1. Abstract

The transmission of proliferative and developmental signals from activated cell-surface receptors to initiation of cellular responses in the nucleus is synergically controlled by the coordinated action of a diverse set of intracellular signalling proteins. The Ras/Raf/MEK/MAPK signalling pathway has been shown to control the expression of genes which are crucial for the physiological regulation of cell proliferation, differentiation and apoptosis. Within this signalling cascade, the Raf protein family of serine/threonine kinases serves as a central intermediate which connects to many of other signal transduction pathways.

To elucidate the signalling functions of the different Raf kinases in motoneurons during development, the expression, distribution and subcellular localization of Rafs in the spinal cord and the facial nucleus in brainstem of mice at various embryonic and postnatal stages were investigated. Moreover, we have investigated the intracellular redistribution of Raf molecules in isolated motoneurons from 13 or 14 day old mouse embryos, after addition or withdrawal of neurotrophic factors to induce Raf kinases activation *in vitro*. Furthermore, in order to investigate the potential anti-apoptotic function of Raf kinases on motoneurons, we isolated motoneurons from *B-raf*$^{-/-}$ and *c-raf-1*$^{-/-}$ mouse embryos and analysed the survival and differentiation effects of neurotrophic factors in motoneurons lacking B-Raf and c-Raf-1.

We provide evidence here that all three Raf kinases are expressed in mouse spinal motoneurons. Their expression increases during the period of naturally occurring cell death of motoneurons. In sections of embryonic and postnatal spinal cord, motoneurons express exclusively B-Raf and c-Raf-1, but not A-Raf, and subcellularly Raf kinases are obviously

colocalized with mitochondria. In isolated motoneurons, most of the B-Raf or c-Raf-1 immunoreactivity is located in the perinuclear space but also in the nucleus, especially after activation by addition of CNTF and BDNF *in vitro*. We found that c-Raf-1 translocation from the cytosol into the nucleus of motoneurons after its activation by neurotrophic factors is a distinct event.

As a central finding of our study, we observed that the viability of isolated motoneurons from *B-raf* but not *c-raf-1* knockout mice is lost even in the presence of CNTF and other neurotrophic factors. This indicates that B-Raf but not c-Raf-1, which is still present in *B-raf* deficient motoneurons, plays a crucial role in mediating the survival effect of neurotrophic factors during development. In order to prove that B-Raf is an essential player in this scenario, we have re-expressed B-Raf in mutant sensory and motor neurons by transfection. The motoneurons and the sensory neurons from *B-raf* knockout mouse which were transfected with exogenous *B-raf* gene revealed the same viability in the presence of neurotrophic factors as primary neurons from wild-type mice.

Our results suggest that Raf kinases have important signalling functions in motoneurons in mouse CNS. *In vitro*, activation causes redistribution of Raf protein kinases, particularly for c-Raf-1, from motoneuronal cytoplasm into the nucleus. This redistribution of c-Raf-1, however, is not necessary for the survival effect of neurotrophic factors, given that *B-raf*$^{-/-}$ motor and sensory neurons can not survive despite the presence of c-Raf-1. We hypothesize that c-Raf-1 nuclear translocation may play a direct role in transcriptional regulation as a consequence of neurotrophic factor induced phosphorylation and activation of c-Raf-1 in motoneurons. Moreover, the identification of target genes for nuclear translocated c-Raf-1 and of specific cellular functions initiated by this mechanism awaits its characterization.

2. Introduction

2.1. Cellular function of the Ras-MAPK signalling pathway

Coordination of cellular function in multi-cellular organisms is regulated by a variety of signals between various types of cells, including direct cell contact molecules, molecules in the extracellular matrix and soluble, secreted signalling molecules. The transmission of proliferative and developmental signals from activated cell-surface receptors to initiation of cellular responses in the nucleus is synergically controlled by the coordinated action of a diverse set of signalling proteins. Among the pathways which transduce these signals are the highly conserved mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK) cascades. The first and best characterized MAPK cascade consists of three sequentially acting protein kinases: Activation of members of the Raf family, which control the MAPK/ERK kinases (MEK1/2) leads to activation of ERK1/2. Upstream of Raf, various forms of Ras are the major regulators of this signalling cascade. This so-called Ras-MAPK pathway has been shown to control the expression of genes which are crucial for the physiological regulation of cell proliferation and differentiation (Daum et al., 1994). Within this signalling cascade, the Raf protein family of serine/threonine kinases serves as a central regulator which connects to many of other signal transduction pathways. Moreover, a variety of signals which impinge upon Raf reiterates the fundamental importance of this protein to signal transduction, by which Raf proteins regulate cellular processes such as proliferation, differentiation and apoptosis.

2.2. Members of the *raf* gene family and their role in intracellular signal transduction

The *raf* family of proto-oncogenes encodes cytoplasmic serine/threonine specific protein kinases (Mark and Rapp, 1984; Moelling et al., 1984) which play a key role in mitogen signal transduction (Heidecker et al., 1989; Rapp et al., 1988a; Rapp et al., 1988b; Li, et al., 1991), functioning to connect upstream tyrosine kinases and Ras with downstream serine/threonine kinases (Avruch et al., 1994). In other words, Raf kinases have been found as critical "gatekeepers" in growth factor signal transduction and oncogenic transformation because they are strategically located at a convergent signalling point in the Ras-MAPK signal transduction pathway (Daum et al., 1994). The Ras oncoprotein, a GTP-activated molecular switch and upstream activator of Raf, interacts directly with the Raf protein kinase to eventually recruit the MAPK and their subordinates. In this signalling cascade, Raf kinases phosphorylate MEK (Kyriakis et al., 1992, 1993; Avruch et al., 1994), and MEK then activates MAPK (Matsuda et al., 1992; Wu et al., 1993). The MAPKs, also known as ERKs are located downstream of MEK, and constitute a family of serine/threonine protein kinases that is involved in the cascade of protein phosphorylation mediated by a serial activation of protein kinases (Pelech and Sanghera, 1992; Davis, 1993). The mammalian MAPK family is comprised of p44 ERK1 and p42 ERK2, p45 and p54 Jun amino-terminal kinases (JNKs, also known as SAPKs) and p38/CSBP (for review, see Marais and Marshall, 1996). The activation of ERK requires the phosphorylation of ERK on both tyrosine and threonine residues. Activated ERKs regulate essential cellular events in many cell types. In summary, in this signal transduction pathway, the Ras/Raf/MEK/MAPK cascade is activated by a wide range of mitogenic and differentiating factors (Marshall, 1995; Kaplan and Miller, 1997; Sugden and Clerk, 1997). After complex formation of Raf and its adaptor proteins, a relaying signal from the GTP-bound form of Ras is transduced to MEK. The subsequent downstream phosphorylation and activation cascade

occurs through MEK and MAPK. MAPKs phosphorylate nuclear transcription factors and thereby lead to the expression of genes necessary for cell division and/or cell differentiation (Avruch et al., 1994; Williams and Roberts, 1994; Marais and Marshall, 1996).

2.3. Raf protein structure and expression

The Raf kinases are highly conserved during evolution. Three mammalian *raf* genes and their corresponding cytoplasmic protein products have been identified to date, namely *A-raf* (Huleihel et al., 1986), *B-raf* (Ikawa et al., 1988), and *c-raf-1* (Bonner et al., 1985). The serine/threonine kinases A-Raf, B-Raf and c-Raf-1 (Daum et al., 1994) are distinguished by their mechanisms of activation (Marais et al., 1997; Vossler et al., 1997) and specific expression pattern in various tissues (Storm et al., 1990). Among these three different Raf isozymes, c-Raf-1 (72~74 kDa) (Bonner et al., 1986) is found to be ubiquitously expressed in almost all tissues, whereas A-Raf (68 kDa) and B-Raf (95 kDa) (Stephens, 1992) show a more specific tissue distribution (Storm et al., 1990) with A-Raf being most abundant in epididymis, urogenital tissue and ovary and B-Raf being predominantly expressed in fetal and adult mouse brain and testis. In addition, the molecular complexity of this family is increased by the presence of at least two c-Raf-1 isoforms (Dozier et al., 1991). On the mRNA level, four alternatively spliced messages for B-Raf have been detected in mouse brain (Barnier et al., 1995). Therefore, various isoforms of these specific kinases exist which probably serve different functions within the signalling networks in the Raf/MEK/MAPK protein kinase cascade.

The three members of Raf family proteins share common structural elements. They possess three conserved regions, CR1, CR2, and CR3, which are embedded in variable regions. The

latter vary strongly between the three Raf isoforms but are evolutionarily highly conserved within the isoforms from different species (Naumann et al., 1996). The CR1 and CR2 domains are part of the regulatory *N*-terminal half of the Raf proteins, whereas CR3 represents the *C*-terminal kinase domain. Deletion of the regulatory half of all three Raf kinases leads to constitutively active forms (Daum et al., 1994; Naumann et al., 1996).

2.4. Mechanisms of Raf activation

The mechanism of activation of these three enzymes, Raf/MEK/MAPK, has been intensively studied (Pages et al., 1993; Troppmair et al., 1994). The best characterized member of Raf protein kinases in this context is c-Raf-1. c-Raf-1 has been crystallized together with Ras, and the 3-D interaction of these two proteins has been determined. Ras-mediated translocation of c-Raf-1 to the plasma membrane is a crucial step in this activation process, and is thought to facilitate phosphorylation by membrane-bound kinases (Leevers et al., 1994; Stokoe et al., 1994; Marais et al., 1995). Ras activation is regulated by multiple components which influence the Ras cycle, which occurs between an inactive, GDP-bound and an active, GTP-bound form of Ras. This transition is mediated by GDP-GTP exchange factors (GEFs) and includes additional regulatory proteins that stimulate the intrinsic GTPase activity of Ras (McCormick, 1993). The Ras cycle is connected to activated receptors by adaptor proteins, such as Grb2, which recruit the GEF Sos, resulting in increased steadystate levels of Ras-GTP (Schlessinger, 1993). Also, the individual Raf isozymes may be differentially regulated by Ras and other upstream factors. For example, A-Raf and c-Raf-1, but not B-Raf, can be activated by Src (Marais et al., 1997), whereas B-Raf and c-Raf-1 are differently activated by cyclic AMP (cAMP) (Erhardt et al., 1995) and nerve growth factor (NGF) (Jaiswal et al., 1996).

Moreover, different Raf isozymes display different potencies in the activation of their substrates MEK and MAPK (Marais et al., 1997; Pritchard et al., 1995).

In the case of activation of Raf by Ras, only the GTP-bound form of Ras can bind Raf protein kinases which leads to their activation (Rodriguez-Viciana, et al., 1994; Hall, 1994). Any physiological activation of c-Raf-1 is paralleled by its hyperphosphorylation. The activation is accomplished by tyrosine phosphorylation (Fabian et al., 1993; Turner et al., 1993), and serine phosphorylation, the latter being independent of protein kinase C (PKC) (Baccarini et al, 1990; Blackshear et al., 1990; App et al., 1991; Ohmichi et al., 1992), and PKC-catalyzed serine phosphorylation (Sozeri et al., 1992; Kolch et al., 1993; Carroll et al., 1994). Following its activation which leads to stimulation of the downstream MEK kinase, c-Raf-1 protein has also been demonstrated to undergo intracellular redistribution. In the case of NIH3T3 cells (Rapp et al., 1988) and rat Ito cells (Lissoos et al., 1993), c-Raf-1 is translocated to the perinuclear space. In gerbil hippocampal pyramidal cells (Olah et al., 1991), c-Raf-1 is translocated to the nucleus. Similar findings have been made with human MO7 leukemia cells (Brennscheidt et al, 1994) and neuronal cells from hypothalamus and brainstem of rats (Lu et al., 1998). So far, the role of intracellular translocation of the activated c-Raf-1 protein kinase to the nucleus is still unresolved.

2.5. Raf-MAPK signalling pathway in the CNS

In the central nervous system (CNS), MAPK proteins are broadly expressed (Boulton and Cobb, 1991; Boulton et al., 1991), particularly in post-mitotic cells (Fiore et al., 1993). Therefore, the MAPK cascade has also been implicated in neuron-specific functions. MAPK kinases phosphorylate numerous substrates in neurons, e.g. cytoskeleton proteins (Morishima-

Kawashima and Kosik, 1996), tyrosine hydroxylase (Haycock et al., 1992) and synapsin (Jovanovic et al., 1996; Matsubara et al., 1996). Moreover, recent data show that activation of MAPK by neurotransmitters plays an important role in synaptic function (Brambilla et al., 1997; English and Sweatt, 1997; Kornhause and Greenberg, 1997). Although the Raf kinases play a fundamental and specific role in the activation of MAPK cascade, the nature of the different Raf protein kinases involved in the activation of the MAPK pathway is still not understood. Instead, only speculations have been put forward that different Raf isoforms cause MAPK activation in different neuronal cell types. So far, it is completely unknown whether differences exist among different types of neurons.

2.6. Expression of members of the *raf* family in the CNS

Previous studies have shown that only *B-raf* and *c-raf-1* mRNAs are expressed in the CNS (Storm et al., 1990). On the basis of observation that B-Raf is the predominant MEK activator in rat and bovine brain (Catling et al., 1994; Moodie et al., 1994; Yamamori et al., 1995), B-Raf is generally considered as the most relevant Raf protein kinase present in the nervous system. Former studies also demonstrated Raf immunoreactivity in certain areas of the brain (Mihaly et al., 1991, 1993, 1996). However, the antibodies employed in these studies did not differentiate between various Raf isoforms. Recently, a comparative study (Morice et al., 1999) of Raf distribution and subcellular localization in adult rat brain revealed that B-Raf and c-Raf-1 protein kinases are present in most brain areas. The two Raf protein kinases have an approximately similar pattern of distribution with a rostro-caudal decreasing gradient of expression. These two protein kinases are colocalized in neurons but they are differentially located in subcellular compartments. B-Raf was found to be widely distributed in the neuronal cell bodies and in the neuronal processes, whereas c-Raf-1 appeared localized mainly in the

cytosolic fraction around the nucleus. These results are difficult to interpret on a functional level, as more than 10 isoforms of B-Raf have been detected so far in the brain. These isoforms are thought to have a differential pattern of distribution, some of them being ubiquitously expressed whereas others being localized in specific brain areas. Also, these isoforms show a clear differential subcellular localization (Morice et al., 1999). These observations suggest that the B-Raf and c-Raf-1 isoforms differentially modulate neuronal signalling pathways in specialized subcellular compartments.

2.7. Raf kinases and apoptosis

2.7.1. General factors of apoptosis

Apoptosis, or programmed cell death (PCD), is a strictly regulated process responsible for the ordered removal of superfluous, aged, or damaged cells (Thompson, 1995; Kroemer et al., 1995). Every second, several millions of cells of the human body undergo apoptosis, i.e. under conditions of homeostasis, each mitosis is compensated by one event of apoptosis. Disturbances in regulation of apoptosis illustrate the importance of apoptosis for normal homeostasis. An abnormal resistance to apoptosis induction correlates with malformations, autoimmune diseases, or cancer due to the persistence of superfluous, self-specific immunocytes, or mutated cells, respectively. In contrast, enhanced apoptotic decay of cells participates in acute pathologies (infection by toxin-producing microorganisms, ischemia-reperfusion damage, or infarction) as well as in chronic diseases (neurodegenerative and neuromuscular diseases, AIDS).

The apoptotic process can be divided into at least three functionally distinct phases: initiation, effector and degradation (Kroemer, et al., 1995). During the heterogenous initiation phase, cells receive a death-inducing stimulus. The biochemical events participating in the initiation phase constitute individually specific pathways in that they depend on the lethal stimulus. It is only the subsequent effector phase that these initiating events are translated into a regular pattern of metabolic reactions and the "decision to die" is taken. Thus, the ultimate fate of the cell during the effector phase is subject to regulatory events. Beyond this stage, during the degradation phase, a series of catabolic enzymes are activated, precluding further regulatory effects. During this last phase the morphology and characteristic biochemistry of apoptosis become apparent. Cells undergoing apoptosis show characteristic morphological features such as condensation of cytoplasmic and nuclear contents, blebbing of the plasma membrane, fragmentation of the nucleus, and ultimately breakdown into membrane-bound apoptotic bodies that are rapidly phagocytosed (Kerr, et al., 1972).

Genetic analysis of the nematode *Caenorhabditis elegans* has identified three genes that control the general apoptotic program. Two genes, *ced-3* and *ced-4*, are required for the apoptotic program (Yuan and Horvitz, 1990). *Ced-3*, which encodes a cysteine protease related to the ICE (interleukin-1β-converting enzyme), or caspase-1, a prototype of family of proteases (caspases) in mammalian cells (Yuan et al., 1993; Alnemri et al., 1996). The closest mammalian homologue of CED-3 is CPP32 (caspase-3) (Fernandes-Alnemri et al., 1994), which cleaves various substrates of the ICE family of proteases, including poly(ADP-ribose) polymerase (PARP) and sterol-regulatory element-binding proteins (SREBPs) in cells undergoing apoptosis (Tewari et al., 1995; Nicholson et al., 1995; Wang et al., 1995, 1996). CPP32 is closely related to CED-3 in terms of sequence identity and substrate specificity (Xue and Horvitz, 1995). CED-4 is homologous to the recently identified human protein Apaf-1

(apoptotic protease activating factor-1), which participates in the activation of the mammalian caspase-3 (Yuan and Horvitz, 1990; Yuan et al., 1993; Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Tewari at al., 1995; Xue and Horvitz, 1995; Zou et al., 1997). *Ced-4* has been determined genetically to function downstream of *ced-9* but upstream of *ced-3* (Shaham and Horvitz, 1996a; Shaham and Horvitz, 1996b). *Ced-9*, which functions upstream of *ced-3* and *ced-4*, negatively regulates the apoptotic program by preventing activation of *ced-3* and *ced-4* (Hengartner et al, 1992). The *bcl-2* family of genes are the mammalian counterparts of *ced-9* (Hengartner and Horvitz, 1994). Overexpression of Bcl-2 prevents mammalian cells from undergoing apoptosis in response to a variety of stimuli (for review see Reed, 1994). The Bcl-2 protein is located primarily on the outer membranes of mitochondria (Monaghan et al., 1992; Krajewski et al, 1993; de Long et al, 1994), suggesting the involvement of mitochondria in apoptosis. Indeed, functional analyses have revealed that changes in mitochondrial membrane are a critical event for the apoptotic process.

2.7.2. Mechanisms of apoptosis

2.7.2.1. Mitochondria and apoptosis

Functional and genetic manipulations indicate that mitochondrial transmembrane potential ($\Delta\Psi_m$) disruption and subsequent nuclear apoptosis are linked. Thus, common signs of apoptosis are invariably preceded by $\Delta\Psi_m$ collapse. The $\Delta\Psi_m$ results from the asymmetric distribution of proton and other ions on both sides of the inner mitochondrial membrane, giving rise to a chemical (pH) and electric gradient which is essential for mitochondrial function (Attardi and Schatz, 1988). Cells induced to undergo apoptosis show a decrease in the $\Delta\Psi_m$, which is a common event of the apoptotic cascade (Zamzami et al., 1995a; Zamzami et al.,

1995b; Castedo et al., 1996). This $\Delta\Psi_m$ disruption can be detected in many cell types, irrespective of the apoptosis-inducing stimulus. Purified cells with a low $\Delta\Psi_m$ rapidly proceed to DNA fragmentation, even after withdrawal of the apoptosis-inducing stimulus, indicating that $\Delta\Psi_m$ collapse marks a point-of-no-return of all death (Zamzami et al., 1995b). Thus, complete $\Delta\Psi_m$ disruption appears to be a specific marker of apoptosis since it is only found in cells that are condemned to death.

The $\Delta\Psi_m$ collapse is caused by the opening of the mitochondrial permeability transition (PT) pores. PT involves a sudden permeability increase of the inner mitochondrial membrane to solutes less or equal to 1500 Da (proton, calcium, glutathione etc.). Speculatively, periodic reversible PT pore opening might allow for the release of calcium from the mitochondrial matrix (Zoratti and Szabo, 1995; Bernardi and Petronilli, 1996). Massive PT culminates in cell death. PT also causes uncoupling of the respiratory chain with collapse of $\Delta\Psi_m$ and cessation of ATP synthesis, matrix Ca^{2+} outflow, depletion of reduced glutathione, depletion of $NAD(P)H_2$ (Zoratti and Szabo, 1995; Bernardi and Petronilli, 1996), hypergeneration of superoxide anion (Zamzami et al., 1995a) and mitochondrial release of an apoptogenic protein (Zamzami et al., 1996). Several of the consequences of PT themselves favor PT pore opening, implying that PT is a self-amplifying process. The exact composition of PT is elusive. Recent data suggest that PT pores are multiprotein complexes formed at the contact sites between the mitochondrial inner and out membrane, and whose opening/closing characteristics are influenced by multiple parameters. Induction of PT suffices to provoke the entire panel of apoptosis-associated metabolic changes, including activation of caspases with caspase 3-like DEVDase activity, redox disequilibria, nuclear DNA fragmentation and phosphatidylserine (PS) exposure on the cell surface (Kroemer et al., 1997). Overall, the PT mediated pre-

apoptotic $\Delta\Psi_m$ collapse constitutes an early and irreversible feature of the apoptotic effector phase. In addition, PT inhibitors can prevent all features of apoptosis, suggesting that PT functions as a central rate-limiting event of the apoptotic cascade. In summary, PT is sufficient to trigger the apoptotic cascade, and inhibition of PT prevents apoptosis, suggesting that PT is a central coordinating event of the apoptotic effector phase. The various "private" pathways of apoptosis converge at the level of PT, in line with the fact that PT can be induced by numerous physiological effectors. Once PT has been triggered, a series of common pathways of apoptosis are initiated, each of which may be sufficient to destroy the cell. Thus, PT causes at least three common, yet dissociable hallmarks of the apoptotic degradation phase: (1) Exposure of PS residues on the cell surface (Castedo et al., 1996); (2) changes in the cytoplasmic redox state, such as depletion of reduced glutathione and enhanced generation of reactive oxygen species (ROS) (Zamzami et al., 1995a); and (3) nuclear apoptosis (Zamzami et al., 1996).

Furthermore, most of the data obtained so far from cell free systems (Zamzami et., 1996; Newmeyer et al., 1994; Susin et al., 1996) are compatible with a two-step model of the apoptotic process. In the first step, different inducers act on mitochondria to induce PT, thereby causing $\Delta\Psi_m$ disruption and release of apoptogenic proteins. Such proteins cause normal, purified nuclei to undergo apoptotic changes *in vitro* in the second step of the assay. Two apoptogenic factors have been identified, namely AIF (apoptosis-inducing factor) and cytochrome c. AIF, a protease of approximately 50 kDa, which is sufficient to cleave and activate caspase-3 and to activate nuclear endonuclease *in vitro*. In contrast, cytochrome c requires two additional cytoplasmic proteins (see below) to induce proteolytic activation of caspase-3 (CPP32) and nuclear apoptosis (Liu et al., 1996; Yang et al., 1997; Kluck et al., 1997; Zou et al., 1997; Li et al., 1997). Translocation of cytochrome c from mitochondria to cytosol has been shown to be a crucial step in the activation of the programmed cell death

machinery in various death models. In our study we have concentrated on the role of the cytochrome c-caspase-activating pathway in motoneurons and other types of embryonic neurons from mice.

