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Raf Serine/Threonine Protein Kinases: Immunohistochemical Localization in the Mammalian Nervous System

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1. Introduction

The Raf protein kinases are members of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway, which links the activation of cell surface receptors to intracellular cytoplasmic and nuclear molecular targets. Raf kinases relay signals from growth factor receptor-coupled GTPases of the Ras family to the MAPK/ERK kinase (MEK). This pathway is very important in developmental processes, cell survival and proliferation (Zebisch & Troppmair, 2006). Three different Raf isoenzymes exist in mammals: C-Raf, B-Raf and A-Raf. They originate from 3 independent genes (for review, see Matallanas et al., 2011). The Raf genes are protooncogenes; they are the cellular counterparts of the v-Raf oncogene, originally discovered in murine sarcoma cell lines (Rapp et al. 1988). The Raf genes are ubiquitously present in different tissues, showing a relatively segregated expression pattern: B-Raf gene is expressed mainly in the brain and testes, C-Raf gene is ubiquitous, although the degree of expression is uneven, A-Raf gene is expressed in gonads, kidney, spleen and bone mainly, as detected in mice (Storm et al., 1990). Accordingly, the Raf proteins are present in three isoforms: the A-Raf, B-Raf and C-Raf are protein kinase isozymes, showing similar molecular structure (Zebisch & Troppmair, 2006). The Raf proteins consist of three conserved regions (CR1, CR2, CR3), which display different functions: CR1 contains the Ras-binding region, which is responsible for the interaction with Ras and membrane phospholipids; CR2 contains phosphorylation site and CR3 is the kinase region of the molecule (Heidecker et al., 1991; Zebisch and Troppmair, 2006). The inactive Raf molecule is in a closed conformation: the N-terminal and C-terminal regions are above each other (Matallanas et al., 2011). When this molecule opens, activation and dimerization can happen (Heidecker et al., 1991; Matallanas et al., 2011; Zebisch & Troppmair, 2006). Activation-inactivation are regulated by phosphorylation-dephosphorylation of the molecule (Matallanas et al., 2011). Activation also needs the recruitment of Raf to the cell membrane (Leervers et al., 1994). Many details of the Raf kinase activation steps have been described in the last twenty years (for review, see Matallanas et al., 2011). The regulation of Raf signaling by intracellular proteins is complicated: (1) because of the membrane recruitment of Raf during activation, scaffolding proteins exert regulatory role on the Ras-Raf-MEK pathway (Matallanas et al., 2011). (2) Recently discovered Raf kinase inhibitor protein (RKIP)

negatively modulates Raf kinase, being important mainly in cancer cells (Klysik et al., 2008; Matallanas et al., 2011). Over the years, B-Raf has been increasingly associated with cancer development (Niault & Baccarini, 2010). Mutations of B-Raf proved to be important in malignant melanoma, thyroid carcinomas and colorectal tumors (Niault & Baccarini, 2010). This underlines the utmost importance of B-Raf in mitogenic signal transduction.

Experimental data suggest, that Raf kinases are activated by growth factor- and cytokine receptors. Platelet-derived growth factor (PDGF; Morrison et al., 1989), epidermal growth factor (EGF; App et al., 1991), nerve growth factor (NGF; Oshima et al., 1991), insulin (Blackshear et al., 1990), interleukins (Carrol et al., 1990), neuronal angiotensin receptor (Yang et al., 1997) and vascular endothelial growth factor (VEGF; Lu et al., 2011) act through the Raf-MEK signaling pathway. Raf mediated signals are able to move in the cytoplasm; the Golgi-apparatus, the mitochondria and the cell nucleus are targets of Raf-mediated phosphorylation signals (Matallanas et al., 2011; Zebisch & Troppmair, 2006). The question arises, if these organelles contain Raf kinase, or Raf kinase translocates to them during the signalling process (Mor & Philips, 2006).

Immunohistochemical studies have revealed that B-raf and C-Raf are widely distributed in central nervous system (CNS) areas including the hippocampus, neocortex and spinal cord (Mihály et al. 1991, 1993; Mihály & Rapp 1993). They are present not only in neurons but also in astrocytes (Mihály & Rapp 1994). Although most CNS neurons express Raf immunoreactivity, the possibility remains that Raf expression varies according to the type of growth factor receptor that a neuron expresses (Mihály & Endrész, 2000). Our ultrastructural immunohistochemical studies proved that Raf protein-like immunoreactivity was localized primarily in postsynaptic densities, dendritic spines, dendrites and soma of the neurons (Mihály et al., 1991). On the basis of these observations the possibility arose, that Raf kinases participated in some receptor-mediated postsynaptic phenomena which might initiate long-term changes in postsynaptic neurons (Mihály et al., 1990). The present review collects the relevant localization data of Raf protein kinases in CNS structures. The localization of the Raf proteins will be discussed in light of some plasticity experiments (Mihály et al., 1990; 1991; 1996).

2. Detection of Raf protein kinases in the brain with Western blotting

The first literature data about the presence of Raf protein kinase in the mammalian brain are those of Mihály et al. (1991). In these studies we used the polyclonal v-Raf antiserum which was produced in the laboratory of Dr. Ulf Rapp. The serum was raised in rabbit, against a large (30 kDa) C-terminal Raf protein, expressed in *E. coli*, by inserting the v-Raf oncogene into the bacterial DNA (Kolch et al., 1988). Adult Wistar rats were deeply anesthetized with halothane, decapitated and the brains were homogenized, lysed and immunoprecipitated. The precipitates were subjected to gel electrophoresis, then proteins were transferred to nitrocellulose membranes and incubated with the polyclonal v-Raf antiserum (Mihály et al., 1991). One conspicuous 95 kDa protein band was detected with this method (Mihály et al., 1991). The same antibody was used on isolated subcellular fractions of the rat brain, where the cytosolic- and microsome fractions displayed positive signals, again at 95 kDa (Mihály et al., 1996). Efforts aiming the detection of C-Raf and B-Raf separately, were also successful (Morice et al., 1999): Western blotting revealed the presence of C-Raf and B-Raf proteins in different areas of the rat brain (Morice et al., 1999). Immunoblotting of guinea pig brain

homogenates also resulted in positive signals at 95 kDa with the aforementioned polyclonal anti-v-Raf serum (Mihály & Endrész, 2000). Several years later, using specific, polyclonal B-raf antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), we detected the 95 kDa protein again in rat brain homogenates (Mihály et al., 2007). Therefore, we conclude, that brain B-Raf kinase is ubiquitous and detectable through Western blotting in the rodent brain, as a 95 kDa protein (Fig. 1).

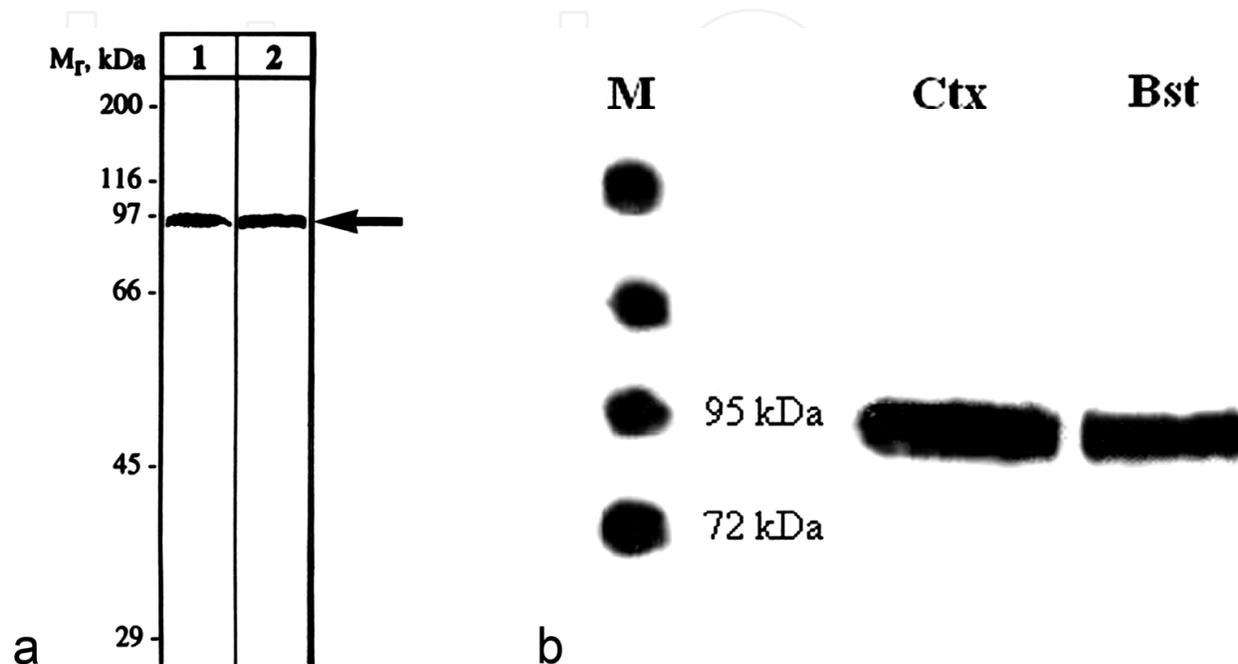


Fig. 1. Rat brain Western blots made with v-Raf-specific (a) and B-raf-specific (b) polyclonal antibodies. Molecular weight markers are on the left side (M_r , kDa; M). Fig. 1a: lane 1 represents the cytosolic fraction, lane 2 the microsome fraction (Mihály et al., 1996). Fig. 1b: „Ctx” represents the signal obtained from brain cortex homogenate; „Bst” represents the signal obtained from brain stem homogenates (Mihály et al., 2007). The two antibodies detect one 95 kDa protein, indicating the presence of B-raf kinase in the rat brain.

