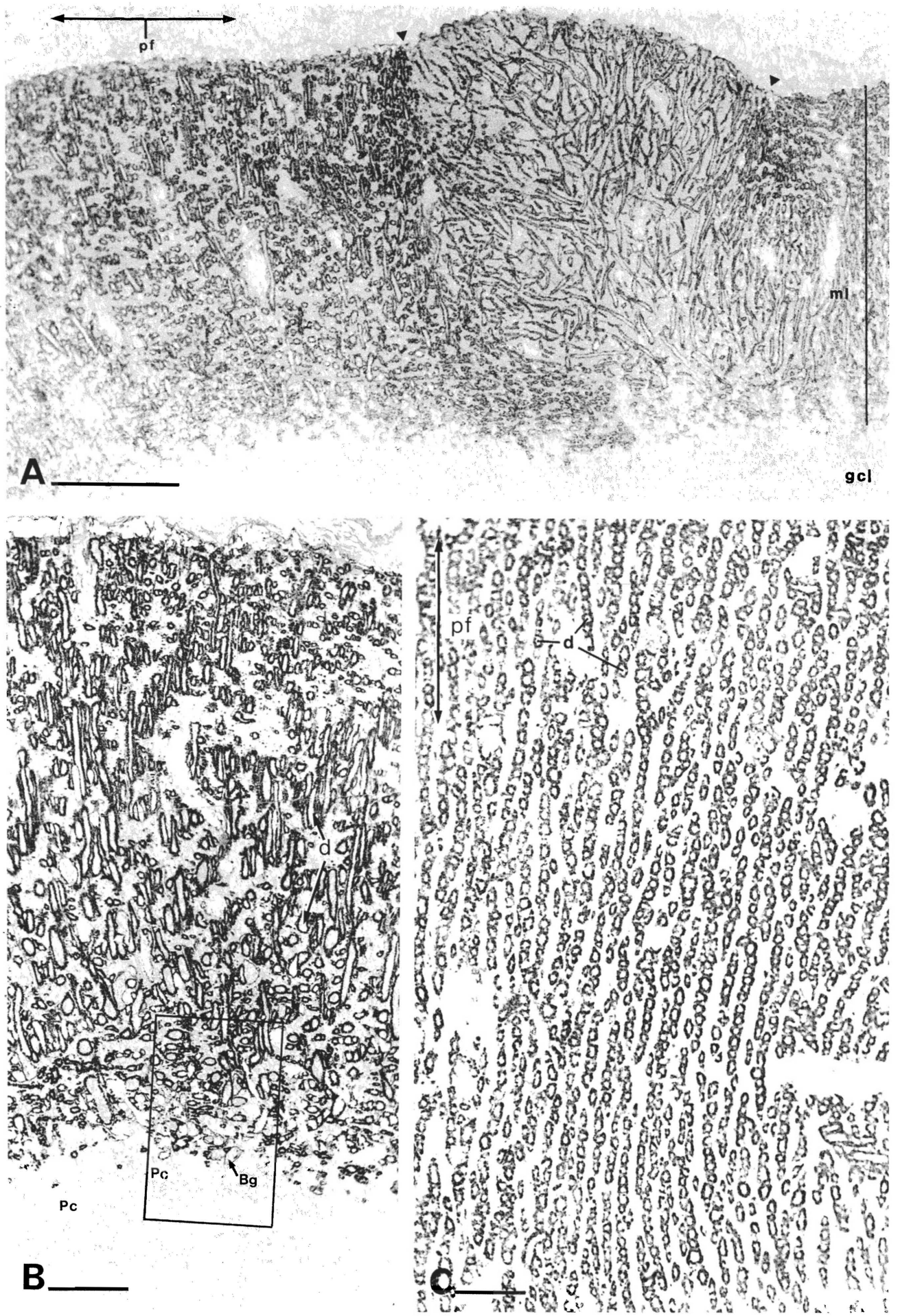
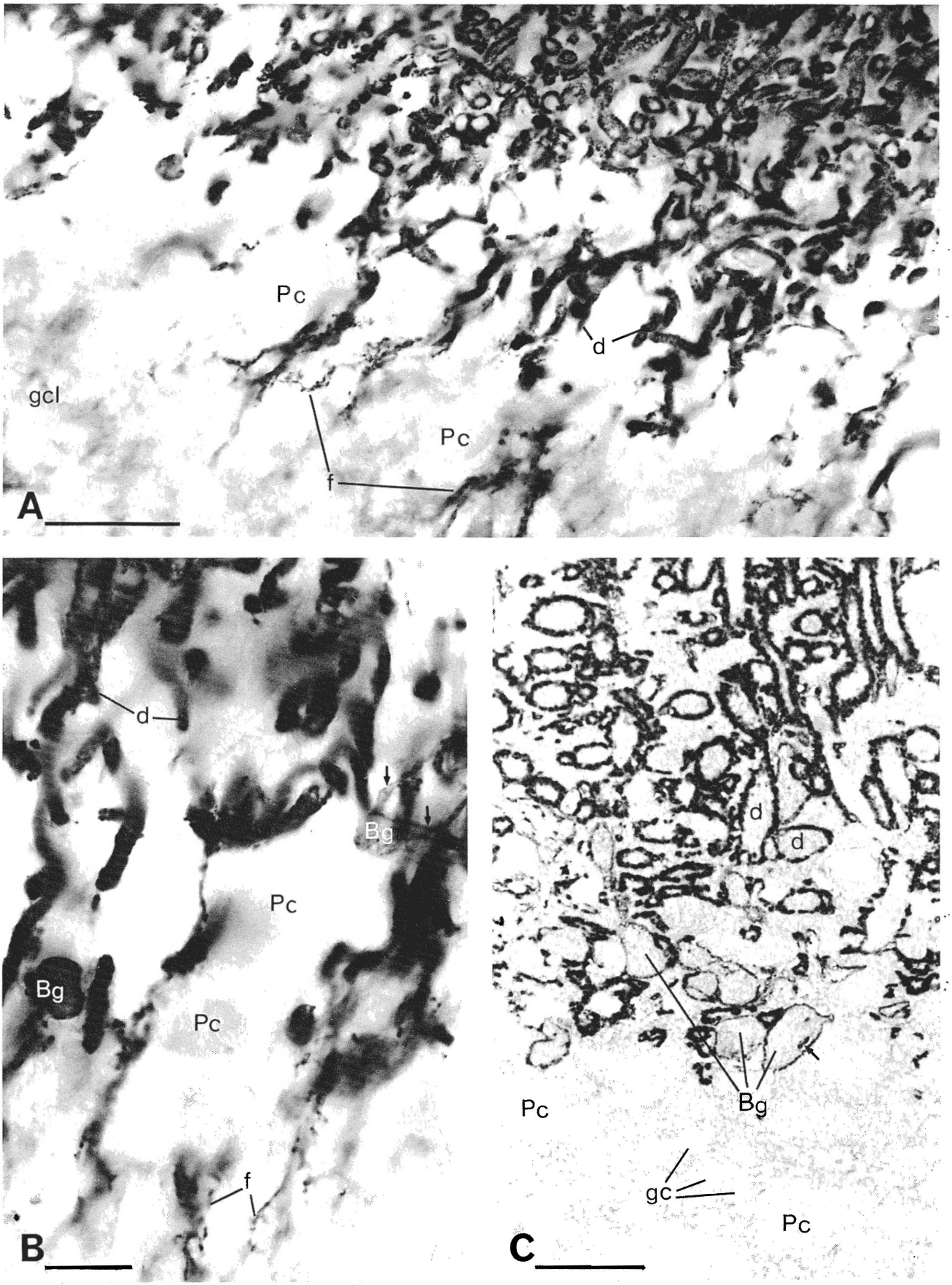


Fig. 3.



