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ARTICLE

Cellular distribution of B-raf protein kinase in the brainstem of the adult rat. A fluorescent immunohistochemical study**

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ABSTRACT The Raf-kinases can be activated by growth factors, cytokines and neurotransmitters. Previous studies in our laboratory proved the presence of Raf proteins in the neurons and astrocytes of the forebrain. The present study investigated the localization of B-Raf protein in the brain stem of the rat. Use was made of polyclonal anti-B-Raf serum which detected B-Raf molecules in our Western blotting experiments. Monoclonal tyrosine hydroxylase and dopamine-ß-hydroxylase antibodies were used to identify catecholaminergic brainstem nuclei and to study the co-localization of B-raf protein in them. We found the widespread distribution of B-raf-protein-like immunoreactivity in every segment of the brainstem. Mainly neurons were stained, but weakly immunoreactive glia-like cells were observed, too. Strongest staining was detected in the motor cranial nerve nuclei, the giant neurons of the reticular formation and in the raphe nuclei. The catecholaminergic structures displayed medium-to-weak B-raf-like immunoreactivity. The neurons of the locus ceruleus and the substantia nigra stood out with a conspicuous B-raf-like immunoreactivity. The B-raf protein was localized in the perinuclear cytoplasm and in thick, dendrite-like processes. The ubiquity of B-raf kinase suggests its important contribution to the regulation of the normal cell cycle in these neurons.
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Protooncogenes are important regulators of cell cycle (Rapp 1991). Their protein products display different localization patterns covering a range of intracellular sites and the outer cell membrane (Rapp 1991). Their main function is to receive and convert extracellular signals, transfer the signal-generated information to the cell nucleus and regulate or modify the expression of different genes (Mihály 1990). These processes are maintained and generated mainly through phosphorylation of specific membrane- and intracellular proteins. Several protein kinases participate in similar intracellular processes, which probably encodes the diversity of the extracellular signals (Zebisch and Troppmair 2006). The different localization of protein kinases could be important in decoding the different kinds of the extracellular information (Mihály et al. 1993). Some of these kinases are able of translocation which further contributes to the versatility of this molecular network (Mihály et al. 1996). The Raf protooncogene products are serine/threonine kinases – different isozymes which exist in different tissues and in different intracellular distribution (Strom et al. 1990; Morice et al. 1999). A very important feature of the cytoplasmic Raf kinases is their ability to translocate from the cytoplasm to the cell membrane (Mihály et al. 1996). The recruitment of Raf to the cell membrane requires the transformation of Ras into a Ras-GTP complex binding Raf molecules, which are then phosphorylated and activated (Kriegsheim et al. 2006). Activated Raf proteins are able to phosphorylate Jun proteins, MAP kinases and MAP kinase kinases (Zebisch and Troppmair 2006). The Northern blotting of mRNAs in different tissues suggested that two isoforms, Raf -1 and B-Raf are present in the brain (Strom et al. 1990; Mihály et al. 1993). B-Raf proteins were detected in rat and guinea pig brain with Western blotting and immunohistochemistry (Mihály et al. 1991, 1993, 1996, 2000; Morice et al. 1999). Mapping of the spinal cord and the cerebral cortex proved the widespread occurrence of Raf-protein-like immunoreactivity (RPI) in neurons and glial cells, mainly astrocytes (Mihály et al. 1993; Mihály and Rapp 1993, 1994; Morice et al. 1999). Ultrastructural immunohistochemistry with preembedding staining proved that the localization of RPI in neurons and astrocytes are mainly cytoplasmic, although postsynaptic densities and dendritic spine apparatuses proved to be immunoreactive, too (Mihály et al. 1991). This preferentially postsynaptic localization points to the importance of Raf kinase in synaptic transmission and in postsynaptic information processing.

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**Dedicated to the honour of Professor Bertalan Csillik, founder of neurohistochemistry in Szeged, on his 80th birthday.

Figure 1. Western blot of homogenates of the neocortex (Ctx) and the brainstem (Bst). Very strong signal is detectable at 95 kDa in cortex and in brainstem samples. M: molecular weight standards.

The present studies were undertaken in order to map the distribution of RPI in the brainstem of the rat. The neurons and their connections in the brainstem are functionally very different; some of the send their axons to the peripheral nervous system (PNS), some of them are of placodal origin (similar to sensory ganglion cells), some are interneurons, some of them have widespread connections to the central nervous system (CNS) – which mean that the chemical informations (trophic factors, transmitters) what they receive are very different, too. It would be interesting to analyze whether these different neurons use similar intracellular molecules in order to process this large diversity of chemical signals. Our presented results, and our previous observations (Mihály et al. 1990, 1991, 1996) suggest, that neurons possess very similar intracellular molecular devices – so probably not the molecules themselves, rather their different intracellular localization, metabolism are responsible for the adaptation of neurons to a changing chemical environment.

