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Brain distribution of myosin Va in rainbow trout *Oncorhynchus mykiss*

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Abstract

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This study presents data on myosin Va localization in the central nervous system of rainbow trout. We demonstrate, via immunoblots and immunocytochemistry, the expression of myosin Va in several neuronal populations of forebrain, midbrain, hindbrain and spinal cord. The neuronal populations that express myosin Va in trout constitute a very diverse group that do not seem to have many specific similarities such as neurotransmitters used, cellular size or length of their processes. The intensity of the immunoreactivity and the number of immunoreactive cells differ from region to region. Although there is a broad distribution of myosin Va, it is not present in all neuronal populations. This result is in agreement with a previous report, which indicated that myosin Va is approximately as abundant as conventional myosin II and kinesin, and it is broadly involved in neuronal motility events such as axoplasmatic transport. Furthermore, this distribution pattern is in accordance with what was shown in rats and mice; it indicates phylogenetic maintenance of the myosin Va main functions.

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Introduction

Class V myosins are one of the most ancient groups of the myosin superfamily. In vertebrates such as rats, mice and humans, the class V myosins include three isoforms, Va, Vb and Vc, which are currently attracting great interest because there is evidence to support their role in the intracellular transport and location of organelles (Langford and Molyneaux 1998; Provance and Mercer 1999; Reck-Peterson *et al.* 2000; Berg *et al.* 2001). Furthermore, the class V myosin presents a broad phylogenetic distribution and it is expressed from yeasts to humans (Reck-Peterson *et al.* 2000). Specifically the myosin Va has been purified and biochemically characterized (Espindola *et al.* 1992; Espreafico *et al.* 1992; Cheney *et al.* 1993; Nascimento *et al.* 1996; Tauhata *et al.* 2001), and current evidence suggests that myosin Va has an important role in neuronal processes, including organelle and vesicle transport within axons, neurotransmitter–vesicle cycling and neurite outgrowth (Prekeris and Terrain, 1997; Evans *et al.* 1998; Tabb *et al.* 1998; Costa *et al.* 1999; Ohyama *et al.* 2001; Casaletti *et al.* 2003).

The essential function of myosin Va is indicated by analysis of the *dilute lethal* and *opisthotonus* mutant in mice and rats, which is null for myosin Va (Evans *et al.* 1997; Bridgman 1999; Futaki *et al.* 2000). This mutation not only affects coat-colour in these animals but it also makes homozygous pups manifest neurological defects such as convulsions as well as posture and balance alterations; they die within 3 weeks of birth. Similar traits are observed in humans with Griscelli's syndrome (Griscelli *et al.* 1978), which presents partial albinism, neurological alterations and immunodeficiency. One of the known causes of this syndrome is a mutation in the gene that codifies the human myosin Va, present in the 15q21 chromosome (Pastural *et al.* 1997).

Several immunohistochemical studies have described the broad distribution of myosin Va in many brain regions (Mercer *et al.* 1991; Espindola *et al.* 1992; Espreafico *et al.* 1992)