3. Light microscopic localization of Raf proteins in the central nervous system (CNS)

Light microscopic immunohistochemistry of brain areas from laboratory rats, laboratory guinea pigs and domestic cats have been performed with help of the polyclonal v-Raf antibodies (Mihály et al., 1991), polyclonal B-raf antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal B-Raf antibodies (produced against the last 12 amino acids of the C-terminal region of B-Raf) and polyclonal C-Raf antibodies (anti-SP 63 serum). The polyclonal anti-v-Raf-, anti-SP 63- and monoclonal anti-B-Raf antibodies were prepared in the laboratories of Dr. Ulf Rapp (National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD, USA; see descriptions of the antibodies in Mihály et al., 1993; 1996). Two human hippocampi were investigated, too. The human brain samples were obtained from autopsy in the Department of Pathology, Szeged University. The autopsy was performed 16 h after death. The hippocampal tissue was kept in fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer) for 12 days, then transverse plane frozen sections were made and stained with the monoclonal B-Raf antibody. Rats,

guinea pigs and cats were deeply anesthetized and perfused through the heart with fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The brains were sectioned with freezing microtome, cryostat and vibratome. The tissue sections were immunostained as glass-mounted- or free-floating sections. Immunofluorescence was performed on cryostat sections, mounted on glass slides. The slides were incubated with primary antibodies (anti-Raf serum), then with secondary antibodies conjugated with fluorescein isothiocyanate (FITC). In peroxidase-based techniques, free-floating sections were used, incubated with the primary antibody, then treated according to the avidin-biotin method, using the Vectastain ABC kits (Vector, Burlingame, CA, USA). Independent from the antibody and method used, the staining pattern of the different brain areas was consistent, showing neuronal and glial localization of Raf kinases. The differences between B-Raf and C-Raf localizations are indicated in the text. No differences were detected in the staining pattern comparing the different species (rat, cat, guinea pig, human). The descriptions below refer to the results obtained with polyclonal v-Raf antibodies, if not indicated otherwise. Staining obtained with v-Raf antibodies will be referred to as B-Raf-like staining, staining with anti-SP 63 will be described as C-Raf-like immunoreactivity. Controls of the immunohistochemical procedure included absorption controls performed with the recombinant v-Raf protein and the SP 63 peptide. One mg of the protein and 10 mg of the peptide were reconstituted in 1 ml of deionized water, mixed with the undiluted antibody (0.01 ml), and kept at room temperature overnight with slow agitation. The mixture was diluted to working concentration (1:500), centrifuged at 70,000xg for 20 min, and used for the immunohistochemical procedure (Mihály et al., 1993). No immunostaining was seen after the procedure (Mihály et al., 1993). Other controls included incubations without the primary anti-Raf sera: in these experiments the primary antibody was omitted, and the sections were incubated in normal horse serum, diluted to 1:100, and secondary antibodies as usual. In these sections, no specific immunostaining was detected (Fig. 6e).

3.1 Localization of B-Raf in the spinal cord and brain stem

Neuronal and glial localization of Raf-protein-like immunoreactivity (RPI) was encountered in the spinal cord of the laboratory rat and guinea pig (Mihály & Rapp, 1993; 1994). Neurons in laminae I, V, VI and IX displayed strong cytoplasmic staining. Glial cells in the white matter and grey substance were stained, too (Fig. 7). Axonal staining (e.g.: in the dorsal horn, where afferent axons terminate in large numbers) was not observed (Mihály et al., 1996). Different levels of the brain stem displayed similar staining pattern: cytoplasmic staining in large- and medium-sized nerve cells. The motor neurons of the cranial nerve nuclei were strongly stained. Scattered RPI was detected in sensory nuclei, such as the superior and medial vestibular nuclei and the ventral cochlear nucleus. The raphe nuclei of the pons and medulla also contained RPI (Mihály & Endrész, 2000; Mihály et al., 2007). Large neurons of the reticular formation and the inferior olive were containing strong RPI (Fig. 3). The neurons of the mesencephalic trigeminal nucleus were strongly stained (Mihály et al., 2007). On the other hand, the spinal trigeminal nucleus displayed only faint staining (except lamina I neurons, which were strongly stained; Mihály et al., 2007). The red nucleus displayed strong staining, whilst the substantia nigra cells contained less RPI (Fig. 3). The immunostaining was always localized in cell bodies, no axonal staining was observed in any of the brain stem areas. Detailed examination of the brain stem of the rat and the guinea pig did not reveal any species differences (Mihály & Endrész, 2000; Mihály et al., 2007).

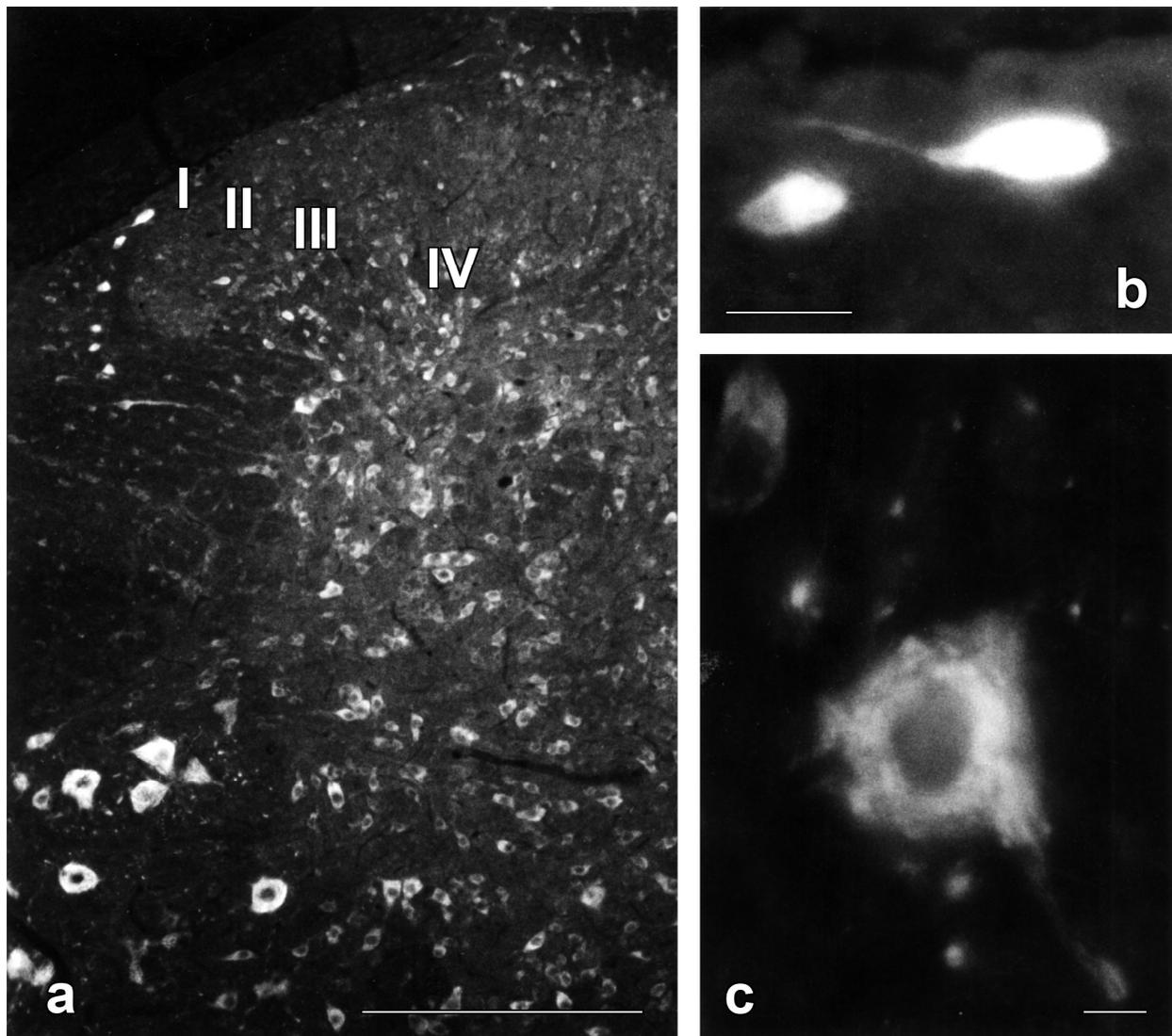


Fig. 2. B-Raf immunoreactivity in the spinal cord of the rat. The RPI is localized in cell bodies and proximal dendrites. No axonal staining is visible. Bar: 0.5 mm. Rexed laminae are indicated with roman numerals (a). Strong staining of lamina I neurons in the dorsal horn is conspicuous. Bar: 10 μ m (b). Large motor neurons of lamina IX are labelled strongly, too. Bar: 10 μ m (c). Immunolabeling was obtained with polyclonal anti-v-Raf serum.