Materials and Methods

Monoclonal tyrosine hydroxylase (TH) antibodies were raised against purified TH, isolated from pheochromocytoma cell lines (Semenenko et al. 1986). The monoclonal dopamine-ß-hydroxylase (DBH) antibodies were raised against rat adrenal medulla DBH (Mazzoni et al. 1991). Both antibodies were raised after intrasplenic injection into mice, and fusion of splenocytes and myeloma cell line cells. The characterization of the antibodies has been described in detail (Semenenko et al. 1986; Mazzoni et al. 1991). The antibodies were donated by Professor John V. Priestley.

For the characterization of antibodies raised against B-raf, brain stem and cerebral cortex homogenates were isolated from Wistar rats (200-250 g). Brains were rapidly removed and chopped with a razor blade and homogenized at 4°C with glass-teflon homogenizer in 10 volumes of 0.3 M sucrose, 20 mM TrisHCl (pH 7.4) containing the following protease inhibitors: 2 mM DL-dithiothreitol (DTT), 1 mM pestatin A, 1 mM iodoacetamide, 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 1 mM 1,10-phenanthroline, 2 mM ethylenediamine tetraacetic acid (EDTA). The homogenate was centrifuged at 10,000 g_{max} for 15 min, and the supernatant was investigated. Protein concentrations were estimated with Qubit™ fluorometer, using protein assay kit (Quant-iT™; Invitrogene). The supernatant was processed for electrophoresis and immunoblotting with the polyclonal rabbit anti-B-raf antibody (1:200 dilution; Santa Cruz Biotechnology). The blots were detected with Super Signal West Pico Chemiluminescent kit (Pierce).

Six male Wistar rats (180-200 g body weight) were anesthetized with sodium pentobarbital (Sagatal, M&B; 6 mg per 100 g body weight), and perfused through the ascending aorta with an oxygenated vascular rinse (pH 7.4), followed by 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The brain and the cervical spinal cord were dissected en bloc, and postfixed overnight in the same fixative. The brainstem was cut in three pieces: above the level of the superior colliculi (upper midbrain), at the level of the inferior colliculi (lower midbrain and pons), and at the medulla, above the level of the vagal trigone (medulla oblongata). From each brainstem segment, transverse plane serial vibratome sections were cut at 40 µm and washed in three changes of phosphate buffered saline (PBS; pH 7.4), overnight. Sections were treated with 0.1% sodium borohydride (Sigma) for 15 min, then washed in PBS. The antibody coctails contained 0.1% TritonX-100 (Sigma). Free-floating sections were incubated in 10% normal goat serum for 1 h, then in primary antibody coctails: upper midbrain – anti-tyrosine hydroxylase (TH; 1:50) + anti-B-raf (1:500); lower midbrain-pons and medulla – anti-dopamine-ß-hydroxylase (DBH; 1:50 + anti-B-raf (1:500) for 24 h, at 4°C, with constant agitation. After extensive washes in PBS, sections were incubated in coctails of secondary antibodies: goat anti-rabbit (rhodamine – TRITC – conjugated; 1:100) and goat anti-mouse (fluorescein isothiocyanate – FITC – conjugated; 1:100). Incubation time: 10-12 h at 4°C. The sections were washed, coverslipped and investigated using a Leitz Laborlux epifluorescent microscope. The identification of the structures was helped by a series of touluidine-blue-stained sections, and the stereotaxic atlas (Swanson 1992). Control sections were incubated without the primary antibody – otherwise the same, as written above (Mihály et al. 1993).

The animal experiments conform with the Declaration of Helsinki and the Guide for the Care and Use of laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996).

Results

The specificity of the anti-B-raf antibody was tested by immunoblot analysis of the rat brainstem and cerebral cortex.