2.7.2.2. A trigger in apoptosis: Cytochrome c release from mitochondria

Cytochrome c is an essential component of the mitochondrial respiratory chain. It is synthesized from two inactive precursor molecules: apocytochrome c and heme. Cytochrome c is encoded by a single copy nuclear gene (Evans and Scarpulla, 1988) in the nucleus, which is translated on cytoplasmic ribosomes as apo-cytochrome c, and undergoes transport across the outer membrane of mitochondria, where it combines with heme group (which is synthesized in mitochondria) of cytochrome c to become the mature protein, fully folded holocytochrome c (Gonzales and Neupert, 1990). The holocytochrome c is a soluble protein that resides within the intermembrane space between the outer and inner membrane of mitochondria (Gonzales and Neupert, 1990), where it snuggles up to the cytochrome c oxidase complex located in the inner membrane. How apo-cytochrome c crosses the outer membrane of mitochondria is enigmatic, thus providing no clues as to how holocytochrome c might escape. One model for how cytochrome c escapes from mitochondria envisions local outer membrane rupture as the culprit, occuring as a secondary consequence of PT pore opening. A second model predicts a specific channel located in the outer membrane that allows release of cytochrome c. A good candidate was identified in Bax, a pro-apoptotic member of the Bcl-2 family. There might be a direct role for this proapoptotic protein in controlling cytochrome c transport.

In many types of cells, loss of cytochrome c from mitochondria has two ways of killing cells: (1) Cessation of mitochondrial electron chain transport with subsequent ATP depletion,

generation of ROS, and related sequela; (2) activation of caspases through Apaf-1. How does cytochome c induce caspase activation once it has been liberated from mitochondria into the cytosol? Recent reports (Zou et al., 1997; Li et al., 1997) show that two isolated cytosolic proteins collaborate with cytochrome c to induce proteolytic processing and activation of caspase-3 (CPP32), a major caspase activation in cells undergoing apoptosis (Faleiro et al., 1997; Martins et al., 1997; Takahashi et al., 1997). In brief, the model of a mechanism for caspase-3 activation begins when caspase-9 (Apaf-3) binds to Apaf-1 (CED-4) in a reaction triggered by cytochrome c (Apaf-2) and dATP. This complex formation between Apaf-1 (CED-4) and caspase-9 (Apaf-3) is mediated by caspase recruitment domain (CARD) on the two proteins. Binding leads to the cleavage of caspase-9, converting it to an active protease. Active caspase-9 (Apaf-3) then cleaves and activates caspase-3 (CPP32), thereby setting in motion the events that lead to DNA fragmentation and cell death. In this model, cytochrome c initiates apoptosis by inducing the formation of the Apaf-1 (CDE-4)/caspase-9 (Apaf-3) complex. Thus, cytochrome c release from mitochondria is a critical step in the apoptotic process in this system. Put together, the elucidation of the correlation between the time points of cytochrome c expression as well as release and the fate of mouse motoneuron survival during development *in vitro* and *in vivo* was one of the goals of our study.

2.7.2.3. The Bcl-2 protein family and apoptosis

Members of the Bcl-2 family can potentially modulate cell death through multiple mechanisms, including caspase-independent effects on mitochondria and regulation of Apaf-1/CED-4-family proteins. Recent discovery demonstrated that in cells overexpressing Bcl-2, or its close family member Bcl-X_L, the release of cytochrome c is blocked, aborting the apoptotic response (Kharbanda et al., 1997; Kim et al., 1997; Kluck et al., 1997; Yang et al., 1997). At least three

general functions for Bcl-2 and some of its anti-apoptotic homologues such as Bcl-X_L have been identified: (1) Dimerization with other Bcl-2 family proteins such as Bax; (2) binding to non-homologous proteins such as Raf kinases (see 2.7.3.); (3) formation of ion-channels/pores (Vaux and Strasser, 1996; Reed, 1997a,b; Green and Reed, 1998; Schendel et al., 1998; Zamzami et al., 1998). Therefore, these data support a model in which Bcl-2, located on the outer membrane of mitochondria, prevents the release of cytochrome c from mitochondria by acting on upstream of this event.

The mechanism by which Bcl-2 blocks the release of proteins from mitochondria and the regulation of the process are still obscure. As mentioned above, mitochondrial depolarization in apoptosis appears to be associated with the PT and events involving the formation of a nonspecific channel in the mitochondrial membrane (Zoratti and Szabo, 1995). Therefore, speculatively Bcl-2 protects mitochondria from these effects (Marchetti et al., 1996; Zamzami et al., 1996; Pastorino et al., 1996). Firstly, mitochondria purified from cells hyperexpressing Bcl-2 are protected against PT induction by several apoptosis inducers, such as pro-oxidants, low doses of Ca^{2+}, the adenosine nucleotide translocation (ANT) ligand atractyloside, protoporphyrin IX and the cytosol of ceramide-treated cells. However, Bcl-2 does not counteract the PT-inducing effect of agents such as high doses of Ca^{2+} (500 µM), the thiol-crosslinking agent diamide and recombinant caspase-1 (ICE) (Zamzami et al., 1996; Susin et al., 1996). Thus, Bcl-2 exerts a direct membrane-stabilizing effect on mitochondria, but with limited spectrum, that can lead to inhibit PT and the release of apoptogenic proteins from mitochondria. Secondly, Bcl-X_L is structurally related to certain bacterial pore-forming proteins (Muchmore et al., 1996), and Bcl-2 probably has a similar structure (Vance et al., 1996). Therefore, Bcl-2 could conceivably block cytochrome c efflux directly; Or, Bcl-2 could prevent this event indirectly by regulating the flow of ions, including Ca^{2+} across the

mitochondrial and endoplasmic reticulum (ER) membranes (Batty et al., 1993; Lam et al., 1994; Zörnig et al., 1995; Distelhorst et al., 1996; Marin et al., 1996; Murphy et al., 1996). Relevantly, the mechanism by which Bcl-2 protein protects motoneuron cell death was also studied in the context of this work by using *bcl-2* deficient mice generated in our group.

2.7.2.4. The downstream mediators of apoptosis: Caspases and their inhibitors

As mentioned above, apoptosis-related proteolysis is largely achieved by a family of at least 14 cysteine proteases which have recently been named caspases. They are cysteine proteases and specifically cleave proteins after Asp residues, hence "caspase" (Alnemri et al., 1996). Caspases appear to be essential components of the apoptotic death machinery. This downstream event of the apoptotic process finally leads to morphologically detectable features of cell death. Caspases operate by cleaving cytoskeletal and nuclear proteins which are critical for maintenance of cell structure (e.g. β-actin, lamin B) (Lazebnik et al., 1995; Mashima et al., 1995; Kayalar et al., 1996), enzymes involved in metabolism (e.g. protein kinase C δ) (Emoto et al., 1995) and repair enzymes such as poly(ADP-ribose) polymerase (PARP) (Lazebnik et al., 1994).

Caspases are related to CED-3, the product of a gene essential for PCD in the nematode *Caenorhabditis elegans* (Ellis and Horvitz, 1986). ICE was the first mammalian homologue of CED-3 which was discovered (Yuan et al., 1993). Since then a number of other mammalian genes with ICE/CED-3 sequence homology have been identified. Inactive proform of caspase requires proteolytic processing to generate the two subunits that form the active enzyme. For instance, caspase-3, a 32 KDa precursor is sequentially cleaved after IETD and ESMD sites to

produce 17 KDa (p17) and 12 KDa (p12) subunits which form the mature enzyme (Han et al., 1997).

With regard to the activation of caspases, the substrate specificities of caspases suggest a cascade of activities and different functional roles: Upstream caspases play a role as initiators of the cascade, whereas downstream caspases (effector caspases) operate to dismantle the cell finally. Importantly, so far there are at least three possible ways in which caspases heve been shown to be activated: (1) As a result of cleavage by upstream proteases; (2) through death-inducing receptors of the TNFR/NGFR family. These receptors contain a death domain (DD) which couples them via adaptor molecules to caspases. For instance, engagement of the Fas receptor and activation of the associated DD cause recruitment of caspase 8 to the complex, then caspase 8 is activated (Medema et al., 1997); (3) translocation of cytochrome c from mitochondria to cytoplasm. This event may be a common signal for apoptosis in non-receptor-mediated pathways (Kluck, et al., 1997; Yang et al., 1997).

On the other hand, protein inhibitors of caspases, in particular the *iap* family of anti-apoptotic genes have been discovered. Besides the Bcl-2 family of proteins, the family of inhibitors of apoptosis (IAPs) is receiving increased attention in the apoptosis field. IAPs were originally identified in baculoviruses and are now known to exist in various organisms as diverse as insects and humans. The anti-apoptotic baculovirus protein p35 is a potent inhibitor of caspases 1~4 (Nicholson and Thornberry, 1997; Bump et al., 1995). The mammalian cell death suppressors also have viral homologues. In addition, human X-chromosome-linked IAP (XIAP) has recently been found to be a direct inhibitor of caspases 3 and 7 (Deveraux et al., 1997). In spinal muscular atrophy patients, a disease which is characterized by spinal cord motoneuron depletion, one of the responsible genes which is called NAIP (neuronal apoptotic

inhibitory protein) (Roy et al., 1995) has sequence similarity to p35 and IAPs (Liston et al., 1996). Current studies showed that although IAPs do not bind or inhibit caspase 8, they do bind to and inhibit its substrate caspase 3, thus arresting the cascade of proteolysis and providing protection from Fas/caspase 8-induced apoptosis (Deveraux et al., 1997, 1998; Roy et al., 1997). In contrast, in the mitochondrial pathway for caspase activation, XIAP, cIAP-1, and cIAP-2 bind directly to the upstream caspase pro-caspase 9, and prevent its processing and activation by cytochrome c, both in intact cells and in cell extracts where caspase activation is induced by addition of exogenous cytochrome c (Deveraux et al., 1998). Recently, a novel gene, *ita* (inhibitor of T cell apoptosis), which is a avian homologue of the viral and mammalian apoptosis inhibitor IAP has been isolated (Digby et al., 1996). In our pilot investigation we found that ITA can prevent apoptosis by inhibition of specific caspases, and induction of ITA expression seems to be an essential signalling event for survival of sympathetic and dorsal root ganglionic sensory neurons in response to NGF (Wiese et al., 1999a). At present, the inhibitors of apoptosis are important target genes which regulate motoneuron survival. These mechanisms were also a target of investigation in the present study.

2.7.3. Activation of Raf kinases and apoptosis

The correlation between activation of Raf kinases and apoptosis on motoneurons is a major focus of our study. Considerable evidence exists that the Raf-MAPK pathway can influence apoptosis, either positively or negatively. The effects of regulation of apoptosis by Raf appear to be complex, less well understood and highly dependent on cell type. In fibroblasts and T lymphocytes, Raf is probably responsible for the apoptosis-promoting effects of Ras but it has also been found to protect from apoptosis in other cell types under some circumstances

(Downward, 1998), possibly depending on its degree of activation. The best characterized case is in neuronal cells, particularly the pheochromocytoma PC12 cells in which the activation of the Raf pathway protects cells from neurotrophic factor withdrawal induced death. Downstream from Raf, a variety of signalling pathways influence survival or apoptosis. The JNK pathway promotes death in PC12 cells (Xia et al., 1995). It has been assumed for a long time, that prolonged activation of the MAPK pathway is necessary for survival of PC12 cells after growth factor withdrawal. This view, however has been challenged by observations that the specific MEK inhibitor, PD98059 does not enhance cell death when added to cultures of NGF stimulated PC12 cells. Light into this complex scenario has been shed by observations that v-Raf can protect myeloid cells from apoptosis (Cleveland et al., 1994) by formation of direct complexes between Raf and Bcl-2 (Wang et al., 1994). The Bcl-2 family of proteins is composed of a large group of anti-apoptotic members and some pro-apoptotic members. The various members of the Bcl-2 family share homology to Bcl-2 in one or more of the Bcl-2 homology regions designated BH1, BH2, BH3, and BH4. Many of the family members have a carboxyl-terminal mitochondrial membrane targeting sequence (Kroemer, 1997). The targeting of c-Raf-1 to the mitochondria, where Bcl-2 is located, results in the protection of these cells from apoptosis, probably by the phosphorylation of Bad, a pro-apoptotic member of the Bcl-2 family (Wang et al., 1996a). Interaction of Bad with Bcl-2 and Bcl-X_L prevents the anti-apoptotic function of these molecules. After phosphorylation of Bad by Raf, the interaction with Bcl-2 and Bcl-X_L is hindered and Bad is released into the cytoplasm of the cell. The loss of the death-promoting function of Bad after phosphorylation by Raf probably involves the formation of a complex with 14-3-3 (Zha et al., 1996; Del Peso et al., 1997; Datta et al., 1997). These data suggest that Raf activation and phosphorylation of Bad could constitute a key mechanism in cell death prevention by neurotrophic factors. Bcl-2 can trap at least one

member of the Raf family to mitochondria and thus suppress apoptosis by a MAPK-independent mechanism.

To analyze this hypothesis in a physiological context, mice with specific deletion of the three Raf kinases have been generated. *A-raf* deficiency leads to post-natal lethality and neurological and intestinal abnormalities in mice (Pritchard et al., 1996). *C-raf-1* mutant mice show developmental defects displaying generalized growth retardation and death around embryonic day 13 (Wojnowski et al., 1998). Importantly, unlike *A-raf* or *c-raf-1* knockout mice, *B-raf* deficient mice die during embryogenesis with specific vascular defects caused in part by enhanced apoptosis of differentiated endothelial cells (Wojnowski et al., 1997). Although this result provides the first genetic evidence for an essential role of *B-raf* gene in the regulation of programmed cell death in particular types of cells, it is unclear whether the apoptotic death of endothelial cells is caused by defects in endothelial growth factor signalling through the Ras/Raf/MEK/MAPK cascade, or is related to the recently discovered interaction of Raf kinases with proteins of the Bcl-2 family at mitochondrial membranes (Wang et al., 1996a). Current attempts focus on the question which specific signalling pathways are responsible for cell death in *B-raf* and *c-raf-1* deficient mice.

2.8. The objective of this study

In order to investigate whether the cell type specific expression of different isoforms of Raf kinases contributes to the diversity of cellular reactions induced by ciliary neurotrophic factor (CNTF) and other neurotrophic factors, this project concentrated on mouse motoneurons which are supported during development and after birth by CNTF (Sendtner, et al., 1994) and other neurotrophic factors (Wiese et al., 1999b). We tried to elucidate the signalling functions

of the different Raf kinases in motoneurons at various stages of development. Therefore, in this study, the expression, distribution and subcellular localization of Rafs in the spinal cord and the facial nucleus in brainstem of mice at different embryonic and postnatal stages were investigated. Specific antibodies against A-Raf, B-Raf and c-Raf-1 kinases were used for immunohistochemistry and Western blot analysis. Moreover, we have investigated the intracellular redistribution of Raf molecules in isolated motoneurons from 13 or 14 day old mouse embryos, after addition or withdrawal of neurotrophic factors. These *in vitro* experiments were designed to induce Raf kinase activation in cultured motoneurons. In addition, on the basis of a previous report that Bcl-2 can target c-Raf-1 to mitochondrial membranes allowing this kinase to phosphorylate Bad (Wang, et al., 1996a), we determined the subcellular distribution of Rafs in motoneurons from *bcl-2*$^{-/-}$ mice. Furthermore, in order to investigate the potential anti-apoptotic function of Raf kinases on motoneurons, we isolated motoneurons from *B-raf*$^{-/-}$ and *c-raf-1*$^{-/-}$ mouse embryos and analysed the survival and differentiation effects of neurotrophic factors in motoneurons lacking B-Raf and c-Raf-1.

2.9. Brief summary of the results

We provide evidence that all three Raf kinases are expressed in mouse spinal cord. Their expression increases during the period of naturally occurring cell death of motoneurons. In sections of embryonic and postnatal spinal cord, motoneurons express exclusively B-Raf and c-Raf-1, but not A-Raf which seems confined to glial cells. Raf kinases are obviously colocalized with mitochondria in motoneurons. In isolated motoneurons, surprisingly, most of the B-Raf or c-Raf-1 immunoreactivity is located in the perinuclear space but also in the nucleus, especially after activation by addition of CNTF and BDNF *in vitro*. We also found that c-Raf-1

translocation from the cytosol into the nucleus of motoneurons after addition of neurotrophic factors is a distinct event.

As a central finding of our study, we observed that the viability of isolated motoneurons from *B-raf* but not *c-raf-1* knockout mice is lost even in the presence of CNTF and other neurotrophic factors. This indicates that B-Raf but not c-Raf-1, which is still present in *B-raf* deficient motoneurons, plays a crucial role in mediating the survival effects of neurotrophic factors during development. In order to prove that B-Raf is an essential player in this scenario, we have re-expressed B-Raf in mutant sensory and motor neurons by transfection. The motoneurons and the sensory neurons from *B-raf* knockout mice which were transfected with exogenous *B-raf* gene revealed the same viability in the presence of neurotrophic factors as primary neurons from wild-type mice.

Our results suggest that Raf kinases have important signalling functions in motoneurons in mouse CNS. *In vitro*, activation causes redistribution of Raf protein kinases, particularly for c-Raf-1, from motoneuronal cytoplasm into the nucleus. This redistribution of c-Raf-1, however, is not necessary for the survival effect of neurotrophic factors, given that *B-raf*$^{-/-}$ motor and sensory neurons can not survive despite the presence of c-Raf-1. We hypothesize that c-Raf-1 nuclear translocation may play a direct role in transcriptional regulation as a consequence of neurotrophic factor induced phosphorylation and activation of c-Raf-1 in motoneurons. Moreover, the identification of target genes for nuclear translocated c-Raf-1 and of specific cellular functions initiated by this mechanism awaits its characterization.

3. Materials and Methods

3.1. Experimental animals and tissue preparation

Wild-type embryonic and postnatal mice (C57BL/6), ranging from E13 to adult, *B-raf* and *c-raf-1* gene mutant mice (E12.5~E13), and *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ adult mice were uesd in this study.

Adult mice (C57BL/6 and *bcl-2* mutants) were fixed by transcardial perfusion under deep ether anaesthesia via the ascending aorta. Following a short rinse with 0.1 M phosphate-buffered saline (PBS, pH 7.4) to remove circulating blood, fixation was carried out with 4% paraformaldehyde in PBS buffer, pH 7.3, for 10 min. After perfusion, brains and lumbar parts of spinal cords were removed, postfixed with the same fixative for 1~2 h at 4 °C. Thereafter, the tissue samples were washed with PBS and infiltrated extensively with 10% sucrose in PBS, pH 7.4, over night at 4 °C. Then the brain and spinal cord tissue specimens were transferred to 20% sucrose in PBS and maintained in the solution till they sunk down to the bottom. After successive dehydration, the tissue samples were frozen on dry ice, and stored at -80 °C. For newborn mice, the manipulation steps were the same as adult mice except for the use of a minipump and omission of PBS for the pre-wash step. For E13 (e.g. *B-raf* mutant mice) and E14 mice (e.g. *c-raf-1* mutatnat mice), the heads and spines were removed, the tissues fixed with 4% paraformaldehyde in PBS over night at 4 °C following by the same procedure as for adult mice.

3.2. Antibodies

Primary antibodies and antisera used for Western blotting included: (a) polyclonal rabbit anti-A-Raf, B-Raf and c-Raf-1 (1:1000; generous gift from Prof. U. Rapp, MSZ, Würzburg University, Germany); (b) monoclonal mouse anti-cytochrome c antibody (1:2000; Pharmingen, USA); (c) monoclonal mouse anti-actin antibody (1:1000; Boehringer Mannheim, Germany). Secondary antibodies used included horseradish peroxidase conjugated goat anti-rabbit IgG antibody (1:5000; Dianova, Hamburg, Germany) and horseradish peroxidase conjugated goat anti-mouse IgG antibody (1:5000; Dianova, Hamburg, Germany).

Primary antibodies and antisera employed for immunochemistry and immunocytochemstry included: (a) polyclonal rabbit anti-A-Raf, B-Raf and c-Raf-1 (1:250~1000; generous gift from Prof. U. Rapp, MSZ, Würzburg University, Germany); (b) monoclonal mouse anti-cytochrome c antibody (1:400; Pharmingen, USA); (c) monoclonal mouse anti-cytochrome c oxidase subunit 1 A-6403 (1:20; Molecular Probes, Inc., USA); (d) monoclonal mouse anti-neurofilament (1:100; Boehringer Mannheim; Germany); (e) monoclonal mouse anti-neurofilament 200 (10 ng/ml, Sigma, USA); (f) polyclonal rabbit anti-mouse $p75^{NTR}$ (1:500; Chemicon, Germany); (g) rat anti-$P75^{NTR}$ (1:100; Chemicon, Hofheim, Germany); (h) monoclonal 39.4D5 antibody against Islet-1 (supernatant of the hybridoma cells diluted 1:1; Developmental Studies Hybridoma Bank, Iowa, USA); (i) monoclonal mouse anti-Islet-1 (10 ng/ml) ascites (DSHB, Iowa, USA); (j) monoclonal mouse anti-HA (10 ng/ml; Boehringer, Mannheim, Germany); (k) polyclonal rabbit antiserum against ITA (1:1000; Digby et al., 1996). Secondary antibodies uesd included Cy3 coupled goat anti-rabbit IgG antibody (1:400; Dianova, Hamburg, Germany), Cy3 conjugated goat anti-mouse IgG antibody (1:400; Rockland, USA), Cy2 coupled goat anti-rabbit IgG (1:100; Rockland, USA), Cy2 coupled

goat anti-mouse IgG (2 ng/ml; Biotrend, Cologne, Germany), Cy2 goat anti-rat IgG (2 ng/ml; Biotrend, Cologne, Germany), biotinlated sheep anti-rabbit IgG (1:500; Boehringer Mannheim, Germany) combined with Cy3 coupled Streptavidin (1:400; Rockland, USA), FITC conjugated sheep anti-mouse IgG (1.100; Boehringer Mannheim, Germany).