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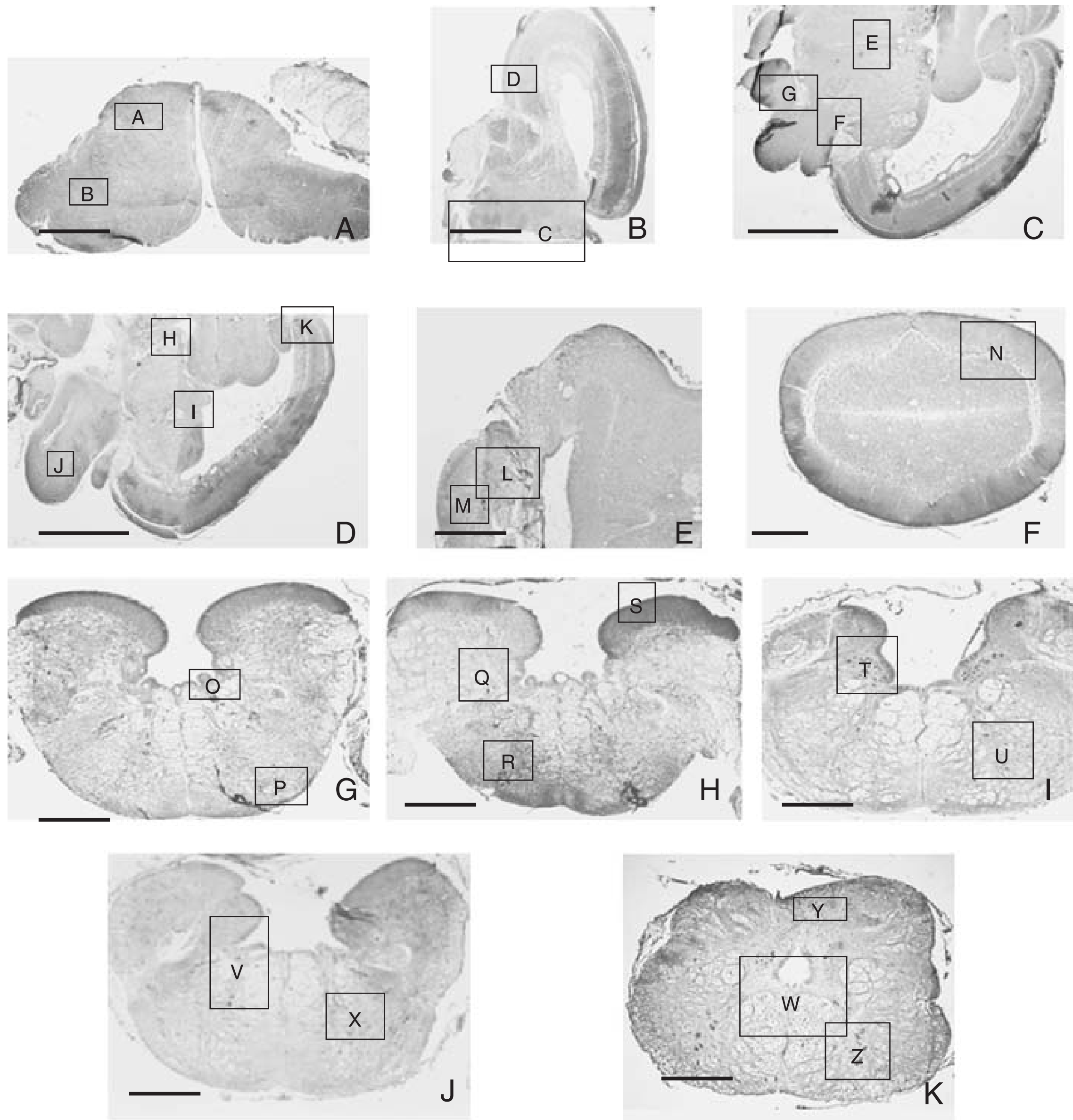

Fig. 1—Panoramic transverse view of sections of trout brain at several levels immunolabelled with anti-myosin Va antibody. Higher magnifications of lettered rectangles are shown in Fig. 3. Levels of transverse sections: —**A**. Telencephalon; —**B**. Diencephalon; —**C**. Rostralmost portion of the mesencephalon; —**D**. Caudalmost portion of the mesencephalon; —**E**. Rostralmost portion of the rhombencephalon; —**F**. Cerebellum; —**G**. Rhombencephalon at level of cranial nerve VII; —**H**–**J**. Rostralmost portion of the rhombencephalon; —**K**. Spinal cord. Scale bars: 400 μm.

and, recently, Tilelli *et al.* (2003) have described a comprehensive map of myosin Va distribution in the central nervous system of the adult rat using immunohistochemistry. These studies contribute to the basic understanding of its role in the nervous system's function and plasticity.