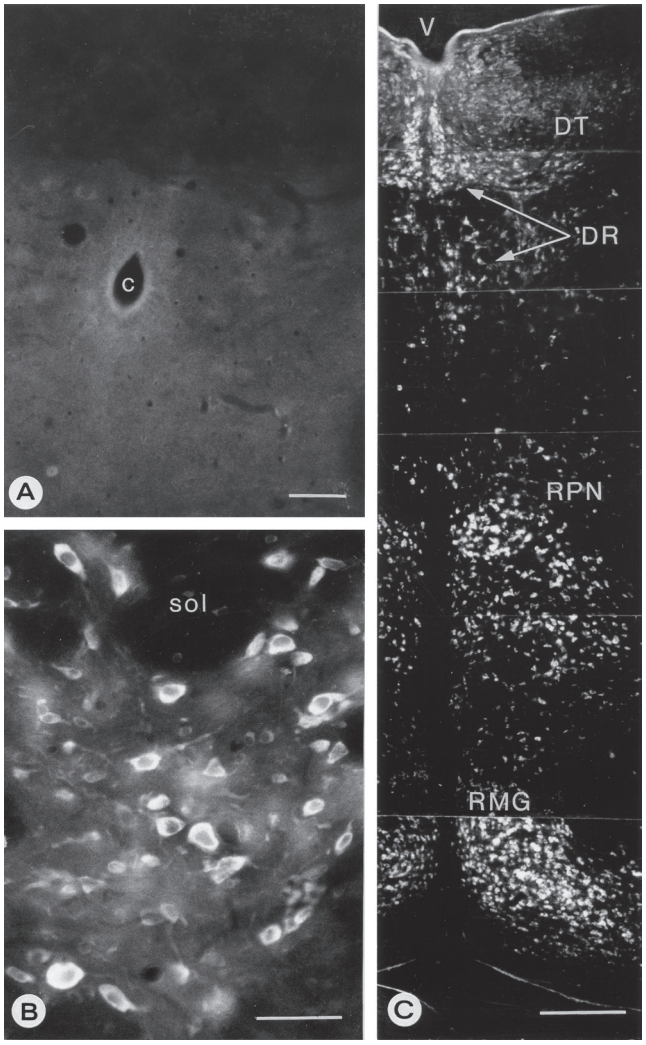

Figure 2. 2A: Section of the lower medulla incubated without primary antibody (c: central canal, bar 50 µm). 2B: in the nucleus of the solitary tract (sol) neurons and neuropil display RPI (bar 50 µm). 2C: pontine raphe displays many RPI-containing neurons (DT: dorsal tegmental nucleus; DR: dorsal raphe nucleus; RPN: raphe pontis nucleus; RMG: raphe magnus nucleus; bar 500 µm).

The antiserum recognized a dominant protein band at 95 kDa in both samples (Fig. 1). The molecular weight of the dominant protein band (95 kDa) corresponds with the previously immunoprecipitated B-raf molecule in brain (Mihály et al. 1991) and cell culture (Kolch et al. 1988). The intensity of the antibody reactions indicated the cytoplasmic localization of the raf protein.

Sections incubated without the primary B-raf serum did not display specific staining (Fig. 2A). Several structures in the medulla displayed RPI. Strong staining of the cell bodies was observed in the nucleus ambiguus, the nucleus retroambigualis and the hypoglossal nucleus. In these structures, mainly the neuronal cell bodies were stained. RPI-containing somata were surrounded by 0.5-2.0 µm thin processes, probably dendrites of motoneurons. The dorsal vagal nucleus and the nuclei of the solitary tract contained strongly- and medium-stained cell bodies (Fig. 2B). Neurons of the inferior olive, and giant neurons of the reticular formation were stained strongly. On the other hand, no cellular immunostaining was observed in the area postrema. In the laminae of the spinal trigeminal nucleus only a few neurons were stained: strongest immunostaining was observed in the marginal layer, where some of the medium-sized neurons contained RPI. The dorsal column nuclei contained scattered immunopositive neurons. The medullary raphe obscurus nucleus contained several strongly-stained neurons.

In the lower pons, the motor nucleus of the facial nerve (Fig. 3D), the abducent nucleus and the lateral vestibular nucleus displayed strong immunostaining. Nuclei of the raphe system contained strongly immunostained neurons as well (Fig. 2C). Neurons of the medial and superior vestibular nuclei and cells of the dorsal cochlear nucleus were medium-weakly stained, with only few RPI-containing neurons present (Fig. 3A,B). The ventral cochlear nucleus contained strongly stained small neurons (Fig. 3C). Neurons of the superior olivary complex and the nucleus of the trapezoid body displayed moderate staining intensity. The large cells of the pontine reticular formation stained more strongly. At higher levels, strong staining was observed in the main motor nucleus of the trigeminal nerve (Fig. 5A). Very few RPI-containing cells were observed in the principal sensory nucleus of the trigeminal nerve (Fig. 5B). The tightly packed neurons of the locus ceruleus (as identified on the basis of strong TH immunoreactivity) displayed medium immunostaining (Fig. 4A,B). The scattered noradrenergic neurons of the subceruleus region and those of the A5 group are brightly stained with TH antibody, but display a weak RPI. Laterally from the locus coeruleus, we observed the first neurons of the mesencephalic nucleus of the trigeminal nerve. These large round cells were strongly stained (Fig. 5C). The large sensory neurons were surrounded by a loose network of TH-containing fibers (Fig. 5D). Many RPI-containing neurons were present in the pontine nuclei.

In the midbrain, motor cells of trochlear and oculomotor nuclei displayed moderate staining. Faintly stained neurons were found in the Westphal-Edinger nucleus. Large cells of the mesencephalic trigeminal nucleus were strongly stained. Strong immunostaining was found in the red nucleus: these neurons stood out due to their staining intensity. Faintly stained small cells were observed in the periaqueductal gray substance or substantia grisea centralis of the midbrain. Scattered stained cells were observed in the substantia nigra and interpeducular nucleus (Fig. 6A-D). The ventral tegmental area contained only a few RPI-positive cells (Fig.