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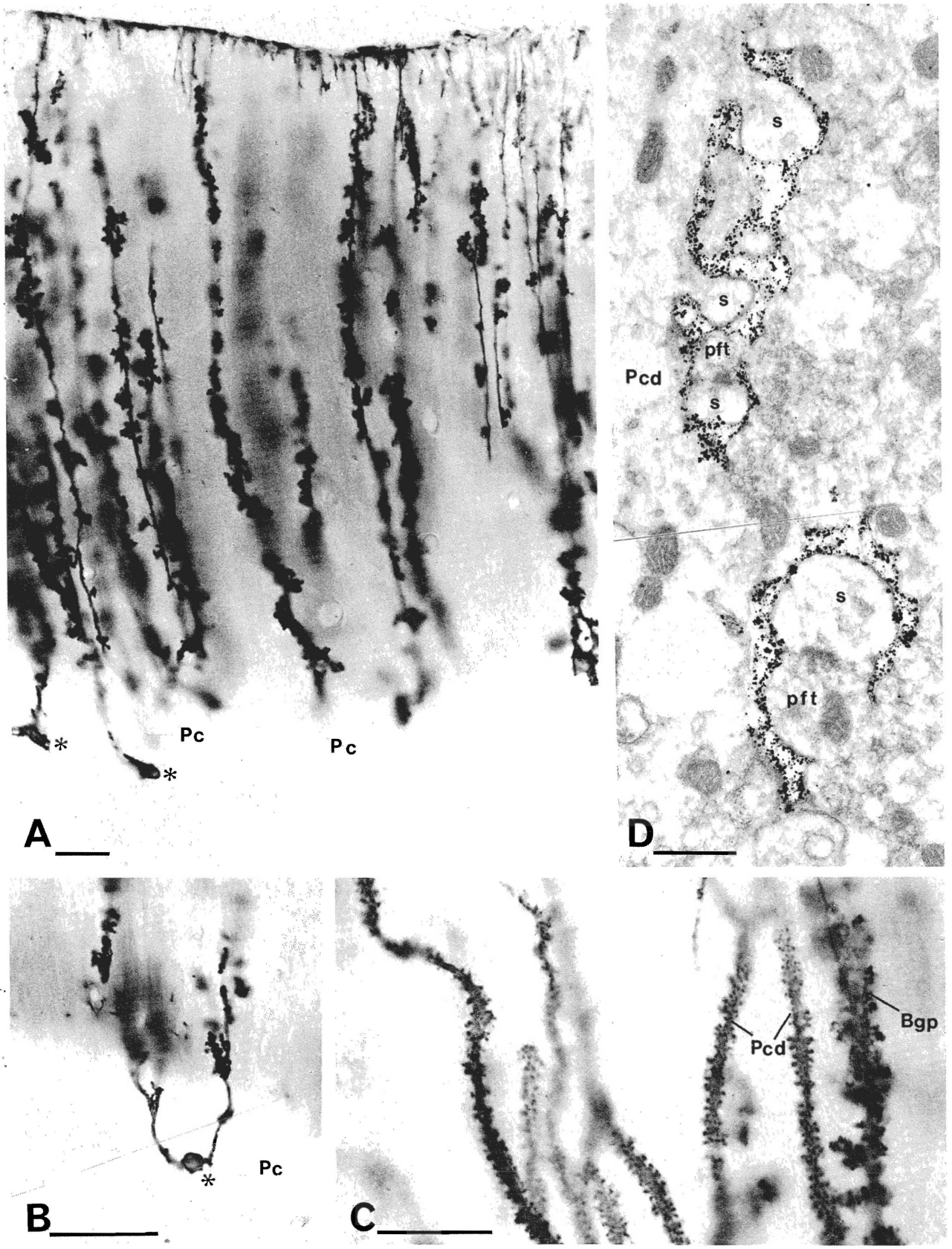

Fig. 5. Individual Bergmann glial cells shown by Golgi impregnation. Chicken cerebellum, eight days old. (A–C) Seventy-micron-thick sections; (D) electron micrograph of an ultrathin section. (A and B) Cell bodies of Bergmann glial cells lie both beneath (asterisks) and above the Purkinje cells (Pc); their thin radial fibres emit leafy processes. (C) Purkinje cell dendrites together with their spines (Pcd) are narrower than gial processes (Bgp). (D) The Golgi-impregnated Bergmann glial processes from a single cell follow both the dendritic shaft (Pcd) and the spines (s) of Purkinje cells, and they ensheath the parallel fibre terminals (pft) as well. Scale bars: A–C = 20 μm; D = 0.5 μm.

well to the immunostaining obtained with antibody IX-50, but the location of the epitope along the Purkinje cell membranes could not be resolved using light microscopy.

Fine structural features of the molecular layer neuropil with relevance to the present study

There are many excellent descriptions of the molecular layer of the cerebellar cortex (for review see e.g. Refs 13, 56, 58, 77), therefore only a few specific features will be reported here. The dominant neuronal elements are the parallel fibres and their terminals making asymmetrical synapses with the Purkinje cell dendritic spines (Figs 6, 10A, 11A and C) as well as with the dendrites of the interneurons (Fig. 8). In the fish the parallel fibre boutons are frequently in direct membrane contact with each other (Fig. 6). The dendrites, the spines and the parallel fibre terminals are surrounded by glial processes which originate from Bergmann glial cells, as shown above for the chick (Fig. 5). Both the leafy processes and their parent stem radial fibres are frequently connected by gap junctions (Fig. 7C and D).

Electron microscopic distribution of immunoreactivity detected by antibody IX-50

Immunoperoxidase staining confirmed that the Bergmann glial cells were immunoreactive. Intracellular immunoreactivity was present in the endoplasmic reticulum (Fig. 7A and B) and in the Golgi apparatus of the chicken. In the fish only the Golgi apparatus was immunoreactive (Fig. 8A and B). In both species, but especially in the fish, membrane limited vesicles and tubules containing immunoreactive material extended into the main glial processes and were frequently aligned with the plasma membrane (Fig. 9A). Occasionally immunoreactivity was also seen in multivesticular bodies and dense bodies representing lysosomes.