3.3. Western blotting

For the Western blot analysis, protein samples were obtained from mice at different time points of embryonic and postnatal development. Spinal cord extracts of mice, and extracts from cultured mouse motoneurons derived from 14 day old embryos, in which the motoneurons were supported by BDNF or without any trophic factor treatment during the culture, were used. After lysis (pH 7.0, 5 mM HNa_2PO_4, 30 mM NaCl, 0.1% SDS), protein samples (50 µg in each lane) were equally loaded onto a 7% (Raf protein kinase detection) or 15% (cytochrome c detection) sodium dodecyl sulphate-polyacrymide gel electrophoresis (SDS-PAGE) gel. Equal quantities of protein samples were verified by staining of the nitrocellulose with Ponceau red and by actin immunodetection with a monoclonal mouse anti-actin antibody (1:1000; Boehringer Mannheim, Germany) on the same nitrocellulose filters after stripping. After appropriate separation, the proteins were electrophoretically transferred onto nitrocellulose filters with a semidry blotter (Biometra, Germany). Non-specific sites on the filters were blocked with 3% non-fat dry milk in TBS-T buffer (50 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.2% Triton-X 100). The filters were incubated with primary antibodies: polyclonal rabbit anti-A-Raf, B-Raf and c-Raf-1 (dilution 1:1000, generous gift from Prof. U. Rapp, MSZ, Würzburg University, Germany) or monoclonal mouse anti-cytochrome c antibody (1:2000; Pharmingen, USA) prepared in TBS-T buffer plus 3% non-fat dry milk. Then horseradish peroxidase conjugated goat secondary anti-rabbit IgG antibody (1:5000;

Dianova, Hamburg, Germany) or horseradish peroxidase conjugated goat anti-mouse IgG antibody (1:5000; Dianova, Hamburg, Germany) were added which were also diluted in blocking solution. Signals on the filter were revealed by chemiluminescence (ECL) according to the manufacturer's instruction (Amersham Life Technologies, Germany). The blots were exposed to film for variable lengths of time (30 sec~2 min) depending upon the intensity of the signal. For antibody stripping, nitrocellulose membranes were incubated with stripping buffer (62.5 mM Tris-HCl, pH 6.0, 2% SDS, 100 mM 2-mercaptoethanol) at 70 °C for 1 h, rinsed three times (30 min each) in TBS-T buffer solution at room temperature and then for 10 min in TBS-T before a new round of detection.

3.4. Raf single, Raf and mitochondrial marker double immunofluorescence *in vivo* and immunofluorescence confocal microscopy

Fourteen µm coronal sections from the facial nucleus region of the brainstem and the lumbar part of spinal cord were prepared with a cryostat microtome (Leica CM1900, Germany). All immunostaining procedures were carried out at room temperature. After three rinses in TGT buffer (pH 7.8, 40 mM Tris-HCl, 0.7% NaCl, 0.04% KH_2PO_4, 0.15% Na_2HPO_4, 0.1% gelatine, 0.2% Triton-X 100), sections were pre-blocked for non-specific binding sites by incubation with 10% goat serum (DAKO, Denmark) in TGT buffer for 30 min, then followed by incubation with the primary polyclonal rabbit anti A-Raf, B-Raf and c-Raf-1 antibodies (dilution 1:250~1000, generous gift from Prof. U. Rapp, MSZ, Würzburg University, Germany) for 1~2 h. The sections were washed three times (10 min each) with TGT buffer, and incubated again with the blocking solution for 20 min followed by incubation with Cy3 coupled secondary goat anti-rabbit lgG antibody (1:400; Dianova, Hamburg, Germany) for 30

min. The sections were then washed with TGT buffer three times for 30 min, coverslipped with PBS containing 50% glycerol (v/v) and sealed with nail polish.

Respectively, the A-Raf, B-Raf or c-Raf-1 double labelling with the mitochondrial marker cytochrome c oxidase subunit I A-6403 (mouse monoclonal, 1:20; Molecular Probes, Inc., USA) was performed according to the procedure described above, except that the sections were simultaneously incubated with different anti-Raf- and anti-cytochrome c oxidase subunit I A-6403 antibodies for 1~2 h. Then, a combination of two different fluorescence dye-labelled secondary antibodies was employed: Cy2 (green) coupled goat anti-rabbit IgG (1:100; Rockland, USA) and Cy3 (red) conjugated goat anti-mouse IgG (1:400; Rockland, USA). When the immunosignals for Raf and A-6403 mitochondrial marker overlapped in the same motoneurons, the colour of the staining became orange.

Immunofluorescence detection was carried out with a Leica fluorescence and confocal laser scanning microscope (TCS, Leica, Heidelberg, Germany) using single and double barrier filters. Images were photographed with the Imagecorder Plus (version 2.03, Focus Graphics, Belmont CA, USA).

3.5. Cytochrome c immunofluorescence

In a separate study, we have investigated cytochrome c distribution in motoneurons of *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ adult mice by immunohistofluorescence. The primary antibody employed was a monoclonal mouse anti-cytochrome c antibody (1:400; Pharmingen, USA). The procedure for immunostaining and immunofluorescence confocal microscopy was the same as described above (**3.4.**).

3.6. Preadsorption controls for immunofluorescence staining

Preadsorption controls were performed with A-Raf, B-Raf and c-Raf-1 peptides (kind gift from Dr. J. Troppmair, MSZ, Würzburg University, Germany), respectively. 12.5 µl peptid solution (10 µg/µl; the final concentration was 5 µl peptid in 200 µl) was mixed with each Raf antiserum (2 µl undiluted antiserum). The mixtures were diluted to working concentration of the antisera (1:250) in a total volume of 500 µl containing 10% goat serum. These solutions were gently rotated at 4 °C over night and followed by a ultracentrifugation at 100,000 × g for 4 h at 4 °C and then the supernants used for immunofluorescence staining. Other controls were performed by incubation of the sections with 10% goat serum in the absence of the primary antibodies.

3.7. Mouse motoneuron cultures and induction of Raf activation in mouse embryonic motoneurons *in vitro*

The motoneuron cultures from mouse embryos were prepared as described (Wiese et al., 1999b). In brief, spinal motoneurons from embryonic day 13 or 14 mice were prepared by a metrizamide cushion centrifugation technique. Adult pregnant mice were sacrificed by deep ether anesthesia and cervical dislocation and the embryos were isolated from the uterus. The ventrolateral parts of the lumbar spinal cords were dissected and transferred to Hank's balanced salt solution (HBSS) (Gibco Life Technologies, Eggenstein, Germany) containing 10 µM 2-Mercaptoethanol. After treatment with trypsin (0.05%, 10 min; Gibco Life Technologies, Eggenstein, Germany), tissues were triturated and the cell suspension passed through a nylon mesh (100 µm pore size). The cells were overlaid on 10% metrizamide in HBSS. The metrizamide cushion was centrifuged for 20 min at 400 g, and cells from the

interphase were taken out, transferred to culture medium without apotransferrin and centrifuged again (400 g, 5 min). Cells were plated at a density of 2000 cells/cm^2 in 4-well culture dishes (Greiner, Nürtingen, Germany), precoated with polyornithine and laminin (Arakawa et al., 1990). Cells were grown in neurobasal medium (Gibco Life Technologies, Eggenstein, Germany) with serum and 500 µM glutamax and 50 µg/ml apotransferrin at 37 °C in a 5% CO_2 atmosphere. Fifty per cent of the medium was replaced at day 1 and then every second day.

Mouse motoneurons (control Balb/C and $p75^{NTR-/-}$) were cultured in the presence of CNTF and BDNF (10 ng/ml each) for 1 or 5 days. To investigate motoneuronal survival and to characterize the cultured motoneurons, the cells were fixed with 4% paraformaldehyde in Tris-buffered saline (TBS) and washed three times with TBS (25 mM Tris-HCl pH 7.4, 0.8% NaCl, 0.2% KCl), 10% goat serum and 0.1% Tween-20. Cells were immunostained for 1 hour with mouse anti-Islet-1 (10 ng/ml) ascites (DSHB, Iowa, USA), rat anti-$P75^{NTR}$ (1:100; Chemicon, Hofheim, Germany) or mouse anti-neurofilament 200 (10 ng/ml; Sigma, USA) antibodies. Controls were performed by omission of the first antibody. Cells were washed three times with TBS containing 10% goat serum and incubated for 30 min with 2 ng/ml Cy2 coupled goat anti-mouse, or Cy2 goat anti-rat antibodies. Cells were washed again three times with TBS and 10% goat serum, then coverslipped with PBS containing 50% glycerol (v/v), observed and photographed under a confocal laser scanning fluorescence microscope (TCS, Leica, Heidelberg, Germany).

The Raf serine/threonine kinase activation was induced by exposure to a variety of mitogens, including growth factors, known to initiate tyrosine, serine, and threonine phosphorylation (Rapp, 1991). Therefore, for subsequent Raf kinases activation experiments, the isolated

motoneurons from E13 and E14 mouse spinal cords, respectively, were cultured with both CNTF and BDNF (10 ng/ml each). In this experiment, the motoneuron cultures were divided into four groups: 6 h culture without neurotrophic factor treatment, 6 h culture with CNTF and BDNF treatment, 3 min treatment with both same factors after 6 h culture, and 4 day culture with CNTF and BDNF treatment. After these treatments, the cultured motoneurons were fixed with 4% paraformaldehyde in PBS, pH 7.3 immediately for 10 min at room temperature, then processed for immunodetection.

3.8. Immunostaining of cultured motoneurons: Detection of members of the Raf family

For the immunostaining purpose, motoneurons were plated onto glass coverslips for 6 h growing during the culture. Mouse motoneurons were then fixed with 4% paraformaldehyde in PBS, pH 7.3 for 10 min at room temperature and washed three times (3 min each) with TGT buffer. After pre-blocking by 10% goat serum (DAKO, Denmark) for 20 min, the immunostaining of the motoneurons was performed by incubation with primary rabbit anti-A-Raf, B-Raf and c-Raf-1 antibodies (1:250; Prof. U. Rapp, MSZ, Würzburg University, Germany) which diluted in TGT containing 10% goat serum over night at 4 °C or for 1 h at room temperature. Controls were performed in the absence of the primary antibodies as well as by pre-adsorption technique as described above. The motoneurons were washed three times with TGT followed by blocking with 10% goat serum for 5 min, then incubated for 30 min with biotinlated anti-rabbit IgG (1:500; Boehringer Mannheim, Germany). The motoneurons were washed again three times with TGT, then non-specific binding sites blocked again with 10% goat serum for 5 min. Afterwards the motoneurons were incubated with Cy3 coupled Streptavidin (1:400; Rockland, USA) for 30 min. After final wash steps with TGT, the glass coverslips on which the motoneurons were grown were turned over and mounted with PBS

containing 50% glycerol (v/v) and analyzed under a confocal laser scanning microscope as described above.

3.9. Determination of mouse motoneuron survival in wild-type and E13 *B-raf*$^{+/+}$ and *B-raf* $^{-/-}$ mice

Embryonic day 14 mouse motoneurons were isolated from wild-type mice. Embryonic day 12.5 or 13 mouse embryos were obtained from *B-raf* heterozygous intercrosses. Most *B-raf*$^{-/-}$ embryos died in the uterus between embryonic day 13 and 14 after fertilization. The motoneurons from wild-type embryos or single *B-raf* mutant embryos were isolated and plated in Greiner 4-well culture dishes at a density of 2000 cells/cm^2. The neurotrophic factors BDNF (1 ng/µl), CNTF (1 ng/µl) and GDNF (1 ng/µl) were added. Culture medium was changed and neurotrophic factors freshly added on day 1 and 3. Initial counting of plated cells was carried out when all cells were attached to the bottom of the well after 4 h in culture. Phase bright cells were counted at day 1 and day 3. A total of 10 fields (1.16 mm^2/field) was counted in each well at each time point. In this study, we could not observe any *B-raf*$^{-/-}$ mouse motoneuron survival after 24 h in culture.

3.10. Immunodoublelabelling of cultured E12.5 *B-raf* mutant motoneurons with B-Raf and neurofilament

E12.5 *B-raf* mutant motoneurons (+/- and -/-) were prepared, cultured and fixed as described above. Subsequently, after 18 h culture with BDNF and CNTF (10 ng/ml each), non-specific binding sites were blocked and cell membranes permeabilized for 20 min with 10% goat serum (DAKO, Denmark) in TGT buffer containing 0.2% Triton-X100. Primary polyclonal rabbit

anti-B-Raf antibody (1:250; Prof. U. Rapp, MSZ, Würzburg University, Germany) and primary monoclonal mouse anti-neurofilament antibody (1:100; Boehringer Mannheim, Germany) were both added over night at 4 °C. The dishes were then washed twice, blocked again for 5 min. Then the motoneurons were incubated for 30 min at room temperature with the corresponding secondary antibodies: Cy3 (red) coupled goat anti-rabbit lgG (1:200; Dianova, Germany) and FITC (green) conjugated sheep anti-mouse IgG (1:100; Boehringer Mannheim, Germany). After a final wash, the motoneurons were mounted with glycerol/PBS (1:1).

3.11. Immunohistofluorescence for Rafs, $p75^{NTR}$ and Islet-1 in E13 *B-raf* and E14 *c-raf-1* mutant mouse spinal cords *in vivo*

Fourteen μm coronal sections from E13 *B-raf* and E14 *c-raf-1* mutant mouse spinal cords (+/+ and -/-) were prepared with a cryostat microtome (Leica CM1900, Germany). Primary antibodies (polyclonal rabbit anti-A-Raf, B-Raf and c-Raf-1 antibodies, 1:250, Prof. U. Rapp, MSZ, Würzburg University, Germany; polyclonal rabbit anti-mouse $p75^{NTR}$, 1:500, Chemicon, Hofheim, Germany; the supernatant of 39.4D5 hybridoma cells producing the Islet-1 antibody, DSHB, Iowa, USA, 1:1 diluted with 10% goat serum in TGT buffer) were respectively added for 1 h at room temperature except for the Islet-1 staining (over night at 4 °C). Afterwards the spinal cord sections were incubated for 30 min at room temperature with secondary antibodies: Cy3 coupled goat anti-rabbit IgG (1:200; Dianova, Germany) for both Rafs and $p75^{NTR}$, and Cy3 conjugated goat anti-mouse IgG (1:400; Rockland, USA) for Islet-1. The rest of procedures for the immunohistofluorescence were performed similarly as described above.

3.12. Calculation and statistics

Single estimation (per cent of surviving neurons relative to the number of originally plated cells in individual wells) from independent experiments were pooled and the results expressed as mean and standard error of the mean (SEM). Statistical significance of differences was assessed by ANOVA followed by Bonferoni's test using the Graph Pad Prism software (San Diego, USA). Survival curves were fitted to the Hill equation: $A * IC_{50}{}^{n} / (IC_{50}{}^{n} + X^{n}) + B$, where A is maximal survival supported by the neurotrophic factors, B is the survival independent from the neurotrophic factor, IC_{50} is the neurotrophic factor concentration at half-maximal survival and n is the Hill coefficient.

3.13. Genotyping of *p75$^{NTR-/-}$*, *B-raf$^{-/-}$* and *c-raf-1$^{-/-}$* mice

Genomic DNA from mouse tissue was isolated by standard methods. *P75$^{NTR-/-}$* mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). A PCR technique was developed for genotyping of the backcrosses with Balb/C mice (Charles River, Sulzfeld, Germany). The primers *p75ex3f* 5´GTGTTACGTTCTCTGACGTTGTG3´ and *p75ex3r* 5´TCTCATTCGGCGTCAGCCCAGGG3´ flanking the targeted exon 3 of *p75^{NTR}* resulted in a 360 bp fragment for the non-targeted allele, and the *3´neo* primer 5´GATTCGCAGCGCATCGCCTT3´ together with the *p75ex3r* primer resulted in a 570 bp fragment for the targeted allele. The PCR was carried out as a multiplex-PCR reaction with 10 pMol of the primers *3´neo* and *p75ex3f* and 20 pMol of the primer *p75fx3r*, 10 mM dNTPs, 100 ng genomic DNA and 5 U Taq-Polymerase (Perkin Elmer, Weiterstadt, Germany) in the appropriate buffer. The DNA fragments were amplified through 35 cycles of 30 sec 95 °C, 30 sec 61 °C and 90 sec 72 °C with a prior and final extension of 95 °C and 72 °C, respectively,

for 5 min each. For *B-raf*$^{-/-}$ mice genotyping, the mice were obtained from Prof. U Rapp's group (MSZ, Würzburg University, Germany). The primers *b-rafmb3l-1* 5´GCCTATGAAGAGTACACCAGCAAGCTAGATGCCC3´ and *b-rafmb-del* 5´TAGGTTTCTGTGGTGACTTGGGGTTGTTCCTGA3´ flanking the targeted exon of *b-raf* resulted in a 220 bp fragment for the non-targeted allele, and the *neo-l* primer 5´ 5´AGTGCCAGCGGGGCTGCTAAA3´ together with the *b-rafmb-del* primer resulted in a 330 bp fragment of the targeted allele. The PCR was carried out as a multiplex-PCR reaction with 10 pMol of the primers *neo-l* and *b-rafmb3l-1*, and 10 pMol of the primer *b-rafmb-del*, 1 mM dNTPs, 100 ng genomic DNA and 1 µl Taq-polymerase (Perkin Elmer, Weiterstadt, Germany) in the appropriate buffer. The DNA fragments were amplified through 35 cycles of 30 sec 95 °C, 30 sec 66 °C and 1 min 72 °C with a prior and final extension of 96 °C and 72 °C, respectively, for 5 min each. For *c-raf-1*$^{-/-}$ mice genotyping, the mice were also obtained from Prof. U Rapp's group. The primers *c-raf-1-r* 5´GGGATTCTGGGAGTTTGGGGCCAGCAGCTA3´ and *c-raf-1-2-2´* 5´CACAGTCCTTTGCTTATTCGG3´ flanking the targeted exon of *c-raf-1* resulted in a 200 bp fragment for the non-targeted allele, and the *neo-l* primer 5´ 5´AGTGCCAGCGGGGCTGCTAAA3´ together with the *c-raf-1-2-2´* primer resulted in a 300 bp fragment of the targeted allele. The PCR was carried out as a multiplex-PCR reaction with 20 pMol of the primers *neo-l* and *c-raf-1-r*, and 20 pMol of the primer *c-raf-2-2´*, 10 mM dNTPs, 100 ng genomic DNA and 1.25 U Taq-polymerase (Perkin Elmer, Weiterstadt, Germany) in the appropriate buffer. The DNA fragments were amplified through 35 cycles of 30 sec 94 °C, 1 min 55 °C and 1 min 72 °C with a prior and final extension of 2 min 95 °C and 10 min 72 °C, respectively.

3.14. Transection of the facial nerve and determination of facial motoneuron numbers in brainstem serial sections

Newborn mice were anesthetized by hypothermia, the right facial nerve exposed and transected as described previously (Michaelidis et al., 1996). NGF was added at 5 µg/2 µl in a small piece of collagen gel foam, and this piece of gel foam was applied to the proximal nerve stump of the lesioned facial nerve. After surgery, mice were kept under a 30 °C humid atmosphere until they had fully recovered, then returned back to their mother. The mice used for this study were kept on a Balb/C genetic background, and mice used were either Balb/C mice (wild-type) or mice obtained from offspring from backcrosses of $p75^{NTR-/-}$ mice (Jackson Laboratories) with Balb/C mice. In a separate experiment, the facial nerve was transected in newborn C57BL/6 mice (Charles River, Sulzfeld, Germany), and NGF (5 µg) was added in gel foam to 2 animals, the other animals from the same litter did not receive NGF and served as controls.

Animals were anaesthetized with a lethal dose of Ketanest/Rompun (100 mg/5 mg per kg body weight i.p.), and perfused with 4% paraformaldehyde in phosphate buffer at 1 week after birth, the brainstem region containing the facial nuclei on both sides was dissected and embedded in paraffin. Serial sections (7 µm) were prepared and motoneurons counted in every fifth section. Counts were corrected for split nucleoli as described (Masu et al., 1993; Sendtner et al., 1997).

3.15. Vector construction

A *Ssp*I-*Bam*HI fragment of the *ita* cDNA coding for the *N*-terminal 365 amino acids and a 117 bp untranslated region was isolated from a chicken brain library (Clontech, Paolo Alto, USA) and ligated to a *Bam*HI-*Eco*RI fragment coding for the *C*-terminal 246 amino acids from a previously described clone (Digby, et al., 1996), then cloned between the *Apa*I and *Eco*RI sites under the control of the cytomegalovirus promotor in the pcDNA3 vector (Invitrogen, Carlsbad, USA). Restriction fragment analysis and DNA sequencing confirmed the identity of the resulting clone. An antisense *ita* expression vector was constructed with a 1755 bp cDNA fragment extending from 180 bp 3´ of the initiation codon to 107 bp 3´ of the termination codon and cloned in reverse orientation into the pcDNA3 vector. In addition, a construct coding for a protein with 2 *N*-terminal haemagglutinin (HA) tags was made by cloning a fragment coding for GYPYPDVPDYAG in tandem into a pcDNA3 vector with *Hind*III/*Kpn*I with an adapter coding for an *N*-terminal methionine. The *ita* cDNA was then cloned into this vector with *Asp*I/*Not*I to generate the pcDNA3-HA-ITA vector.

The expression vector for a truncated ITA lacking the 328 *N*-terminal amino acids of the ITA protein which includes the three BIR domains was generated by a polymerase chain reaction strategy using *Pfu* polymerase (Stratagene). The *Kpn*I-containing forward primer 5´GCGCGGTACCATGGACTACAAAGATGACGACGACAAACTGCTTCGTGTGAAAGG AGGAG3´ (designed to contain a FLAG epitope tag) together with the reverse primer 5´TAGCTGAAAGTAAACTCCCAAGG3´ was employed to synthesize an ~0.5 kb product. After digestion of the polymerase chain reaction product at the *Kpn*I and *Aoc*I sites, the resulting ~0.4 kb fragment was cloned into a *Kpn*I/*Mro*I digested pcDNA3 ITA expression

vector to yield pcDNA-ITAΔBIR. All constructs were sequenced to verify that they contained no polymerase chain reaction-generated errors.

A full-length *bcl-2* cDNA (gift from Susan Cory, Walter and Eliza Institute, Melbourne) was cloned as an *Eco*RI-*Taq*I fragment encoding the entire protein into the *Eco*RI-*Cla*I sites of pcDNA3. The pCH110 *lacZ* expression vector was obtained from Amersham Pharmacia.

3.16. Primary neuronal culture

Sympathetic chain ganglia (SCG) were prepared from 11-day-old chick embryos and dorsal root ganglia (DRG) were isolated from 8-day-old chick embryos as described (Sendtner et al., 1988). The ganglia were collected within 60 min and kept in PBS at 4 °C, then incubated in PBS containing 0.1% trypsin (Worthington) for 30 min at 37 °C, subsequently washed three times with F14 media supplemented with 10% horse serum (Linaris, Wertheim, Germany) and triturated to produce single cell suspensions. Cells were preplated for 3 hours on tissue culture plates (Nunclon) and non-adhering cells were then collected and counted. These neuron enriched populations were plated on tissue culture plates coated with poly-DL-ornithine (PORN)-laminin and grown in F14 media plus 10% horse serum at 37 °C in a 3% CO_2 containing humidified atmosphere.

