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Monoclonal antibody anti-type I and anti-zebrin II labelling in siluriform fishes: the role of shared lineage versus shared function in polypeptide co-distributions

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Abstract

Two monoclonal antibodies (mabs), the newly generated mab anti-type I and the previously described mab anti-zebrin II, were reacted with brainstem sections of two ostariophysan siluriforms, the gymnotoid *Rhamphichthys rostratus* and the siluroid *Ictalurus punctatus*. Mab anti-type I recognizes a 47 kDa polypeptide present in the dendrites and soma of projection neurons. Mab anti-zebrin II recognizes a 36 kDa polypeptide present throughout the neuronal cytoplasm, including the axon. Strongly type I immunopositive cells include: all cerebellar Purkinje cells; pyramidal cells of the nucleus medialis, electrosensory lateral line lobe and tectum; pacemaker relay cells; Mauthner neurons; lateral line ganglion cells; cells of the inferior olive; and large neurons of the reticular formation and lateral reticular nucleus. Weakly reactive type I cells include: neurons in the torus semicircularis, medial and efferent octavolateralis nuclei, the magnocellular and lateral tegmental nuclei; and the motor neurons of the Vth, VIIth and Xth cranial nerves. Most type I positive cells are brainstem projection neurons. Zebrin II expression is restricted to subsets of two cell types which also express the type I antigen – Purkinje cells and acousticolateralis pyramidal cells. Both of these neuronal types develop from the region of the rhombic lip. While the mutual expression of the type I antigen can be explained by the shared function of projection neurons, the common expression of the zebrin II antigen is most likely due to a shared embryological and/or phylogenetic lineage.

Keywords: Immunocytochemistry; Purkinje cell; Pyramidal cell; Projection neuron

1. Introduction

Comparative neurobiologists have traditionally relied upon morphological criteria to establish homology among neuronal populations within or among species [8,20,36,37]. For example, Johnston [22] and Larsell [28] used morphological similarity to hypothesize that cerebellar Purkinje cells evolved from brainstem pyramidal cells early in vertebrate phylogeny [33]. Immunocytochemical criteria provide a second technique for assessing homologous neuronal populations. The shared presence of macromolecules or epitopes will be recognized through the co-distribution of specific immunoreactivities. Macromolecules common to distinct neuronal types can reflect two types of relationships: either a shared function or a shared embryological and/or phylogenetic ancestry [36]. For example, the zebrin II antigen offers both functional and phylogenetic information. Adult mammalian cerebella form alternating parasagittal zebrin II$^+$ and zebrin II$^-$ compartments [6,7,26]. These compartments, defined by immunocytochemical methods, reflect differences in afferent input. Thus, in mammals, zebrin II distribution corresponds to a functional segregation; it serves as a marker for afferent compartments, although zebrin II has no direct role in segregating these inputs [6,7,16].

The distribution of zebrin II also provides phylogenetic information. In the lateral line system of electrosensory teleosts, the first order lateral line nuclei have traditionally been divided into a mechanoreceptive nucleus and an electroreceptive nucleus [9,11,12,

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30,33]. Siluroids (catfishes) are passively electroreceptive. They have a mechanoreceptive nucleus medialis (nM) and an electrosensory lateral line lobe (ELL) that receives inputs from ampullary electroreceptors [11,13,38]. Gymnotoids (South American weakly electric fishes) were derived from a siluroid-like ancestor [14] about 90–100 million years ago (personal communication, J. Albert). In addition to having a mechanoreceptive nM, gymnotoids have an ELL which receives inputs from both ampullary organ and tuberous organ electroreceptors [4,9,19]. Mab anti-zebrin II not only recognizes a subset of cerebellar Purkinje cells, but also the pyramidal cells in the developing nM (mechanoreceptive) and in the medial segment of the ELL (ampullary organ electroreceptive) [23]. Therefore, mab anti-zebrin II does not recognize the traditional functional mechanoreceptive-electroreceptive division of the lateralis region as defined by morphologists and physiologists. Instead, zebrin II expression in developing gymnotoids is consistent with the lateral line organization of siluroids [25], which represents the gymnotoid ancestral, or primitive, condition [11,14].

In the present report, we extend these observations of shared Purkinje and pyramidal cell immunocytochemical labelling and ask whether these patterns reflect shared function or shared lineage. In particular, we examine the labelling pattern of a newly generated antibody, monoclonal antibody (mab) anti-type I, in *Rhamphichthys rostratus* (Gymnotoidei: Rhamphicthyidae) and in *Ictalurus punctatus* (Siluriodei: Siluridae). In a preliminary study using the gymnotoid *Apteronotus* [24], the type I antigen was shown to be present in all Purkinje cells, pyramidal cells, Mauthner neurons and pacemaker relay cells. For comparison, we conducted a parallel series of experiments using mab anti-zebrin II on both *Rhamphichthys* and *Ictalurus*, as detailed below.

2. Materials and methods

Two species of fishes in two suborders within the ostariophysan order Siluriformes [14] were examined: the trumpet nose knife fish, *Rhamphichthys rostratus* (Gymnotoidei) and the channel catfish, *Ictalurus punctatus* (Siluroidei). Gymnotoid phylogeny is according to J. Albert (personal communication), ictalurid phylogeny according to Lundberg [29]. In addition, two adults of a second species of gymnotoid, the brown ghost knife fish *Apteronotus leptorhynchus* previously studied by Brochu et al. [6,7] and Lannoo et al. [24], were used as positive controls. Eight adult trumpet nose knife fish and two brown ghost knife fish, 15–20 cm total length, were purchased through commercial tropical fish suppliers. Ten adult channel catfish, 9–13 cm total length, were obtained from Aquatic Control (Seymour, IN). Fish were housed under light (12 h light/12 h dark) and temperature (22–26°C) conditions similar to their summer season. Brainstem nuclei were identified according to the nomenclature of Maler et al. [32] for gymnotoids and Finger [11], Tong and Finger [38] and McCormick and Braford [34] for siluroids.