3.2 Localization of B-Raf and C-Raf proteins in the cerebellum

The C-Raf and B-Raf proteins were detected in the vermis of the guinea pig cerebellum, using polyclonal anti-SP 63 (anti-C-Raf), and polyclonal anti-v-Raf (Mihály et al., 1993) antibodies. The B-Raf-like staining was very strong in neuronal cell bodies and glia-like cells. The staining was localized in neuronal cell bodies and dendrites. Dendritic staining was conspicuous in Purkinje cells, mainly in primary and secondary, large dendrites. Cell bodies of Purkinje cells were stained, too. Strong RPI was seen in the granular layer, where cell bodies of the granule cells and Golgi cells were immunoreactive. Small groups of glia cells stained in the white matter, and strong RPI was detected in the neurons of the fastigial nucleus (Fig. 4).

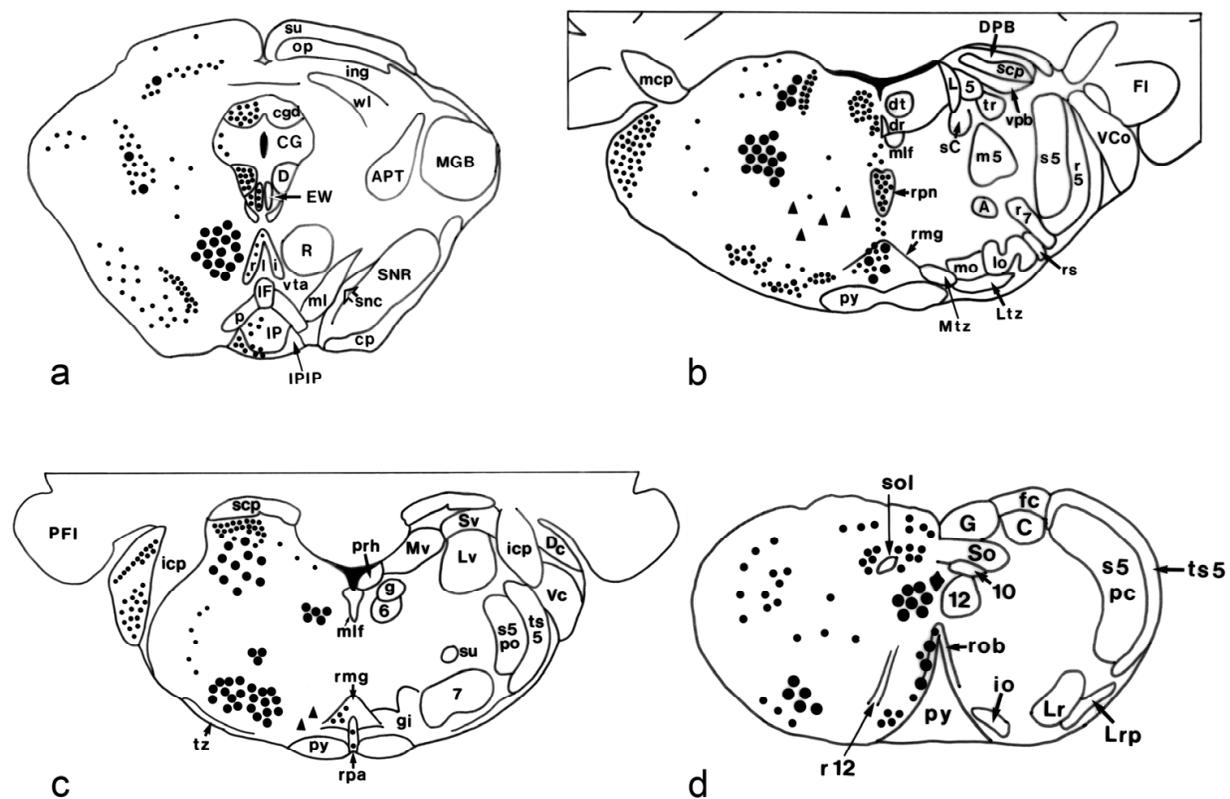


Fig. 3. Representative cross sections of the rat brain stem (Swanson, 1992), showing the cellular distribution of Raf-protein-like immunoreactivity. Large dots represent strongly stained neurons, small dots represent medium or faint staining. Triangles represent large nerve cells with outstanding RPI. The upper mesencephalon (a), the upper pons (b), the lower pons (c) and the lower medulla (d) are represented (Mihály et al., 2007).

Abbreviations: 5: mesencephalic nucleus of trigeminal nerve; m5: motor nucleus of trigeminal nerve; s5: principal sensory nucleus of trigeminal nerve; r5: radix of trigeminal nerve; s5po: spinal trigeminal nucleus pars oralis; s5pc: spinal trigeminal nucleus pars caudalis; ts5: spinal trigeminal tract; 6: abducens nucleus; 7: facial motor nucleus; g: genu of facial nerve; r7: radix of facial nerve; 10: dorsal nucleus of vagus nerve; 12: hypoglossal nucleus; r12: radix of hypoglossal nerve; D: Darkschewitsch nucleus; CG, cgd: periaqueductal gray; su, op, ing, wl: layers of the superior colliculus; R: red nucleus; SNR, snc: substantia nigra; EW: Edinger-Westphal nucleus; APT: anterior pretectal nucleus; MGB: medial geniculate body; IP, IPIP: interpeduncular nucleus; vta: ventral tegmental area; IF: interfascicular nucleus; p: paranigral nucleus; cp: cerebral peduncle; rli: raphe rostral linear nucleus; rmg: raphe magnus nucleus; rpn: raphe pontis nucleus; rpa: raphe pallidus nucleus; rob: raphe obscurus nucleus; dt: dorsal tegmental nucleus; mlf: medial longitudinal fasciculus; Lr, Lrp: lateral reticular nucleus; py: pyramidal tract; mo, lo: medial and lateral superior olivary nucleus; Mtz, Ltz: medial and lateral nucleus of the trapezoid body; Vco, Vc: ventral cochlear nucleus; Dc: dorsal cochlear nucleus; DPB, vpb: dorsal and ventral parabrachial nucleus; scp: superior cerebellar peduncle; mcp: middle peduncle; icp: inferior cerebellar peduncle; rs: rubrospinal tract; Mv, Lv, Sv: medial, lateral, superior vestibular nucleus; prh: prepositus hypoglossal nucleus; gi: gigantocellularis reticular nucleus; su: superior salivatory nucleus; sol: solitary tract; So: nucleus of the solitary tract; io: inferior olivary nucleus; Lr, Lrp: lateral reticular nucleus; G: gracile nucleus; C: cuneate nucleus; fc: fasciculus cuneatus; Fl: flocculus; PFI: paraflocculus.

The C-Raf staining was pale, displaying Purkinje cells and their large (primary and secondary) dendrites. Other neuronal elements (e.g.: granule cells, basket cells, Golgi cells) did not stain. C-Raf-like staining was seen in the nerve cells of the fastigial nucleus, and faint labeling was detected in some glia-like cells in white matter (Fig. 5).

3.3 Localization of B-Raf and C-Raf proteins in the cerebral cortex

RPI has been detected with polyclonal anti-v-Raf antibody and polyclonal anti-SP 63 antiserum. The latter is specific for C-Raf (Schultz et al., 1985; 1988; Morice et al., 1999). The RPI was detected in the neocortex (Fig. 6), cingular, pyriform, perirhinal, entorhinal areas and in the hippocampus (Mihály et al., 1993). RPI was localized in neurons and glia-like cells. No attempt was made to identify the cell types with double immunostainings. However, on the basis of the shape of the RPI-containing cells, we state that pyramidal and non-pyramidal cells of the Ammon's horn, granule cells of the dentate fascia and multipolar cells of the hilum of the dentate fascia were strongly stained (Mihály et al., 1993). Similar localizations were detected with the two antibodies, although C-Raf-like staining was faint compared to the B-Raf-like immunoreactivity. The intensity differences of the staining obtained with serial dilutions of the antibodies indicated that the cytoplasmic concentration of B-Raf kinase was much higher than that of the C-Raf in every region of the cerebral cortex. Detailed analysis of C-Raf-like staining and localization was not performed (Mihály et al., 1993). The brains of three domestic cats were also studied, using vibratome sections of the motor neocortex (sigmoid gyrus). Similarly to rats and guinea pigs, RPI has been detected in neuronal and glial cells bodies. Layer V pyramidal cells were stained in their cell bodies and proximal, large dendrites (Fig. 6). Scattered, small, glia-like cells were labeled, too. The human hippocampus displayed faint immunostaining with the B-Raf antiserum. Neuronal cell bodies were stained, which were similar to pyramidal cells, located in the stratum pyramidale of CA1 (Fig. 6).

3.4 Localization of Raf proteins in glia cells

B-Raf immunostaining was observed in small, glia-like cells, measuring 8-12 μm , in the cerebral cortex (gray and white matter), cerebellum (mentioned above), brain stem and spinal cord. Glial cells in the grey substance and in the white matter were regularly seen (Fig. 4; Fig. 7). Marginal astrocytes contained RPI in the spinal cord. Radial glia processes were stained in the cerebellar cortex (Fig. 4). No double immunolabelling was attempted in order to identify the cells. RPI was localized in the cytoplasm of the cell body and the processes (Fig. 7). No systematic study was made for the exploration of RPI in other glial cell types (oligodendroglia, microglia).