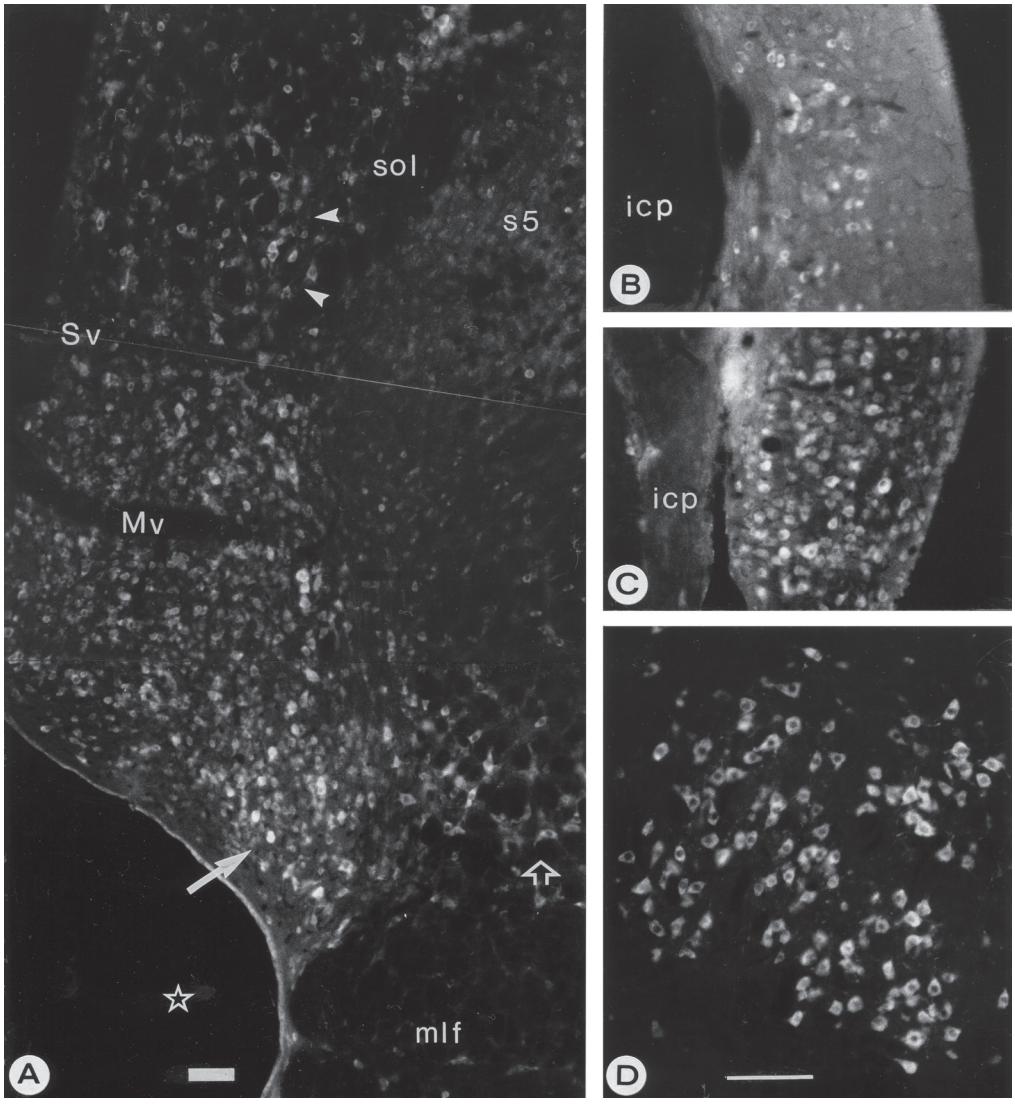

Figure 3A-D. Lower pons structures with RPI. A: superior (Sv) and medial (Mv) vestibular nuclei. Solid arrow: prepositus hypoglossal nucleus; star: fourth ventricle; empty arrow: reticular formation; sol: solitary tract; s5: spinal trigeminal nucleus. B: dorsal cochlear nucleus with a few immunostained cells. C: ventral cochlear nucleus with numerous stained neurons (icp: inferior cerebellar peduncle). D: facial motor nucleus with strongly stained neurons. Bars: 50 µm.

6B). In the superior and inferior colliculi, only a few cells were stained. These neurons appeared to be the larger neurons and were found in the deep layers of the superior colliculus (not shown). Scattered large RPI-containing cells were present in the lateral reticular formation.

The general features of RPI-positivity of cranial nerve nuclei and other brainstem structures are summarized in Figure 7. As a general rule, motor nuclei displayed stronger staining than sensory nuclei. However, the mesencephalic nucleus of the trigeminal nerve, the lateral vestibular nucleus and the ventral cochlear nucleus were exceptions: these sensory structures contained strongly stained neurons. Other structures related to the brainstem auditory pathway, *i.e.* the superior olivary complex and the nucleus of the trapezoid body, similarly contained RPI. Staining was detected in dorsal column nuclei, the locus ceruleus, pontine nuclei, the superior colliculus and the ventral tegmental area. Strong RPI was observed in magnocellular and parvocellular parts of the red nucleus. The neurons of the substantia nigra were moderately stained. All parts of the brainstem reticular formation (including raphe nuclei) contained RPI-positive neurons. When higher dilutions of the antibody were used, staining of perikarya faded more rapidly than that of neuropil (not shown), indicating that the concentration of the antigen was much higher in dendritic processes than in the perikaryon. Finally, stain-