Fish were anesthetized with 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, A-5040) at a concentration of 1:15,000 and perfused through the heart with 10–15 ml 0.1 M phosphate buffered saline (PBS) pH 7.2, followed by 15–20 ml Zamboni's fixative [26,40]. Shallow midsagittal and transverse incisions were made in the skull, and the intact brain, lateral line ganglia and rostral spinal cord were removed and processed for immunocytochemistry, as follows. Tissue was postfixed for 2–4 h in refrigerated (6°C) Zamboni's fixative, then transferred to a refrigerated 30% sucrose solution (g/ml dissolved in 0.1 M PBS) overnight or until the tissue equilibrated (sunk).

Brains were placed with ganglia and peripheral nerves extended in a cooled, 10% liquid gelatin solution (g/ml dissolved in 0.1 M PBS), which was then allowed to harden. Prior to sectioning the gelatin-embedded tissue, the olfactory tract and telencephalon were severed and the excess gelatin trimmed. Transverse sections, 30–35 μm thick, were cut on a International Equipment Company (Damon) Minotome cryostat at -20°C. Individual sections were placed in separate wells (maximum volume 2 ml) in 24-well tissue culture plates. Following sectioning, plates were removed from the cryostat and 0.1 M PBS was added to each well containing a section. These free-floating sections were then reacted with primary and secondary antibodies as detailed below.

2.1. Immunocytochemistry

Two monoclonal antibodies (mabs) were used: mab anti-type I and mab anti-zebrin II. Both mabs were generated in Balb/c mice immunized with a crude homogenate of hindbrain from *Apteronotus leptorhynchus*. Mab anti-zebrin II has been previously described in other species [6,7,23,25,25]. This antibody recognizes a single 36 kDa polypeptide which is present throughout the cell body, axon and dendrites, but absent in the cell nucleus [6,7]. Mab anti-zebrin II hybridoma supernatant was diluted 1:2 in 0.1 M PBS plus 0.1% Triton X-100. These relatively high concentrations produce specific labelling without generating non-specific reactivity. Full strength mab anti-type I plus 0.1% Triton X-100 was required to produce a similar level of reactivity.

Brainstem sections were incubated overnight at room temperature in supernatant containing either mab anti-type I or mab anti-zebrin II. Following incubation, sections were rinsed twice with 0.1 M PBS then incubated for a minimum of 2 h in secondary antibody (peroxidase-conjugated, rabbit anti-mouse immunoglobulin; DAKOPATTS, Denmark, P260) diluted 1:200 in 5% powdered milk solution (g/ml in 0.1 M PBS). Tissue sections were rinsed and incubated in 0.06% 4-chloro-1-naphthol plus 0.01% hydrogen peroxide in 0.1 M PBS [17,18]. The reaction was followed visually until sections were labelled optimally (i.e., a robust specific reaction), a process that usually took 5 to 15 minutes. Sections incubated in Triton X-100 and milk in the absence of primary antibody served as negative controls and did not label. During each reaction, wells containing only secondary antibody were tested with the 4-chloro-1-naphthol chromagen to provide a positive control. Labelled sections were mounted and coverslipped in 80% glycerol. These were examined and photographed within 24 h of the chromagen reaction.

2.2. Photography

Low power photographs were made with a Nikon SMZ-U photodissecting microscope. Higher power photographs of labelled cells and tracts were photographed with a Zeiss Jenalumar compound microscope. Kodak T-max 135 mm film, 100 ASA, was used.

2.3. Electrophoresis

The molecular weight of the type I antigen was determined using standard $NaDodSO_4$/polyacrylamide gel electrophoresis [15]. West-

ern blotting was completed according to the procedures of Towbin et al. [39] as modified in Beesley et al. [3].

3. Results

3.1. Monoclonal antibody anti-type I characteristics and labelling

Western blots of gymnotoid brain homogenate reveal that mab anti-type I appears to recognize a major polypeptide band at 47 kDa (Fig. 1), which is weaker than the 36 kDa band labelled by mab anti-zebrin II. In neurons, the type I polypeptide is present throughout the cell body and its dendrites, but is not found in either the cell nucleus or its axon. Depending on the cell type, labelling is either strong or weak. Neurons displaying weak reactivity consistently label and this reactivity is consistently weak, suggesting the presence of low levels of the antigen, rather than artifactual labelling. Mab anti-type I recognizes various types of projection neurons throughout the midbrain and hindbrain of *Rhamphichthys rostratus* and *Ictalurus punctatus*, as follows (shown schematically in Figs. 2–4).

3.2. Strong type I labelling

In both *R. rostratus* and *I. punctatus*, mab anti-type I labels all Purkinje cells, including cells of the corpus cerebelli (Fig. 5A), the medial and lateral portions of the valvula (shown schematically in Figs. 3C and 4A) and all three divisions of the eminentia granularis (EGa; Fig. 5B), EGm and EGp shown schematically in Figs. 3I,K and 4E,G). Further, mab anti-type I labels pyramidal cells, including pyramidal cells in the electrosensory lateral line lobe (Fig. 5C,D), nucleus medialis (shown schematically Figs. 3I,K and 4G,I) and tectum (Fig. 5E) in both species. Mab anti-type I also labels lateral line ganglion cells (Fig. 5F).