For the first time in fish, the present study reveals the presence of one member of the myosin V class expressed in brain (myosin Va) and its localization in different areas of the central nervous system of rainbow trout, which shows that in fish the myosin Va is broadly expressed, suggesting its *involvemen*t in neuronal processes.

Materials and Methods

Protein quantification, electrophoresis and immunoblots

Adult rainbow trout were killed with an overdose of benzocaine and their brains were dissected, immediately frozen in

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liquid nitrogen and stored at –20 °C until prepared for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Samples were homogenized in 40 mM Tris–HCl buffer, pH 6.8, containing 1% SDS, 1 mM benzamidine, 1 mM aprotinin, 2 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, 1 mM pefabloc, and 5 mM ATP. The protein content was estimated by the Bradford method (Braford 1976), by using bovine serum albumin as a standard. Samples containing approximately 20 µg of protein were submitted to SDS–PAGE 7% (Laemmli and Favre 1973) and stained with Coomassie Brilliant Blue R. For Western blots, approximately 60 µg protein was loaded on the gels and, following SDS–PAGE, was blotted to nitrocellulose membranes (Towbin *et al.* 1979). The membranes were probed with primary antibody rabbit immunoglobulin G (IgG) anti-chick myosin Va tail (1 : 100), followed by incubation with secondary antibody peroxidase-conjugated goat IgG anti-rabbit IgG (1 : 1000), following standard procedures for peroxidase methods. The primary antibody recognizes an epitope located between amino acids 1117 and 1435 and it was previously characterized by Espindola *et al.* (1992) and Espreafico *et al.* (1992) in extracts of chicken brain.

Immunocytochemistry

Rainbow trout were anaesthetized with benzocaine and the brains were fixed in Bouin's fluid for 8 h at room temperature. Eight brains of male and female trout were analysed from July to December They were washed in several changes of phosphate buffer (100 mM sodium phosphate, 400 mM NaCl, pH 7.4), dehydrated in a graded ethanol series, diaphanized in xylene, embedded in paraffin and semi-serially and transversely sectioned (10 µm thickness). The sections were submitted to microwave retrieval of antigenic sites (Martins *et al.* 1999), blocked for non-specific binding sites in 100 mM Tris–glycine buffer, pH 7,4 for 1 h at room temperature and in phosphate-blocking buffer (100 mM sodium phosphate, pH 7.4, with 0.3% Triton X-100, 400 mM NaCl, 5% bovine serum albumin and 10% normal goat serum) for 4 h at room temperature. Subsequently, the sections were incubated overnight at 4 °C with primary antibody anti-myosin Va (1 : 25) diluted in phosphate-blocking buffer, washed for 1 h in phosphate buffer (100 mM sodium phosphate, pH 7.4, with 0.3% Triton X-100), incubated for 2 h at room temperature in secondary antibody (1 : 1000) goat IgG anti-rabbit IgG biotinylated, and processed following standard procedures for avidin–biotin–peroxidase methods (Vectarstain, Vector Laboratories, Ltd., Peterborough, UK). Controls were performed by omission of the primary antibody. The comprehensive atlas of trout brain cytoarchitecture published by Meek and Nieuwenhyus (1998) was used for topographical interpretations. Panoramic views of all encephalic portions analysed in this work are showed in Fig. 1. Micrograph images were obtained under a Zeiss Axiophot microscope. The images were digitalized, converted to

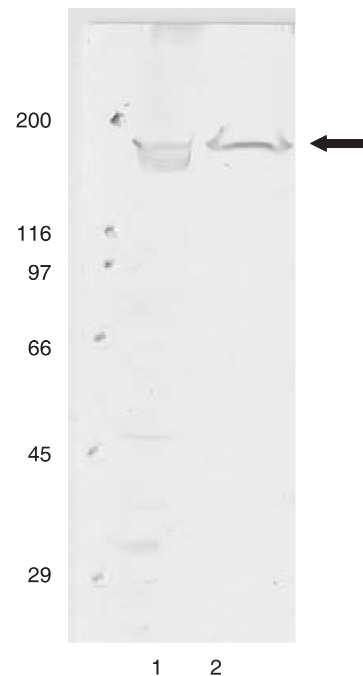

Fig. 2—Western blot of the brain homogenates of chicken (lane 1) and trout (lane 2) probed with polyclonal antibody anti-tail domain of myosin V showing a single band (arrow) of the 190 kDa (in chicken and trout) coincident with the molecular weight of myosin Va of higher vertebrates. Molecular mass markers are indicated on the left.