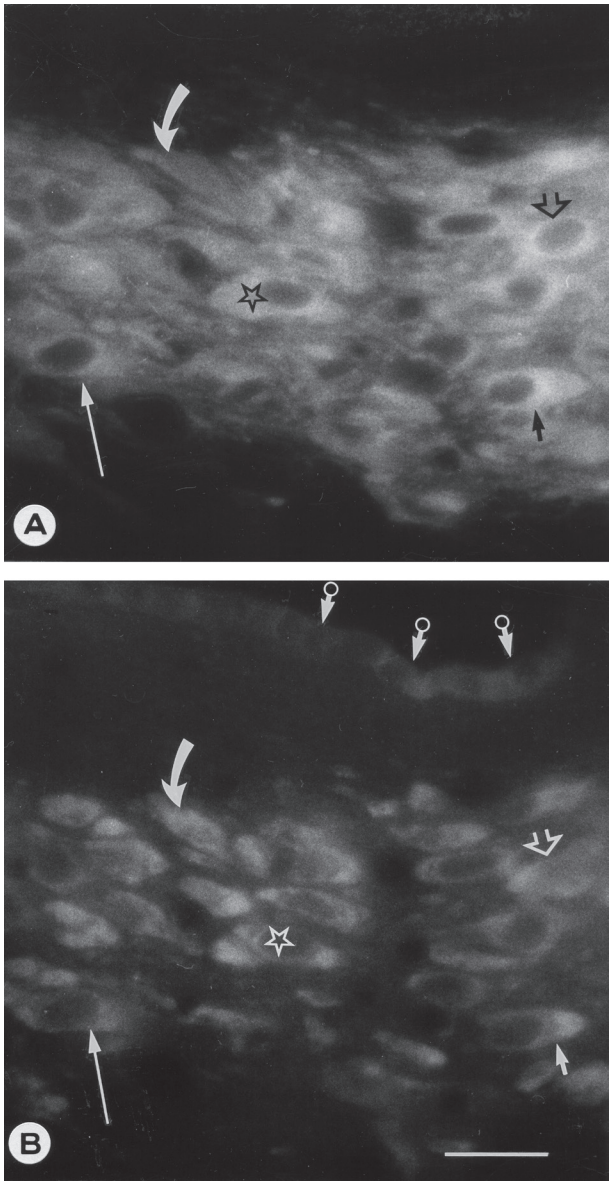

Figure 4A-B. Single section of the locus ceruleus with TH antibody (A) and B-raf serum (B). Star and arrows point to identical neurons. Small arrows with circle on B point to RPI-containing ependyma cells. Bar: 20 μm.

ing intensity depended on the size of cells: larger neurons regularly displayed stronger staining than medium-sized and small neurons.

Discussion

Data are scarcely available on the distribution patterns and possible roles of protooncogenes in the brainstem. The transcription regulator genes are activated and their protein products detected predominantly after stimulation of cranial nerves (Rutherfurd et al. 1992). B-raf belongs to another group of cellular oncogenes, *i.e.* the intracellular, cytoplasmic signal transducers (Rapp 1991). A very important feature of cytoplasmic Raf kinases is their ability to translocate from the cytoplasm to the cell membrane. Recruitment of B-raf to the cell membrane requires transformation of Ras into a Ras-GTP complex binding Raf molecules, which are then phosphorylated and activated (Morrison and Cutler 1997). Activated Raf kinases are able to phosphorylate Jun proteins, MAP kinases and MAP kinase kinases - the latter kinases are often abbreviated as MEK (mitogen- or extracellular-regulated kinase) (Heidecker et al. 1992; Roy et al. 1997). The possibility that they phosphorylate MAP kinases is particularly interesting because this function could explain the presence of B-raf in neuronal processes (Mihály et al. 1991, 1993). The immunohistochemical studies of Fiore et al. (1993) revealed that the localization of p42 MAP kinase was strikingly similar to that of Raf proteins in the forebrain of rat (Fiore et al. 1993; Mihály et al. 1993). The immunohistochemical localization of MEK isoforms also exhibits a distribution pattern that is identical to that of Raf in the brainstem of rat (Flood et al. 1998), with marked immunostaining of neuronal cell bodies in cranial nerve nuclei and other brainstem structures, such as the red nucleus, periaqueductal gray substance, reticular formation, superior olivary complex, inferior olive and dorsal column nuclei (Flood et al. 1998). These findings support the validity of our present observations and point at the importance of the B-raf - MAP kinase cascade in neuronal signal transduction.