Several cell types are immunopositive in gymnotoids but immunonegative in siluroids. In two cases, immunopositive cell types are derived cells associated with an active electrosense and therefore are only found in gymnotoids. These are the relay cells of the pacemaker nucleus (Fig. 6A) and the spherical cells of the ELL (Fig. 6B). Other cell types are present within both species, but are only recognized in gymnotoids. These include large neurons of the reticular formation (Fig. 6C) and the lateral reticular nucleus (Fig. 6D), Mauthner neurons (Fig. 6E) and cells of the inferior olive (Fig. 6F).

3.3. Weak type I labelling

In gymnotoids, weak mab anti-type I reactivity is present in the torus semicircularis, motor nuclei of the Vth, VIIth and Xth cranial nerves, the magnocellular

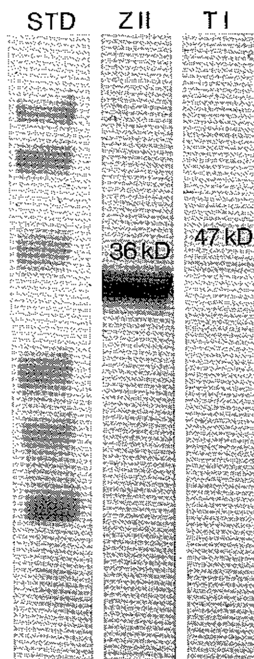

Fig. 1. Western blots of gymnotoid hindbrain homogenate indicating the 36 kDa zebrin II polypeptide and the 47 kDa type I polypeptide.

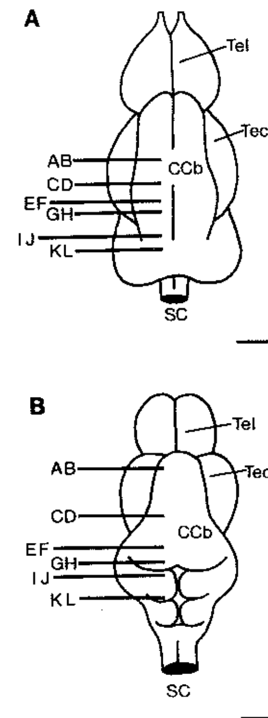

Fig. 2. Dorsal views of the brains of A (*Rhamphichthys rostratus*) and B (*Ictalurus punctatus*) with the levels of transverse sections indicated. Levels indicated refer to Fig. 3 (A) and to Fig. 4 (B). Abbreviations: Tel, telencephalon; SC, spinal cord. Scale bars = 1 mm.

and lateral tegmental nuclei and the large cells of the efferent and medial octavolateralis nuclei (shown schematically in Figs. 3 and 4). In siluroids, only the motor neurons of the Xth and perhaps the IXth, cranial nerve are weakly labelled (Fig. 4K).

3.4. Monoclonal antibody anti-zebrin II characteristics and labelling

Zebrin II reactivity in adults of the gymnotoid *R. rostratus* and the siluroid *I. punctatus* is present only in the Purkinje cells of discrete cerebellar regions. In both species, all Purkinje cells in the corpus cerebelli (CCb), the lateral valvula cerebelli (VCbl) and the anterior eminentia granularis cerebelli (EGa) express zebrin II. In the gymnotoid, all other regions of the cerebellum, including the medial valvula cerebelli (VCbm) and the medial and posterior eminentia granularis cerebelli (EGm and EGp) are immunonegative. In the siluroid, mab anti-zebrin II reactivity is present in the Purkinje cells of the VCbm. This labelling is essentially identical to that shown for the gymnotoids *Apteronotus* and *Eigenmannia* and the siluroid *Ameiurus* [6,7,23,26] and is shown schematically in Figs. 3 and 4.