Phase bright neurons were counted under a phase contrast microscope in at least ten fields (each 1.16 mm^2), corresponding to 14.2% of the total culture dish area at various time points after plating. Each experiment was repeated at least three times, and values shown are either data from one representative experiment (Western blot analysis) or mean ± SEM from at least three independent experiments.

3.17. Transfection by trituration

Plasmid DNA was introduced into primary neurons by a modification of a method used to introduce proteins into primary neurons (Borasio et al., 1993). Ganglia (5~8 DRG, or 2~3 sympathetic chains) were trypsinized for 30 min at 37 °C, then centrifuged for 5 min at 400 x g, and the supernatant removed. After gently washing in 1.5 ml F14 media with 10% horse serum, ganglia were transferred to 1.5 ml trituration buffer containing 0.8 % NaCl, 35 mM KCl and 1 µM 2-Mercaptoethanol. After centrifugation, ganglia were transferred to 50 µl of fresh trituration buffer containing 50 nM plasmid DNA for 5 min at room temperature. Ganglia were then triturated using a 200 µl Gilson pipet with the tip opening placed in the bottom of the tube, and bent against the opposing wall to prevent formation of a tight seal. A volume of 40 µl was used for 35 strokes, and the cells were left in the buffer containing the plasmid at room temperature for 10 min. Cells were then preplated and treated as described above (the complete procedure of the transfection by trituration is illustrated in Fig. 23).

3.18. Staining for LacZ expression

Cells were washed once with PBS, fixed for 5 min with 2% paraformaldehyde in PBS and washed three times with PBS. Cells were then incubated at 37 °C in PBS containing 500 g/ml X-Gal, 2.5 mM potassium ferricyanide, 2.5 mM potassium ferrocyanide and 2 mM $MgCl_2$. Blue staining developed after 4~6 hours and cells were photographed after 24 or 72 hours of incubation.

3.19. Immunostaining and fluorescence microscopy of cultured sensory neurons

Rabbit polyclonal antisera were raised against a peptide corresponding to the 580 *C*-terminal residues of the ITA protein (Digby et al., 1996). Polyclonal antibodies were partially purified by Protein G Sepharose affinity chromatography. Preadsorption of the rabbit anti ITA antiserum was carried out by incubation of the antiserum with the recombinant peptide. A 1:1000 dilution of the antiserum was incubated with 500 µg recombinant peptide. The solution was mixed overnight at 4 °C and then ultracentrifuged. The supernatant was used in parallel with the ultracentrifuged non-adsorbed antibody for the immunodetection of ITA.

Dorsal root ganglionic neurons and sympathetic chain neurons were transfected with *ita* or *ha-ita* plasmids or mock transfected and cultured for 18 hours in the presence or absence of NGF. The cells were washed with TBS containing 10% goat serum and 0.05% Tween-20 and incubated for 1 h with mouse anti-HA (10 ng/ml; Boehringer, Mannheim, Germany) or a rabbit antiserum against ITA (1:1000). Controls were performed in the absence of the first antibody (HA) or after preadsorption of the ITA-antiserum (1:1000) with the peptide used for immunization (580 *C*-terminal amino acid residues of the ITA sequence, at 500 µg/ml). After preadsorption (overnight at 4 °C), the antibodies were ultracentrifuged and the supernatant was used for the experiment. After incubation with the first antibody, cells were washed three times with TBS containing 10% goat serum and then incubated for 30 min with 2 ng/ml Cy2 coupled goat anti-mouse (Biotrend, Cologne, Germany), or Cy3 coupled goat anti-rabbit (1:400; Dianova, Hamburg, Germany) secondary antibodies, respectively. Cells were washed again three times with TBS containing 10% goat serum, then covered with PBS containing 50% glycerol (v/v) and observed under a Leica confocal laser scanning microscope (TCS, Leica, Heidelberg, Germany). Scans were performed with BP-FITC or LPS590 barrier filters.

3.20. Western blot analysis and semiquantitative analysis of ITA expression

For each time point of our analysis, at least 15 dorsal root ganglia from embryonic day 8 chicken or 5 sympathetic chain ganglia from E11 chicken were prepared. Either ganglia were taken directly for Western blot analysis or cells were isolated from the ganglia, enriched by preplating and cultured as described above.

Cells were washed with PBS and treated with lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 µg/ml Aprotinin, 100 µg/ml phenylmethylsulfonylfluoride (PMSF)], the lysate was frozen in liquid nitrogen and thawed at 37 °C for 5 cycles. Debris was removed by 5 min centrifugation (600 x g). Proteins were separated by SDS-polyacrylamide gel electrophoresis under denaturing conditions and transferred by electroblotting to nitrocellulose. The membrane was stained with Ponceau S (Sigma, USA), and the lanes and position of molecular mass standard proteins were marked. Blots were blocked at room temperature for 3 h with 6% Blotto (BioRad, Germany), 0.01% bovine serum albumin fraction V, 0.1% polyvinylpyrrolidone (PVP), 0.1% horse serum, 0.1% fetal calf serum, 0.01% goat serum and 0.005% Tween-20 in PBS. The blots were then incubated overnight at 4 °C with a 1:1000 dilution of the affinity purified ITA antiserum in blocking buffer. ITA-immunoreactive bands were detected with a goat anti-rabbit horseradish peroxidase conjugated secondary antibody (Biotrend, Cologne, Germany) which was incubated with the blot at room temperature for 1 h at a concentration of 260 ng/ml in phosphate free buffer. The membrane was then reacted with ECL reagent (Amersham, Braunschweig, Germany) and exposed to X-ray film. Films were scanned with an INTAS Duo-Store image analysis system (Göttingen, Germany) and the ITA-immunoreactive bands were analysed with the Gel-Pro Analyzer software (INTAS, Göttingen,

Germany). At least three blots from three independent experiments were used, and the data were statistically analysed with the GraphPad Prism software (San Diego, CA, USA).

3.21. RT-PCR analysis

For each time point of our analysis, thirty DRGs from 6 or 8 day old chick embryos, or ten sympathetic chain ganglia from 8, 11 or 16 day old chick embryos were prepared. Either ganglia were taken directly for RNA preparation and RT-PCR, or cells from E8 DRG or E11 sympathetic chain ganglia were isolated, enriched by preplating and cultured as described above. Tissue or cells were lysed in TRIZOL (Life Technologies, Germany) according to the manufacturer's protocols. The RNA concentration was measured photometrically, and 1.0 µg of total RNA was used for each RT-PCR reaction.

The primers 5´TATCCTAGCTGCAGTTTTGTTC3´ and 5´CCAGGTCATCAAGTCCAGC3´ according to the published sequence of ITA in the area of the BIR domains amplified the expected 342 bp product. After an initial denaturing step for 5 min at 95 °C, the amplification was carried out for 25 or 30 cycles at 95 °C for 30 sec, 60 °C for 30 sec, 72 °C for 30 sec. A final amplification for 5 min at 72 °C finished the PCR. The product was cloned into the pAdv vector (Clonetech) and sequenced to confirm the specificity of the RT-PCR reaction. The primers 5´GTGCGGTAACGCAAGCGAT3´ and 5´ACACCCTCTCCCCCGGATT3´ amplifying a 200 bp fragment of chick 16s RNA were used for RT-PCR to confirm the quantity and the quality of the RNA. After an initial denaturing step of 5 min at 95 °C, the amplification was carried out for 25 cycles at 95 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec. A final amplification for 5 min at 72 °C finished the PCR.

4. Results

4.1. Effects of neurotrophic factors on isolated motoneurons

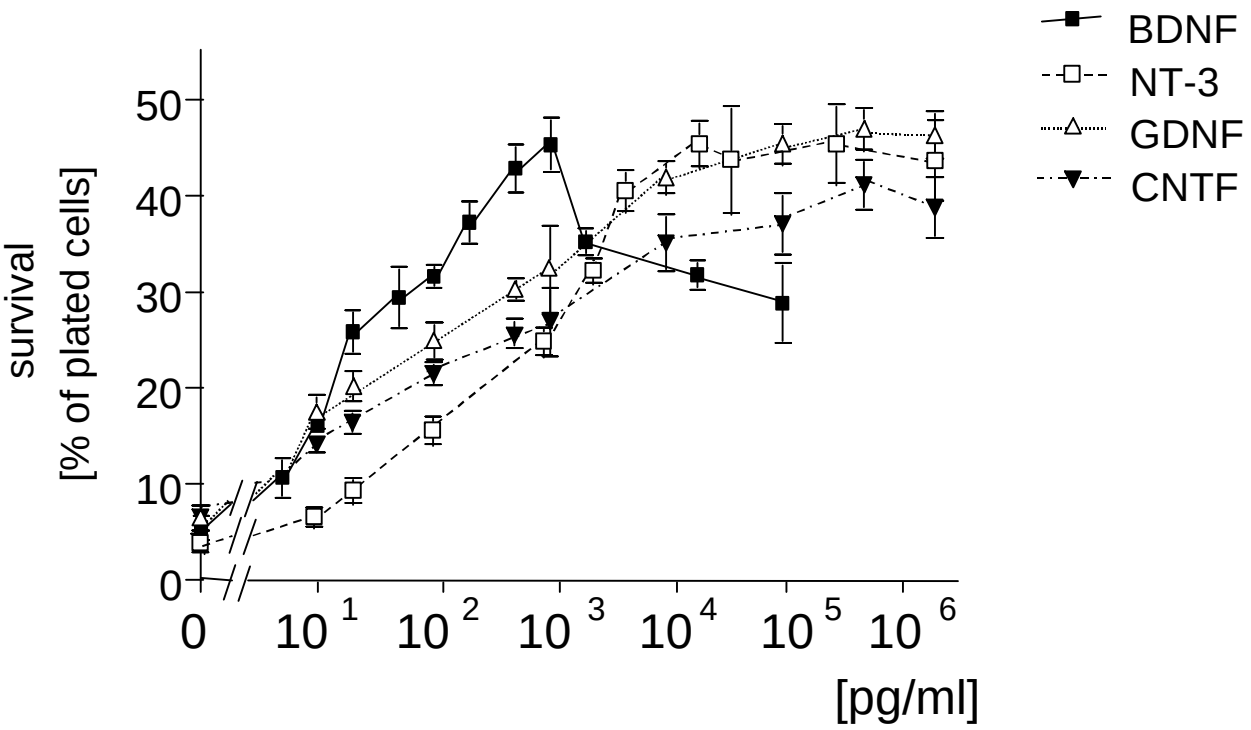
4.1.1. Isolation of motoneurons from embryonic mice

In order to investigate the effects of neurotrophins and other neurotrophic molecules on mouse motoneurons with specific gene alterations, we have adapted techniques previously used for isolation and culture of chick and rat motoneurons (Arakawa et al., 1990). Motoneurons from E14 mouse spinal cord were enriched by metrizamid cushion centrifugation. Enrichment of motoneurons was determined in a window ranging from 6~12% (w/v) metrizamid. As a parameter, the relative number of Islet-1-positive cells was determined: the optimal metrizamid concentration was 10% (w/v). At this concentration, 85 ± 3.5% of all cells stained positive for Islet-1 after one day in culture (data not shown) and could thus be identified as motoneurons. Islet-1 immunostaining was also investigated at day 5 in culture: at that time point, 92 ± 1.5% of all cells were positively labelled (Fig. 1A), confirming that the motoneurons were highly enriched by the techniques applied here. These neurons grew out neurites which were strongly stained both with antibodies against $p75^{NTR}$ (Fig. 1C,D) and neurofilament (Fig. 1E). Virtually all cells stained positively with anti-neurofilament antibody after 5 days *in vitro*, indicating that non-neuronal cells were absent in our cultures (data not shown).

Fig. 1 is on this page and the text is on the following page.

Fig. 1. Mouse motoneurons in cell culture. (A,B) After 5 days in culture, most (92 ± 1.5%) cells stain positive for Islet-1 (A). (B) Corresponding phase contrast picture. (C,D) Motoneurons grown for 5 days with BDNF (C) or GDNF (D) at 1 ng/ml show comparable $p75^{NTR}$ immunoreactivity. (E) Neurofilament immunocytochemistry in mouse motoneurons after 5 days in culture. Bar in (A) valid for (A,B), bar in (C), valid for (C,D), bar in (E): 10 µm.

Isolated mouse motoneurons could survive in the presence of BDNF, NT-3, CNTF and GDNF, but not NGF, as shown in Fig. 2. Half-maximal survival with BDNF and NT-3 was observed at 1.2 x 10^{-12} M and 7.1 x 10^{-12} M, respectively. Similarly, CNTF and GDNF supported half-maximal survival at 0.6 x 10^{-12} M and 0.5 x 10^{-12} M (Fig. 2). NGF had no significant survival effect on spinal motoneurons at 3 and 5 days in culture (Table 1). However, transiently increased survival was observed in *$p75^{NTR-/-}$* motoneurons with 100 pg/ml NGF (80.3 ± 2.8%) in comparison with untreated cells (42.8 ± 1.8%) after 1 day in culture (data not

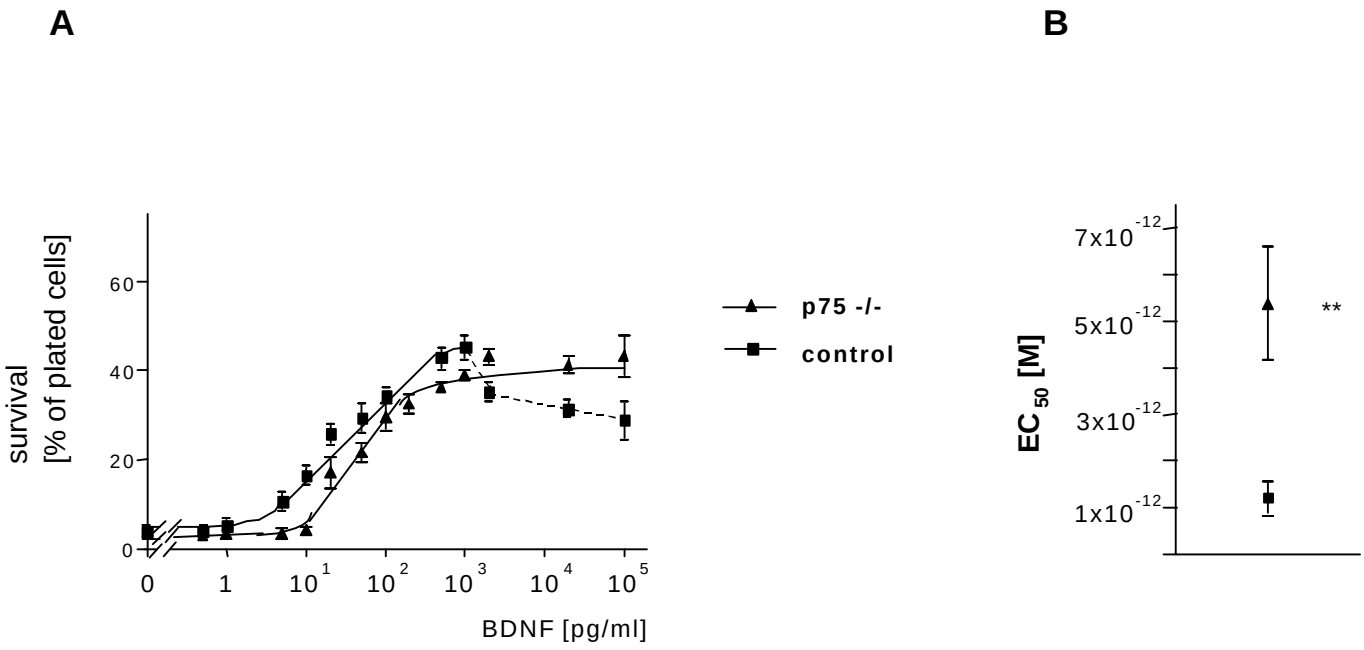


shown).

Fig. 2. Survival of isolated E14 mouse motoneurons after 5 days in cell culture. BDNF, NT-3, GDNF and CNTF were added at concentrations shown. Data were obtained from at least three independent experiments.

4.1.2. The effect of BDNF on cultured motoneurons from control and *p75*$^{NTR-/-}$ mice

In order to characterize the role of the low-affinity neurotrophin receptor in embryonic motoneurons, we have investigated the survival response of motoneurons derived from wild-type Balb/C and *p75*$^{NTR-/-}$ mice to neurotrophins. Interestingly, motoneurons from wild-type mice showed a bell-shaped survival response in the presence of BDNF with decreased survival when cultured at concentrations higher than 1 ng/ml (Fig. 3A). This drop in the dose-response curve was not seen in *p75*$^{NTR-/-}$ motoneurons. In addition, half-maximal survival responses were shifted from 1.2 ± 0.4 x 10^{-12} M in control cultures to 5.4 ± 1.2 x 10^{-12} M in *p75*$^{NTR-/-}$ motoneurons (Fig. 3B). This indicates that BDNF interacts with p75^{NTR} on motoneurons, and that p75^{NTR} can alter the survival response of motoneurons in the presence of BDNF.



C

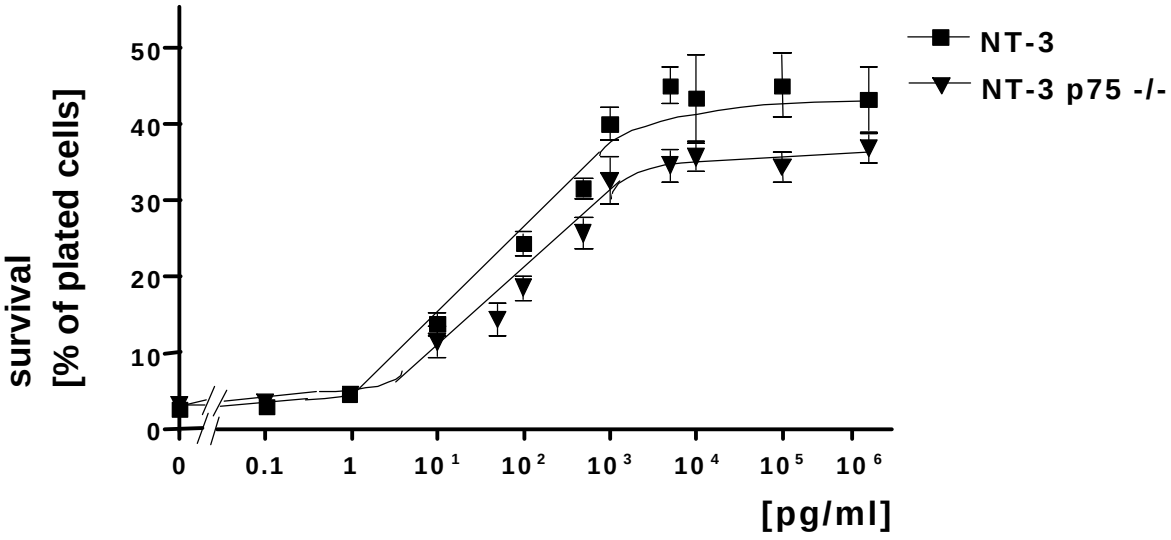


Fig. 3. Effect of BDNF and NT-3 on survival of WT and *p75*^{NTR}-deficient motoneurons after 5 days in culture. (A) BDNF-mediated survival of WT motoneurons is maximal at 0.5 ng/ml, and decreases at higher concentrations. The difference in motoneuron survival at 0.5 ng/ml from the survival at concentrations higher than 20 ng/ml was statistically significant ($P<0.05$, unpaired Student's *t*-test). (B) The EC_{50} for BDNF-mediated survival of motoneurons was compared between WT and *p75*$^{NTR-/-}$ motoneurons. About five times higher concentrations of BDNF were necessary for half-maximal survival of *p75*$^{NTR-/-}$ in comparison to WT motoneurons. (C) NT-3-mediated survival of WT and *p75*$^{NTR-/-}$ motoneurons. Although survival of *p75*$^{NTR-/-}$ motoneurons appeared slightly reduced in comparison to WT, this difference did not reach statistical significance ($P>0.05$, unpaired Student's *t*-test).

4.1.3. Characterization of the neurotrophin effects in cultured *p75*^{NTR}-deficient motoneurons

In order to investigate how p75^{NTR} is involved in mediating neurotrophin survival responses in mouse motoneurons, we have compared the survival effects of BDNF and NT-3 in the absence and presence of various concentrations of NGF.

In wild-type motoneurons, concentrations of NGF which are known to saturate $p75^{NTR}$ receptors on the cell surface significantly reduced the survival response to BDNF and NT-3 (Table 1). This effect could be interpreted in two ways: (1) NGF actively induces cell death through $p75^{NTR}$, as shown before in embryonic chick retinal ganglionic cells (Frade et al., 1996), in trigeminal mesencephalic sensory neurons (Davey and Davies, 1998) or other cell types in culture (Casaccia Bonnefil et al., 1996; Dechant and Barde, 1997). (2) Binding of NGF to $p75^{NTR}$ interferes with the binding of BDNF to its cellular high-affinity receptor on motoneurons.

Effect of NGF on BDNF- and NT-3-mediated survival of WT motoneurons after 5 days in culture

	Motoneuron survival (% of originally plated cells)				
	Concentration of NGF (pg/ml)				
	0	10	100	1000	10000
BDNF (pg/ml)					
0	3.8 ± 0.9	5.4 ± 1.2	3.4 ± 1.0	5.2 ± 0.8	3.8 ± 1.0
10	16.1 ± 0.7	2.3 ± 0.6	0.0 ± 0.0	ND	ND
100	31.6 ± 1.2	26.4 ± 2.8	0.0 ± 0.0	ND	ND
1000	45.2 ± 3.8	ND	38.3 ± 1.8	3.2 ± 1.3	0.0 ± 0.0
NT-3 (pg/ml)					
10	13.8 ± 1.5	12.4 ± 1.0	0.0 ± 0.0	ND	ND
100	21.3 ± 1.5	ND	2.5 ± 1	0.0 ± 0.0	ND
1000	40.0 ± 2.2	ND	30.8 ± 2.1	6.3 ± 0.9	0.0 ± 0.0

Table 1. Values represent mean ± SEM of at least three determinations. The experiment was repeated five times. ND, not determined.

In order to investigate which of these possibilities applies to our system, we have tested whether NGF addition could also reduce survival of these motoneurons in the presence of CNTF or GDNF. As a result, NGF at 20 ng/ml did not alter maximal survival in the presence of these factors at 0.5 ng/ml (data not shown).

Addition of NGF at increasing concentrations to BDNF- and NT-3-treated cultured motoneurons showed that NGF could reduce the survival already at very low concentrations. For example, 100 pg BDNF/ml supported 31.6 ± 1.2% of plated motoneurons after 5 days in culture. This survival effect was significantly reduced ($p<0.05$) by the addition of 100 pg/ml NGF to 12.5 ± 1.3% at 3 days (data not shown) and undetectable levels at 5 days. Moreover, as shown in Fig. 4, BDNF and NGF were added at concentrations indicated at 10 or 100 pg/ml. The survival effect of BDNF is significantly reduced in the presence of NGF at equimolar concentrations, and completely abolished when NGF is given at doses 10 times higher than BDNF after 3 adys in culture (Sendtner et al., 2000). Similarly, 100 pg/ml NGF reduced the survival effect of 100 pg/ml NT-3 from 21.3 ± 1.5% to 2.5 ± 1.0% of originally plated cells (Table 1). These data suggest that NGF could interfere with BDNF and NT-3 supported survival of cultured motoneurons at concentrations in the range of high-affinity binding to cellular receptors.