Our previous studies of guinea pig spinal cord indicated that motor neurons of the ventral horn, and some medium-sized cells in the dorsal horn (cells in lamina I, nucleus proprius and base of the dorsal horn) were immunopositive with Raf antibodies (Mihály and Rapp 1993). Primary afferent nerve endings were not positive (Mihály et al. 1996). In rats, a large variety (though not all) of dorsal root ganglion (DRG) cells contained B-raf; double labelling revealed that these cells were from every size class (Mihály et al. 1996). On the other hand, we did not find B-raf in the sensory nerve endings of skin and cornea (Mihály et al. 1996). Ligation of the sciatic nerve did not result in accumulation of B-raf on either side, indicating that B-raf molecules are not transported in peripheral nerves in the sensory and motor axons (Mihály et al. 1996). Extrapolation of these results to brainstem allows the conclusion that the B-raf in the cranial nerve nuclei functions in the central nervous system, and does not participate in peripheral signal transduction processes. The signals which travel from the periphery have to reach the cell body first, where they can interact with the B-raf kinase system (Mihály et al. 1996). The strong immunostaining of the trigeminal mesencephalic nucleus is very similar to that of dorsal root ganglion cells (Mihály et al. 1996). On the

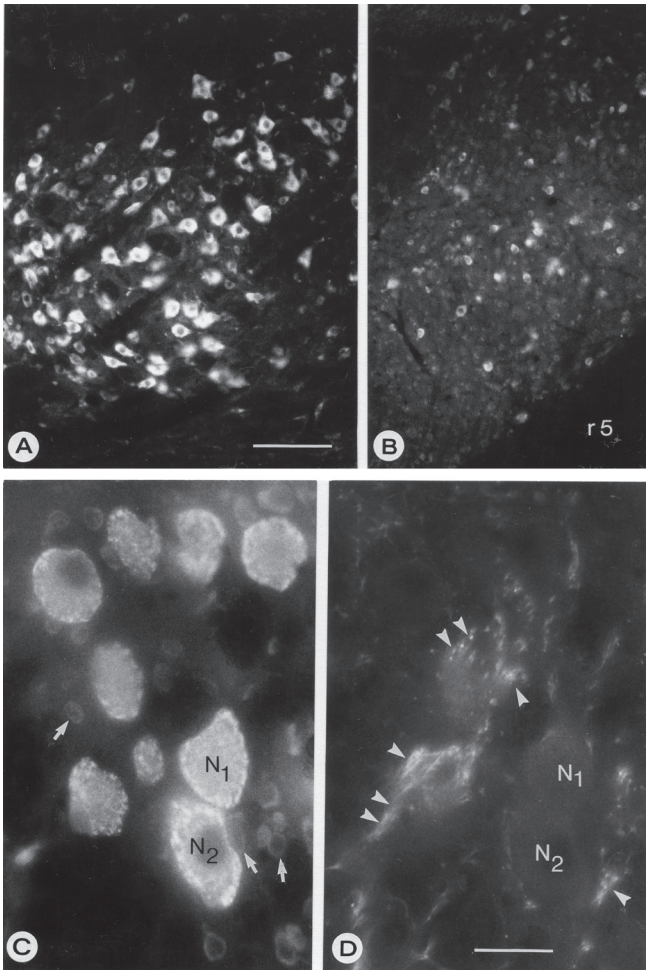

Figure 5A-D. Trigeminal nuclei in upper pons. A: motor nucleus (bar 100 µm). B: principal sensory nucleus (r5: radix of trigeminal nerve; same magnification as A). C: mesencephalic nucleus. Note large sensory cells (N1 and N2) with strong RPI. Small cells (probably glia cells) with weak staining are visible, too (arrows). D: axon network around large sensory neurons contain TH (arrowheads). N1 and N2 are identical with cells on C (bar 50 µm).