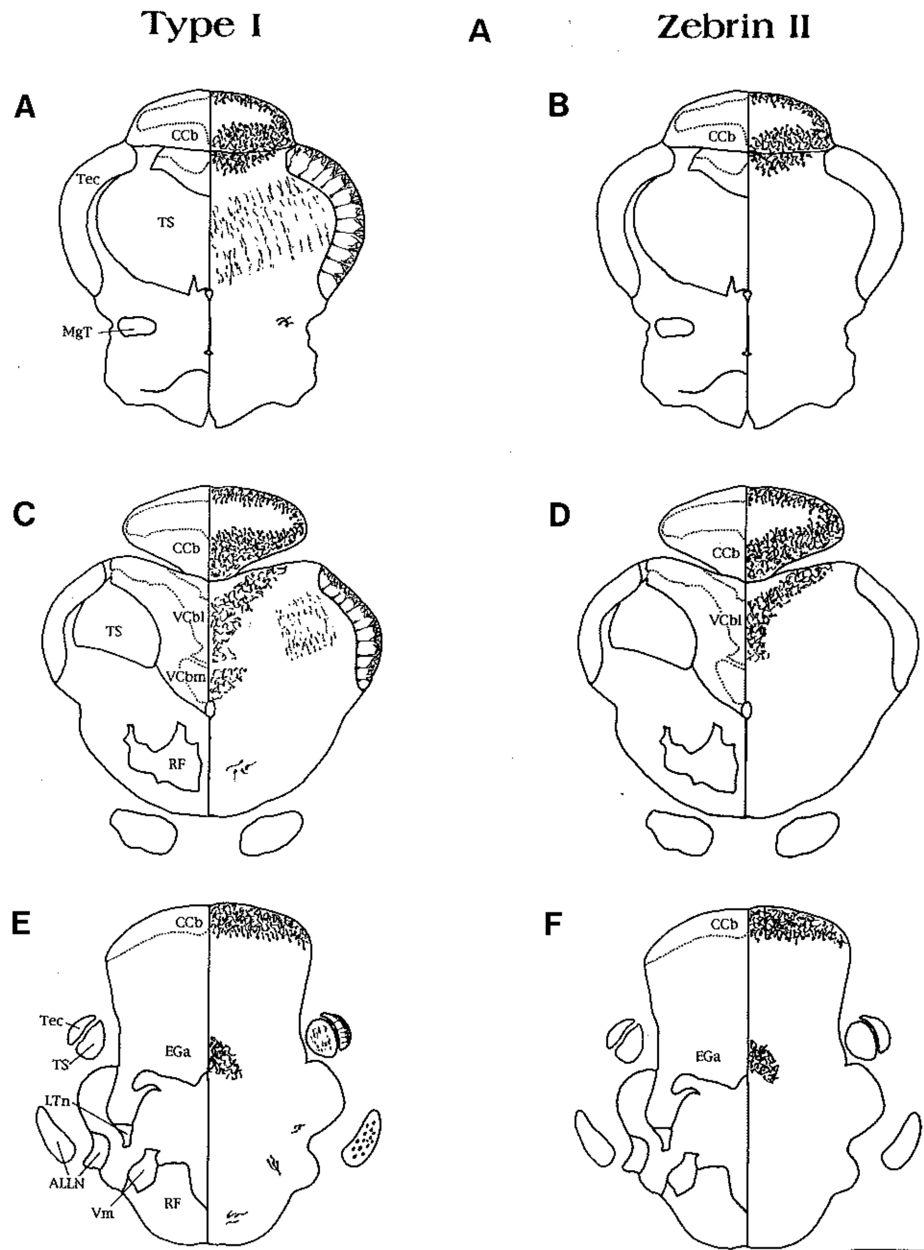

Fig. 3. Mab anti-type I and anti-zebrin II labelling in the adult *Rhamphichthys rostratus*. Sections proceed rostral to caudal (A–L) as indicated in Fig. 2A. Scale bar = 1 mm.

In both species, mab anti-zebrin II reveals the presence of local circuit and projecting Purkinje cell axons. Local circuit Purkinje cells are observed as they course to and synapse on, eurydendroid cells in the proximal granule cell layer. Extracerebellar axons originating from projecting Purkinje cells in the CCb and the EGa can be followed in the tractus cerebello-acousticolateralis (tCb-Al) as they terminate in the nucleus medialis and the dorsal octavolateralis nucleus (DO; Figs. 3J,L and 4H,J,L).

4. Discussion

The type I and zebrin II antigens are present in the gymnotoid *Rhamphichthys rostratus* and the siluroid *Ictalurus punctatus*. Within immunopositive neurons, both antigens are present in the cell soma and dendrites, but not the nucleus. The zebrin II antigen is present in the axon, the type I antigen is not. Across immunopositive neurons, the differential distribution of the type I and zebrin II antigens offers clues to the relationships of neuronal cell classes within the siluriform brainstem.

4.1. Type I expression

Mab anti-type I recognizes the largest neurons within a brainstem nucleus, independent of their afferent, associative or efferent functions. Immunopositive cells are observed in sensory nuclei (lateral line ganglia, ELL spherical and pyramidal cells, nM pyramidal cells