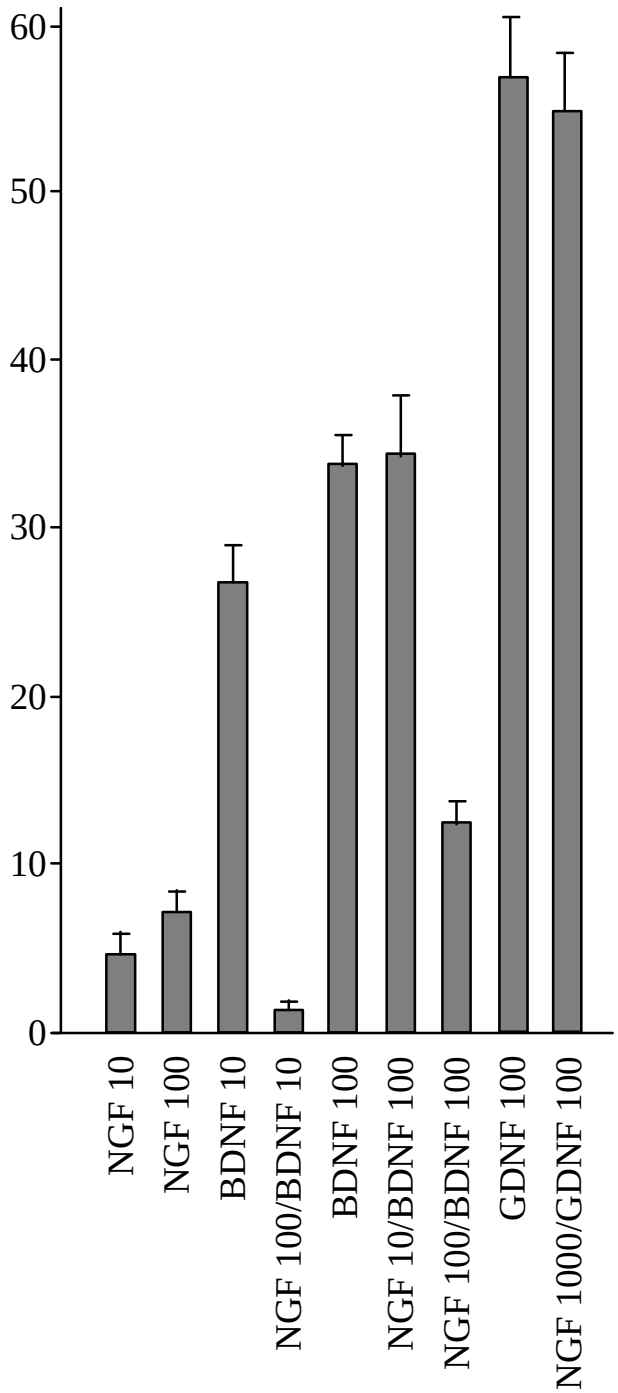


Fig. 4. NGF antagonizes BDNF-mediated survival of mouse motoneurons in culture. Motoneurons were isolated from lumbar spinal cord of 14-day-old mouse embryos (Wiese et al., 1999b). Survival of motoneurons was determined by counting surviving motoneurons after 3 days in culture. Values shown are percentages of initially plated neurons in the culture dish. BDNF and NGF were added at concentrations indicated at 10 or 100 pg/ml. The survival effect of BDNF is significantly reduced in the presence of NGF at equimolar concentrations, and completely abolished when NGF is given at doses 10 times higher than BDNF. Note that the maximal concentration of NGF in this experiment was 100 pg/ml, which is below the concentration thought to saturate low-affinity neurotrophin receptors. NGF could not reduce the survival response initiated by GDNF, even when the concentration was increased to a level of 1000 pg/ml, thus suggesting that NGF acts as a specific antagonist, probably at the receptor level, rather than acting as an independent cell death effector on these cultured motoneurons.

In order to investigate whether $p75^{NTR}$ plays a role in the observed effects, the same experiments were performed with motoneurons isolated from *$p75^{NTR-/-}$* mice. The results (presented in Table 2) clearly demonstrate that NGF does not reduce survival of motoneurons cultured with BDNF or NT-3 when $p75^{NTR}$ is absent. Therefore, we conclude that NGF can

interfere at high-affinity binding sites for BDNF and NT-3 at the cell surface of motoneurons, and that the $p75^{NTR}$ is probably part of these receptor complexes.

NGF does not reduce BDNF- and NT-3-mediated motoneuron survival in $p75^{NTR}$ -/- motoneurons after 5 days in culture

	Motoneuron survival (% of originally plated cells)				
	Concentration of NGF (pg/ml)				
	0	10	100	1000	10000
BDNF					
[pg/ml]					
0	3.4 ± 1.3	4.2 ± 1.2	5.1 ± 1.3	3.8 ± 1.0	3.2 ± 1.1
10	4.1 ± 0.8	2.3 ± 0.6	6.3 ± 1.1	ND	ND
100	29.5 ± 3.1	32.4 ± 2.0	33.8 ± 1.9	31.4 ± 2.3	ND
1000	38.9 ± 1.3	ND	39.0 ± 2.8	43.2 ± 3.1	39.5 ± 4.1
NT-3					
[pg/ml]					
10	13.0 ± 1.1	12.1 ± 1.0	13.2 ± 0.9	ND	ND
100	21.3 ± 2.0	ND	22.3 ± 1.8	20.7 ± 2.5	ND
1000	39.8 ± 3.0	ND	42.5 ± 2.5	40.0 ± 3.6	38.4 ± 2.6

Table 2. Values represent mean ± SEM of at least three determinations. The experiment was repeated five times. ND, not determined.

4.1.4. NGF reduces facial motoneuron survival after axotomy: Role of $p75^{NTR}$ *in vivo*

Previously, it has been observed that NGF can reduce motoneuron survival after axotomy (Miyata et al., 1986; Sendtner et al., 1992) in newborn rats. After facial nerve transection in newborn Balb/C mice, 83 ± 4% lesioned motoneuron degenerate within 1 week. This rate of degeneration is increased in Balb/C mice when NGF is added at 5 µg in gel foam to the proximal nerve stump (Table 3). Similar results have been observed with newborn rats (Sendtner et al., 1992). In order to investigate whether the $p75^{NTR}$ receptor component is involved, the same experiment was performed with *$p75^{NTR}$* knockout mice. On the lesioned side, 21 ± 16% of motoneurons survived in the absence of added factors and 36 ± 9% of lesioned motoneurons survived in the presence of NGF. Thus, survival after NGF addition was significantly ($P<0.005$) higher in *$p75^{NTR-/-}$* mice in comparison to control Balb/C mice (Table 3). Interestingly, the number of motoneurons on the unlesioned side was significantly reduced in *$p75^{NTR-/-}$* mice, indicating that the role of $p75^{NTR}$ in motoneurons differs from other neuronal populations, e.g. the septal cholinergic neurons, whose number is enhanced in *$p75^{NTR}$*-deficient mice (van der Zee, 1996; Yeo et al., 1997).

Number of facial motoneurons in 1-week-old WT and p75^{NTR}-/- mice after unilateral facial nerve lesion

	unlesioned side	lesioned side	% survival
control BalbC (n=4)	2897 ± 98**	488 ± 67	17 ± 4
control BalbC (n=3) +NGF to lesioned nerve	2583 ± 68**	54 ± 9	2 ± 0.1*
p75 ^{NTR}-/- (n=7)	1988 ± 77**	451 ± 112	21 ± 16
p75 ^{NTR}-/- (n=4) +NGF to lesioned nerve	2221 ± 136**	806 ± 148	36 ± 9*
control C57Bl/6 (n=1)	1662	362	22%
control C57Bl/6 (n=2) +NGF to lesioned nerve	1897	35	1.8%

Table 3. Values shown are mean ± SEM for animals with Balb/C genetic background. *P<0.005, Student's t-test. **Statistical comparison of motoneuron cell numbers on the unlesioned side between control Balb/C (n = 7) and p75^{NTR}-/- mice (n = 11) revealed a significant difference (P<0.0005, Student's t-test).

4.2. The role of Bcl-2 for mediation of neurotrophic factor signals on motoneuron survival

4.2.1. Expression and cellular localization of Raf kinases in *bcl-2* deficient motoneurons

Immunostaining of B-Raf and c-Raf-1 in *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ adult mouse spinal motoneurons showed that B-Raf and c-Raf-1 are confined obviously to the cytoplasm of the motoneurons (Fig. 5) in the spinal cord. The immunointensities of B-Raf and c-Raf-1 are the same as in wild-type mice (Fig. 10). There is no difference between *bcl-2*$^{+/+}$ (Fig. 5A,C) and *bcl-2*$^{-/-}$ (Fig. 5B,D) mice.

4.3. The role of cytochrome c: Developmental expression in mouse spinal cord and immunolocalization in motoneurons in the presence and absence of *bcl-2* gene

4.3.1. Developmental expression of cytochrome c in normal mouse spinal cord and its expression in *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ mouse spinal cords by Western blot analysis

Western blot analysis of cytochrome c from mouse spinal cord showed that during development, cytochrome c could be detected as early as at E13, but the expression is extremely weak (Fig. 6), it starts to be slightly upregulated around at E14 and maintains the same low level before birth. After birth (P1), the expression of cytochrome c is increased. It is upregulated to maximal level at P7 and continuously expressed thereafter to adulthood at the same high level (Fig. 6). In *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ adult mouse spinal cords, the expression of cytochrome c is indistinguishable from wild-type adult mouse and there is no difference in expression between *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ mice (Fig. 6).

In cultured motoneurons from E14 mouse spinal cord, expression of cytochrome c was not detectable by Western blotting. The results from BDNF treated cultured motoneurons and untreated motoneurons did not differ (data not shown).

Fig. 5 and the text are on this page.

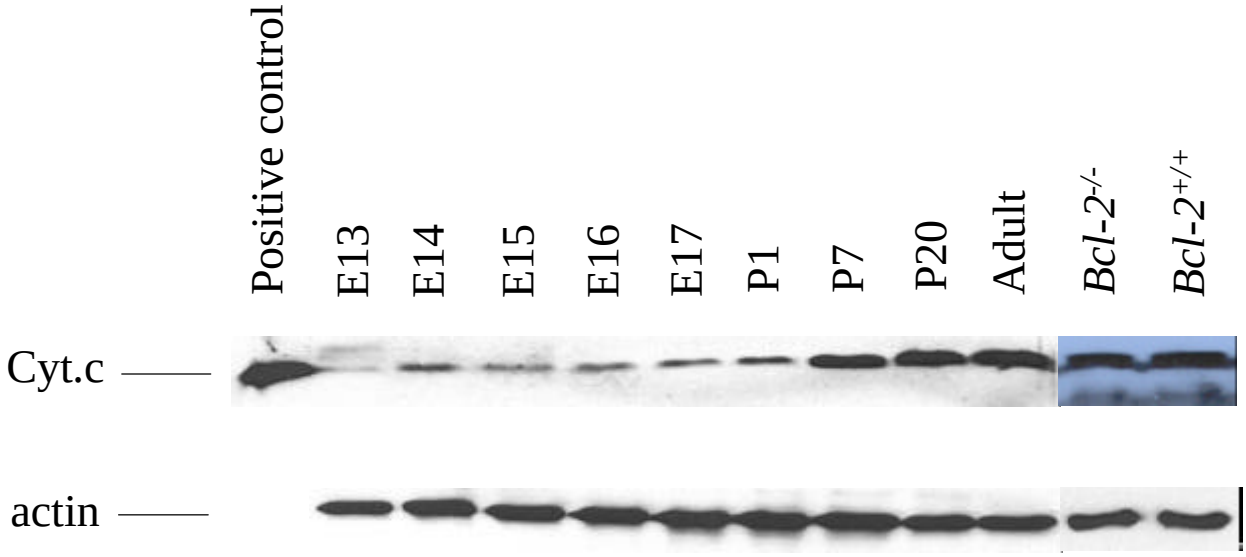


Fig. 6. Western blot analysis of cytochrome c from different embryonic and postnatal stages of wild-type and *bcl-2* mutant mouse spinal homogenates. Fifty micrograms of proteins of each stage were loaded onto each lane, separated on 15% SDS-PAGE and transferred to nitrocellulose. Membranes were probed with a cytochrome c antibody and, after stripping, with an actin antibody. Immunoreactivity was revealed by ECL detection. This Western blot is representive of five independent experiments. Note that during development, cytochrome c could be detected as early as at E13 but the expression was extremely weak (a very faint band). It starts to be slightly upregulated around at E14 and maintained the same level before birth. After birth (P1), the expression level of cytochrome c is increased. It is upregulated to the maximal level at P7 and continuously expressed thereafter to adulthood at the same level. In addition, in *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ adult mouse spinal cords, the expression of cytochrome c is similar as in wild-type adult mice and there is no difference in expression between *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ mice.

4.3.2. Cytochrome c immunostaining in *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ mouse spinal and facial motoneurons

Since cytochrome c release from mitochondria is a crucial step in apoptosis induction, and Bcl-2 can block the release of cytochrome c from mitochondria in this scenario, we have investigated the expression of cytochrome c in motoneurons of *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ mice.

Cytochrome c immunostaining in *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ mouse spinal and facial motoneurons demonstrates that cytochrome c is confined to specific intracellular compartments (mitochondria) (Fig. 7A,C). Interestingly, the immunosignal for cytochrome c in the cytosol of motoneurons appears and more diffusely distributed in *bcl-2*$^{-/-}$ motoneuorons (Fig. 7B,D) in comparison with *bcl-2*$^{+/+}$ ones (Fig. 7A,C).

4.4. Expression and localization of Rafs in the developing spinal cord and in motoneurons

4.4.1. Development of techniques for immunolocalization of Rafs in motoneurons: Specificity of Raf antibodies for their corresponding Raf isoforms

Preadsorption of A-Raf, B-Raf and c-Raf-1 antibodies with peptides and recombinant Raf proteins supressed or specifically decreased the immunostaining observed in mouse lumbar spinal sections (Fig. 8B,E,H). Sections incubated with 10% goat serum in the absence of the primary antibodies appeared unstained (Fig. 8 C,F,I). For the both controls, no specific signal was detectable.

Fig. 7 and the text are on this page.

Fig. 8 and the text are on this page.

4.4.2. Expression of A-Raf, B-Raf and c-Raf-1 proteins in mouse spinal cord at different embryonic and postnatal stages

Western blot analysis of Raf-kinases from extracts of the mouse spinal cords showed that the A-Raf, B-Raf and c-Raf-1 protein kinases are expressed in the mouse spinal cord during all stages of development. The expression of all three Rafs is maintained until adulthood but their isoforms are differentially distributed and expressed (Fig. 9). The two higher molecular weight isoforms of A-Raf (Fig. 9A) are weakly expressed at E13, E16 and P20 and found throughout adulthood. The expression of the lower molecular isoform of A-Raf is much more intense than the higher ones: corresponding bands are already present at E13 and increase at E16. The same level of expression is maintained until adulthood. For B-Raf (Fig. 9B), two isoforms are expressed in the mouse spinal cord. The two isoforms of B-Raf are constantly present at different stages from E13 to adult. For c-Raf-1 (Fig. 9C), the expression of the higher molecular weight isoform of the protein is present at P20 and the adult. The lower molecular weight isoform of c-Raf-1 is already expressed at E13 mouse spinal cord and the expression is maintained at the same intensity during embryonic and postnatal stages, but in the adulthood its expression is slightly decreased. These experiments were repeated five times and similar results obtained.

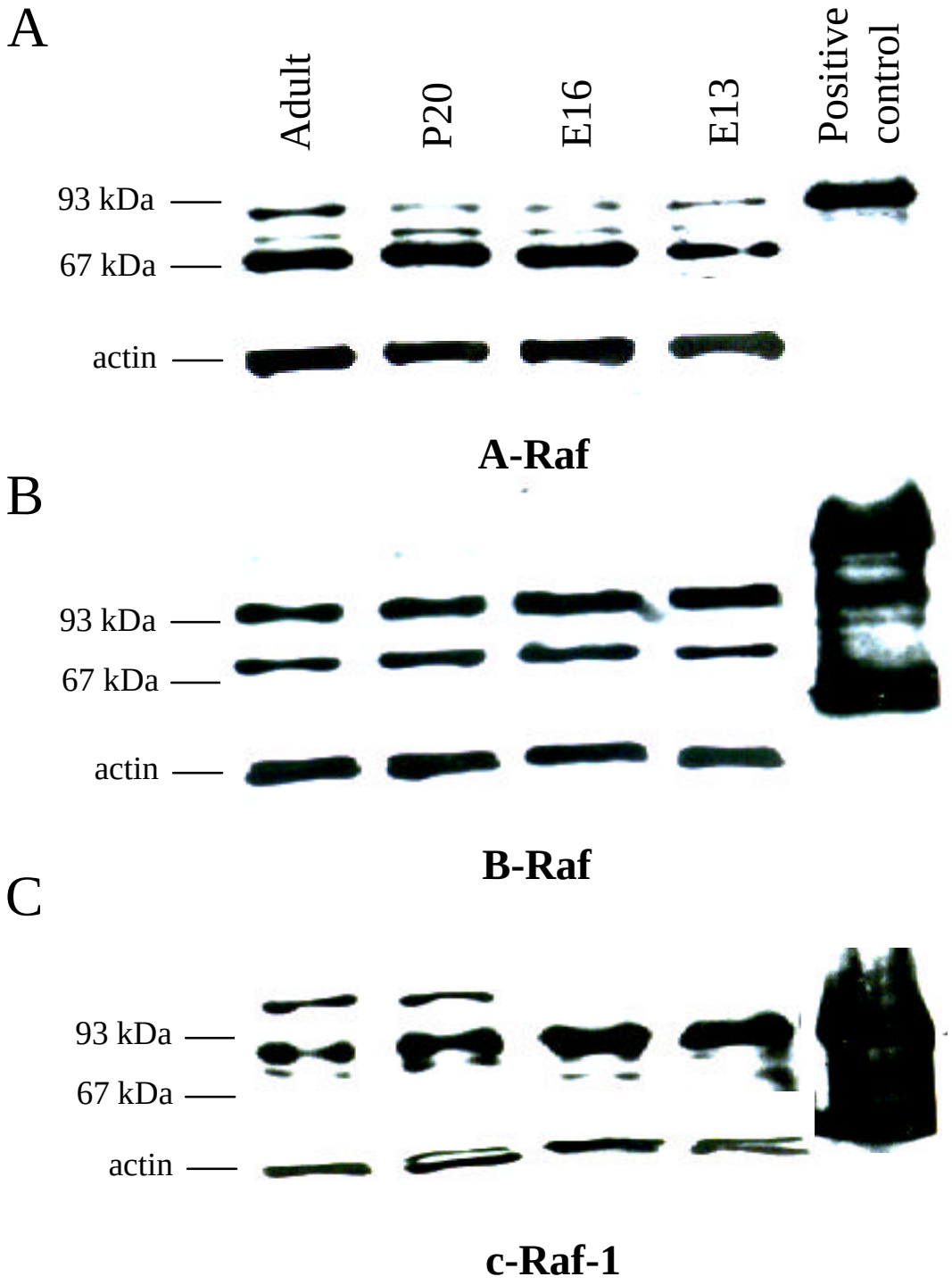


Fig. 9. Western blot analysis of A-Raf (A), B-Raf (B) and c-Raf-1 (C) protein kinases in mouse spinal cord from different embryonic and postnatal stages. Fifty micrograms of proteins of each stage were loaded onto each lane, separated on 7% SDS-PAGE and transferred to nitrocellulose. Membranes were probed with A-Raf, B-Raf and c-Raf-1 antibodies and, after stripping, with an actin antibody. Immunoreactivity was revealed by ECL detection. The position of molecular weight (kDa) standards is indicated on the left side of each gel. These Western blots are representive of five independent experiments. Note that the three Raf proteins are expressed at all stages of development in mouse spinal cord. For A-Raf (A) and c-

Raf-1 (C), their isoforms appear differentially distributed and expressed at different mouse stages. Two isoforms of B-Raf (B) are constantly present at all stages.

4.4.3. Detection of the distribution of A-Raf, B-Raf and c-Raf-1 protein kinases in mouse motoneurons within the ventral horn of the spinal cord and the facial nucleus

Staining for the different Raf protein kinases showed that motoneurons in the postnatal mouse express exclusively B-Raf and c-Raf-1. The expression of A-Raf is very weak (data not shown) and mostly confined to glial cells. B-Raf and c-Raf-1 proteins are detectable by immunostaining in motoneurons within the same regions of the CNS (facial nucleus and lumbar spinal cord) but with slightly differential staining intensity. B-Raf immunoexpression appears stronger than c-Raf-1 (Fig. 10, Fig. 11). The immunoreactivities detected both by conventional (Fig. 10) and confocal laser scanning microscopy (Fig. 11) showed that B-Raf and c-Raf-1 are localized in the cytoplasm of the motoneurons. Both B-Raf and c-Raf-1 staining was widespread in both motoneuron cell bodies and processes but the nucleus of these cells appeared unstained. In lumbar spinal sections, some distinct staining patterns were found in cytoplasmic compartments of motoneurons resembling mitochondria (Fig. 10C,D) (Wang et al., 1996a). When single motoneurons in the same regions were selected and analysed under the confocal laser scanning microscope, we found expression of B-Raf and c-Raf-1 widespread within cytosol of the motoneurons. Moreover, the staining was concentrated at punctate structures. Apparently, both B-Raf and c-Raf-1 are associated with cytoplasmic organelles (Fig. 11A,B,C,D). This observation further suggests association with mitochondria (see below). In addition, strong B-Raf immunoreactivity was also located in association with the nuclear membrane (Fig. 11C).

Fig. 10 and the text are on this page.

Fig. 11 and the text are on this page.

4.4.4. B-Raf and c-Raf-1 are colocalized with A-6403 mitochondrial marker cytochrome c oxidase subunit I in mouse motoneurons

To explore the association of Raf protein kinases with mitochondria in motoneurons within the facial nucleus or spinal cord, the A-6403 antibody against the mitochondrial marker cytochrome c oxidase subunit I was used for the co-immunolocalization detection. It was found the Raf-kinases co-locolize with this specific mitochondial marker in motoneurons. This could be clearly observed by double immunofluorescence with B-Raf or c-Raf-1 and A-6403 mitochondrial marker, respectively (Fig. 12C,F). In order to investigate the subcellular colocalization of c-Raf-1 and A-6403 in more detail, 10 single motoneurons in the same section stained with c-Raf-1/Cy2 (green) and A-6403/Cy3 (red) were selected, scanned and 3-D-reconstructed by confocal laser scanning microscopy (TCS, Leica, Heidelberg, Germany) using double filters for visualization of combined fluorescence (orange, Fig. 13). Our results show that c-Raf-1 and A-6403 immunorecativities colocalized within the mitochondrial compartments of the motoneurons (Fig. 13A,B). Moreover, not all of c-Raf-1 immunoreactivities (green) were localized at mitochondria, staining also appeared in other cytoplasmic regions of the motoneurons (Fig. 13A,B).