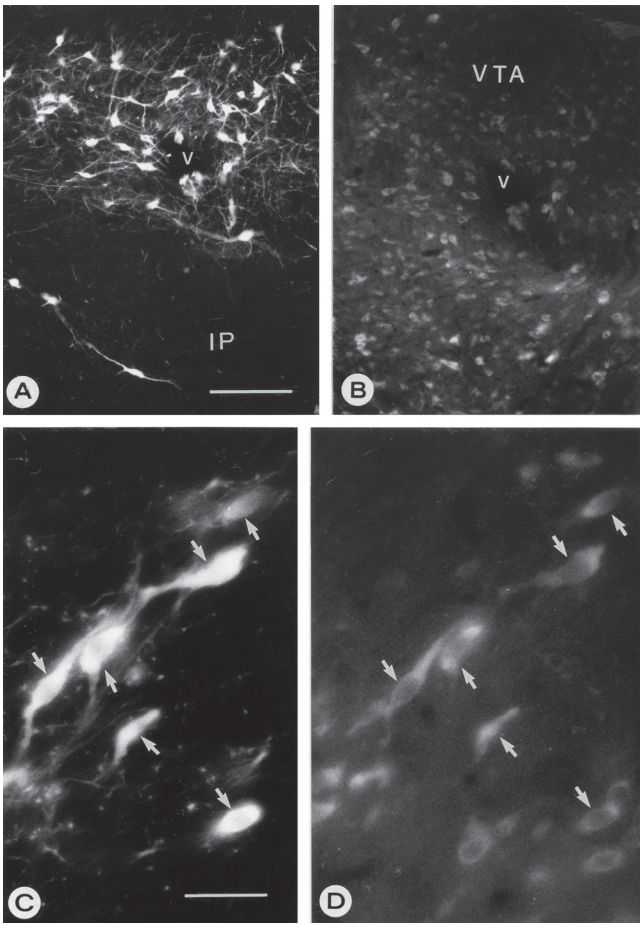

Figure 6A-D. Dopaminergic cells of the midbrain contain RPI (A,B and C,D are identical sections). A: dopaminergic neurons in the ventral tegmental area (VTA; bar for A,B 100 µm). B: some of the dopaminergic cells and neurons of the interpeduncular nucleus (IP) contain RPI (v: blood vessel). C: substantia nigra neurons stained with anti-DBH (arrows; bar for C,D 50 µm). D: the same cells (arrows) contain weak B-raf-like staining.

basis of previous observations (Mihály and Rapp 1994; Mihály et al. 1996; Vossler et al. 1997; Wartmann et al. 1997) and the present data, it can be suggested that B-raf kinase is a cytoplasmic signal transducer, which in the case of neurons is not transported through cranial and spinal nerves to the periphery. It seems that it resides in the cell body and dendrites and receives molecular signals from presynaptic terminals or axons coming from the peripheral nervous system; or coming from the vicinity from other neurons or glial cells. In previous experiments we cut the sciatic nerve, and observed that cytoplasmic B-raf undergoes a characteristic translocation in DRG neurons from the cytoplasm to the periphery of the cell body (Mihály et al. 1996), indicating the sensitivity of B-raf to certain factors arriving via peripheral nerves. This translocation was similar to the membrane translocation described in cell cultures (Leevers et al. 1994). Membrane translocation has been explained on the basis of Raf activation by Ras (Leevers et al. 1994). We think, the our results suggest the ubiquity of B-raf kinase in the central nervous system, indicating that this cytoplasmic signal transducer is not specifically related to synaptic transmission, but is related to other signals coming through 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 (Carroll et al. 1990) and neuronal angiotensin receptor (Yang et al. 1997) have been found to activate the Ras-Raf-MAP kinase pathway. The question arises, whether the neurons of the brainstem