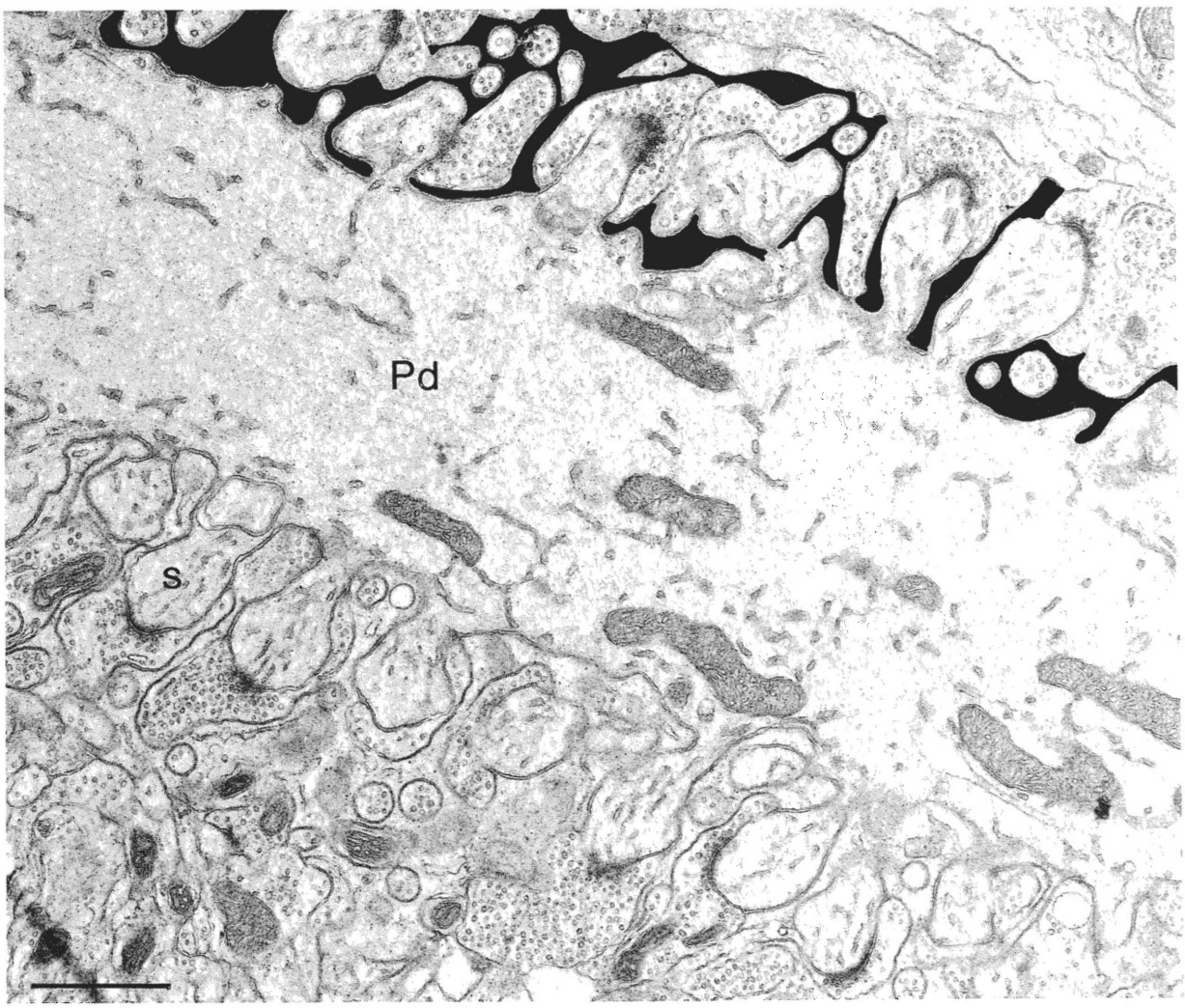

Fig. 6. Electron micrograph of the molecular layer in the fish cerebellum. A Purkinje cell dendrite (Pd) with regularly spaced spines (s), forming synapses with parallel fibre terminals, is ensheathed with Bergmann glial lamellae (shaded on one side). Scale bar = 1 μm.

The immunoperoxidase reaction also showed that the plasma membrane of Bergmann glial cells was immunopositive in both species. This was very obvious in the fish, where the membrane of the cell bodies was strongly positive (Fig. 8A). Only weak reactivity was present on the plasma membrane of the cell bodies in the chick (Fig. 7A and B). In both species the plasma membrane of glial processes was covered with peroxidase reaction endproduct. The reaction was not continuous (Figs 8 and 9), which was

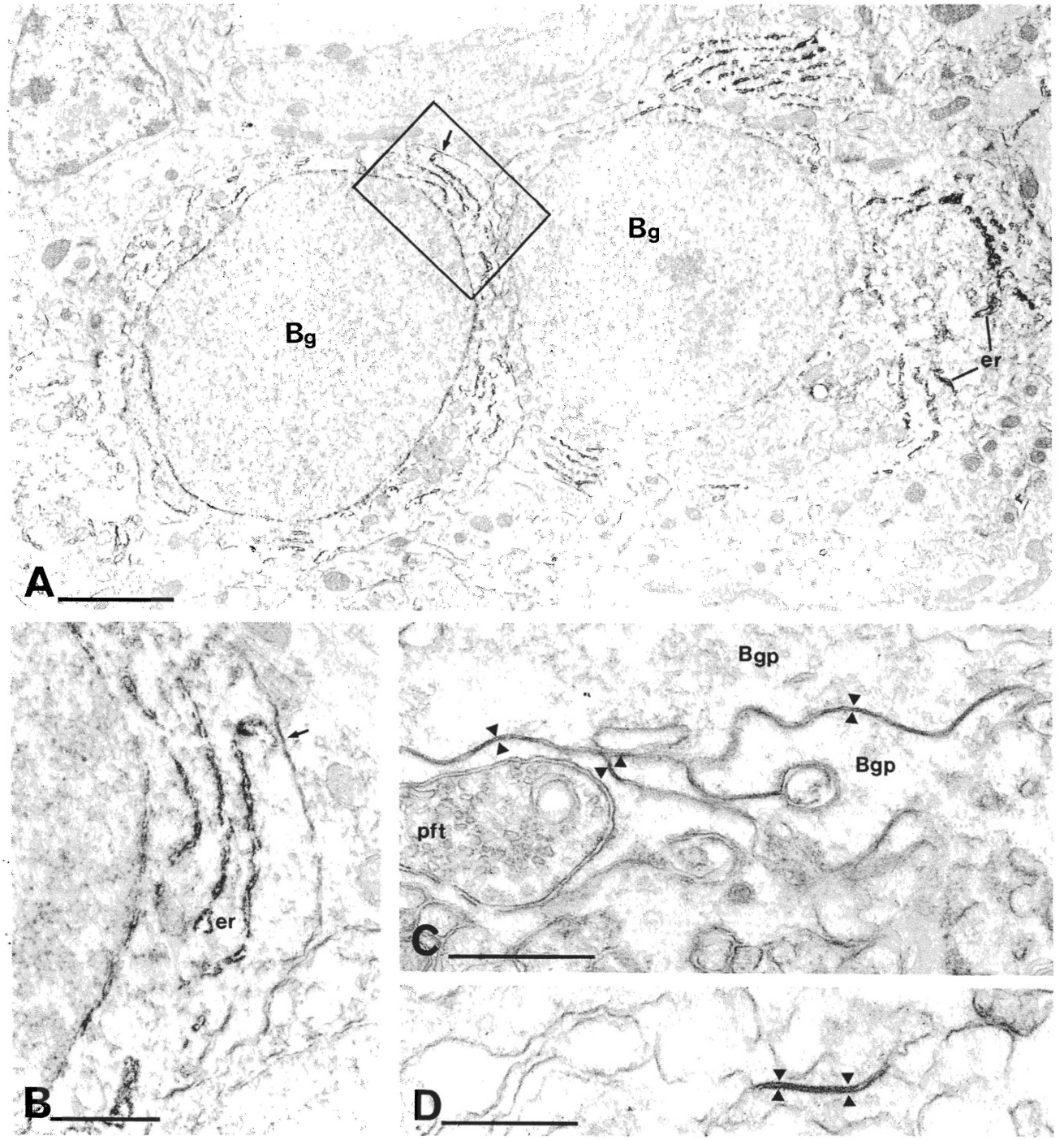

Fig. 7. (A and B) Electron micrographs of Bergmann glial cells (Bg) in the chick (17-day-old) showing immunoreactivity with antibody IX-50 in the endoplasmic reticulum (er) and also associated with the plasma membrane (arrows). (C) Extensive areas of gap junctions (e.g. arrowheads) between Bergmann glial processes (Bgp) in the 28-day-old chick. Note the usual width of extracellular space between glia and a parallel fibre terminal (pft). (D) Gap junction (arrowheads) between Bergmann glial processes (Bgp) in the fish. Scale bar: A = 2 μm; B–D = 0.5 μm.