4. Electron microscopic localization of RPI in neurons

Ultrastructural immunohistochemistry was performed on vibratome sections of rat and cat cerebral cortex, using peroxidase-based preembedding methods (Mihály et al., 1991; Mihály & Rapp, 1994). These reports were the first in the literature, describing the electron microscopic localization of Raf kinases in the brain (Mihály et al., 1991; Mihály & Rapp, 1994). Comparison of the Raf kinase localization in a rodent and a carnivore, did not reveal differences: the localization pattern of RPI was consistent and similar in the two species.

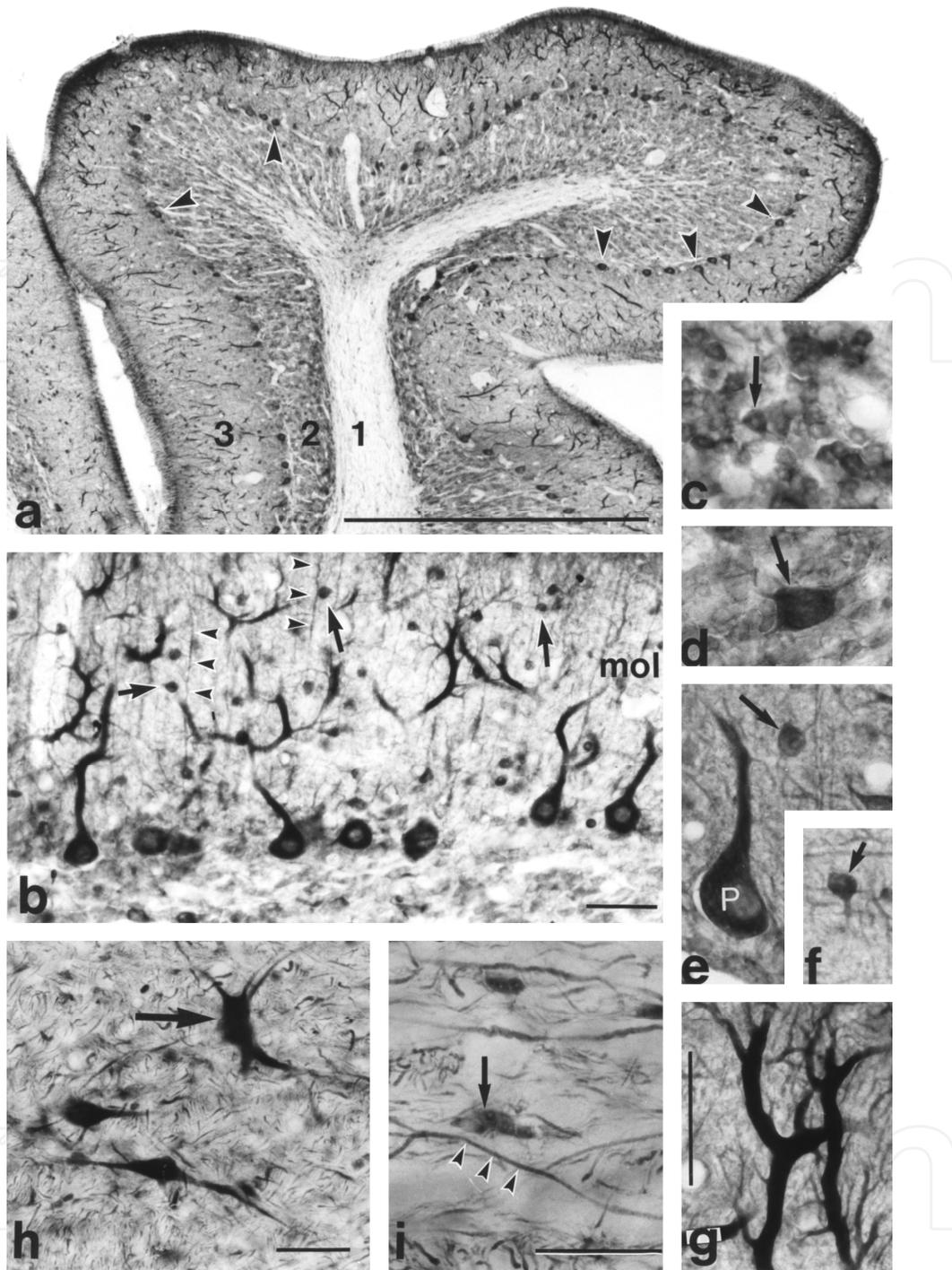


Fig. 4. Cerebellar immunostaining with polyclonal v-Raf serum in the guinea pig. Fig. 4a: Immunostaining depicts the layers of the cerebellar cortex: (1) white matter; (2) granular layer; (3) molecular layer; arrowheads: Purkinje cell layer; bar: 0.5 mm. Fig. 4b: the Purkinje cell RPI was detected not only in cell bodies, but also in dendrites (Fig. 4g). Arrowheads: radial glia processes; arrows: small neurons in the molecular layer (mol). Bar: 50 μm in Fig. 4b; 5 μm in Fig. 4g. The RPI in granule cells (Fig. 4c), Golgi cells (Fig. 4d), Purkinje cells (Fig. 4e) and outer stellate cells (Fig. 4f) are pointed by arrows. Bar as in Fig. 4g. Fig. 4h: the B-Raf staining of the neurons of the fastigial nucleus (arrow). Bar: 10 μm . Fig. 4i: white matter RPI apparently localized in glia cells (arrow). Arrowheads point to white matter axons containing RPI. Bar: 50 μm .

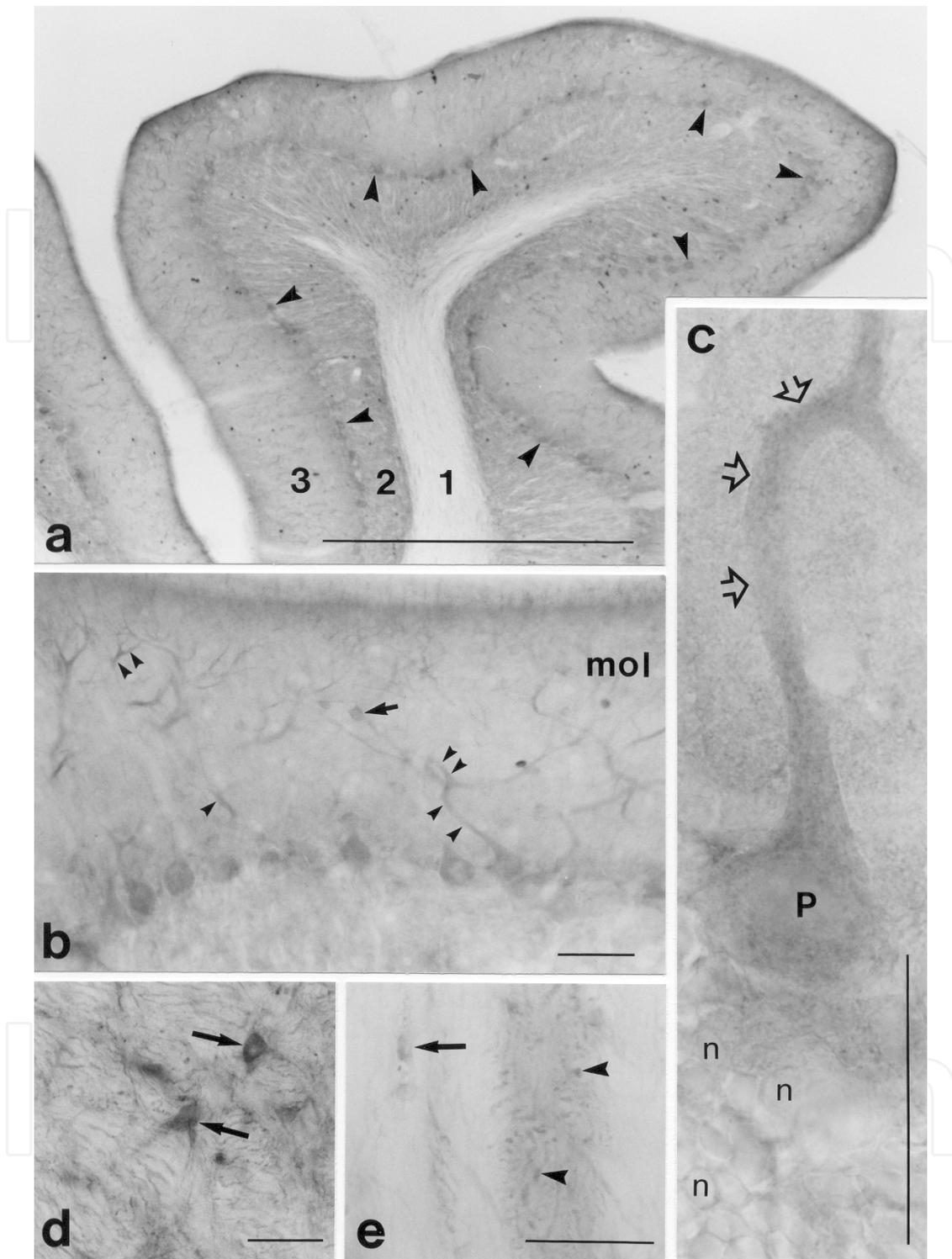


Fig. 5. Cerebellar immunostaining with anti-C-Raf serum in the guinea pig. Fig. 5a shows the overall weak staining (arrowheads: Purkinje cell layer; 1,2,3: as in Fig. 4a; bar: 0.5 mm). Fig. 5b: Purkinje cells contain C-Raf, which is localized in cell bodies and dendrites (arrowheads). Arrow points to outer stellate cell, which displays weak staining. Bar: 50 μ m. Fig. 5c: Purkinje cell (P) staining with high magnification. Arrows point to dendrites (n: unstained granule cells; bar: 50 μ m). Fig. 5d: neurons of the fastigial nucleus (arrows) containing C-Raf. Fig. 5e: white matter containing weakly stained glia cell (arrow) and weak axonal staining (arrowheads). Bars: 50 μ m.