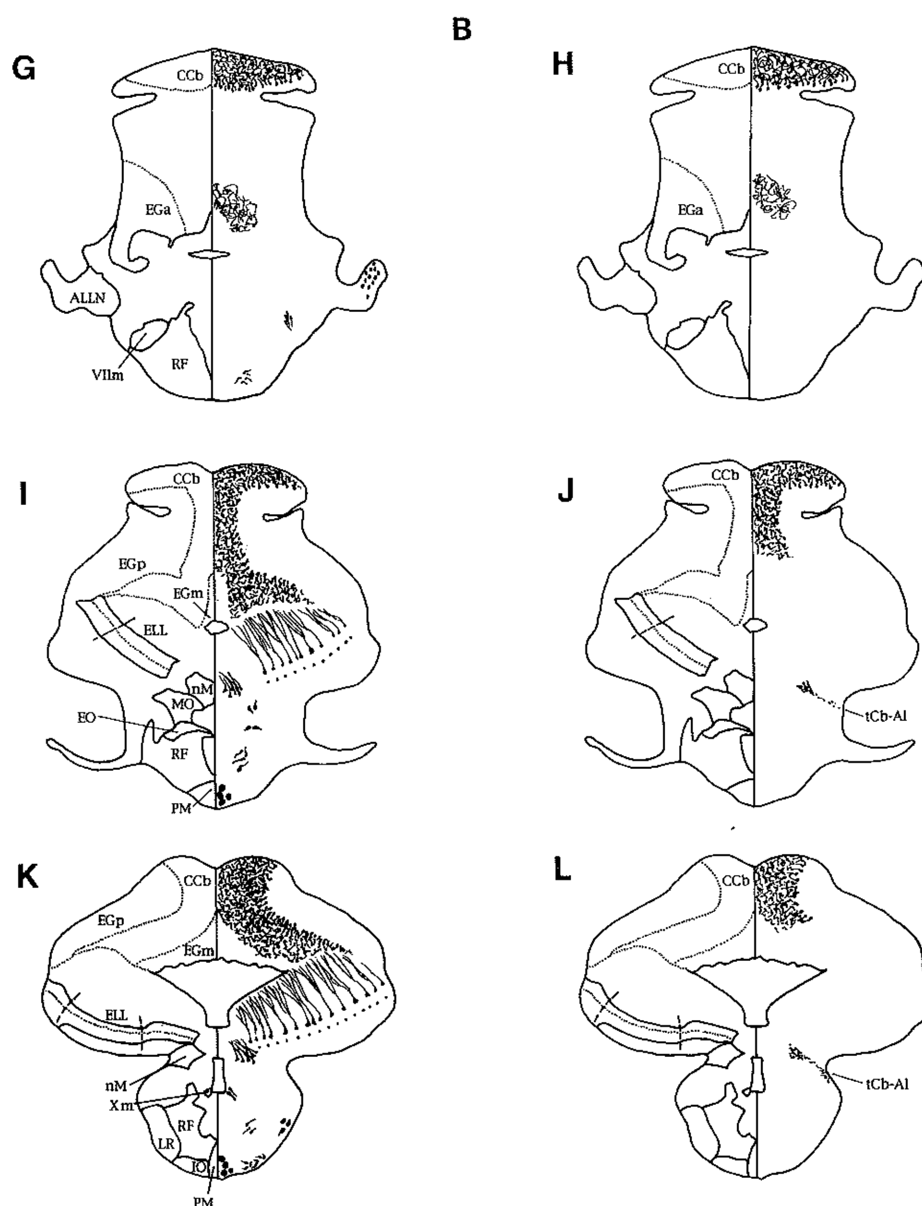

Fig. 3 (continued).

and the medial and efferent octavolateralis nuclei), motor nuclei (motor neurons in the Vth, VIIth and Xth nuclei, pacemaker relay cells and Mauthner neurons) and associative nuclei (Purkinje cells, large reticular neurons and olivary cells). These large neurons tend to be projection neurons, which are characterized by short dendrites and long axons, and are designed for receiving and sending signals from one brain region to another [21]. It appears that some aspect of these features of projection neurons corresponds to type I expression. The exception to this observation that type I^+ cells are projection neurons is local circuit Purkinje cells. In fishes, local circuit Purkinje cells contact nearby eurydendroid cells by using short axons that remain within the cerebellum.

While most type I immunopositive neurons are projection neurons, not all projection neurons are type I^+. Therefore, the type I polypeptide does not appear to be a mandatory component of all projection neurons. Among gymnotoids, notable type I^- neurons include cells in the isthmal nucleus preeminentialis, spinal motor neurons and all cells in the diencephalon. Further, several types of projection neurons are type I^+ in gymnotoids but not siluroids. These cells include torus semicircularis cells, Mauthner neurons, large neurons of the reticular formation and lateral reticular nucleus,