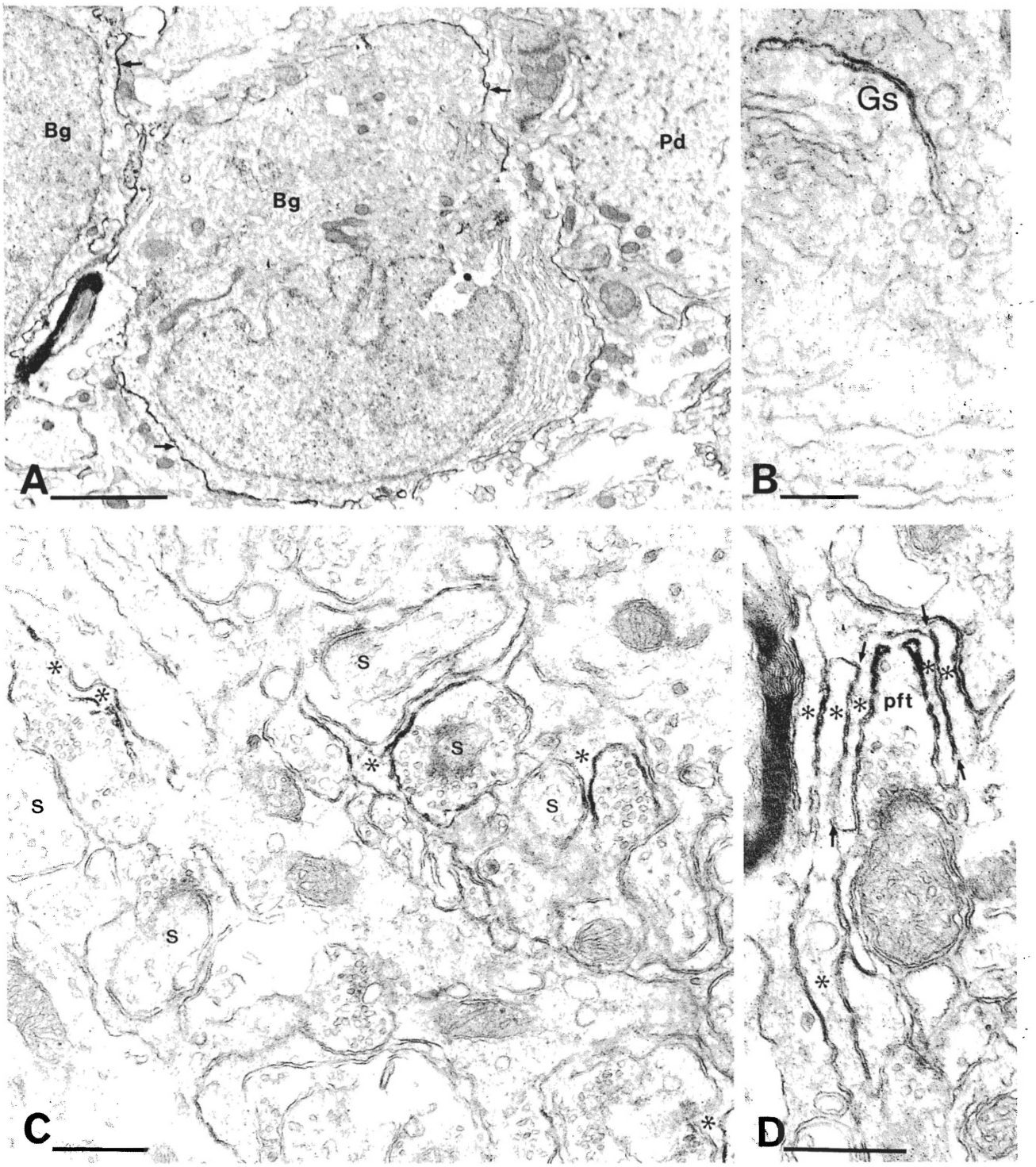

Fig. 8. Immunoperoxidase reaction with antibody IX-50. Electron micrographs of fish cerebellum. (A) Bergmann glial cells (Bg) outlined by electron-dense immunoreaction endproduct (arrows). (B) Immunoreactivity in a Golgi saccule (Gs) of a Bergmann glial cell. (C) Immunoreactivity along glial lamellae (asterisks) surrounding parallel fibre/spine (s) synaptic complexes. (D) Presumed parallel fibre terminal (pft) in synaptic contact with a dendrite. Note that the glial membranes are immunoreactive along their contact with the terminal as well as along faces where they contact other glial lamellae (arrows). Scale bars: A = 2 μm; B = 0.25 μm; C and D = 0.5 μm.