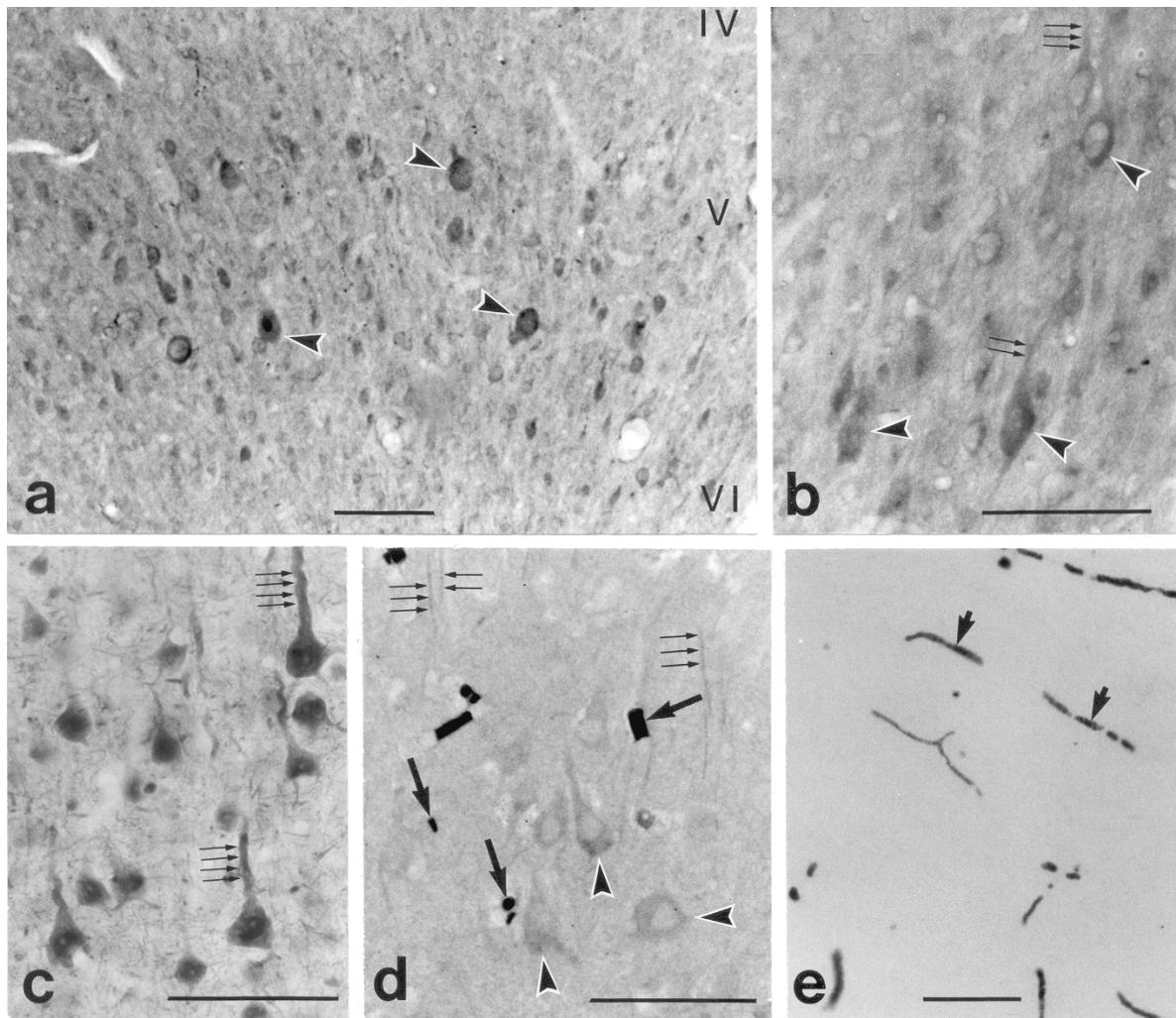


Fig. 6. B-Raf localization in the cat neocortex (a, b), rat neocortex (c) and human hippocampus (d, e). Bars: 50 μ m. Roman numerals show neocortex layers. Arrowheads point to large nerve cells with immunostaining. Thin arrows in (b), (c) and (d) point to immunostained apical dendrites. Thick arrows in (d) and (e) point to red blood cells remaining in capillaries. Fig. 6e: control section was incubated in normal horse serum, the anti-B-Raf serum was omitted.

Neurons contain strong Raf kinase immunoreactivity in the cytoplasm of the cell body and dendrites (Figs 8-11). Not every neuron did stain: in the granule cell layer of the dentate fascia, cca. 20% of the granule cells remained unstained. This feature was visible already under the light microscope, and was applicable to other cortical areas, as well (Mihály et al., 1993). As to the staining of the dendrites, very strong RPI was seen in dendritic spines (Figs 8-9; Fig. 11). The Raf-kinase-like staining was strong in postsynaptic densities, and in spine apparatuses (Fig. 9; Fig. 11; Mihály et al., 1991). Not only the spine apparatuses, but also dendritic subsynaptic cisternae contained RPI (Fig. 10d). In these membrane cisternal organelles the immunoprecipitate was localized between the membrane cisternae; the cavity of the cisternae did not contain RPI (Fig. 11). We did not observe immunostained axon terminals in the tissues studied so far. None of the synapsing axons terminating on dendrites, dendritic spines or neuron somas did contain Raf-kinase-like immunostaining (see Figs 8-11). There are no comprehensive electron microscopic data about the ultrastructural localization of C-Raf, so this issue requires and deserves further, extensive experimentation.

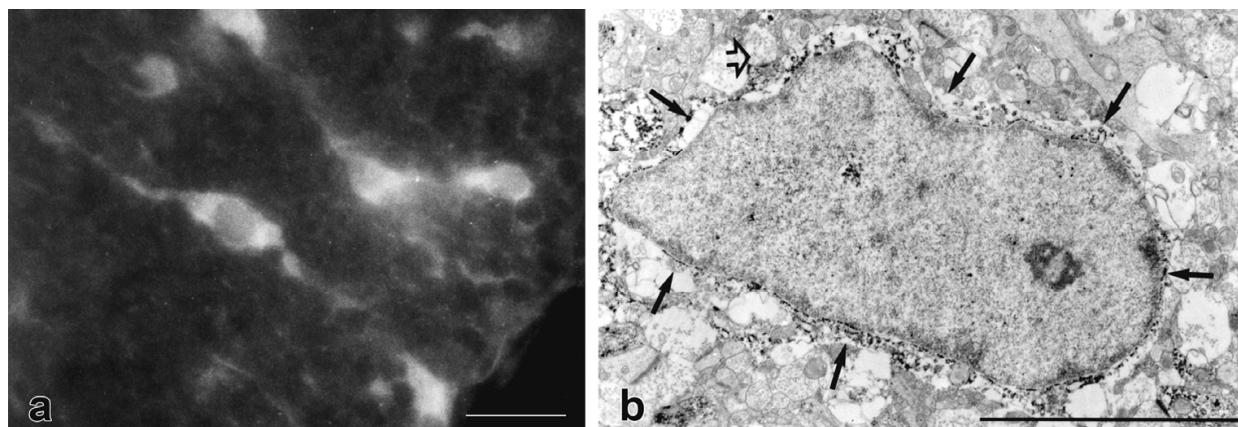


Fig. 7. Glial B-Raf kinase localization in rat spinal cord (a) and rat hippocampus (b). Fig. 7a displays RPI in astroglia-like cells of the white matter (bar: 10 μm). Fig. 7b shows the ultrastructural localization of B-Raf immunoreactivity in the cytoplasm of astrocyte-like cell of the rat hippocampus (solid arrows point to the contours of the astrocyte; empty arrow point to a synapse in the vicinity of the astrocyte; bar: 5 μm).

4.1 Electron microscopic localization of RPI in astrocytes

We observed several immunostained astrocytes in the cerebral cortex of rats (Fig. 7) and cats (Mihály & Rapp, 1994). The cytoplasmic staining of the cell body was conspicuous: very often in the vicinity of bundles of intermediate GFAP filaments (Mihály & Rapp, 1994). RPI was also observed in perivascular glial processes, and glial processes in the neuropil, around synapses (Mihály & Rapp, 1994). The RPI containing astrocytes were detected in the white matter, too – similarly to light microscopy observations. No RPI containing oligodendroglia cells were observed – in some sections we have seen RPI containing astrocytes together with unstained oligodendroglial cells (Mihály & Rapp, 1994). However, no systematic studies were performed to prove or disprove the lack of Raf proteins in oligodendroglia cells. The endothelial cells and pericytes were not labelled with any of the antibodies.