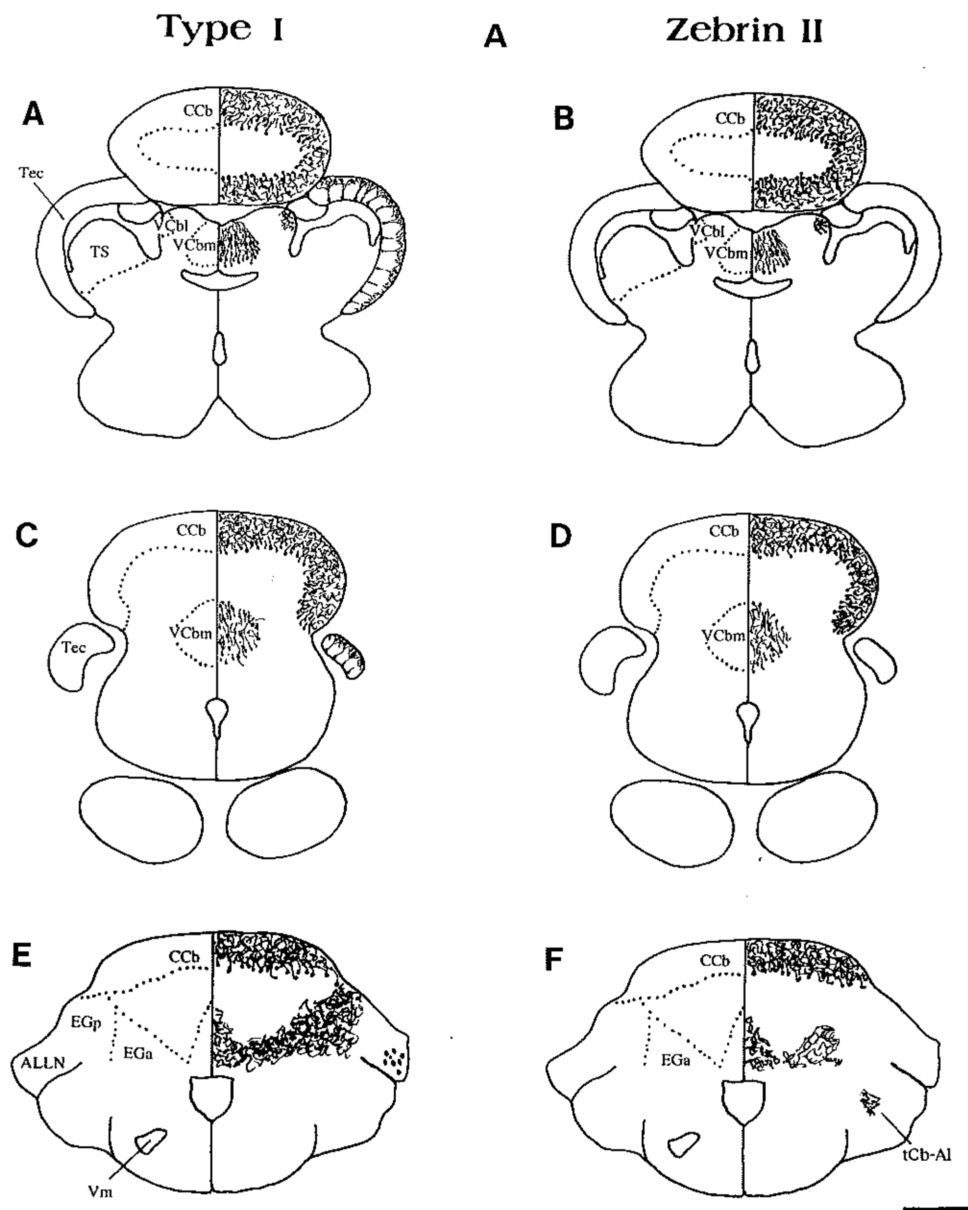

Fig. 4. Mab anti-type I and anti-zebrin II labelling in the adult *Ictalurus punctatus*. Sections proceed rostral to caudal (A–L) as indicated in Fig. 2B. Scale bar = 1 mm.

cells of the inferior olive, motor neurons of the Vth and VIIth cranial nerves, cells of the magnocellular and lateral tegmental nuclei and cells of the medial and efferent octavolateralis nuclei.

The shared feature of most type I^+ neurons – that they project – appears to be a functional characteristic rather than a shared phylogenetic or developmental characteristic. To argue for phylogenetic linkage, derived neurons must have recently shared a common ancestral cell type. Therefore, one might reasonably expect immunopositive cell types to share at least the early portion of their developmental history. For example, in mammals, Berrebi et al. [5] suggest a developmental linkage between cerebellar Purkinje cells and cartwheel cells of the dorsal cochlear nucleus by demonstrating that developmental mutations that affect Purkinje cells also affect cartwheel cells. Both cell types originate from neighboring regions of the embryonic metencephalon (rostral rhombencephalon). In this germinal zone, Purkinje cells arise from the medial portion, cartwheel cells from the lateral portion. In our study, type I immunopositive cells demonstrate no such developmental (or phylogenetic) similarities. Labelled cells trace their development back to different areas of the neuraxis (mesencephalon or rhombencephalon), arise at different times (motor neurons typically precede sensory neurons [21]) or develop from different precursor cells (neuroblasts or placodal cells).

4.2. Zebrin II expression

In adults of both species, zebrin II expression occurs in the Purkinje cells of the CCb, VCbl and EGa; EGm and EGp cells do not label. VCbm Purkinje cells are zebrin II^- in gymnotoids; cells corresponding in position to the VCbm are zebrin II^+ in siluroids. In both species, zebrin II^+ projecting Purkinje cell axons form the tCb-Al as they course to the nM and DO. Similar differences in distributions that fall out along subordinal lines are reported for species in two other gymnotoid genera (*Apteronotus leptorhynchus* and *Eigenmannia virescens* [6,7,23]) and in one other siluroid genus (*Ameiurus melas* [26]). The difference in zebrin II reactivity between the VCbm of gymnotoids (zebrin II^-) and siluroids (zebrin II^+) is consistent and problematical [7]. Morphologically, catfish lack a clear divi-