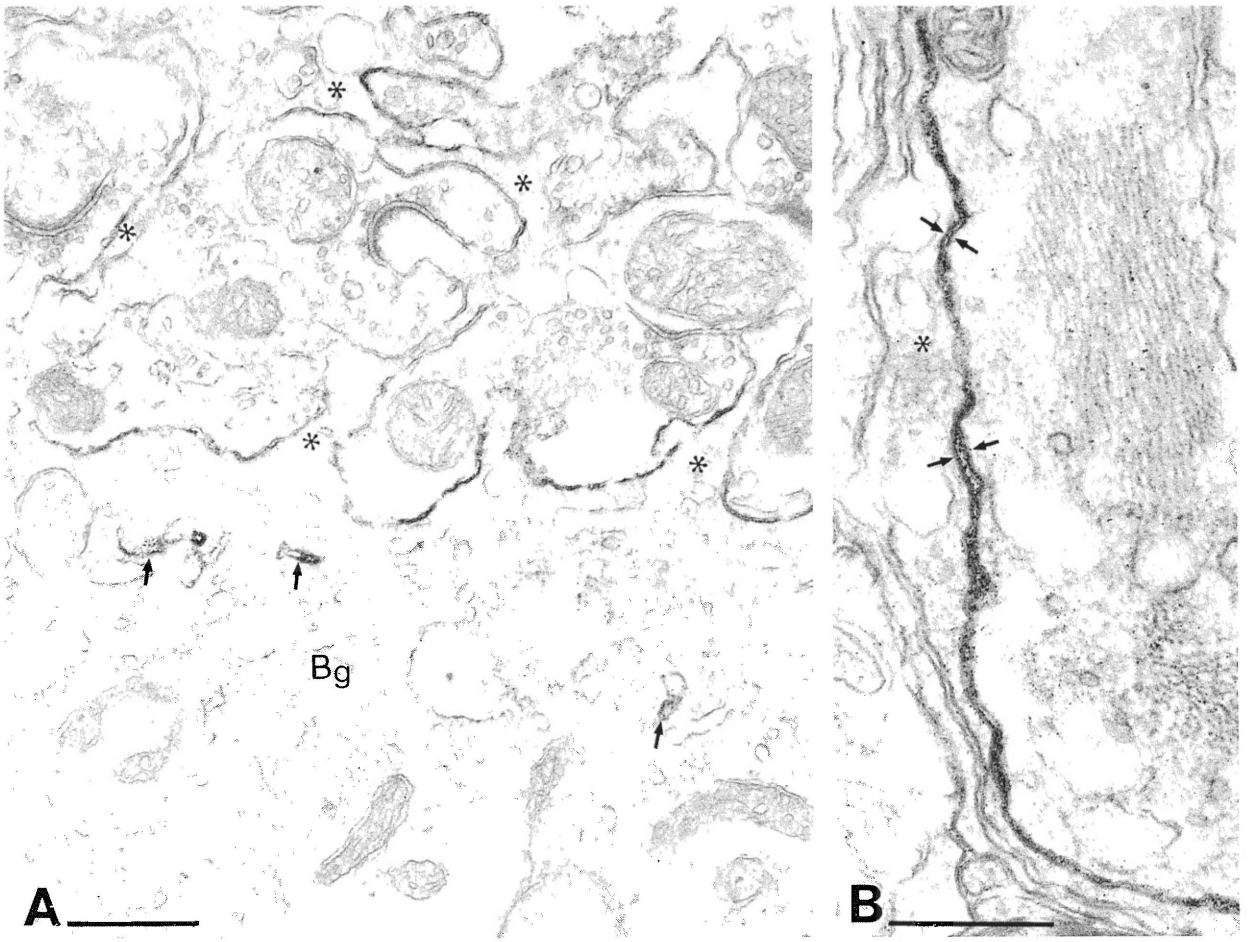

Fig. 9. Immunoperoxidase reaction with antibody IX-50. Electron micrographs of fish cerebellum. (A) A Bergmann glial (Bg) process emitting thin lamellae (asterisks). Immunoreactivity is present along the plasma membrane and in some of the smooth tubules (arrows) which usually lie parallel with the plasmalemma. Some lamellae (asterisk) are immunoreactive in the neuropil. (B) Immunoreactivity between a Bergmann glial process and another glial process (to the right), probably belonging to an astrocyte. The reaction product is on the outer surface of the glial membranes (e.g. at arrows) indicating the extracellular location of the epitope. Scale bars = 0.5 μm.

probably due to the limited penetration of antibodies. The deeper the level which was studied from the thick Vibratome section, the shorter the immunopositive plasma membrane segments that were found. The exact location of the epitope recognized by the antibody could not be established in the chicken. However, in the fish the reaction endproduct was deposited in the extracellular space along the outer electron-dense lamina of the trilaminar plasma membrane (Fig. 9B), indicating that the epitope was extracellular.

The immunoperoxidase reaction endproduct diffuses from the site of the enzyme and is deposited on both plasma membranes facing each other across the extracellular space (see e.g. Fig. 9B). With this technique it is therefore impossible to decide which of the two membranes carries the epitope. Reaction endproduct was found between plasma membranes of glia/parallel fibre terminal (Figs 8C, D and 9A), glia/dendritic spine (Fig. 8C), glia/dendritic shaft and, most significantly, glia/glia contacts. Glial fibrillary protein bundles could be found in some of the glial processes participating in glia/glia contacts (Fig. 9B), or in both processes originating from thin sheets of Bergmann glia (Fig. 8D). The presence of immunoreactivity between two adjoining Bergmann glial processes shows unequivocally that the glial membrane carries the immunoreactive protein.

The membrane contacts, including the synaptic junctions, between parallel fibre terminals with Purkinje and other dendrites were invariably devoid of immunoreactivity. However, using the pre-embedding technique it is possible that the lack of reactivity resulted from the lack of antibody penetration. The synaptic cleft in particular is filled with extracellular material which might limit the access of antibodies to molecules in the junctional membrane. Therefore a postembedding immunogold method, that overcomes penetration problems, was also used at the electron microscopic level in the chicken.

Immunoreactivity was not obtained without etching of the resin, or in material treated with osmium tetroxide. The best result was obtained with sodium ethanolate etching, but the identification of the cellular elements is difficult in these sections. Therefore serial sections were used and the cellular processes covered by the gold particles were identified in the adjoining non-reacted sections (Figs 10 and 11). The shift in the position of membranes, even between two serial sections, together with the scatter of gold resulting from the two-layer procedure, prevented the allocation of gold particles to single membrane. Nevertheless, it could be established that the gold particles were selectively deposited along glial lamellae, irrespective of the neuronal elements adjoining them (Figs 10 and 11). The distribution of gold particles confirmed the glial location of the epitope; the synaptic junctions were completely free of immunogold. The rarely seen dense bodies within glial processes were always densely covered by gold particles (Fig. 10C).

Electron microscopic distribution of glutamate immunoreactivity

Glutamate is one of the endogenous excitatory amino acids that might act as an agonist on the putative kainate receptor recognized by antibody XI-50, and localized on Bergmann glial cells as shown above. It has been shown by quantitative immunocytochemistry that the terminals of parallel fibres are rich in glutamate.[71] Only a qualitative study was carried out here in the chicken and in the fish to see if the distribution of immunoreactive glutamate is similar to that described in mammals. The highest density of immunogold was deposited over parallel fibre terminals (Fig. 12), followed by parallel fibre axonal bundles, Purkinje cells and other dendrites. The mossy fibre terminals in the granule cell layer were also strongly reactive for glutamate in both the fish and the chicken. The glial processes contained a very low density of gold particles (Fig. 12), indicating their low glutamate content.

DISCUSSION

The identity of the antigen recognized by antibody IX-50

The monoclonal antibody IX-50 recognizes a polypeptide of apparent molecular weight of 49,000 on immunoblots following the separation of proteins by sodium dodecylsulphate–polyacrylamide gel electrophoresis. This protein is present both in the goldfish and in the chick and to some degree also in the rat brain.[19] The chick polypeptide with $M_r =$ 49,000, a similar polypeptide with $M_r = 49{,}000$ from pigeon brain,[45] and a polypeptide with $M_r = 48{,}000$ from frog brain[24] were shown to carry the kainate binding site. The widespread conservation of the epitope recognized by antibody IX-50 across species strongly suggests that the same protein is recognized also in the brain of the rainbow trout. The epitope for antibody IX-50 is also present in a $M_r = 93{,}000$ polypeptide in the chick brain.[19] Similarly, the monoclonal antibody directed against the kainate binding protein of the frog brain also recognized a $M_r = 99{,}000$ protein.[24] In the latter study, reducing agents were shown to regulate the relative amounts of the $M_r = 48{,}000$ and $M_r = 99{,}000$ polypeptides, suggesting the possibility of a monomer–dimer relationship involving disulphide bond formation.

The protein identified by antibody IX-50 binds kainate with low affinity. Since it is generally believed that glial cells participate in the uptake of neuronally released glutamate (for review see Refs 33, 38), and kainic acid is able to block to some extent this uptake,[37] it could be argued that the protein is not a receptor but a glutamate carrier. According to this argument the high density of glutamate releasing terminals in the molecular layer might call for an equally high density of glutamate carrier. Although this possibility cannot be ruled out, there are several arguments against it.

1. The protein has a restricted distribution. If it was a glutamate carrier, one would expect to find it on most astroglial cells. Although the protein was detected in greatly varying amounts by immunoblots in several brain regions including the chicken forebrain,[19] and also in non-neuronal tissues, we could not detect it immunocytochemically in the chicken forebrain (unpublished observation). This shows that it is unlikely to be a common glial membrane protein, or that different populations of glial cells possess greatly varying levels of this protein.

2. Kainate and quiscalate are not taken up by the glutamate carrier in tissue slices[37] or into cultured glial cells.[44] Although both kainate and its analogue dihydrokainate are able to interact with the glutamate carrier, dihydrokainate is about twice as potent as kainate in inhibiting glutamate uptake.[37] However, kainate displaces a ligand, kainyl–serum albumin with very high binding affinity, from cerebellar kainate binding sites with a potency at least 10^4 times higher than that displayed by dihydrokainate for the same sites.[15]

3. The amino acid sequence of the chick kainate binding protein, deduced from the sequence of its encoding gene, shows great similarities, both in its putative transmembrane domains and in the localization of the latter within the protein sequence, with that of the nicotinic acetylcholine receptor protein.[19a] These data suggest that the kainate binding protein localized in the present study belongs to the family of ligand gated channel proteins.