a grey scale, and adjusted for brightness and contrast using COREL PHOT-PAINT 8 program. Photomontage and lettering were performed by using POWERPOINT program.

Results

Expression of myosin Va in homogenates of brains

Western blots of homogenates of chick (positive control) and trout brain were probed with anti-myosin Va. Both samples showed that this antibody recognized a single 190-kDa band, which corresponded to the well-defined position of the myosin Va heavy chain (Fig. 2). This result indicated the presence of myosin V, similar to the myosin Va of chicken or rat, in trout brain.

Immunolocalization of myosin Va in brain sections

The myosin-Va-immunoreactive (MVa-ir) neuronal populations were broadly distributed in the adult trout brain, which was the basal plate of mesencephalon and rhombencephalon, the regions with a major number of immunoreactive populations. The controls showed total absence of reaction product (not shown).

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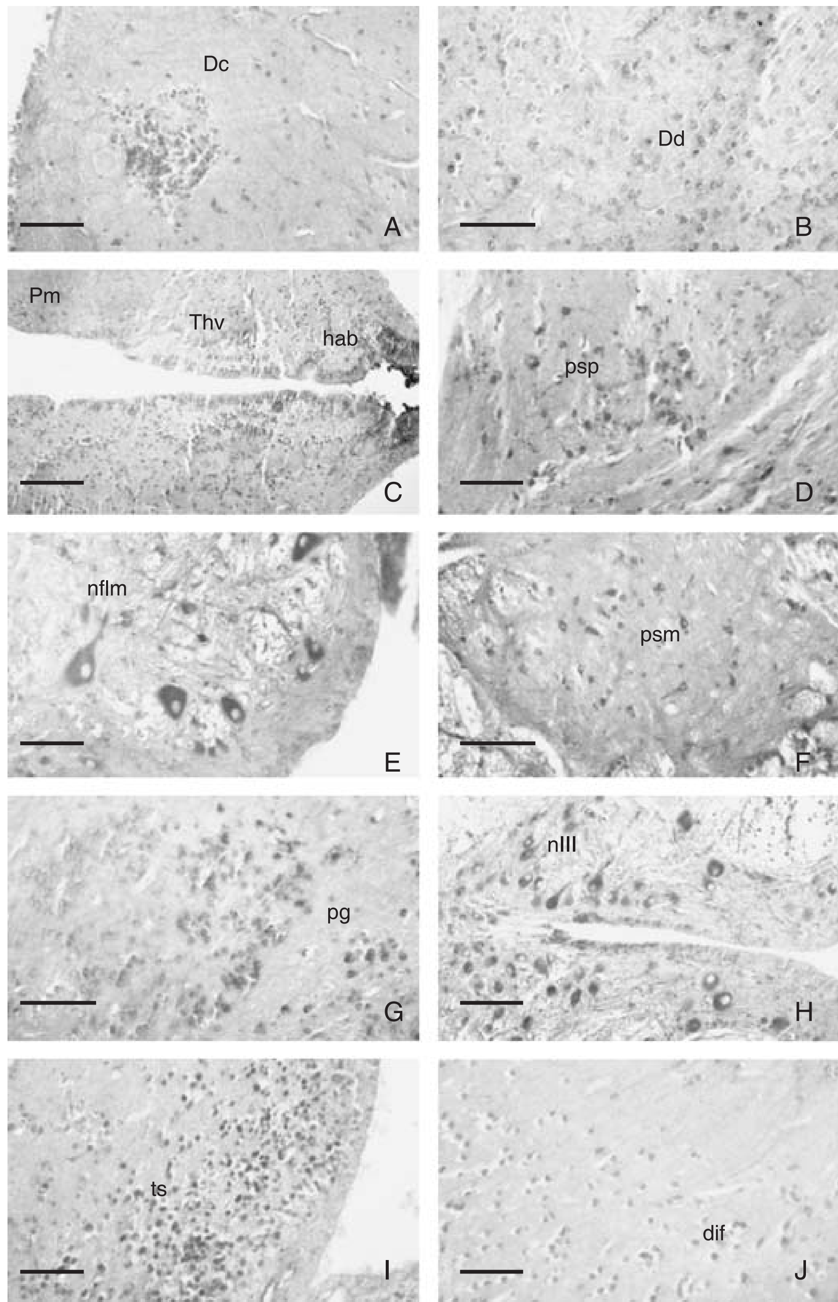