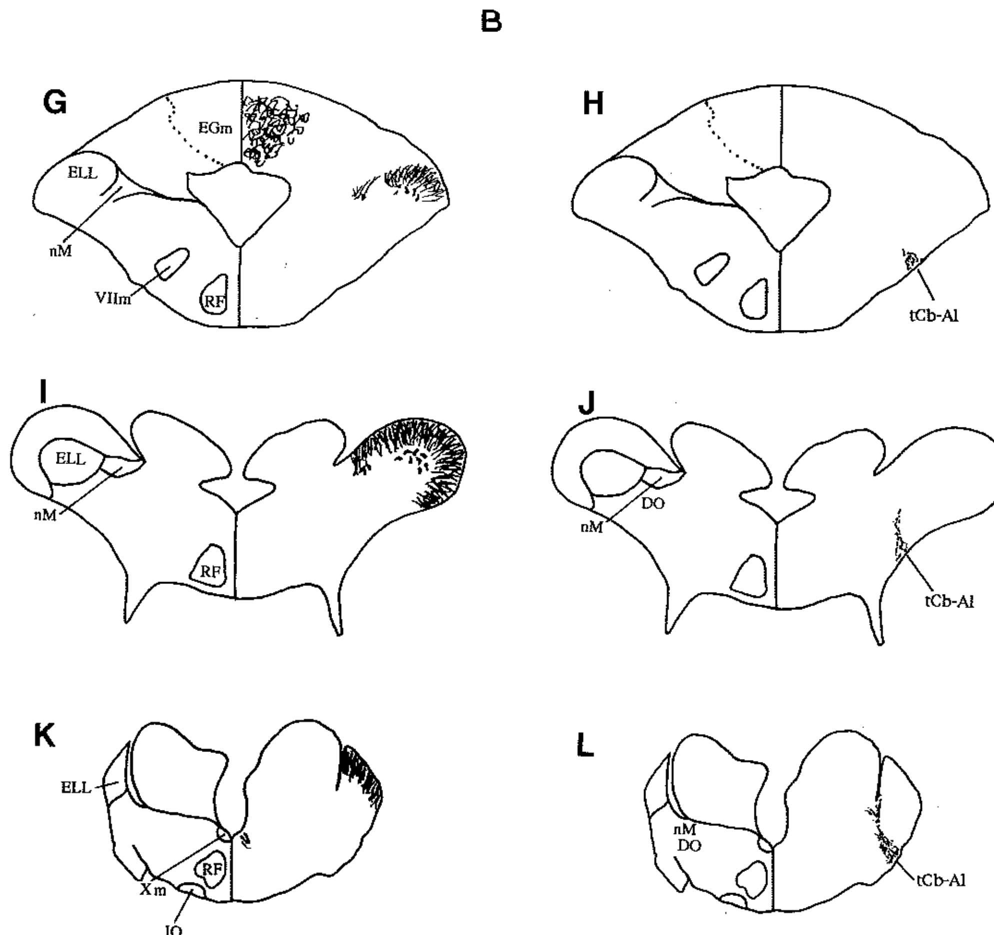

Fig. 4 (continued).

sion between VCbm and VCbl [10]. In weakly electric mormyrid fishes, mab anti-zebrin II distribution has been used to redefine cerebellar regions originally based on histological criteria [35]. Perhaps a similar revision will be necessary in siluroids.

In gymnotoids, zebrin II expression may reflect a

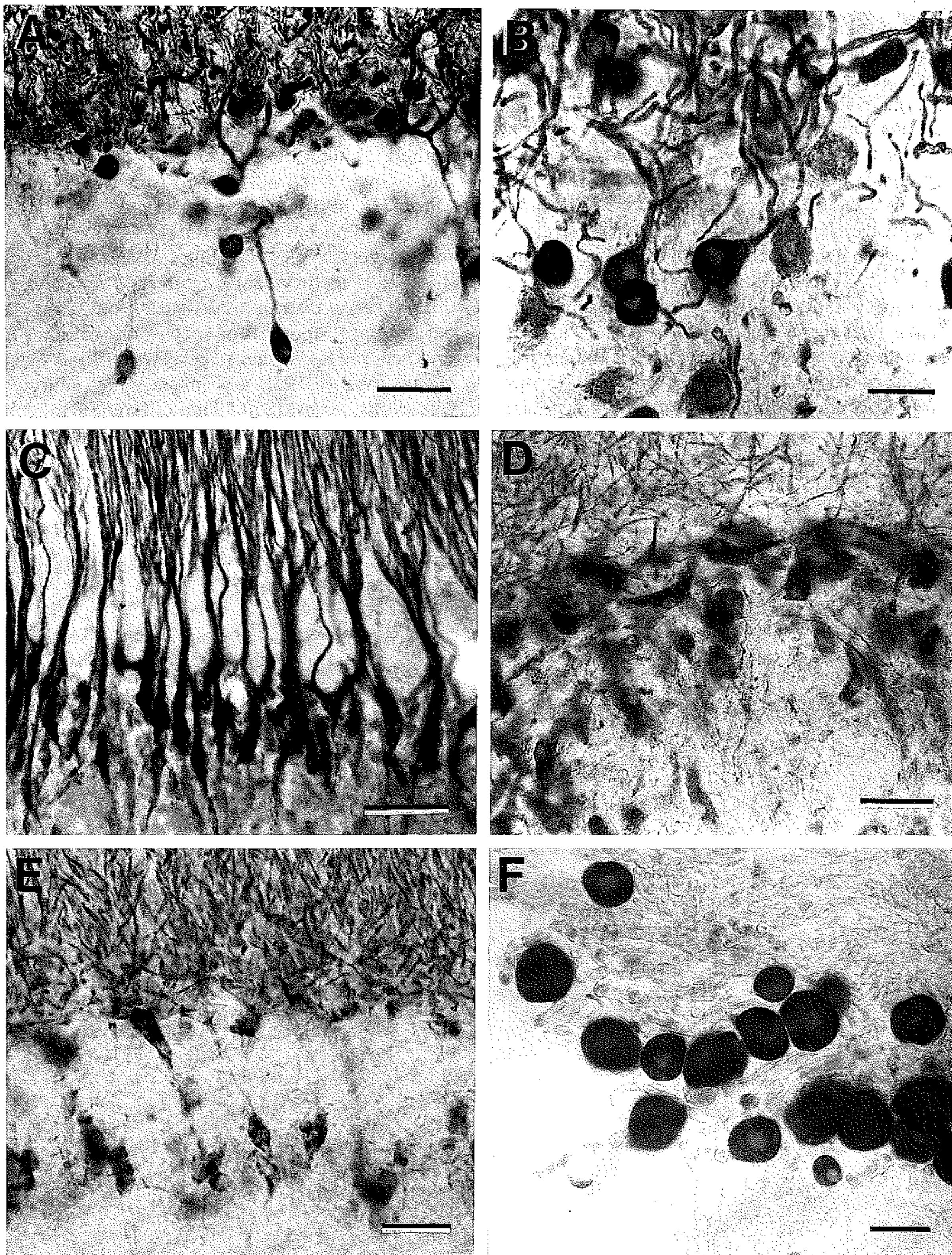

Fig. 5. Mab anti-type I immunopositive cells. A: siluroid corpus cerebelli Purkinje cells. B: gymnotoid anterior eminentia granularis Purkinje cells. C: gymnotoid electrosensory lateral line lobe (ELL) pyramidal cells. D: siluroid ELL pyramidal cells. E: gymnotoid tectal pyramidal cells. F: gymnotoid lateral line ganglion cells. Scale bars: A = 75 μm; B–F = 25 μm.