Selective cellular distribution of the putative receptor

The present study confirmed preliminary antibody binding results with the IX-50 antibody in the chick,[19] and immunohistochemical results using antibodies to kainyl–BSA,[15] which showed that most of the immunoreactivity was located in the molecular

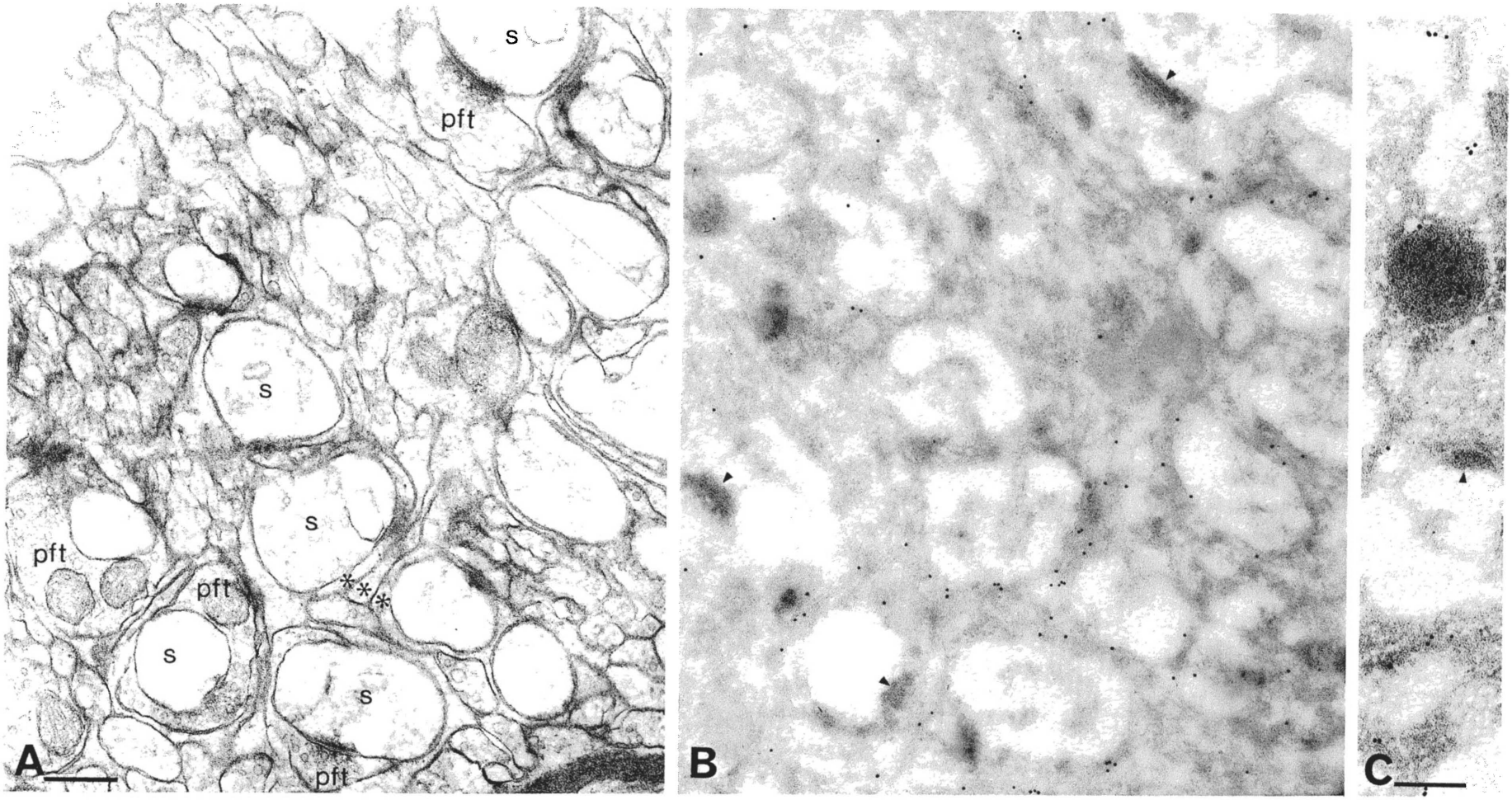

Fig. 10. Postembedding immunogold reaction with antibody IX-50. Electron micrographs of chicken cerebellum (26-day-old). (A and B) Serial sections; the section shown in B was immunoreacted. The gold particles are associated with the glial lamellae surrounding the spine (s) parallel fibre terminal (pft) complexes. One of the immunopositive sites has three glial lamellae (asterisks). Synaptic junctions (arrowheads) are free of gold. (C) High density of immunogold in a glial lysosome. Other gold particles are mainly over glial ridges and a synaptic junction is free of gold. Scale bars = 0.25 μm.