5. Comparison of B-Raf and C-Raf brain immunolocalization patterns

The localization of the two isozymes did not show substantial differences. The main observation in this respect was, that C-Raf kinase was expressed only in a few neurons and glia cells – compared to the expression of B-Raf. The staining was also weaker in most of the experiments. The C-Raf staining was specific, because the absorption controls did not show immunostaining (Mihály et al., 1993). The C-Raf was detected in the cerebellum and the cerebral cortex: these structures are compared in Table 1. B-Raf antibodies stained several neurons, irrespective of the size and type. However, staining was stronger in large cells (Figs 2-4). The B-Raf was also localized in astrocytes, as strong cytoplasmic staining. Under the electron microscope, the neuronal immunostaining was cytoplasmic, and exceptionally strong in dendritic spines, spine apparatuses and postsynaptic densities. Axon terminals did not stain. The C-Raf was localized in large neurons and their proximal dendritic branches. A few glia-like cells also displayed faint C-Raf-like staining (Fig. 5). We did not perform systematic electron microscopic studies for the localization of C-Raf kinase: only one series of experiments was done, in which we investigated the ultrastructural localization of C-Raf

in guinea pig hippocampal slices (Mihály et al., 1991). In this electron microscopic study we observed neuronal and dendritic localization of C-Raf, thus corroborating the light microscopy findings (Mihály et al., 1991).

Isozymes	Neuronal localization	Glial localization	Other (e.g.: endothelium)
B-Raf	++++	++++	None
C-Raf	+	+	None

Table 1. Comparison of B-Raf and C-Raf localization and expression in the cerebellum and cerebral cortex of the rat and the guinea pig. The staining intensities are characterized by (+) symbols; (++++) means strong immunostaining; (+) means weak, but consistent staining.

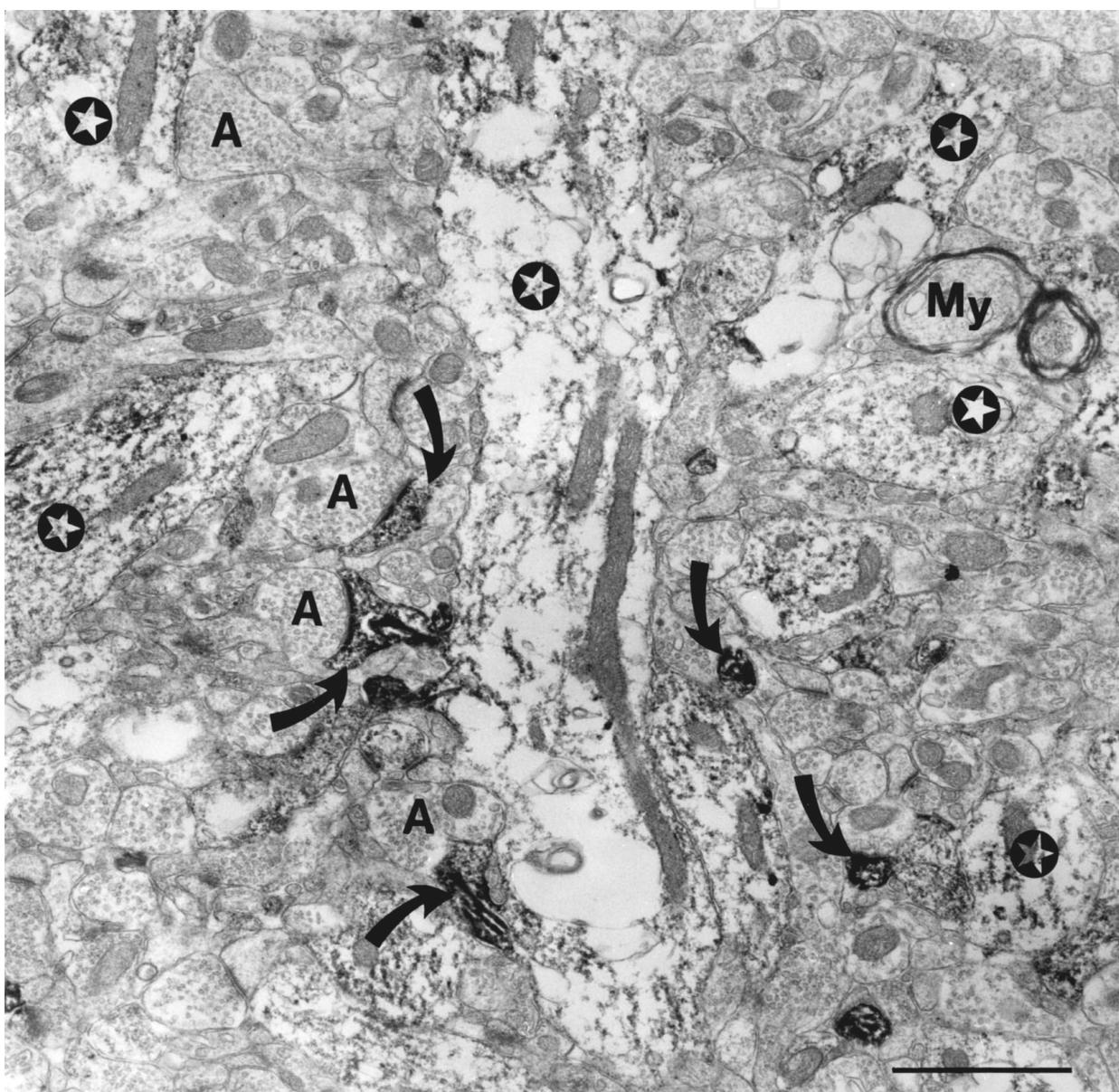


Fig. 8. B-Raf immunostaining in the rat cerebral cortex. Dendrites (stars) are labeled, axons (A) do not stain. The immunostaining of the dendritic spines (arrows) is extremely strong. My: myelinated axon. Bar: 1 μ m.

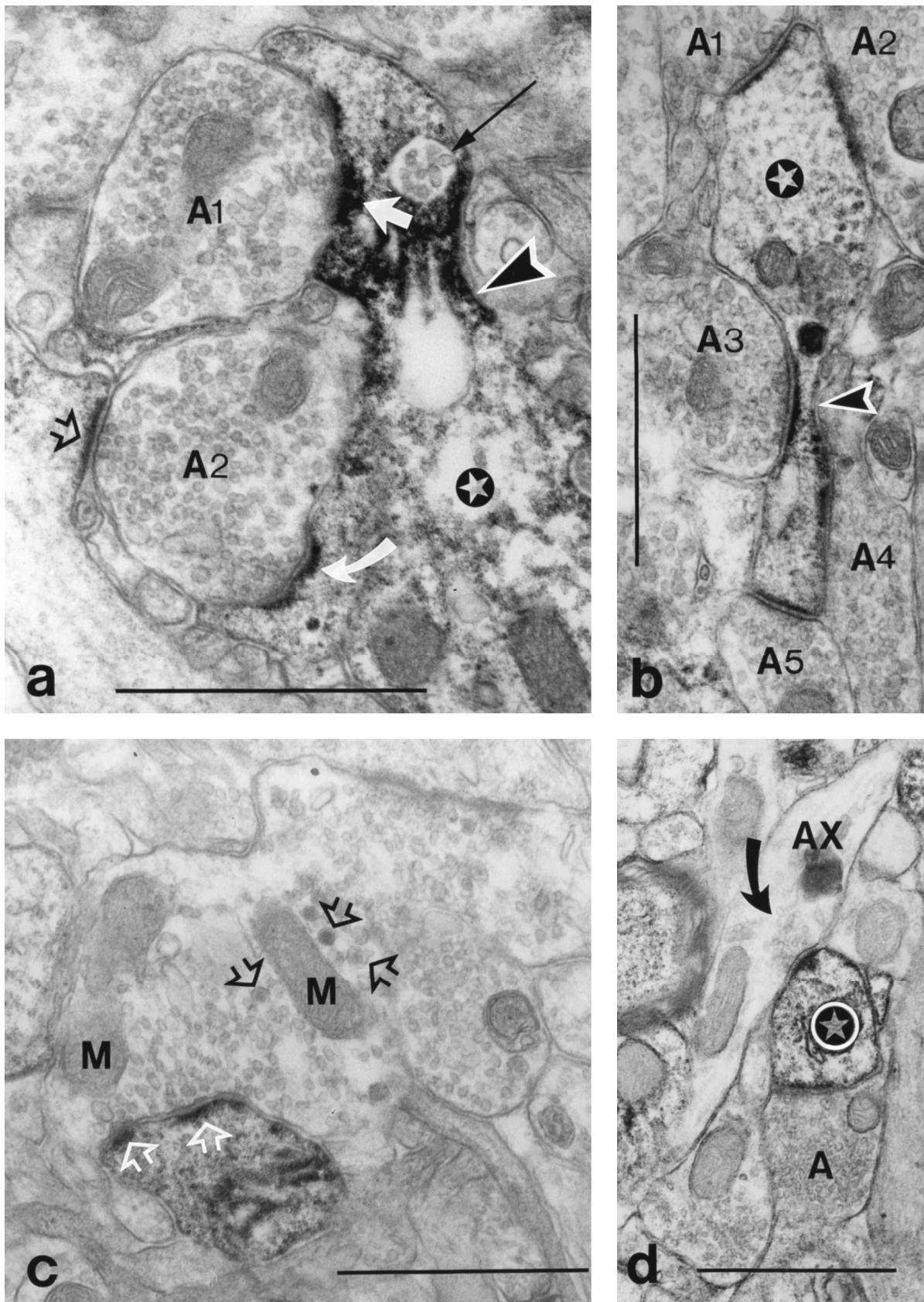


Fig. 9. a-d. Immunohistochemical localization of B-raf kinase in the hippocampus of the rat. In every case, postsynaptic dendrites and dendritic spines are immunoreactive. In (a) thick

arrows point to postsynaptic densities (thin arrow shows multivesicular body); arrowhead points to neck of spine, asterisk is in dendritic stem (A1, A2 are axon terminals); bar: 1 μ m. In (b) dendrite (asterisk) is seen from which spine originates (arrowhead); A1-A5 are presynaptic axons; bar: 1 μ m. In (c) mossy fiber terminal is unstained (M: mitochondria, black arrows point to dense core vesicles), postsynaptic dendrite (spine) is strongly immunoreactive (white arrows point to postsynaptic densities); bar: 1 μ m. In (d) unstained axons (AX, A) synapsing with strongly stained small dendrite (asterisk); bar: 1 μ m.