common embryological and/or phylogenetic lineage. Among generalized (i.e., primitive) ostariophysans, such as cypriniforms, zebrin II is expressed in the CCb, VCb and EGa of the cerebellum [26]. In electroreceptive siluriforms, two divisions of the cerebellum primarily involved with electroreception and probably derived

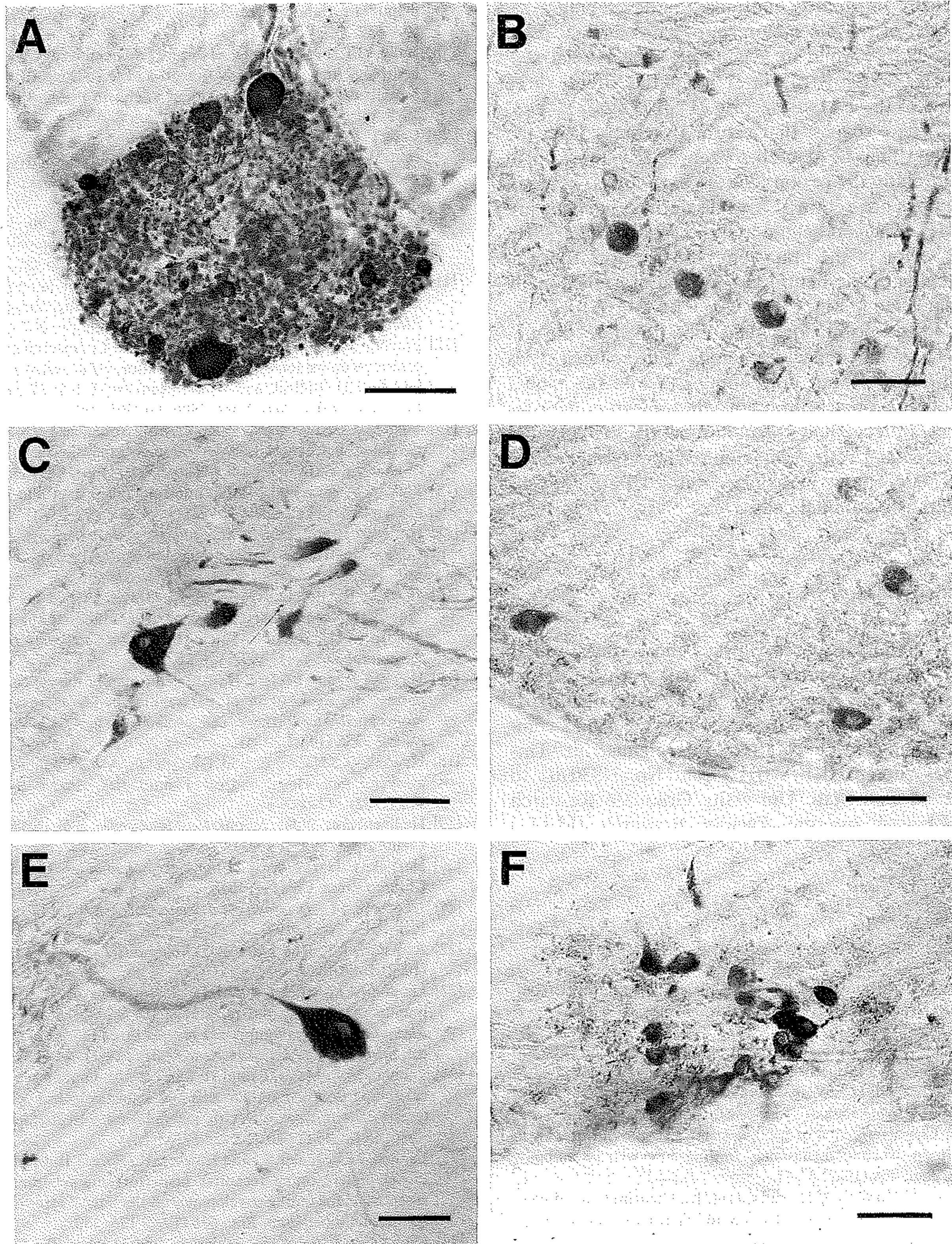

Fig. 6. Mab anti-type I immunopositive cells in gymnotoids. A: pacemaker relay cells. B: ELL spherical cells. C: large reticular neurons. D: lateral reticular neurons. E: Mauthner neuron. F: olivary cells. Scale bars: A = 150 μm; B = 50 μm;, C–F = 25 μm.

from the EGa – the EGm and EGp – do not express zebrin II Thus the generalized regions of the cerebellum are zebrin II$^+$ while the derived regions which tend to be associated with electrosensation are zebrin II$^-$ [1,7,10]. Further, in siluroids, the developing pyramidal cells of the mechanoreceptive nM and the ELL are zebrin II$^+$, while in gymnotoids, the medial segment of the ELL is zebrin II$^+$, but the lateral three segments of the ELL are zebrin II$^-$ [27].

These data suggest that zebrin II expression in the cerebellum may be related to zebrin II expression in the octavolateralis region [26]. In both areas, generalized regions are zebrin II$^+$, while derived portions are zebrin II$^-$. The cerebellum and the acousticolateral-recipient region of the brainstem both develop from the same region of the neural axis [5] and are functionally interconnected [2,31].

In conclusion, monoclonal antibodies have the potential to reveal similarities among neuronal cell types by unmasking shared polypeptides. While the type I polypeptide is present in brainstem projection neurons and likely reflects their common function, the zebrin II polypeptide may reflect common embryological and/or phylogenetic origins among generalized Purkinje cells and lateralis pyramidal cells.

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