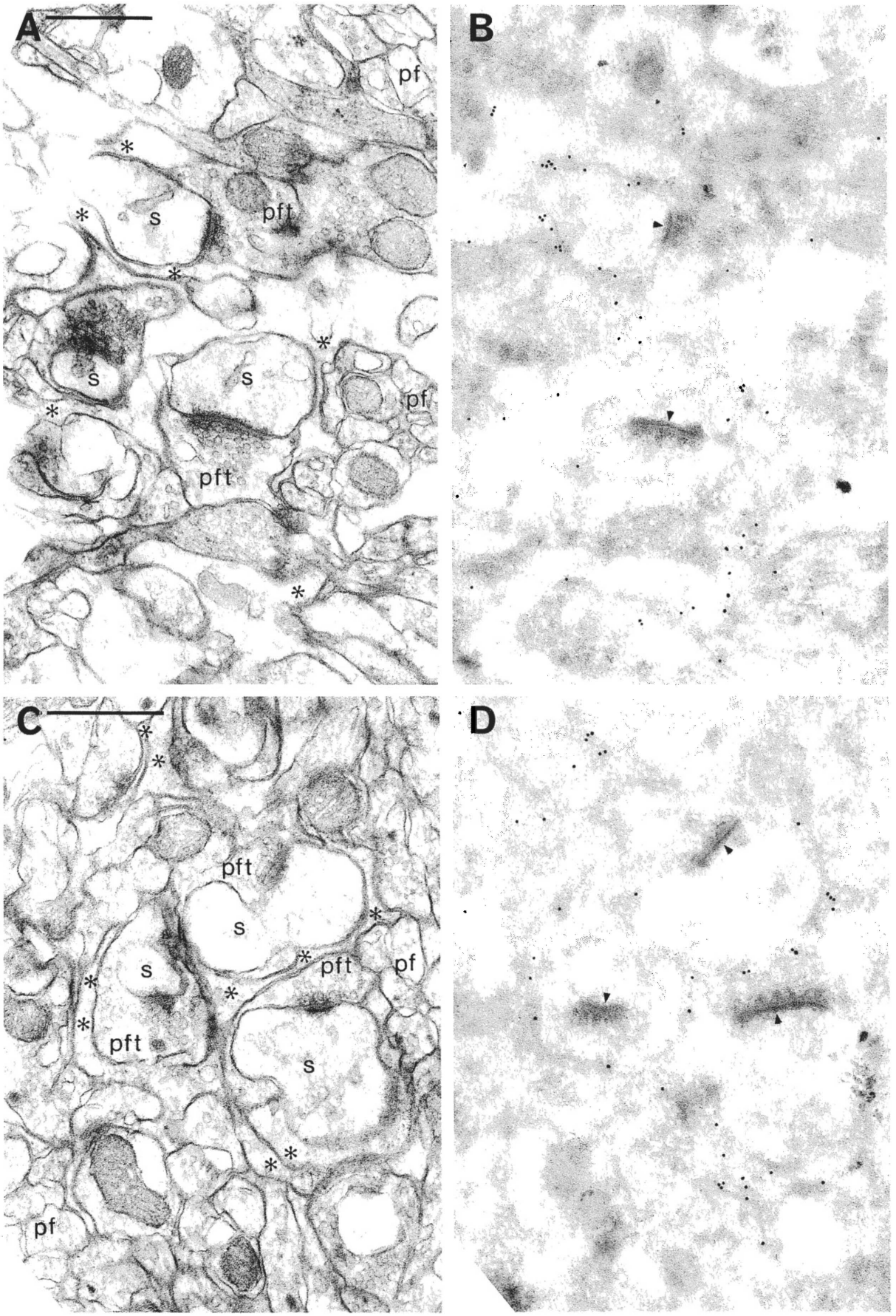

Fig. 11. Postembedding immunogold reaction with antibody IX-50. Electron micrographs of chicken cerebellum (26-day-old). (A–D) Serial sections; the sections shown in B and D were immunoreacted. The gold particles are deposited along glial processes (asterisks) irrespective of whether they surround spines (s) or parallel fibre terminals (pft). Synaptic junctions and areas covered by cross-sectioned parallel fibres (pf) are free of gold particles. Scale bars = 0.5 μm.

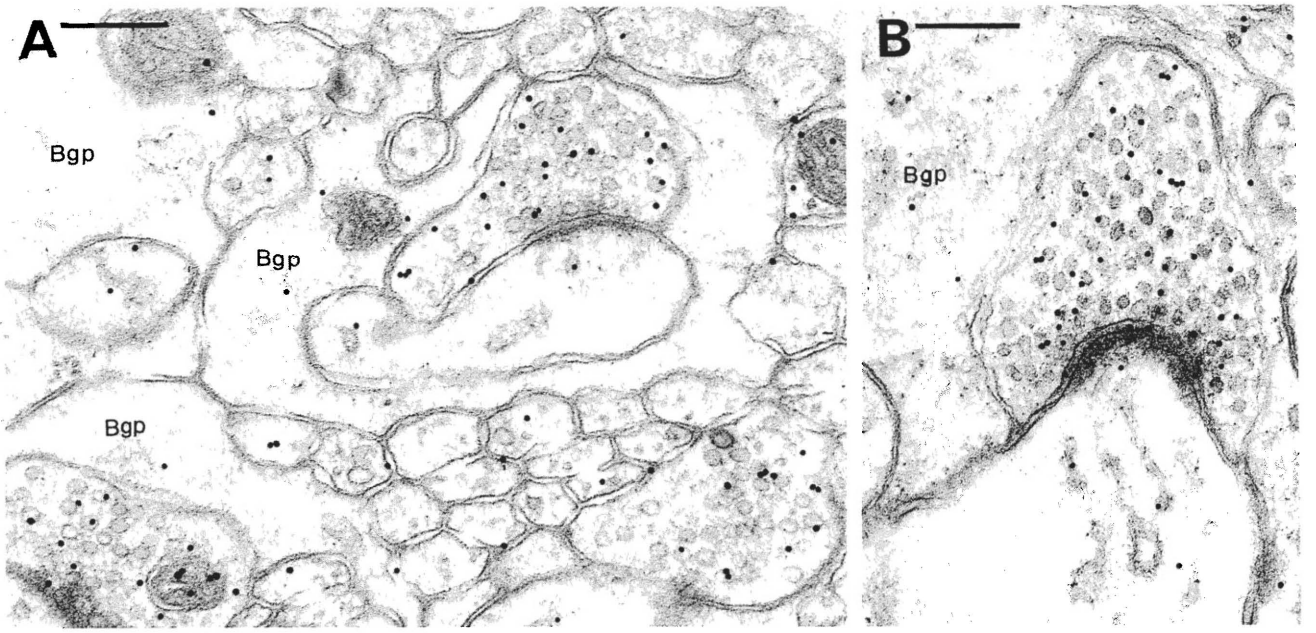

Fig. 12. Postembedding immunogold reaction with antiserum to glutamate. Electron micrographs of chicken (A, 8-day-old), and fish (B) cerebellum. The concentration of gold particles over parallel fibre terminals indicates their high glutamate content. Note the sparsity of gold particles over Bergmann glial processes (Bgp). Scale bars = 0.25 μm.

layer. Studies with radiolabelled kainate[27] and kainyl–BSA[15] binding have also shown that in birds most of the binding sites are in the molecular layer.

This layer has some structural and biochemical features that are unique in the central nervous system. It contains postsynaptic elements which, without exception, belong to GABAergic neurons, and it contains the highest density of glutamatergic synapses and nerve terminals. These biochemical characteristics may call for a unique glial involvement and contribution to neurotransmission, which may result in the high density of a putative excitatory amino acid receptor on the Bergmann glial cells. This localization requires an examination of the properties of this class of glial cell.

The position of Bergmann glia in the astrocyte classification is not straightforward since both the presence[5,49] and absence[65] of glial fibrillary acidic protein has been reported in Bergmann fibres. There are at least two classes of astrocyte which contain glial fibrillary acidic protein.[61] Type 2 astrocytes are thought to be present only in the white matter, therefore it could be argued that Bergmann glial cells have type 1 characteristics; however, this has not been confirmed *in situ*. Their unique shape and position may indicate that they do not fall in the type 1/2 classification. The classification is not only an issue of nomenclature because, in addition to biochemical differences, type 1 and 2 astrocytes have different electrophysiological properties (see below), and it would be useful to relate these to the presence of the putative kainate receptor identified in this study.

The characteristic shape and the leafy processes of Bergmann glial cells develop fully only *in vivo*. Therefore, it is difficult to predict which, if any, of the established cerebellar glial cultures will carry the receptor protein. It has been suggested that a so-called type II glial cell line from the mouse cerebellum might correspond to Bergmann glial cells.[1] Unfortunately, similar cultures are not available from chicken cerebellum. Co-culturing of neuronal and glial cells might lead to the development of morphologically recognizable Bergmann glial cells.[25a]

The distribution of kainate binding sites is different in mammals, where radiolabelled kainate binds to both the molecular and the granule cell layer.[26,55,59] The antibody IX-50 used in the present study also binds to the granule cell layer in mouse cerebellum,[14] but it is not yet known whether glial cells carry any of the binding sites. Previous studies using mutant mouse strains deficient in Purkinje or granule cells were interpreted as indicating a presynaptic localization of the receptor on granule cell axons.[20] A glial localization could also explain the results if the expression of the glial receptors depended on the presence of functional parallel fibres. The developmental appearance of the receptor protein suggests that this might be the case in the rat[69] and chicken cerebellum.[19]

It is surprising that no immunoreactivity could be detected on neurons. Kainate induces large inward currents in cultured Purkinje cells,[34] and the effect of glutamate released from granule cell terminals is thought to be mediated by non-NMDA-type excitatory amino acid receptors.[21,34,41] A conceivable

explanation could be that the neuronal receptor does not carry the same epitope as the glial protein.