Fig. 12 and the text are on this page.

Fig. 13 and the text are on this page.

4.4.5. Redistribution of Raf protein kinases to the perinuclear space and to the nucleus of motoneurons after addition of neurotrophic factors *in vitro*

In order to investigate subcellular redistribution of Raf protein kinases after activation of cell surface receptors, motoneurons from E14 mouse spinal cords were isolated and divided into four groups: (1) 6 h culture without neurotrophic factor treatment (Fig. 14A,B,C); (2) 6 h culture with CNTF and BDNF addition for induction of Raf kinase activity (Fig. 14D,E,F); (3) 3 min treatment with both neurotrophic factors after 6 h culture (Fig. 14G,H.I); (4) continuous 4 d culture with CNTF and BDNF (Fig. 14J,K,L). In contrast to our *in vivo* study for subcellular localization of Raf protein kinases which revealed a widespread cytoplasmic localization pattern, most of the A-Raf and B-Raf immunoreactivities after 6 h *in vitro* in motoneurons were distributed predominantly in the perinuclear space as well as in the nuclei, especially after addition of CNTF and BDNF (Fig. 14A,B,D,E,G,H,J,K). Interestingly, for the distribution of c-Raf-1 immunoreactivity, we observed that after 6 h of culture, irrespective of addition of the neurotrophic factors, c-Raf-1 dramatically translocated from cytoplasmic region to the nucleus of the motoneurons (Fig. 14C,F,I,L). The same staining for c-Raf-1 in the nucleus was also observed in CNTF and BDNF treated cultured motoneurons (Fig. 14,F,I,L). The same result was obtained from cultured E13 mouse motoneurons as well (data not shown).

Fig. 14 and the tex are on this page.

4.4.6. Intracellular involvement of B-Raf kinase signalling in anti-apoptotic effects via neurotrophic factors in motoneurons

Previous study has shown that *B-raf* deficient mice die during embryogenesis with vascular defects caused in part by enhanced apoptosis of differentiated cells of the endothelial lineage (Wojnowski et al., 1997). This result provides the first genetic evidence for an essential role of *B-raf* gene, but not *A-raf* and *c-raf-1* genes, in the regulation of programmed cell death. We then speculated that cell death prevention by neurotrophic factors via the Raf kinase pathway might occur a MAPK pathways independent form. Therefore, to investigate the potential anti-apoptotic role of B-Raf kinase on motoneurons, we have isolated motoneurons (Wiese et al., 1999b) from *B-raf*$^{-/-}$ mouse embryos and analyzed the survival and differentiation effects of neurotrophic factors in these motoneurons of lacking B-Raf.

4.4.6.1. Immunohistochemical detection of A-Raf, B-Raf and c-Raf-1 protein kinases in E13 B-raf$^{+/+}$ *and B-raf*$^{-/-}$ *mouse spinal motoneurons, and in E14 c-raf-1*$^{+/+}$ *and c-raf-1*$^{-/-}$ *mouse spinal motoneurons*

4.4.6.1.1. Expression in E13 B-raf$^{+/+}$ *and B-raf*$^{-/-}$ *mouse spinal motoneurons*

Breeding of *B-raf* heterozygous mice can not produce viable homozygous mutant (*B-raf*$^{-/-}$) offspring. Most homozygous embryos die in uterus before E14 after fertilization. This overall growth deficiency because also manifest as a diminished embryonic weight. Former study (Wojnowski et al., 1997) has clarified that this growth deficit could not be attributed to a developmental arrest. Rather, the *B-raf*$^{-/-}$ embryos have vascular abnormalities due to apoptotic endothelial cell death.

During development, the ventral horn region of E13 mouse spinal cord contains a high number of motoneurons since at this time point the period of naturally occurring cell death has not started yet. Our results demonstrate that *B-raf*$^{-/-}$ mouse completely lacks the B-Raf protein (Fig. 15B), as shown by immunohistochemistry indicating the protein is completely lacking and not expressed in a truncated form. In contrast, B-Raf immunostaining in *B-raf*$^{+/+}$ mouse showed distinct high expression in the motoneurons (Fig. 15A). The intensities of A-Raf and c-Raf-1 immunostaining in the motoneurons were not different in both *B-raf*$^{+/+}$ and *B-raf*$^{-/-}$ mice (Fig. 15C,D,E,F). Our results also demonstrate that the intensities of the immunostainings with B-Raf (in *B-raf*$^{+/+}$ mouse), c-Raf-1 and A-Raf (both in *B-raf*$^{+/+}$ and *B-raf*$^{-/-}$ mice) antibodies decreased progressively during development in the spinal ventral horn (Fig. 15A,C,D,E,F).

4.4.6.1.2. Expression in E14 c-raf-1$^{+/+}$ *and c-raf-1*$^{-/-}$ *mouse spinal motoneurons*

In *c-raf-1*$^{-/-}$ mouse spinal motoneurons, c-Raf-1 immunostaining is negative (Fig. 16B). The intensities of the immunosignals for B-Raf (both *c-raf-1*$^{+/+}$ and *c-raf-1*$^{-/-}$ mice) (Fig. 16E,F), c-Raf-1 (*c-raf-1*$^{+/+}$ mouse) (Fig. 16A), and A-Raf (both *B-raf*$^{+/+}$ and *B-raf*$^{-/-}$ mice) (Fig. 16C,D) in turn decreased successively. Also, the intensities of A-Raf and B-Raf immunoexpression in both *c-raf-1*$^{+/+}$ and *c-raf-1*$^{-/-}$ animals were not different.

Fig. 15 and the text are on this page.

Fig. 16 and the text are on this page.

4.4.6.2. Islet-1 and p75^{NTR} immunoreactivities in motoneurons are decreased in B-raf$^{-/-}$ mouse but no differences are detectable between c-raf-1$^{+/+}$ and c-raf-1$^{-/-}$ mice

4.4.6.2.1. Expression in E13 B-raf$^{+/+}$ and B-raf$^{-/-}$ mouse spinal motoneurons

Since developing motoneurons express high levels of Islet-1 (Ericson et al., 1992) and p75^{NTR} (Ernfors et al., 1989) at embryonic day 13, we have investigated the expression of these proteins in order to test whether differentiation of motoneurons is abnormal before embryonic day 13 in *B-raf$^{-/-}$* mice. Islet-1 has been found as a key regulator of motoneuron development (Pfaff et al., 1996), p75^{NTR} is also highly expressed in developing motoneurons (Ernfors et al., 1989), although its physiological role as a receptor protein in motoneurons during this developmental period is not clear. We have examined the immunoreactivities for these two proteins in E13 *B-raf$^{+/+}$* and *B-raf$^{-/-}$* mouse spinal motoneurons. Islet-1 (Fig. 17A,B) and p75^{NTR} (Fig. 17C,D) immunoreactivities in spinal motoneurons were both found at slightly lower levels in *B-raf$^{-/-}$* mouse in comparison with *B-raf$^{+/+}$* mouse.

4.4.6.2.2. Expression in E14 c-raf-1$^{+/+}$ and c-raf-1$^{-/-}$ mouse spinal motoneurons

In E14 *c-raf-1$^{+/+}$* and *c-raf-1$^{-/-}$* mouse spinal motoneurons, both Islet-1 and p75^{NTR} immunoreactivities in the motoneurons within the ventral horn region displayed indistinguishable between *c-raf-1$^{+/+}$* mouse and *c-raf-1$^{-/-}$* animals (Fig.18.A,B,C,D).

Fig. 17 and the text are on this page.

Fig. 18 and the text are on this page.

4.4.6.3. Absence of B-raf gene but not c-raf-1 leads to loss of viability of mouse motoneurons in vitro

We have investigated the survival response of isolated motoneurons derived from E12.5 *B-raf* mutant mice to neurotrophic factors. The *B-raf*$^{+/-}$ and *B-raf*$^{+/+}$ motoneurons could survive in the presence of BDNF and CNTF for more than 5 d *in vitro* at normal rates as observed in our previous studies employing wild-type motoneurons from 14 day old mouse embryos (Wiese et al., 1999b). Fig. 19 A and B show that *B-raf*$^{+/-}$ mouse motoneurons display marked expression of B-Raf immunoreactivity after 24 h culture, which was distributed mainly in the perinuclear space, some staining was also found in the nucleus. The same result had been obtained with motoneurons from wild-type E13 and E14 mice (Fig. 14B,E,H,K). In addition, in order to demonstrate that the motoneurons had properly developed and reached a normal level of maturity at E12.5, the cultured motoneurons were immunodouble-labelled with B-Raf (Fig. 19C) and neurofilament (Fig.19D) in *B-raf*$^{+/-}$ mice. The result demonstrated that both proteins are normally expressed (Fig. 19E).

Fig. 19 and the text are on this page.

Surprisingly, the isolated motoneurons from E12.5 *B-raf*$^{-/-}$ mice could not survive after 6 h in culture under the same condition as *B-raf*$^{+/-}$ motoneurons in response to BDNF and CNTF (1 ng/ml each growth factor). Our investigation also showed that no motoneurons immunostained with B-Raf and/or neurofilament antibodies could be found after 6 h *in vitro* (Fig. 19F). All cells did obviously undergo cell death until this time point. Fig. 20 shows the quantification of survival of isolated motoneurons from E12.5 *B-raf* mutant mice after 3 days in culture. The results demonstrate that CNTF, BDNF or GDNF can not support *B-raf*$^{-/-}$ motoneuron survival *in vitro* (Fig. 20). In contrast, almost 80% motoneurons from *B-raf*$^{+/-}$ (Fig. 20) and *B-raf*$^{+/+}$ (data not shown) mice can survive with the same different single neurotrophic factors after 3 days in culture.

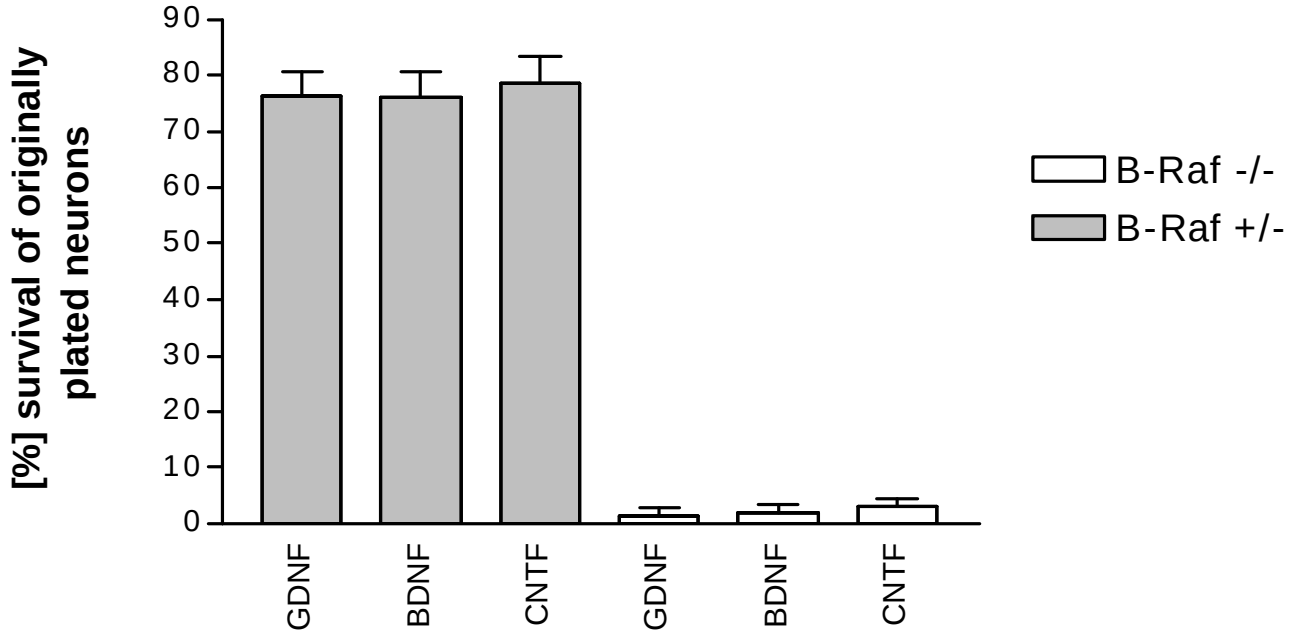


Fig. 20. Survival of motoneurons from E12.5 *B-raf* mutant mice after 3 days in culture. Surviving motoneurons were counted after 3 days in culture in the presence of neurotrophic factors. CNTF, BDNF and GDNF can not support *B-raf*$^{-/-}$ motoneuron survival *in vitro*. In contrast, almost 80% motoneurons from *B-raf*$^{+/+}$ and *B-raf*$^{+/-}$ mice can survive in the presence of these neurotrophic factors after 3 days in culture. The data represent mean ± SEM from three independent experiments.

Conversely, CNTF, BDNF and GDNF can support both E12.5 *c-raf-1*$^{+/-}$ and *c-raf-1*$^{-/-}$ motoneuron survival after 5 days *in vitro*. Almost 50% motoneurons from both *c-raf-1*$^{+/-}$ and *c-raf-1*$^{-/-}$ mice can survive in response to various neurotrophic factors after 5 days in culture (Fig. 21). The survival of motoneurons from E12.5 *c-raf-1* heterozygous and knockout mice after 5 days in culture is not statistically different.

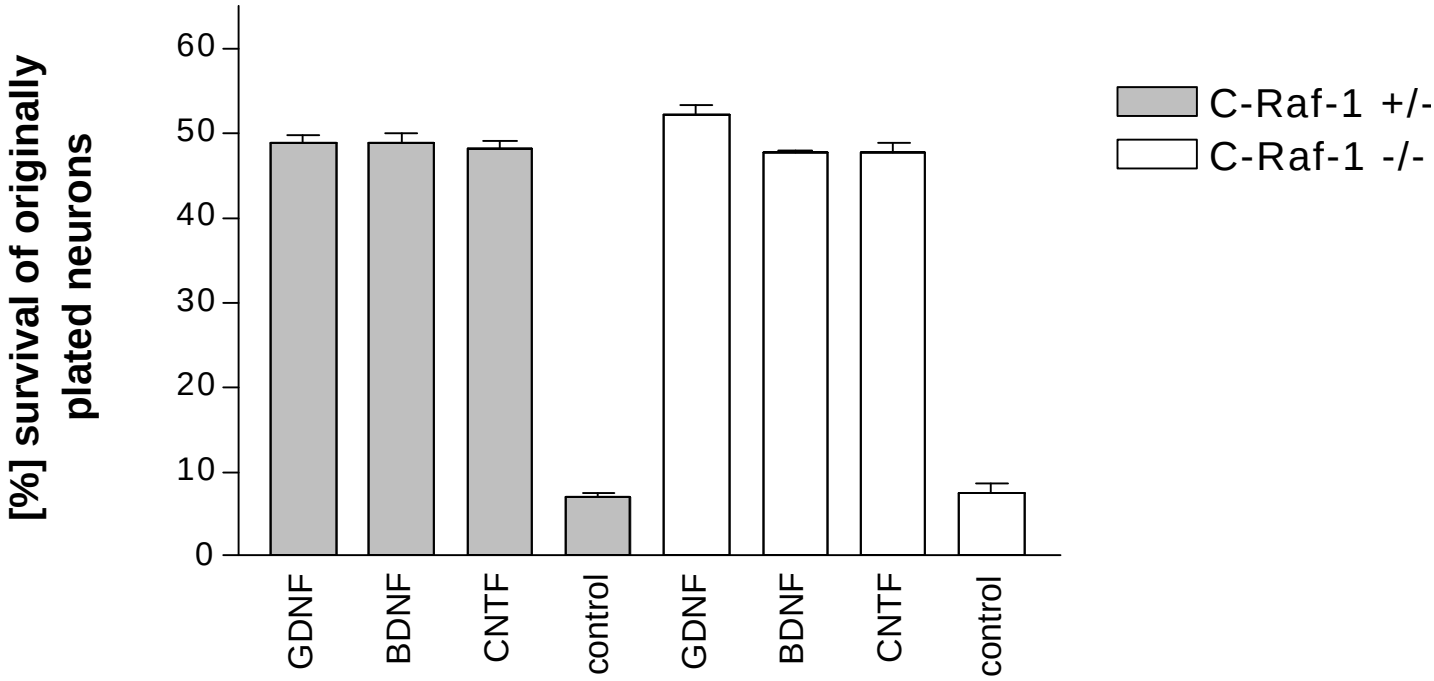


Fig. 21. Survival of motoneurons from E12.5 *c-raf-1* mutant mice after 5 days in culture. Surviving motoneurons were counted after 5 days in culture in the presence of neurotrophic factors. In contrast to *B-raf*$^{-/-}$ motoneurons (Fig. 20), CNTF, BDNF and GDNF can support both E12.5 *c-raf-1*$^{+/-}$ and *c-raf-1*$^{-/-}$ motoneuron survival after 5 days *in vitro*. Almost 50% motoneurons from both *c-raf-1*$^{+/-}$ and *c-raf-1*$^{-/-}$ mice can survive with neurotrophic factors after 5 days in culture. The survival rates of motoneurons from E12.5 *c-raf-1* heterozygous and knockout mice after 5 days in culture are no statistically different from wild-type mouse motoneurons. The data represent mean ± SEM from three independent experiments.

The similar results also obtained from sensory neurons which were isolated from both 12.5 day old *B-raf* and *c-raf-1* mutant mouse embryos (data not shown).

4.4.6.4. Transfection of B-raf expression plasmids to B-raf$^{-/-}$ motoneurons and sensory neurons rescues neurotrophic factor-mediated survival

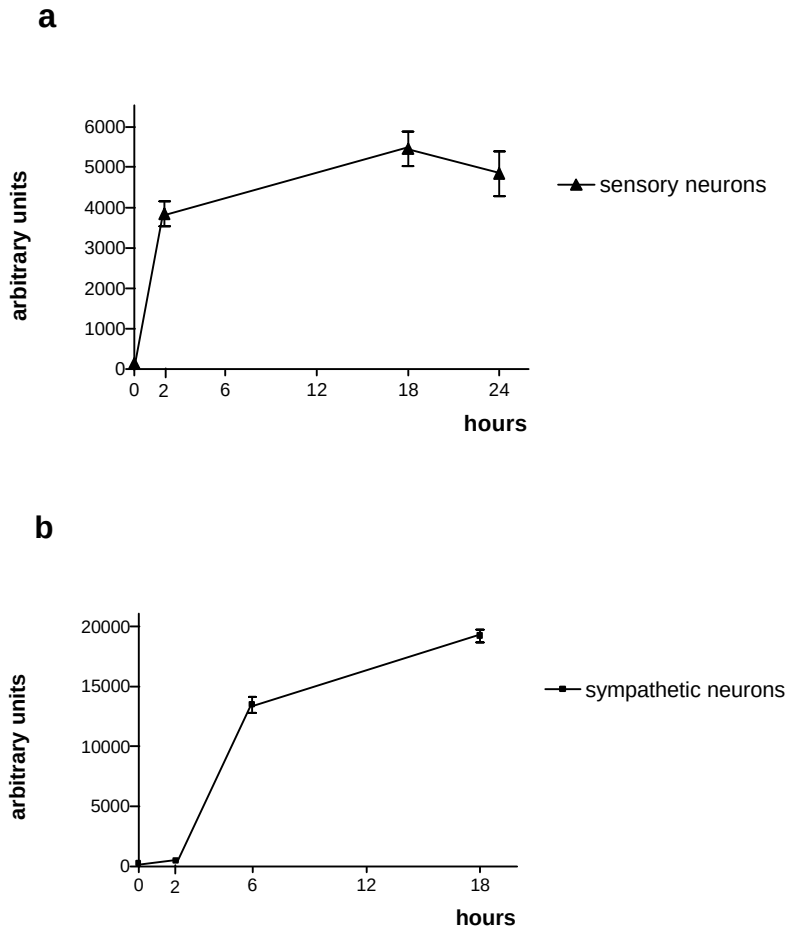
Based on these data that the viability of isolated motoneurons from *B-raf* knockout mice is lost in the presence of neurotrophic factors, as well as in order to further analyse and prove the anti-apoptotic effects of B-Raf kinase for supporting motoneuronal survival during development, we transfected exogenous *B-raf* gene into the motoneurons and sensory neurons from E12.5 *B-raf*$^{-/-}$ mice. We found that transfected neurons could respond again to neurotrophic factors and the viability of the cells could be recovered by B-Raf.

4.4.6.4.1. Development of techniques for plasmid transfection of primary sensory and sympathetic neurons

A. NGF upregulates ITA expression via PI-3 pathways in cultured neurons

In order to investigate downstream signalling processes which mediate the survival effect of neurotrophic factors, we have studied ITA expression in response to NGF in developing sensory and sympathetic neurons. Dorsal root ganglia (DRG) were isolated from 8-day-old chick embryos and paravertebral sympathetic chains from 11-day-old embryos. After dissociation of the ganglia and neuronal enrichment by preplating, the neurons were cultured for periods up to 18 h with or without NGF or CNTF. We chose 18 h as the longest culture period because sensory and sympathetic neurons started to die between 18 and 20 hours in the absence of NGF (data not shown) and can no longer be rescued by delayed addition of NGF (unpublished observations; Sendtner et al., 1988). Thus, under our specific experimental conditions, after 18 h an irreversible cell death process was switched on. Cells were harvested

and lysed, and the extracts were analyzed by Western blot analysis using polyclonal antibodies against ITA. The specificity of the ITA antibodies was tested by preadsorption of the antiserum with the immunising peptide (580 amino acids from the *C*-terminal part of the ITA protein). The ITA-immunoreactive band in Western blots of E8 DRG neurons was completely abolished by preadsorption (Fig. 22e). The ITA protein is expressed in E8 chick dorsal root ganglia, but expression drops below the detection level after isolation and during preplating of the freshly prepared neuronal cells (Fig. 22e). Without NGF (at 5 h after preparation or 0.5 and 18 hours after plating), ITA protein levels remained low. However, 2 hours of NGF exposure led to a 26-fold increase of ITA protein content in dorsal root ganglionic sensory neurons (Fig. 22a). A similar increase (51-fold) was detectable in sympathetic neurons at 6 h after NGF exposure (Fig. 22b,c).