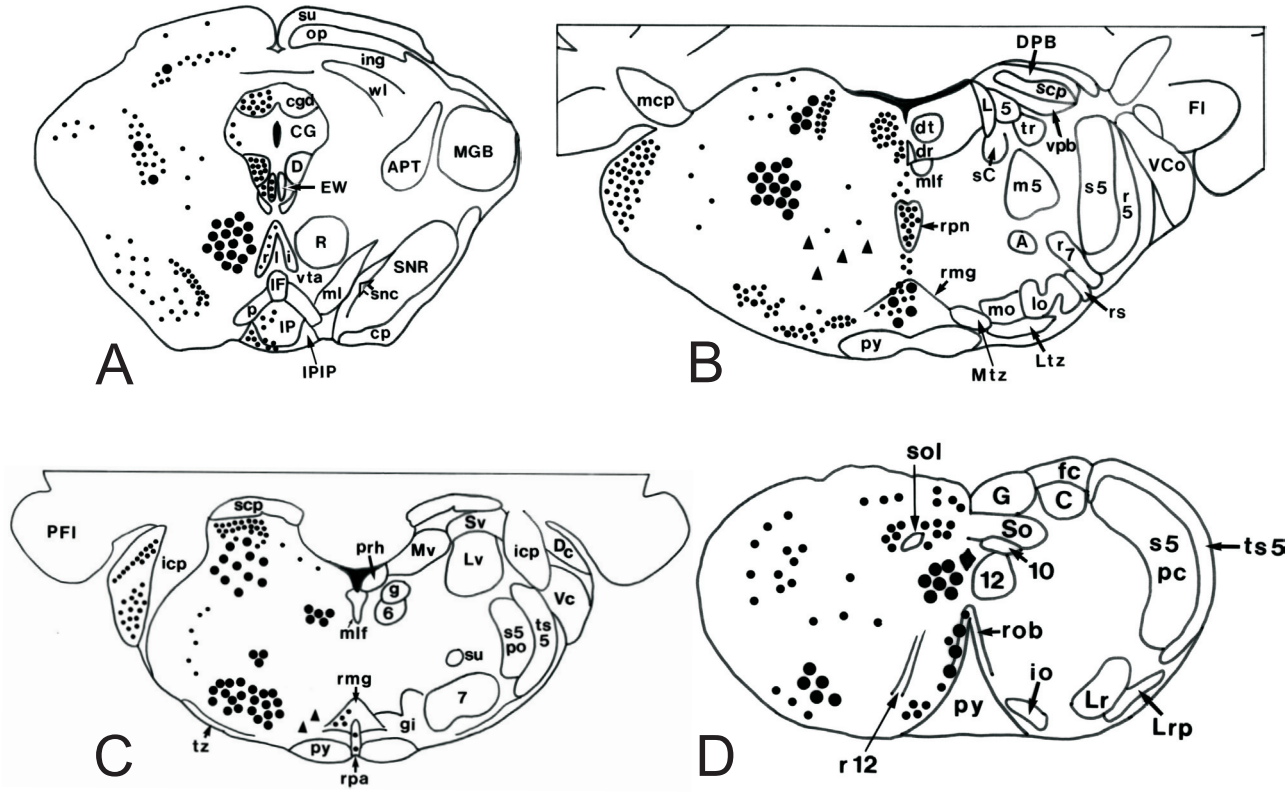

Figure 7A-D. Representative cross sections of the rat brainstem (Swanson 1992). Large dots represent strongly stained neurons, small dots represent medium or faint staining. Triangles represent large nerve cells with outstanding RPI content. A: upper mesencephalon; B: upper pons; C: lower pons; D: lower medulla (closed part). Abbreviations of Fig. 7. 5: mesencephalic nucleus of trigeminal nerve; m5: motor nucleus of trigeminal nerve; s5: principal sensory nucleus of trigeminal nerve; 6: abducens nucleus; g: genu of facial nerve; 7: facial nucleus; 7r: facial nerve root; 10: dorsal motor nucleus of vagus; 12: hypoglossal nucleus; r12: hypoglossal nerve root; APT: anterior pretectal nucleus; A: A5 noradrenaline cells; L: locus ceruleus; sC: subceruleus nucleus; su: superficial gray matter of the superior colliculus (Fig. 7A); op: optic nerve layer of the superior colliculus; ing: intermediate gray layer of the superior colliculus; wl: white layer of the superior colliculus; EW: Edinger-Westphal nucleus; D: nucleus of Darkschewitsch; R: red nucleus; SNR: substantia nigra pars reticulata; snc: substantia nigra pars compacta; MGB: medial geniculate body; IP, IPIP: interpeduncular nucleus; cp: cerebral peduncle; CG, cgd: substantia grisea centralis; rli: raphe, rostral linear nucleus; vta: ventral tegmental area; IF: interfascicular nucleus; p: paranigral nucleus; py: pyramidal tract; dt: dorsal tegmental nucleus; dr: dorsal raphe nucleus; rpn: raphe pontis nucleus; rmg: raphe magnus nucleus; mlf: medial longitudinal fasciculus; mo, lo: medial and lateral superior olivary nucleus; Mtz, Ltz: medial and lateral nucleus of trapezoid body; Vco, Vc: ventral cochlear nucleus; Dc: dorsal cochlear nucleus; DPB: dorsal parabrachial nucleus; vpb: ventral parabrachial nucleus; scp: superior cerebellar peduncle; icp: inferior cerebellar peduncle; mcp: middle cerebellar peduncle; rs: rubrospinal tract; Mv, Lv, Sv: medial, lateral, superior vestibular nucleus; prh: prepositus hypoglossal nucleus; rpa: raphe pallidus nucleus; gi: gigantocellular reticular nucleus; s5po: spinal trigeminal nucleus, pars oralis; s5pc: pars caudalis; ts5: spinal trigeminal tract; su: superior salivatory nucleus (Fig. 7C); sol: solitary tract; So: nucleus of the solitary tract; io: inferior olivary nucleus; rob: raphe obscurus nucleus; Lr, Lrp: lateral reticular nucleus; G: gracile nucleus; C: cuneate nucleus; fc: fasciculus cuneatus.

depend on these growth factors and cytokines, too.

NGF receptors have been found in some of the RPI-containing nuclei: in the mesencephalic trigeminal nucleus, superior colliculus, nucleus ambiguus, medullary raphe nuclei, reticular formation and medial vestibular nucleus in rat (Pioro and Cuello 1990), and visceral solitary tract nucleus in ferret (Henderson et al. 1991). Angiotensin receptors have been detected in brainstem neurons (Richards et al. 1999), and basic fibroblast growth factor (bFGF) in developing and adult brainstem (Grothe et al. 1991). Most of these structures contain RPI as we have shown in the present study. Recently, it was demonstrated that neuronal differentiation and probably regeneration in the adult is mediated by bFGF, and intracellular pathways include the Ras-Raf-MEK pathway (Kuo et al. 1997). This mechanism could also be important in the brainstem auditory system, which has regeneration capacity in rat (Ito et al. 1999). Our present study demonstrated the presence of B-raf in most of the brain stem auditory structures. A further step towards understanding of functions of Raf is the discovery that activated B-raf kinase plays a role in responses to transmitter receptors: cell culture studies have revealed that stimulation of M3 muscarinic receptors with carbachol activates cytoplasmic Raf kinase (Kim et al. 1999). A similar

activation can be achieved with norepinephrine via α- and β-adrenoceptors (Yamazaki et al. 1997). The significance of these findings in adult brainstem is far from understood, but they underline the significance of Raf kinases in neuronal signal transduction, and validate our observations on the widespread localization of the enzyme. It seems that B-raf is a key enzyme in the cell membrane-to-cell nucleus pathway; a number of receptors depend on it, most of them regulating development and regeneration.

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