Fig. 3—Myosin V immunoreactivity in rainbow trout brain. —**A**, **B**. Transverse section of the telencephalon. —**A**. Detail of the pars centralis (Dc). —**B**. Detail of the pars dorsalis (Dd). —**C**, **D**. Transverse section of the diencephalon. —**C**. Detail of the magnocellular pre-optic nucleus (Pm), habenular nucleus (hab) and ventral thalamus (Thv). —**D**. Detail of the parvocellular superficial pretectal nucleus (psp). —**E**–**G**. Transverse section of the mesencephalon rostralmost portion. —**E**. Detail of the fasciculus longitudinalis medialis nucleus (nflm). —**F**. Detail of the magnocellular pretectal superficial nucleus (psm). —**G**. Detail of the preglomerular complex (pg). —**H**–**K**. Transverse section of the mesencephalon caudalmost portion. —**H**. Detail of the oculomotor nerve nucleus (nIII), —**I**. Detail of the semicircular torus (ts). —**J**. Detail of nucleus diffusus neurones of the inferior hypothalamic lobe (dif).

Forebrain. In the trout telencephalon, faint Mva-ir cells were observed in areas such as the pars ventralis and lateralis, pars centralis and pars dorsalis (Fig. 3A,B). In the diencephalon, Mva-ir was observed in the magnocellular pre-optic nucleus, in the neurones of the parvocellular superficial pretectum, suprachiasmatic nucleus, habenular nucleus and ventral thalamus (Fig. 3C,D).

Midbrain. Several populations of cells were Mva-ir in the mesencephalic portion. Immunoreactivity was observed in the neurones of the oculomotor nucleus, semicircular and longitudinal torus, nucleus diffusus of the inferior hypothalamic lobe and in cells of the midbrain roof (periventricular stratum) (not shown) (Fig. 3H–K). In mesencephalic rostral portions and in mesencephalic–diencephalic transition Mva-ir was observed in large neurones of the fasciculus longitudinalis medialis, hypothalamic magnocellular nuclei (not shown), magnocellular pretectal superficial nuclei, in preglomerular complex cells and in neurones of the mesencephalic nucleus of trigeminal nerve (not shown) (Fig. 3E–G).