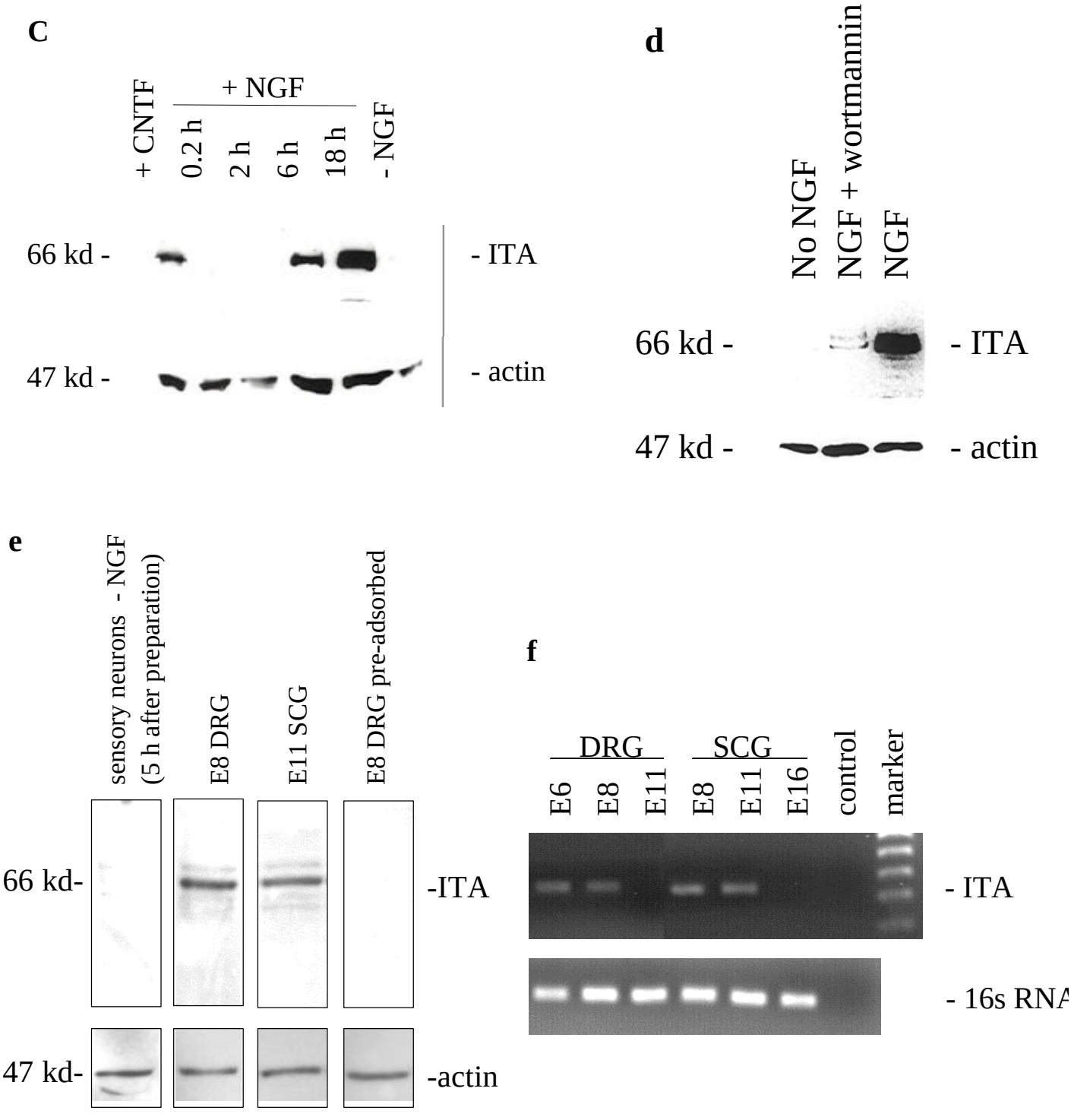


Fig. 22. Time course of NGF-induced upregulation of ITA in primary sensory and sympathetic neurons from developing chick embryos. (a) Time course of ITA upregulation in primary dorsal root ganglionic sensory neurons after addition of NGF. The relative intensity of the ITA bands were quantitated from Western blots. (b) Time course of ITA upregulation in primary sympathetic neurons after addition of NGF. (c) Western blot showing ITA protein content in

cultured sympathetic neurons at various time points after addition of NGF or in its absence. (d) Blockade of PI3-K by Wortmannin (50 nM) abolishes NGF induced upreglation of ITA in primary sympathetic neurons after NGF addition. (e) Expression of ITA in E8 dorsal root ganglia (DRG) and E11 sympathetic chain ganglia (SCG). ITA protein expression was compared in 100.000 neurons obtained directly after the preplating step (about 5 h after isolation). For comparison, 15 E8 DRG ganglia containing approximately the same number of neurons were homogenized directly after preparation and the protein extract loaded on the same gel. A strong immunoreactive band for ITA was detectable in the extract of the DRG ganglia but not in the isolated neurons, indicating that ITA protein levels dropped during preparation, trypsin incubation and preplating (approximately 5 h). In the same experiment, preadsorption of the antiserum with ITA peptide abolished the signal obtained with an extract from 15 E8 dorsal root ganglia. (f) Expression of *ita* mRNA in dorsal root and sympathetic ganglia during development. RT-PCR (25 cycles) showed significant expression of ITA *in vivo* at E6 and E8 in lumbar dorsal root and at E8 and E11 in paravertebral sympathetic ganglia. At later stages (E11 in dorsal root and E16 in sympathetic ganglia), expression was reduced.

In vivo, *ita* mRNA expression was detected by semiquantitative RT-PCR in dorsal root ganglia from E6 and E8 and in sympathetic chains from E8 and E11 chick embryos. At later developmental stages (E11 in sensory and E16 in sympathetic ganglia), *ita* mRNA levels were decreased (Fig. 22f).

Signalling pathways involving PI-3K have been shown to play an important role in NGF-mediated survival at least of sympathetic neurons (Crowder et al., 1998). To investigate whether the involvement of PI3-K mediated pathways in the upregulation of ITA expression, we inhibited PI3-K activity with Wortmannin in NGF treated cultures. Wortmannin (50 nM) completely abolished the upregulation of ITA expression in sympathetic neurons after 18 h in culture (Fig. 22d). Similarly, a recent observation also showed that Wortmannin at a concentration of 100 nM inhibits NGF-mediated survival of primary rat sympathetic neurons (Crowder et al., 1998 and references therein).

B. Overexpression of ITA promotes neuronal survival in primary sensory and sympathetic neurons

In order to investigate whether ITA is essential for neuronal survival in response to NGF, we developed a mechanical trituration methold for transfection of primary neurons with expression plasmids (Fig. 23). This method allowed us to transfect about 56 ± 8% of isolated sensory neurons and 48 ± 6% of sympathetic neurons, as revealed by a *lacZ* reporter plasmid in NGF-supported neuronal cultures at 24 (Fig. 24f) and 72 hours (Fig. 25) after plating. Transfection with *ita* supported survival of 21.7 ± 1.4% of the cultured sensory neurons at 72 h in culture. At this time point, only 7.7 ± 1.2% of the originally plated mock-transfected cells could survive without NGF. Addition of NGF to *ita*-transfected sensory neurons supported 48.4 ± 2.0% survival, which was not significantly different from the survival of mock-transfected cultures with NGF (42.2 ± 2.2%). As a positive control for assessment of transfection efficacy, a *bcl-2* expression plasmid was introduced into parallel cultures, supporting survival of 16.8 ± 1.0% of the cells at the same time point. These data indicate that about 50% of the NGF-responsive sensory neurons were transfected, and could be supported by ITA in the absence of NGF. Thus ITA is at least as effective in supporting survival of primary neurons as mammalian IAPs or neuronal apoptosis inhibitory proteins (NAIPs) (Liston et al., 1996; Simons et al., 1999).

Transfection technique for primary embryonic chick sympathetic and sensory neurons

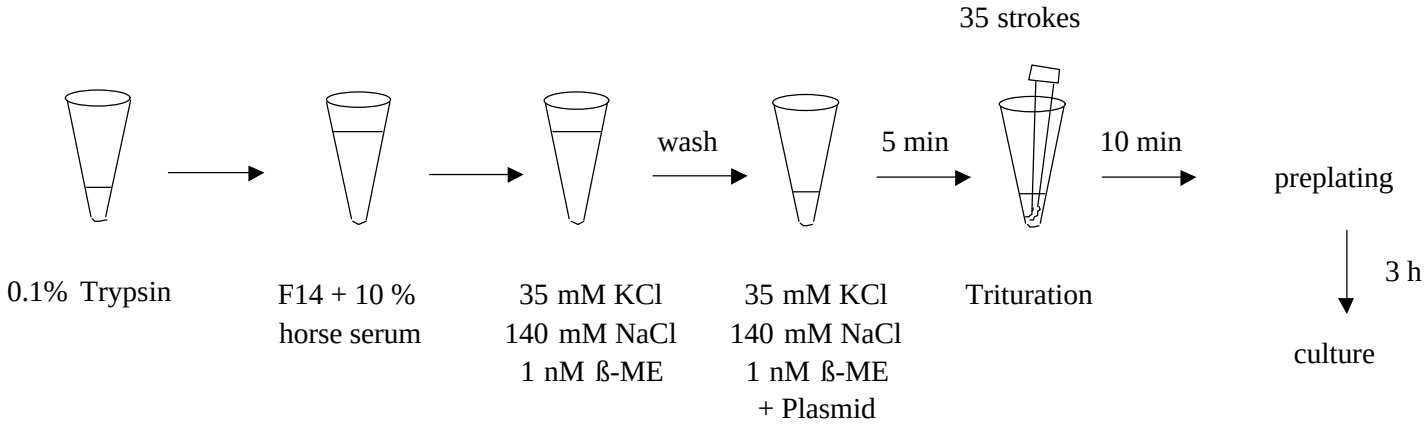


Fig. 23. Technique for transfection of primary sensory and sympathetic neurons with expression plasmids. Ganglia (5~8 DRG, or 2~3 sympathetic chains) were trypsinized for 30 min at 37 °C, then centrifuged for 5 min at 400 x g, and the supernatant removed. After gently washing in 1.5 ml F14 media with 10% horse serum, ganglia were transferred to 1.5 ml trituration buffer containing 0.8 % NaCl, 35 mM KCl and 1 µM 2-Mercaptoethanol. After centrifugation, ganglia were transferred to 50 µl of fresh trituration buffer containing 50 nM plasmid DNA for 5 min at room temperature. Ganglia were then triturated using a 200 µl Gilson pipet with the tip opening placed in the bottom of the tube, and bent against the opposing wall to prevent formation of a tight seal. A volume of 40 µl was used for 35 strokes, and the cells were left in the buffer containing the plasmid at room temperature for 10 min. Cells were then preplated and cultured accordingly to related text in this dissertation.

Fig. 24 is on this page and the text is on the following page.

Fig. 24. Morphology of primary embryonic chick dorsal root ganglionic sensory neurons after transfection with plasmids coding for *ita*, antisense *ita*, *itaDBIR*, *bcl-2* or *lacZ*. (a) Cells transfected with a control vector (pcDNA3 without insert) after 24 h in culture with NGF. (b) Cells transfected with a *bcl-2* transfection vector (pcDNA3 vector, positive control) after 24 h in culture without NGF. (c) Cells transfected with an antisense-*ita* vector after 24 h in culture with NGF. (d) Cells transfected with a sense-*ita* vector after 24 h without NGF. (e) Cells transfected with an expression vector coding for a truncated ITA lacking the BIR domains (ITAΔBIR). Cells were grown for 24 h without NGF. (f) Cells transfected with a *lacZ* expression plasmid after 24 h in culture with NGF. Scale bar in (a), 50 µm, for figures a~f.

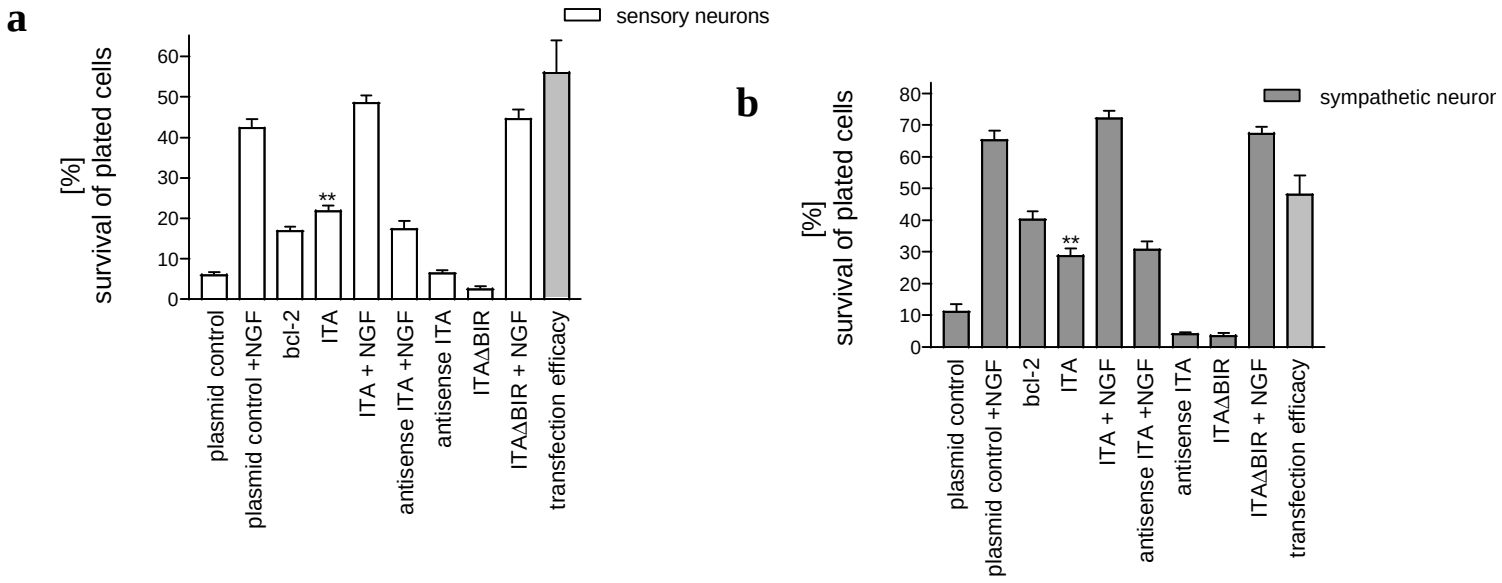


Fig. 25. Neuronal survival of sensory and sympathetic neurons after transfection with control and *ita* expression plasmids. (a) Survival of E8 chick dorsal root ganglionic sensory neurons with or without NGF, after transfection with plasmids coding for *bcl-2*, *ita*, antisense-*ita* or a truncated ITA protein lacking the 328 *N*-terminal amino acids that contain the three BIR domains (ITAΔBIR). (b) Survival of E11 chick sympathetic neurons with or without NGF, after transfection with the same expression plasmids as in (a). Transfection efficacy was determined in parallel cultures with the *lacZ* expression plasmid in the presence of NGF. The culture period for all sensory neurons was 72 h and for all sympathetic neurons 96 h. Surviving cells were counted under the phase contrast microscope. The number of lacZ-positive cells was determined after fixation and visualization of transfected cells by X-gal staining.

We have also tested whether the positive survival effect of ITA involves the BIR domains, as recently shown in other cellular contexts (Takahashi, et al., 1998). As the result, transfection of

a construct coding for a truncated ITA protein lacking the BIR domains into primary sensory and sympathetic neurons failed to prevent cell death without NGF (Fig. 24e, Fig. 25).

When *ita*-transfected sensory neurons were grown for 18 hours, strong ITA immunoreactivity was observed in the surviving neurons (Fig. 26e,f). To distinguish between endogenous ITA and ITA from plasmid transfection in NGF-treated cultures, we expressed a haemagglutinin-tagged ITA and detected it with antibodies against the tag. In this experiment, 62% of the cells stained with antibodies against the tag (Fig. 26a,b), and this proportion increased to 95% when the cells were grown in the absence of NGF (Fig. 26c,d). The few unstained cells lacked neurites, were small and probably reflect dying cells. Specificity of the antibody for ITA was tested by preadsorption with the immunizing peptide, which abolished ITA immunostaining in *ita*-transfected neuronal cultures (Fig. 26g,h).

Fig. 26 is on this page and the text is on the following page.

Fig. 26. ITA immundetection in transfected sensory neurons. Sensory neurons were transfected either with an *ita* expression plasmid or a *ha-ita* expression plasmid. The cells were cultured for 18 h with or without NGF. (a,b) Cells transfected with *ha-ita* and cultured with NGF. Immunostaining (a) was done with an anti-mouse-HA antibody (Boehringer, Mannheim). Three of six cells in the corresponding phase-contrast picture (b) are strongly labelled. Unlabelled cell bodies are indicated by arrows in (b). Similar staining was observed in 248 out of 400 cells individually investigated and counted, corresponding to 62 % labelled cells. This probably reflects the transfection efficacy in this particular experiment. In three parallel control groups with a *lacZ* expression plasmid, 58 ± 5 % of the cells appeared labelled. (c,d) Cells transfected with *ha-ita* and cultured without NGF. Virtually all cells with long neurites expressed ITA, as detected by the haemagglutinin tag antibody. Small shrunken cells without processes (indicated by arrows in d) were not or were only faintly stained with the HA antibody. (e,f) ITA Immunoreactivity in transfected cells cultured without NGF. Cells were immunostained with a polyclonal antiserum against ITA. (g,h) Controls were incubated with the peptide-preadsorbed antiserum, which did not stain the cells. Scale bar, 20 µm.

C. *Ita* antisense expression abolishes NGF-mediated survival in primary sensory and sympathetic neurons

An *ita* antisense plasmid was constructed and transfected into E8 DRG sensory neurons. This led to a reduction of the NGF survival response from 42.2 ± 2.2% to 17.2 ± 2.1% in these cell cultures (Fig. 25a). Because only about half of the NGF-sensitive cells were transfected, this suggests that *ita* antisense expression abolishes NGF-mediated survival in virtually all transfected cells.

We tested the *ita* antisense plasmid for specificity with an ITA-overexpressing PC12 cell line. The same antisense plasmid which was used in our experiments with primary neurons significantly reduced ITA protein content in ITA-overexpressing PC12 cells (data not shown), without any effect on cell survival, thus demonstrating that the *ita* antisense plasmid can specifically suppress ITA protein expression in transfected cells.

We have also tested the *ita* antisense plasmid in cultured E8 sensory neurons. The antisense plasmid leads to reduction of ITA as revealed by Western blot analysis with a specific ITA antiserum (Fig. 27a). The blots shown in Fig. 27a was stripped and reincubated with an antibody against α actin as a control for equal amounts of protein in the individual lanes.

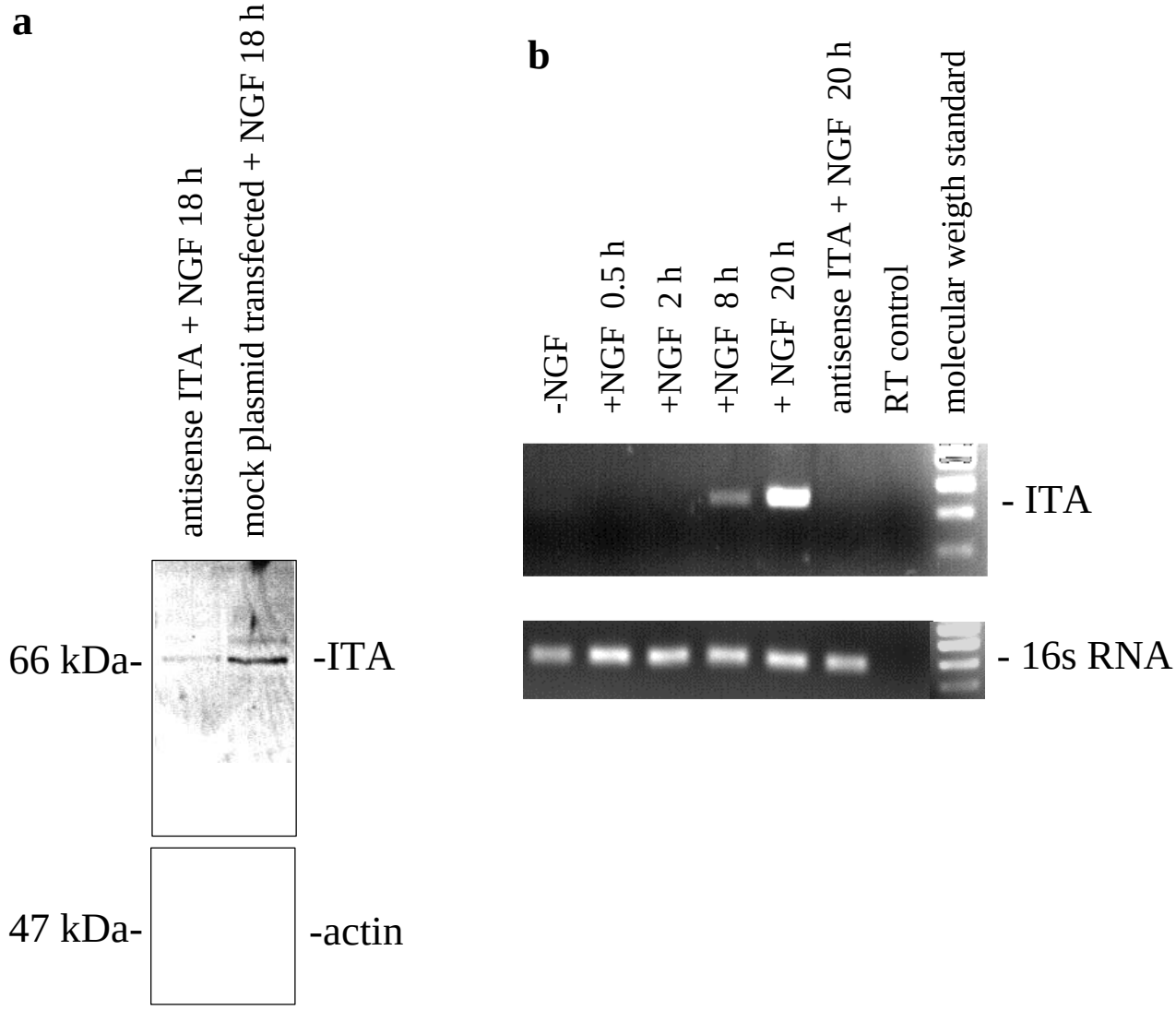


Fig. 27. The *ita* antisense plasmid leads to reduction of ITA protein and *ita* mRNA content in primary neurons. (a) Transfection of *ita* antisense plasmid into cultured sensory neurons reduces ITA levels as revealed by Western blot analysis with a specific ITA antiserum. The blots were stripped and reincubated with an antibody against α actin as a control for equal amounts of protein in each lanes. (b) Transfection of *ita* antisense plasmid reduces *ita* mRNA in cultured sympathetic neurons. Upregulation of *ita* expression was investigated by RT-PCR (25 cycles). The *ita* mRNA expression was clearly detectable at 8 h after initiation of NGF treatment, and the strong signal seen at 20 h was markedly reduced by the antisense treatment.