Subcellular distribution

The putative kainate receptor seems to be evenly distributed along the plasma membrane; the glial lamellae that contact the spine/parallel fibre complexes showed immunoreactivity similar to the membrane of the cell bodies. However, the peroxidase method is not suitable for the detection of quantitative differences. A similar seemingly uniform distribution has been observed for some subunits of the $GABA_A$/benzodiazepine receptor complex in the plasma membrane of neurons.[75]

The intracellular distribution as detected by the antibody shows probable sites of synthesis in the rough endoplasmic reticulum, of glycosylation in the Golgi apparatus and of degradation in lysosomes. The protein binds to Concanavalin A, supporting its glycoprotein nature.[19] In contrast to the chick, only the agranular endoplasmic reticulum was immunopositive in the Bergmann glia of the fish. Interestingly, in the fish cerebellum no $M_r = 93{,}000$ protein could be detected in immunoblots.[19] The $M_r = 93{,}000$ protein recognized by antibody IX-50 could be a precursor and its abundance in the chick is in line with the strong staining of the granular endoplasmic reticulum, the appropriate compartment for a precursor.

Architecture of the molecular layer and the function of Bergmann glia

Bergmann glial cells are thought to play an important guiding role in the migration of granule cells during development,[57,62] but in the adult no specific roles other than those displayed by astrocytes have been associated with them. The presence of very high levels of a putative kainate receptor in the Bergmann glial membrane, but not in the other glial cells in the cerebellar cortex, suggests that the Bergmann glia plays a particular role in the excitatory events in the molecular layer. The association of the Bergmann processes with the Purkinje cell surface described earlier[22,58,77] suggests that this role is related to the Purkinje cells. The selective association between these two classes of cells is strikingly revealed by the complete visualization of the Bergmann membrane, which provides a negative image of Purkinje cells. It has also been noticed that in the spine/parallel fibre bouton complexes it is the spine which is more completely covered.[13,22,58,77,78] Thus, in the adult the Bergmann glia provides a membrane cover for the Purkinje cell dendritic and spine membrane.

Possible role of the putative kainate receptor

The intimate association of Bergmann glial cell processes with parallel fibre Purkinje cell contacts predicts a role for the putative glial kainate receptor in the context of synaptic transmission in the molecular layer. Electrophysiological recordings from identified Bergmann glial cells *in situ* have not been carried out. However, kainic acid induces fluxes of both radioactive sodium and rubidium ions from preloaded chick cerebellar glial cells in culture, and these cells exhibit the epitope for antibody IX-50 (A. Ortega and V. I. Teichberg, unpublished observations). Furthermore, excitatory amino acids have been shown to activate second messenger mechanisms (for review see Ref. 60) and also depolarize other types of astrocytes *in vitro*.[7,43,79] Kainate opens Na^+/K^+ channels on both type 1[76] and type 2[81] astrocytes. It is not known if Golgi epithelial/Bergmann glial cells can be distinguished morphologically from the other astrocytes *in vitro*, or whether they show type 1 or type 2 characteristics *in vivo*. Nevertheless, the high density of the putative kainate receptor on Bergmann glial cells suggests that similar responses would be evoked by agonists in these cells as well. What is the endogenous substance acting on these receptors and what is its source?

Glutamate is an obvious candidate and the immunocytochemical results in the present and previous studies[71] show that it is abundant in parallel fibre terminals. Although glial cells were also suggested to synthesize glutamate[8] using the enzyme glutamate dehydrogenase (EC 1.4.1.3), present in high concentration in Bergmann glial cells,[2,40,85] the present and previous immunocytochemical studies indicate that glial cells store relatively low levels of glutamate. Therefore, at present a neuronal source of glutamate, either from parallel fibre terminals or possibly from Purkinje cell dendrites, seems more likely.

Glial excitatory amino acid receptors may be activated by neuronally released glutamate.[4,18,81] As suggested earlier,[18] the resulting depolarization of glial cells could lead to the release of neuroactive substances that act on the neighbouring neuronal elements. Kainate and other excitatory amino acids have been shown to evoke the release of preloaded GABA and aspartate from cerebellar type 2 astrocytes in culture.[18] Bergmann glial cells *in vivo* accumulate both excitatory amino acids[86] and GABA.[42] However, the low levels of glutamate that can be detected in cerebellar glia[71] and the high levels of glutamate metabolizing enzymes particularly in Bergmann glia[2,51,85] do not favour a significant glial glutamate release. Glial cells are known to release accumulated GABA on depolarization[18,33,36] through the activation of a reverse transport due to the increase in intracellular sodium.[18] Gap junctions between the glial cells, demonstrated previously (for review see Ref. 56) and also in the present study, could facilitate synchronization of glial depolarization. GABA released from the Bergmann glial cells and acting on $GABA_A$ receptors could provide a local control over the degree of spine depolarization caused by glutamate. In support of this hypothesis, $GABA_A$ receptors have recently been localized on the dendritic spines of Purkinje cells,[75] which are in direct contact with Bergmann glial processes, but never

receive GABAergic neuronal input. The mechanism outlined above may not necessarily operate under all conditions, but could be activated at high levels of parallel fibre activity, tonically regulating the gain of the parallel fibre synapses.

Are glial receptors involved in the neurotoxic action of kainate?

The localization of putative kainate receptors on Bergmann glia raises the question of their role in the toxicity of kainic acid. As in other parts of the brain (for review see Ref. 12), kainic acid destroys neurons in the cerebellum of mammals with different thresholds for the different types of cells.[12,32,46,80] Similarly, in species with a high density of kainate binding sites in the molecular layer, such as the goldfish, kainic acid also causes the degeneration of stellate and Golgi neurons and some of the Purkinje cells.[82] In the pigeon, neurons of the molecular layer were found to be very sensitive, while Purkinje cells were only affected in the immediate area of the injection.[64] Although the pigeon cerebellum contains a much higher density of kainate binding sites than the rat cerebellum, the toxic effects of kainate were reported to be comparable in the two species.[27] Since, by analogy with the trout and the chick, the kainate receptors in the goldfish and the pigeon cerebellum are probably also located on Bergmann glial cells, the neuronal toxicity of kainic acid requires explanation. Although the present study does not reveal immunoreactivity to antibody IX-50 on neurons, it is possible that the density of neuronal kainate receptors is below the sensitivity of our methods, but sufficient for receptor-mediated toxicity. Kainate may also act on neuronal receptors that are not recognized by antibody IX-50.

Unfortunately, the effect of kainate on Bergmann glial cells has not been reported, thus glial involvement can only be inferred from other systems. Excitatory amino acids do not destroy glial cells in mouse cortical cell cultures,[9] or in the chick retina,[11] therefore a lack of toxicity may also apply to Bergmann glial cells. This would not be surprising since, unlike that of other excitotoxins, the action of kainate is not thought to be mediated by postsynaptic kainate receptors alone.[11,17] Since kainate receptors have been assumed to be present only on neurons, the toxic effects have been explained only via pre- and postsynaptic neuronal mechanisms.[11] Toxicity depends on the presence of a glutamate-containing neuronal innervation of the affected area.[6,53] In view of the glial localization of putative kainate receptors, it is possible that excessive kainate activation disrupts the normal function of glial cells, leading to neuronal damage.

Acknowledgements—The authors are grateful to Miss Jane Ellis and Mr Frank Kennedy for excellent technical assistance, and to Mrs Pamela Twynam for secretarial help. The gift of antibodies from Drs P. Petrusz and J. G. Richards is gratefully acknowledged. Nomi Eshhar thanks the European Science Foundation for a travel Fellowship. V. I. Teichberg holds the Louis and Florence Kutz-Cohen Professorial Chair in Neuropharmacology.

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(*Accepted* 11 *December* 1989)