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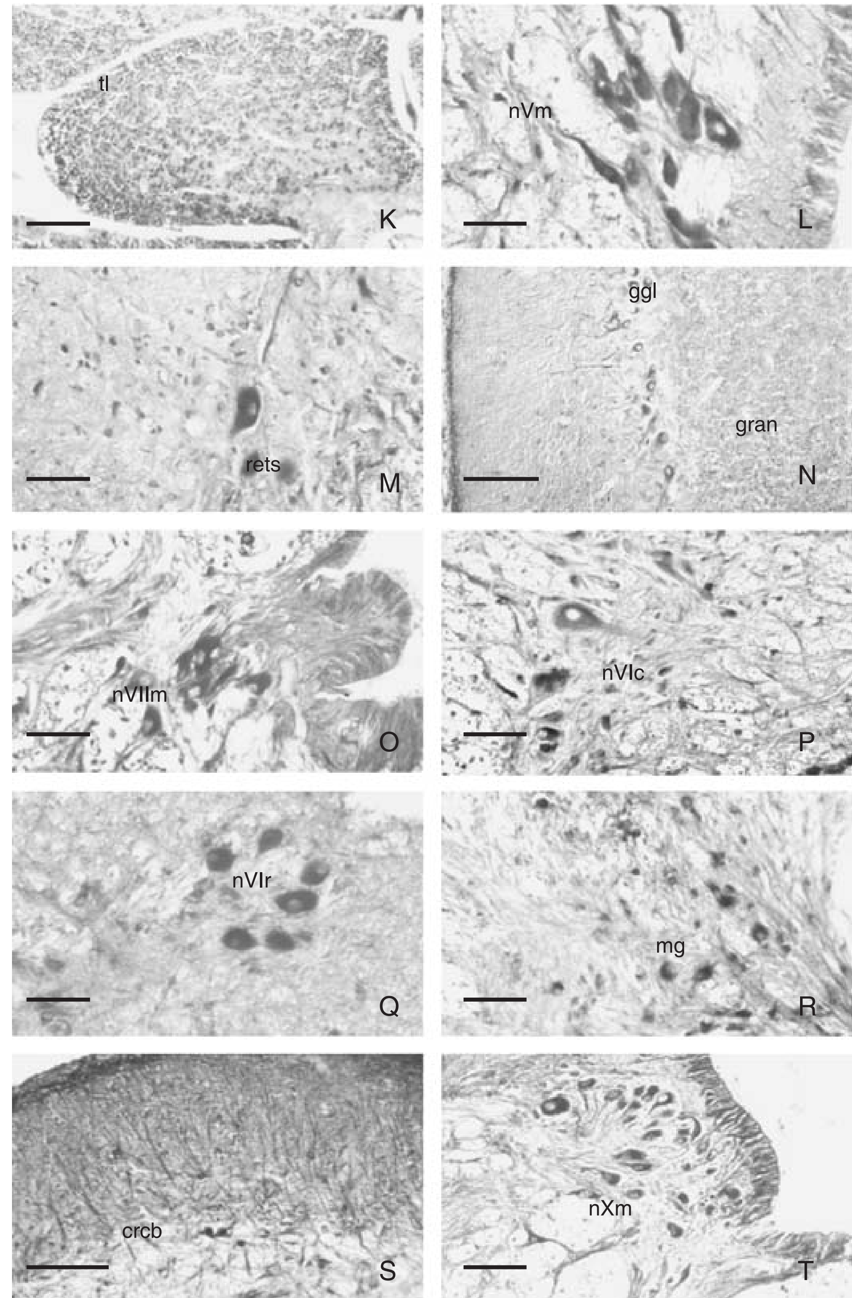

Fig. 3—**K**. Detail of the longitudinal torus (tl). —**L**–**N**. Transverse section of the rhombencephalon rostralmost portion, —**L**. Detail of the trigeminal nerve nucleus (nVm). ——**M**. Detail of the neurones in the superior portion of reticular formation (rets). —**N**. Transverse section of the cerebellum showing the molecular granular (gran) and ganglionar (ggl) layers; magnification × 100. —**O**, **P**. Transverse section of the rhombencephalon at level of cranial nerve VII. —**O**. Detail of the facial nerve motor nucleus (nVIIm). —**P**. Detail of the abducen nerve nucleus pars caudalis (nVIc). —**Q**–**X**. Transverse section of the rostral portion of the rhombencephalon. —**Q**. Detail of the abducen nerve nucleus pars rostralis (nVIr). —**R**. Magnocellular octaval nucleus (mg). —**S**. Detail of the cerebellar crest (crcb). —**T**. Vagal motor nucleus neurones (nXm).