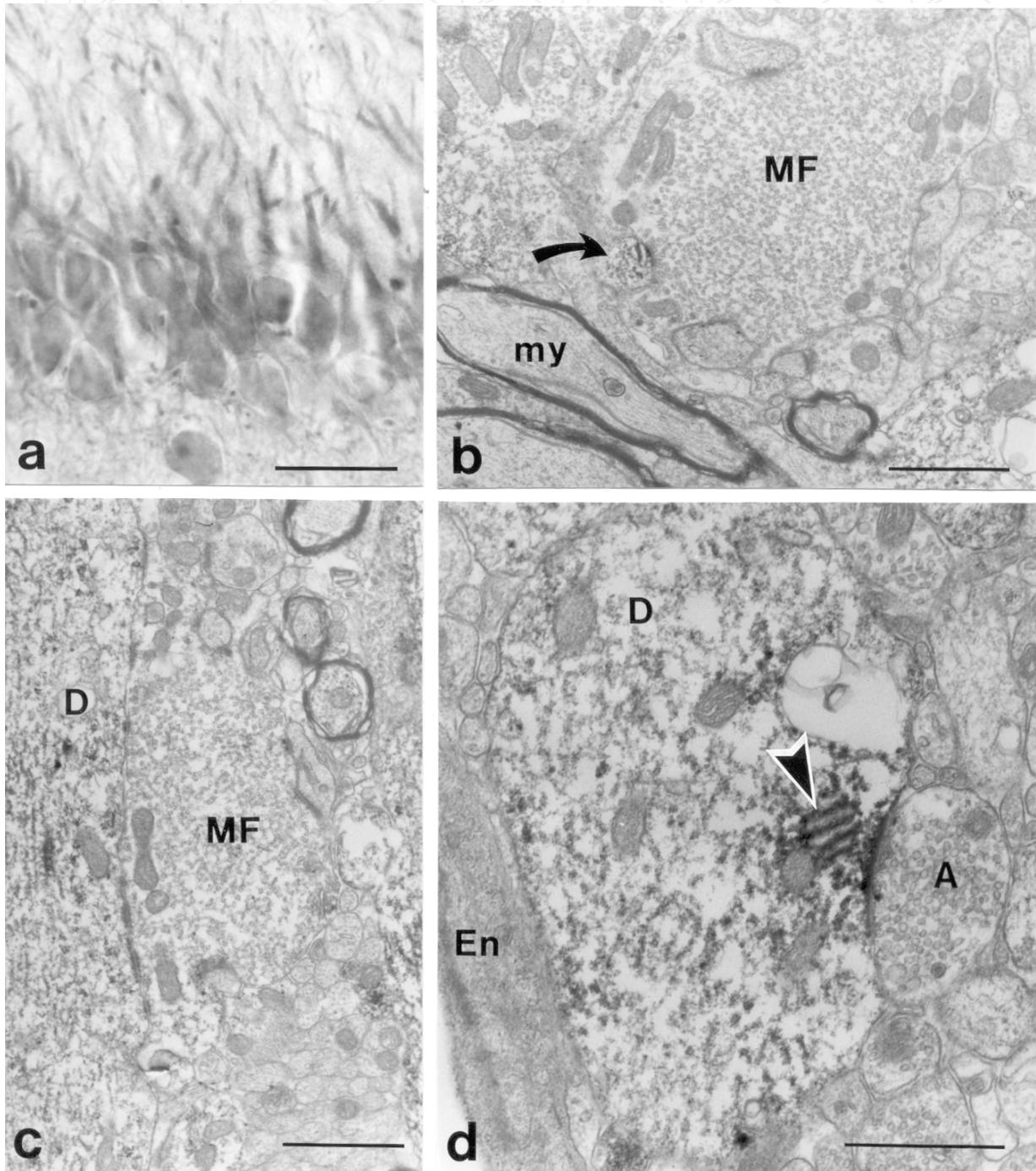


Fig. 10. a-d. Ultrastructural localization of RPI in the CA3 sector of the rat hippocampus. Fig. 9a displays the light microscopic appearance of the stratum pyramidale and stratum

lucidum after staining with polyclonal v-Raf antibodies. Cell bodies and apical dendrites are immunostained; mossy fibers do not stain (bar: 100 μm). Fig. 9b and c show two unstained mossy fiber ending (MF) synapsing with RPI containing dendrites (arrow and D). My: myelinated axon (unstained). Bars: 1 μm . Fig. 9d: large dendrite (D) containing RPI and immunostained subsynaptic cisternae (arrowhead). Synapsing axon (A) is unstained. En: unstained endothelial cell, bar: 1 μm .

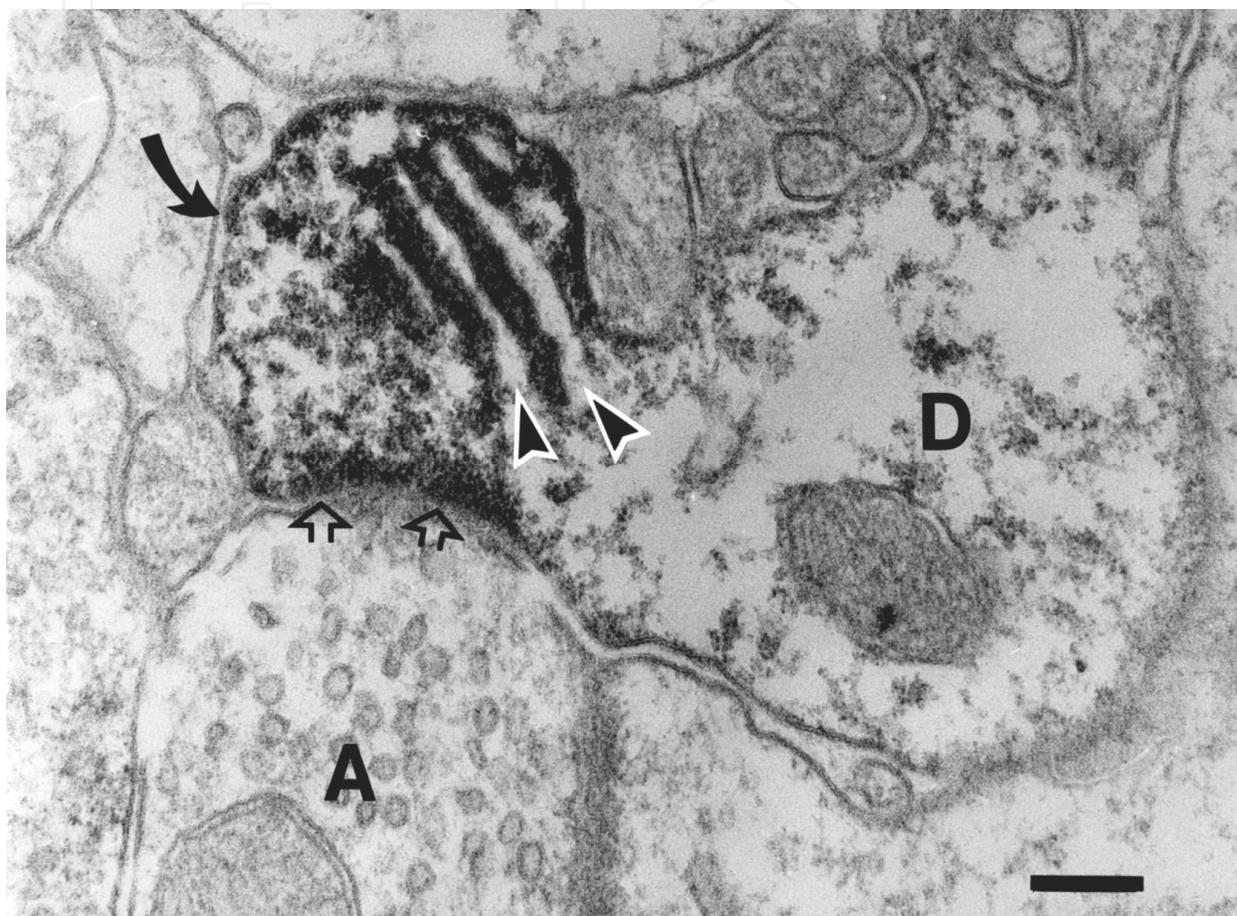


Fig. 11. Typical spine apparatus localization of B-Raf kinase in the rat cerebral cortex. The dendritic spine is pointed by the large solid arrow. The membranes of the spine apparatus are pointed by arrowheads. The postsynaptic density (empty arrows) is containing RPI, too. A: axon terminal; D: dendrite, containing RPI. Bar: 0.1 μm .