Hindbrain. In the rhombencephalon at the level of cranial nerve VI, Mva-ir cells were observed in the abducen nucleus pars rostralis (Fig. 3Q), medial octavolateral nucleus, magnocellular octaval nucleus (Fig. 3R) and in neurones of the cerebellar crest (Fig. 3S). At the level of cranial nerve V, Mva-ir cells were observed in the trigeminal nerve nucleus and in the superior portion of the reticular formation (Fig. 3L,M). Other regions, such as the facial nerve motor nucleus (Fig. 3O), the abducen nucleus pars caudalis (Fig. 3P), the vagus nerve motor nucleus (Fig. 3T) and the glossopharyngeal nerve motor nucleus, (Fig. 3V) showed immunoreactivity for myosin Va. In the cerebellar region, Mva-ir cells and neurites were observed in the granular and ganglionic layers (Fig. 3N).

Spinal cord. In the dorsal horn, Mva-ir cells were observed in the fusiform and intermediate-sized neurones (Fig. 3X) and in the Cajal commissural nucleus cells at the obex region level (Fig. 3Y). In the ventral horn, the motoneurones showed myosin Va immunoreactivity (Fig. 3Z).

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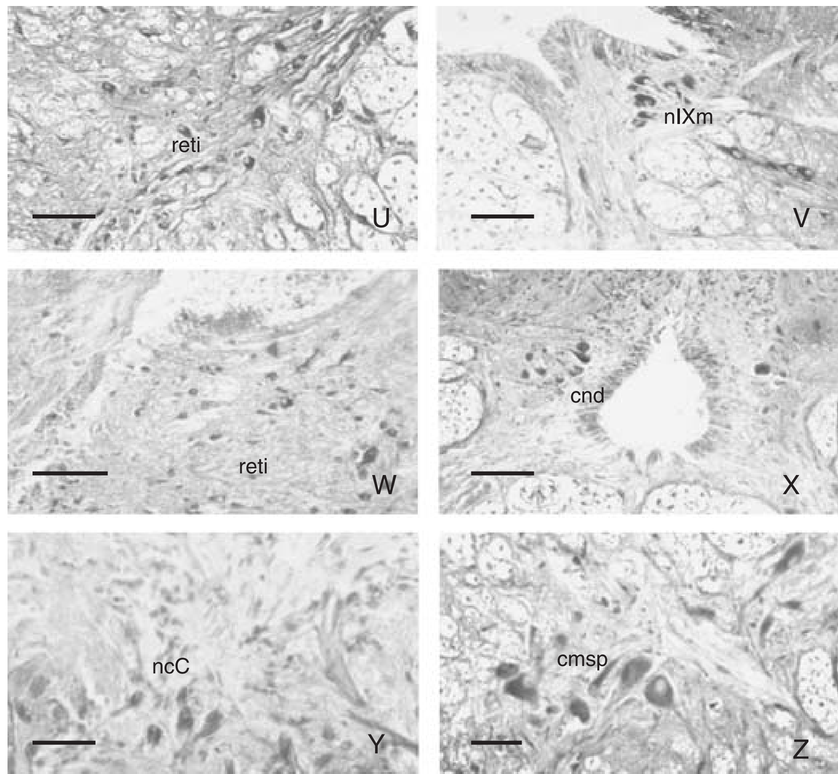

Fig. 3—**U**. Reticular formation neurones (reti). —**V.** Detail of the glossopharyngeal nerve motor nucleus (nIXm). —**W**. Detail of the reticular formation (reti) at level of cranial nerve IX motor nucleus, × 160. —**X**–**Z**. Transverse section of the spinal cord. —**X**. Detail of the spinal dorsal horn neurones (cnd). —**Y**. Detail of the Cajal commissural nucleus (ncC). —**Z**. The spinal ventral horn neurones (cmsp). Scale bars: (C) = 200 μm; (A, I, J, K) = 100 μm; (B, D, F, G, H, N, T, U, V, X, W) = 50 μm; (E, L, M, O, P, Q, R, S, Y, Z) = 25 μm.