6. Discussion of the immunohistochemical findings

6.1 Neuronal B-Raf kinases

The immunolocalization of Raf kinases shows consistent patterns in different mammalian species (rat, guinea pig, cat and human). The main Raf molecule of the brain is certainly the B-Raf isozyme. The 95 kDa protein was detected in rat and guinea pig (Mihály et al., 1991; 1996; 2007; Mihály & Endrész, 2000). This observation conforms the literature data which give the same molecular weight in other experiments (Stephens et al., 1992; Dwivedi et al., 2006). Based on the specificity of the polyclonal v-Raf serum we refer to the immunostaining detected as to the B-Raf kinase immunostaining. This statement is strongly supported by the

repeated Western blot experiments, which gave very consistent results: the polyclonal v-Raf serum and the B-Raf antibodies resulted in the same 95 kDa band in brain homogenates (Mihály et al., 1991; 2007; Mihály & Endrész, 2000). We did not perform immunoblotting with the C-Raf antibody because the polyclonal anti-SP 63 serum was thoroughly investigated (Schultz et al., 1985; 1988; Morice et al., 1999). The synthetic SP 63 peptide is the C-terminal part of the C-Raf protein (Schultz et al., 1985; 1988). This 12 amino acids peptide was used for immunization in rabbits (Schultz et al., 1985; 1988). The polyclonal serum was affinity purified, tested with immunoprecipitation and was found to be specific for the murine and human C-Raf terminal sequences in cell cultures and in tissue sections (Schultz et al., 1985; 1988; Mihály et al., 1993). This immunostaining is therefore referred to as the immunolocalization of C-Raf kinase. Neuronal B-Raf and C-Raf localization at the light microscopic level was found to be very similar in other experiments, years later (Morice et al., 1999). We think therefore, that our descriptions concerning the brain localization of Raf kinases are the first in the literature, and give a precise picture of neuronal B-Raf kinases (Mihály et al., 1991; 1993).

The main features of neuronal B-Raf kinase localization are as follows:

1. Cytoplasmic localization in the cell body was a general feature. This reflects the existence of a standard pool of the B-Raf kinase in the neuron. In case of growth factor signals, this cytoplasmic kinase pool can be mobilized quickly, in form of recruitment to the cell membranes or to the nucleus, if necessary.
2. Localization in dendrites was another characteristic feature of neuronal B-Raf. Dendritic staining was extremely strong in dendritic spines and in postsynaptic densities, meaning that a certain amount of B-Raf was already attached to the membrane, in proximity of the synaptic receptors. This localization suggested a very effective signal transduction process, mediated by the B-Raf.

6.2 Neuronal signal transduction with B-Raf: experimental proofs

The first observations pointing to the possible significance of Raf kinases in learning and memory were those of Mihály et al., (1990). In these experiments long term potentiation (LTP) was induced in rats, then the animals were subjected to immunohistochemical detection of Raf kinase with the polyclonal v-Raf antibodies (Mihály et al., 1990). The RPI was investigated in the granule cell layer of the dentate gyrus, by counting the immunoreactive neuronal cell bodies (Mihály et al., 1990). The number of RPI-containing cell bodies increased significantly in successfully potentiated animals (Mihály et al., 1990). This observation has been discussed either as the post-LTP increase of Raf kinase expression in dentate granule cells; or as the translocation of the Raf kinase from the distal dendrites to the cell body, following LTP (Mihály et al., 1990). We were not able to decide between these two possibilities in these *in vivo* experiments (Mihály et al., 1990). Years later, with help of *in situ* hybridization, it turned out that 24 h following LTP, the B-Raf expression in the dentate gyrus increased significantly (Thomas et al., 1994). Recently, experiments on B-Raf knockout mice proved, that the B-Raf is absolutely necessary for learning and memory consolidation (Chen et al., 2006; Valluet et al., 2010). However, the detailed mechanisms of the participation of B-Raf in LTP are unknown.

6.3 The possible intracellular translocation of B-Raf in neurons

Since the early immunohistochemical experiments on LTP (Mihály et al., 1990), the possibility of intracellular Raf translocation was an important issue, because the translocation could be one intracellular mechanism for signal transduction. The translocation was observed *in vitro* (Leevers et al., 1994) and *in vivo* (Oláh et al., 1991) in different experimental conditions. The recruitment to the cell membrane is considered to be a necessary step of Raf activation (Matallanas et al., 2011). However, the translocation to the cell nucleus is difficult to explain, although the experimental facts seem to be firm (Oláh et al., 1991). Another, translocation-like phenomenon was observed in sensory ganglion cells (Mihály et al., 1996). Primary sensory neurons contain B-Raf in their cell body, in form of homogeneous immunoreactivity (Mihály et al., 1996). Two-eight days after the transection of the peripheral nerve, this cytoplasmic staining pattern displays translocation towards the periphery, and at 8 days, most of the RPI was localized beneath the cell membrane of the sensory ganglion cells (Mihály et al., 1996). The significance of this phenomenon is not known, possibly it is connected to the process of chromatolysis (Mihály et al., 1996). The phenomenon of the translocation has been proved in cell cultures (see Mor & Philips, 2006), but not *in vivo*, in plasticity or pathological conditions. Further experiments are needed for the understanding of the possible *in vivo* translocation processes.

6.4 Possible functions of C-Raf in the neuron

Although C-Raf was found to be ubiquitous (Storm et al., 1990; Matallanas et al., 2011), not every neuron displayed immunoreactivity in our studies. These findings are in conform with other immunohistochemical studies (Morice et al., 1999). On the other hand, it seems, that C-Raf expression can be induced by some (yet unknown) conditions. Hippocampal slices maintained *in vitro* do not express C-Raf at the beginning of the *in vitro* incubation. Incubation of the slice for 2-4 h in oxygenized environment induced the appearance of the C-Raf staining in the nerve cells (Mihály et al., 1991). Upregulation of C-Raf was observed in Alzheimer-brains, too (Mei et al., 2006). *In vitro* grown neurons were protected by C-Raf inhibitors against the toxicity of amyloid peptides (Echeverria et al., 2008). It seems therefore, that the upregulation of C-Raf in the cell causes cell damage and death: it can happen through the activation of apoptosis signals (Echeverria et al., 2008). No data exist about the causes of C-Raf upregulation: excess excitatory amino acids (excitotoxic effects), hypoxic conditions, and ageing may operate through unknown signaling pathways in brain cells.

7. Conclusions

Raf protein kinases are widespread in the mammalian brain and spinal cord. Raf protein kinases are activated by several cytokines and growth factors through their membrane receptors. We investigated the immunohistochemical localization of B-Raf kinase and C-Raf kinase in neurons and glial cells. The B-Raf kinase is localized in the cell body, in dendrites, dendritic spines, spine apparatuses, subsynaptic cisternae and postsynaptic densities. The localization does not depend on neuronal type, but the intensity of the immunostaining is greater in large neurons. Neuronal B-Raf was not found in presynaptic axons, suggesting, that in these cells B-Raf kinase is coupled to postsynaptic signaling pathways. The

similarities of the presence and localization of B-Raf in three mammalian orders (rodents, carnivores and hominids) suggest that this protein kinase has an important, and phylogenetically conserved function in normal, adult neurons. Data are available that learning and memory consolidation are processes in which B-Raf participates importantly. The C-Raf kinase has a similar CNS localization, but the amount of C-Raf in the cells is generally lower, than that of B-Raf. On the other hand, results from different experiments suggest, that the neuron adapts and alters the C-Raf expression, and thus regulates the amount of C-Raf present in the cytoplasm and in the membrane compartments. Therefore, we can state, that the regulation of the two isozymes (B-Raf and C-Raf) are different (although the downstream cellular events are similar):

1. CNS cells have a constant cytoplasmic pool of B-Raf, which translocates to different neighbouring organelles, in order to participate in signaling. The short-term regulation of B-Raf depends on membrane receptor stimulation.
2. CNS cells have another signaling molecule, the C-Raf, which displays low cytoplasmic concentration in normal neurons. However, the amount of cytoplasmic C-Raf is probably regulated by gene expression, the mechanisms of which are largely unknown. Therefore, C-Raf can be regulated twice: first by gene expression (upstream), second by membrane receptor activation (downstream). The significance and effects of this double regulation in the brain is not known: the effects probably manifest on the system level (e.g.: as alterations of the viability of neuronal networks).

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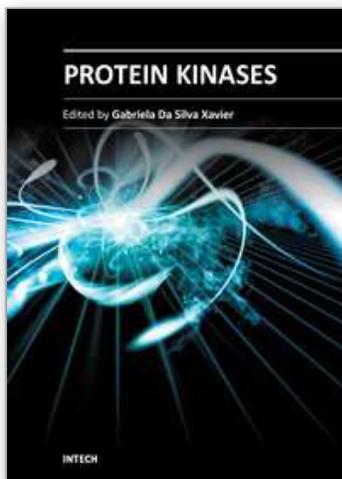
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Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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