Discussion

This work reports the tissue distribution of myosin Va in the central nervous system of rainbow trout. The presence of myosin V class members has been shown in several organisms through the phylogenetic scale (Reck-Peterson *et al.* 2000). However, this is the first description in fish.

Although there is evidence that supports the notion that forms of myosin V are molecular motors and have a specific role in organelle transport and location, there is still a great deal of uncertainty about the exact functions in cellular processes. Specifically, myosin Va is highly expressed in melanocytes and neurones and is clearly associated with the transport of melanosomes (Wu *et al.* 1998; Rogers *et al.* 1999) and membrane receptors in neurones (Takagishi *et al.* 1996). Furthermore, its association with the synaptic vesicles and associated proteins (Prekeris and Terrian 1997; Costa *et al.* 1999; Ohyama *et al.* 2001), localization in brain nerve terminals (Mani *et al.* 1994; Prekeris and Terrian 1997), and its involvement with organelle movement in axons (Evans *et al.* 1998; Tab *et al.* 1998) indicated that myosin Va has a specific role in organelle transport during synaptic function.

The immunohistochemical studies show that myosin Va is broadly distributed in the vertebrate nervous system. In mice, intense labelling was reported in specific cell types such as Purkinje cells of the cerebellum (Espindola *et al.* 1992; Espreafico *et al.* 1992), in cells of the retina (Schlamp and Williams 1996) and in a subset of neurones in the myenteric ganglia (Drengk *et al.* 2000). Tilelli *et al.* (2003) produced a comprehensive map of myosin Va distribution in the central nervous system of the rat showing several neuronal cells and immunoreactive nuclei. Our results show that myosin Va is amply expressed throughout the central nervous system of rainbow trout, which indicates a general role in neurone function. The populations that express myosin Va in trout constitute a very diverse group that do not seem to have many specific similarities in neurotransmitters used, cellular size or length of their processes. This fact, together with its broad distribution, is indicative of myosin Va participation in basic neuronal activities. It is not present in all neuronal populations or similarly distributed in neurones. Thus, alternative mechanisms should be operating in different cells by other myosins (Zhao *et al.* 1996). This distribution pattern is in accordance with what was evidenced in rats and mice, and it indicates phylogenetic maintenance of the main functions of myosin Va, such as axoplasmatic transport. The similarities are only related to broad distribution, intensity variation and diversity of neuronal types. Regional patterns are difficult to identify because of differences in the cytoarchitecture of the nervous system. Furthermore, in these extensive pieces of work there is a great variation in the examined and documented areas. For example, Tilelli *et al.* (2003) does not show analyses in the rat spinal cord as we do in trout. This portion of the vertebrate nervous system is a useful model for

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comparative studies because it shows more similarities than differences. Some regional similarities for myosin Va positivity can be observed in the cerebellum (Purkinjie cells and granular layer) and mesencephalon (hypothalamic magnocellular nuclei, mesencephalic nucleus of trigeminal nerve). Comparative studies focusing on restricted areas of the nervous system need to be performed to clarify adaptations and specializations in teleosts and amniotes. Furthermore, this result is in agreement with a previous report that indicated that myosin Va was approximately as abundant as conventional myosin II and kinesin (Cheney *et al*. 1993).

Although there is a broad distribution of the myosin Va in the trout brain, the intensity of immunoreactivity and the number of immunoreactive cells differ from region to region. For example, all the nuclei of cranial nerves show strong immunostaining for myosin Va, to the detriment of the weak immunostaining of cells in the diencephalic and telencephalic regions. This same pattern is observed as the number of reactive cells.

In conclusion, this work describes for the first time the presence of the myosin V class in fish and it supports the idea of phylogeneticaly conserved functions of brain myosin V as well as contributing to the basic understanding of the role of myosin V in brain function, particularly related to its nature as a molecular motor.

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