To demonstrate that the *ita* antisense plasmid specifically suppresses ITA expression in primary neuronal cultures from E11 sympathetic ganglia, we performed a RT-PCR for *ita* with a specific 3´ primer which allowed us to amplify the cDNA corresponding to *ita* mRNA. Antisense treatment leads to significant reduction of *ita* mRNA expression after 20 h in culture in the presence of NGF (Fig. 27b). The mRNA for *ita* in antisense-treated cultures was only detectable at significant levels when the PCR for *ita* mRNA was performed with 30 instead of 25 cycles (data not shown).

Similar results were also obtained with E11 chick sympathetic neurons. In this cell population, *ita* antisense expression decreased NGF-mediated survival from 60.3 ± 2.2% to 30.7 ± 2.5 % (Fig. 25b). ITA overexpression supported 28.7 ± 2.3% of the cells in the absence of NGF. Bcl-2 overexpression supported 40.2 ± 2.5%, corresponding to our observation that only about half of the isolated cells could be transfected. Thus, ITA overexpression supports survival of primary sensory and sympathetic neurons, and antisense expression abolishes NGF-mediated survival of these cells.

4.4.6.4.2. Transfection of B-raf expression plasmids to B-raf$^{-/-}$ motoneurons and sensory neurons rescues neurotrophic factor-mediated survival

We have investigated the survival of E12.5 sensory neurons of dorsal root ganglia from *B-raf*$^{+/+}$ and *B-raf*$^{-/-}$ mice, which were transfected with exogenous *B-raf* expression plasmids and cultured with BDNF and CNTF for 18 h. The sensory neurons were immunostained with specific antibodies against B-Raf and neurofilament by employing combination of two fluorochrome-labelled secondary antibodies. After transfection of the *B-raf* expression plasmids, sensory neurons from *B-raf*$^{+/+}$ and *B-raf*$^{-/-}$ animals survived for 18 h in culture with

BDNF and CNTF, which was the period for our investigation *in vitro*, and both showed marked expression of B-Raf immunoreactivity (Fig. 28A,B,E.F). In contrast, non-transfected individual sensory neurons among the DRG neurons from *B-raf*$^{-/-}$ mouse were undergoing cell death (Fig. 28E,F, arrowheads) in these cultures. In addition, for verifying the specificity of the primary antibody used in this experiment, controls were employed in which the primary antibody was omitted (Fig. 28I,J,K,L). Independent controls in which the primary antibody was preadsorbed (Fig. 28M,N,O,P) are also shown.

Fig. 28 and the text are on this page.

Fig. 28 (continued) and the text are on this page.

5. Discussion

The Raf family of serine/threonine protein kinases are major signalling molecules in virtually all cells which transduce signals from the cell surface to the nucleus. However, very little is known so far about their function in the nervous system. We have studied the distribution and subcellular localization of Raf kinases in mouse motoneurons *in vivo*, and investigated the role of these kinases in motoneurons *in vitro* specifically after activation via neurotrophic factor receptors. In addition, we have tried to define the function of A-Raf, B-Raf and c-Raf-1 in regulating neuronal survival and axon growth.

5.1. Expression of Raf protein kinases during embryonic and postnatal development

As a first step of our analysis, we have investigated Raf protein expression and localization in motoneurons with specific antibodies which recognize the 3 known isoforms of Raf (Fig. 8). As shown in Fig. 9, Western blot analysis of mouse spinal cord obtained at different developmental stages showed no cross-reactivity of the antisera for their specific antigens. The three Raf protein kinases show different patterns and various isoforms of expression in mouse spinal extracts, indicating that their expression is regulated independently and that they might be involved in separate signalling pathways in motoneurons and their specialized subcellular compartments. Although previous studies (Storm et al., 1990; Morice et al., 1999) have shown that A-Raf mRNA and protein expression is restricted to urogenital tissues and is poorly detectable in the CNS of adult rodents, by using specific A-Raf antibody we could detect A-Raf protein kinase expression at different stages of mouse development in spinal extracts. There are two higher molecular weight isoforms of A-Raf faintly present from E13 until

adulthood. Also one intensely expressed lower molecular weight isoform of A-Raf is already present at E13 and its expression is increased at E16 and maintained at the same level of expression until adulthood (Fig. 9A). On the other hand, at the immunohistochemical level, A-Raf expression in motoneurons is relatively weak.

Previous studies have shown that B-Raf protein kinase exists in more than 10 isoforms in most areas of the rat brain, some of them being ubiquitously distributed but others showing region specific distribution, whereas for c-Raf-1 expression, only one isoform is detectable (Morice et al., 1999). The B-Raf isoforms are generated by alternative splicing of the exons 8b and 10 or correspond to two different *N*-termini of the protein, and they are identified by several sera specific for alternative exons (exons 1-2, exon 8b, exon 10). These studies also found various B-Raf isoforms differentially localized at the subcellular level (Barnier et al., 1995; Morice et al., 1999). Our results show that there are only two isoforms of B-Raf protein expressed in mouse spinal cord. The two isoforms of B-Raf are expressed at similar rate and they are constantly present from E13 to adult. Our results are consistent with former findings at mRNA level that B-Raf protein kinase is the most abundant Raf isoform during embryonic and postnatal development in the CNS (Storm et al., 1990).

C-Raf-1 is expressed as two isoforms and significant amounts of c-Raf-1 protein are found both during development and adulthood in mouse spinal cord. The expression of the higher molecular weight isoform of c-Raf-1 is similar at P20 and in the adult. Its lower molecular weight isoform is already expressed at E13 and is maintained at the same intensity during embryonic and early postnatal development but its expression is slightly decreased during adulthood. We conclude that all three Raf protein kinases are expressed in mouse spinal motoneurons. Their expression increases during the period of natually occurring cell death of

motoneurons.

5.2. Immunohistochemical distribution of three Raf protein kinases in mouse motoneurons within the spinal ventral horn and the facial nucleus

Former immunohistochemical studies have revealed that B-Raf and c-Raf-1 are widely distributed in the CNS including the hippocampus, neocortex and spinal cord, and their immunoreactivities are confined mainly to neuronal cell bodies and dendrites (Mihaly et al., 1991, 1993; Mihaly and Rapp, 1993). However, another study has shown that they are present not only in neurons but also in astrocytes (Mihaly and Rapp, 1994). Our study on the immunohistochemical distribution of the Raf kinases in mouse spinal and facial motoneurons showed that the neuronal cells expressed exclusively B-Raf and c-Raf-1 (A-Raf immunoreactivity is very faint in motonerons). Also, the staining intensity with B-Raf and c-Raf-1 antibodies indicates that the concentration of B-Raf protein in the motoneuronal cytoplasm tended to be higher than that of c-Raf-1 kinase. The immunosignals observed both by conventional and confocal laser scanning microscopy reveal that B-Raf and c-Raf-1 are confined obviously to the cytoplasm of the motoneurons. A previous report has exhibited that B-Raf and c-Raf-1 immunostaining is mostly confined to neurons and that these two kinases are coexpressed in most of the neurons (Morice et al., 1999). Other investigators have suggested that these two kinases have ubiquitous distribution with a decreasing antero-posterior gradient of expression which is similar to the distribution pattern for MEK and ERK proteins (Ortiz et al., 1995). Our results demonstrate that B-Raf and c-Raf-1 immunoreactivities are localized in the cytosol of motoneurons suggesting that B-Raf and c-Raf-1 protein kinases play a role in intracellular signalling of motoneurons. Although both B-Raf and c-Raf-1 kinases are present in the CNS, only B-Raf was detected as a MEK activator in bovine brain (Catling et al., 1994; Yamamori et al., 1995). The question still remains

whether B-Raf also signals through another pathway which does not require the phosphrylation of MEK in neurons. In contrast, several studies support the view that c-Raf-1 is the major player within this protein family which regulates at least ERK-driven entry in the cell cycle. However, some investigations also implicate that c-Raf-1 could act in other signalling pathways which do not require MEK/ERK activation (Galaktionov et al., 1995; Kuo et al., 1996; Lu et al., 1998).

5.3. Subcellular localization of Raf protein kinases: colocalization with the A-6403 mitochondrial marker in mouse motoneurons

Our results indicate that the immunoreactivities for B-Raf and c-Raf-1 are concentrated at cytoplasmic organelles, which most probably are mitochondria (Wang et al., 1996a). We have investigated this observation at more detail using the specific A-6403 mitochondrial marker. The result proved that subcellularly Raf kinases are localized at mitochondrial compartments in motoneurons *in vivo*. This result also indicates that Raf kinase signalling in motoneurons might be restricted to specific subcellular compartments such as mitochondria.

Unlike traditionally known roles of Raf protein kinases as promoters of cell proliferation and differentiation within the context of the Ras signalling pathway, recent evidence also showed that Bcl-2 protein can target c-Raf-1 to mitochondria, where Bcl-2 is located, through an interaction that depends on the BH4 domain of Bcl-2 and the catalytic domain of c-Raf-1 (Wang et al., 1996a). Consequently, Bcl-2 targets c-Raf-1 to mitochondria as an effector protein kinase which mediates resistance to apoptosis. Our data show that Raf kinases are also localized at mitochondria of motoneurons within the facial nucleus, in particular when the tissue sections are analyzed. Previous studies have demonstrated that after targeting by Bcl-2

to mitochondria, c-Raf-1 may become activated by Bag-1 (Wang et al, 1996b). Bag-1 is a Bcl-2- and c-Raf-1-interacting protein that enhances the anti-apoptotic effects of Bcl-2 (Wang et al., 1996b). C-Raf-1 can phosphorylate and inactivate Bad and possibly also other protein substrates involved in apoptosis regulation (Wang et al., 1996a). Since active Bad binds to Bcl-2 instead of c-Raf-1 and induces a signalling cascade which causes cell death (Gajewski and Thompson, 1996; Kroemer, 1997; Zha et al., 1996), phosphorylation of Bad by c-Raf-1 seems to be an important signal preventing its binding to Bcl-2 and Bcl-X_L, thus relieving repression of these anti-apoptotic proteins. This hypothetical mechanism of protection of cells from apoptosis appears as an alternative pathway compared with the currently intensively investigated Ras/PI3/PKB/Akt pathway (Yao and Cooper, 1995; Marte and Downward, 1997; Khwaja et al., 1997; Marte et al., 1997): After activation of PKB/Akt via PI3-kinase, PKB/Akt phosphorylates Bad, preventing its interaction with, and inhibition of, the apoptosis inhibitory proteins Bcl-2 and Bcl-X_L (Zha et al., 1996; Del Peso L et al., 1997; Datta et al., 1997). Moreover, Raf kinases have been reported to exert anti-apoptotic as well as pro-apoptotic effects. The regulation of apoptosis appears to be complex and highly dependent on cell types (Downward, 1998). Therefore, we suppose that the localization of Raf kinase to the mitochondria in motoneurons *in vivo* might play a role in suppressing apoptosis by interaction with Bcl-2 in a MAP kinase cascade- or PKB/Akt pathway-independent manner. Further experiments with Raf knockout mice focusing on the investigation of Raf kinases as an effector kinase of Bcl-2 protein leading to Bad phosphorylation could be helpful to define such functions in motoneurons. Thus the involvement of Raf kinases in mediating survival and differentiation in motoneurons could be investigated.

5.4. Intracellullar redistribution of Raf protein kinases in motoneurons after activation by neurotrophic factors *in vitro*

Previous study have shown that c-Raf-1 undergoes intracellular redistribution in cultured cells following its activation (Rapp et al., 1988; Lissoos et al., 1993; Olah et al., 1991; Brennscheidt et al., 1994; Lu et al., 1988). On the contrary, another report showed that no detectable movement of c-Raf-1 protein to the perinuclear or nuclear space can be observed in a fibroblastic cell line (Rat-1) (Prouty et al., 1998). Up to now, the mechanism of signal-induced intracellular redistribution of the c-Raf-1 protein kinase is not well known. Thus the question is still open whether c-Raf-1 which has been redistributed to the nucleus is involved in the regulation of gene expression or whether this redistribution effect is merely a general phenomenon without specific function. We suspect that this redistribution event may play an important role in transcriptional regulation of target genes. However, further studies have to clarify whether this is the case. Therefore, in the present study we have investigated the intracellular fate of Raf kinases after stimulation with neurotrophic factors in motoneurons. This corresponds to our data on the situation *in vivo* which did not reveal c-Raf-1 or B-Raf immunoreactivity in the nucleus in motoneurons (Fig. 10, Fig. 11). However, this could be due to the fact that the motoneurons are not exposed to excessive concentrations of neurotrophic factors. Our *in vitro* analysis was designed to induce Raf kinase activation by addition of both CNTF and BDNF to cultured motoneurons, subsequently the intracellular redistribution and translocation of Raf kinases in the cultured motoneurons was detected by confocal laser scanning microscopy.

These experiments showed that after 6 h culture most of A-Raf and B-Raf immunoreactivities were located in the perinuclear space and nuclei of E14 mouse motoneurons, especially when

CNTF and BDNF were added to the cultures. Interestingly, we observed c-Raf-1 being dramatically translocated from the cytoplasma into the nuclei of motoneurons after 6 h culture, irrespective of addition of neurotrophic factors. This could be due to the possibility that c-Raf-1 is activated by other signals than neurotrophic factors. However, isolated motoneurons depend on neurotrophic factors for their survival, and motoneuron cell death starts around 6 h in culture in the absence of neurotrophic factors. This indicates that the nuclear translocation of c-Raf-1 is not involved in mediating the survival effects of neurotophic factors.

What could be the function of c-Raf-1 in the nucleus of motoneurons? Raf protein kinases neither contain an established or putative nuclear localization signal sequence nor nucleolar localization sequences (Boulikas, 1993; Szebeni et al., 1995; Schlenstedt, 1996). Moreover, the amino acid sequence of Raf kinase does contain a consensus appropriate for DNA binding, while the high proportion of hydrophobic residues may allow for lipid-protein or protein-protein interactions. Such properties might contribute to the intracellular and intranuclear redistribution (Rapp et al., 1988a). The consequence of nuclear translocation of c-Raf-1 after factor-induced phosphorylation and activation has been shown in previous work (Rapp et al., 1988b). Treatment of serum-deprived NIH3T3 cells with the mitogen platelet-derived growth factor (PDGF, maximal at 5 min) or with the phorbol ester tumor promoter TPA (maximal at 20 min) provokes a rapid redistribution of c-Raf-1 from the cytosolic fraction to the perinuclear area (Rapp et al., 1988b). Olah et al. (1991) demonstrated that an ischemic insult causes redistribution of Raf protein kinase into the cell nucleus of hippocampal neurons. Increased amounts of Raf protein in the nuclei of pyramidal cells following ischemia were also detected by Western blot analysis of isolated nuclear fractions of neuronal cells. This result indicated that in the hippocampal neurons the intracellular redistribution patterns of Raf kinases seem to coincide with the ongoing pathological processes. Thus the subcellular localization of

Raf protein kinases is thought to constitute a sensitive indicator of postischemic cellular damage. It has been suggested that this nuclear scaffold-bound pool of Raf in the hippocampal cells may be more resistant to proteolytic breakdown evoked by transient ischemic attack (Olah et al., 1991). Another study (Lu et al, 1998) has shown that angiotensin II (Ang II) stimulates c-Raf-1 targeting into the neuronal nucleus, suggesting that this translocation may play a direct role in regulating synthesis and processing of the ribosomal RNA involved in the control of protein synthesis relevant to the neuromodulatory and other cellular actions of angiotensin II in neurons. With regard to the mechanism of the translocation, it is likely that c-Raf-1 is transported across the nuclear membrane accomplished by its binding to another carrier protein. One such carrier could be MAPK since MAPK kinase inhibitor can block Ang II-induced nuclear translocation of both MARK and c-Raf-1 (Lu et al, 1998). Future studies are needed to better define the precise role of the cellular effects of nuclear translocation of Rafs. Our study indicates that this translocation is not associated with neuronal survival. However, other functions in gene expression, possibly also in the initiation of pathways which ultimately lead to cell death are feasible and need further characterization in future studies.

5.5. B-Raf kinase and apoptosis in motoneurons

B-raf deficient mice die during embryogenesis with vascular defects caused by enhanced apoptosis of differentiated cells of the endothelial lineage. This finding is the first genetic evidence for an essential role of *raf* genes in the regulation of programmed cell death (Wojnowski et al., 1997). In addition, *B-raf* knock-out mice show subtle anatomical abnormalities in neuronal tissue with disturbed growth and differentiation of neuroepithelium (Wojnowski et al., 1997). Because B-Raf is predominantly expressed in mouse brain (Storm et al., 1990) and in motoneurons as revealed by our experiments, *B-raf* deficient mice represent

an ideal tool for studying its specific role in motoneurons. As the regulation of apoptosis by Raf is highly dependent on cell type (Downward, 1998), we suspect that cell death prevention by neurotrophic factors via Raf kinase pathways could play an important role and might involve both MAPK-dependent and MAPK-independent mechanisms. Therefore, the potential role of B-Raf in mediating anti-apoptotic signals in motoneurons was investigated in this study. We analyzed the survival and differentiation effects of neurotrophic factors on the E12.5 mouse motoneurons isolated from *B-raf* deficient mice. In order to make sure that these mice do not express alternatively truncated B-Raf protein or B-Raf from another gene which is not targeted in these mice, we have performed immunohistochemistry with B-Raf specific antibody. These experiments clearly demonstrated that at the histochemical level, the *B-raf*$^{-/-}$ mouse used for the study completely lacks the B-Raf protein, whereas A-Raf and c-Raf-1 expression was normal (Fig. 15).

Our experiments showed that at E13, the latest time point when we could obtain alive *B-raf* mutant mice, Islet-1 and p75^{NTR} immunoreactivities were both decreased in *B-raf*$^{-/-}$ mice compared with *B-raf*$^{+/+}$ mice. Islet-1 is a transcription factor specifically expressed in developing motoneurons (Tsuchida et al., 1994). During development, the differentiation of motoneurons is dependent on the expression of Islet-1. Spinal motoneurons fail to develop in Islet-1 null mutant embryos, thus establishing Islet-1 as a key regulator of motoneuron development (Pfaff et al., 1996). This LIM homeodomain transcription factor is the first marker of postmitotic motoneurons and is expressed soon after their exit from the cell cycle (Ericson et al., 1992). All motoneurons pass through a period of Islet-1 expression, but not all motoneurons remain Islet-1-positive (Tsuchida et al., 1994).

BDNF, neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5) which belong to the nerve growth factor (NGF) family promote embryonic motoneuron survival at later developmental stages

(Oppenheim et al., 1992; Henderson et al., 1993; Becker et al., 1998). Very little is known about the requirements of motoneurons for their survival before they become dependent on these neurotrophins. One recent study (Dutton et al., 1999) has shown that in isolated spinal cord precursor and in explant cultures, purified *N*-terminal Sonic hedgehog (Shh-N), a neural tube ventralizing factor, can cooperate with NT3 but not with other neurotrophic factors resulting in Islet-1/2 expression in almost all neurons generated. This result suggests that NT3 is the endogenous factor that acts specifically with Shh-N to induce Islet-1. This could indicate that NT3 signalling and Shh-regulated signalling pathways do not only support differentiation but also survival before the peroid of physiological motoneuron cell death. NT3 is expressed in spinal cord and in skeletal muscle, the regions where motoneurons receive signals for differentiation and survival. In particular the spinal fusimotor neurons are lost in NT3 knockout animals (Kucera et al., 1995). Correspondingly, mice lacking a functional TrkC receptor also show a 30% loss of motoneuron fibers in the ventral root of spinal cord, indicating a similar reduction in motoneuron numbers (Klein et al., 1994). Our results show that Islet-1 immunoreactivity within the spinal ventral horn is reduced in E13 *B-raf*$^{-/-}$ mouse. In this respect, this could mean that NT3 produced by surrounding cells (e.g. embryonic myotubes) (Hughes et al., 1993; Oppenheim, 1996) during this developmental stage could not normally transmit signals when the B-Raf kinase is lacking, and thus could not synergically cooperate with Shh to induce Islet-1 expression. On the other hand, cell-culture experiments and the knockout phenotype of mice lacking individual Raf genes suggest that the three Raf isoforms also have overlapping functions (Hagemann and Rapp, 1999): For example, B-Raf and c-Raf-1 mRNA are both highly expressed in embryonic mouse brain (Storm et al., 1990). The results from the knockout mice suggest a role for B-Raf (Wojnowski et al., 1997) in addition to c-Raf-1 (Wang et al., 1996a) as a survival mediator. Thus, B-Raf and c-Raf-1 could cooperate

during early embryonic development and display functional redundancy (Hagemann and Rapp, 1999).

We have also investigated the expression of $p75^{NTR}$ in *B-raf*$^{-/-}$ and control mice because this low-affinity neurotrophin receptor is also highly expressed in developing motoneurons, long before they become dependent on neurotrophins for their survival (Ernfors, et al., 1989). We observed that $p75^{NTR}$ immunoreactivity is also reduced in E13 *B-raf*$^{-/-}$ mice when compared to *B-raf*$^{+/+}$ animals. However, $p75^{NTR}$ expression is not absent, and we speculate that this could be due to the fact that the signalling mechanisms involving c-Raf-1 are still active and lead to upregulation on $p75^{NTR}$ expression, at least to a certain level. Experiments with chick embryos have shown that $p75^{NTR}$ could act as a cell death receptor early during development before retinal ganglionic cells and motoneurons have reached their target tissue, optic tectum and the skeletal muscle, respectively (Frade et al., 1996; Frade, 2000). Recent findings indicate that at later stages an antagonistic cross-talk between Trk and $p75^{NTR}$ comes up, with Trk being able to silence the cell death promoting $p75^{NTR}$ signalling pathways (Dobrowsky et al., 1994). As well, investigations on sympathetic neurons led to the conclusion that $p75^{NTR}$ could vice versa downregulate at least some TrkA functions (Miller, 1994). In line with the concept that these two receptors both functionally and biochemically antagonize each other in some situations, the ultimate outcome for a individual motoneuron is likely to be determined by cross-talk between Trk receptors and $p75^{NTR}$ pathways (Kaplan and Miller, 1997). We assume that the lower expression of $p75^{NTR}$ seems to be balanced by a reduced capacity of Trk tyrosine kinase signalling in *B-raf*$^{-/-}$ mice, which allows relatively normal development of motoneurons up to the developmental stage (E12) when they become dependent on neurotrophins (BDNF, NT-4) for their survival.

In order to investigate the role of B-Raf in motoneurons during later developmental stages (later than E12) when motoneurons depend on neurotrophic factors for their survival, we have isolated motoneurons from E12.5 *B-raf*$^{+/+}$, *B-raf*$^{+/-}$ and *B-raf*$^{-/-}$ mice and investigated their further development in culture. Isolated motoneurons from E12.5 *B-raf*$^{-/-}$ mice could not survive after 6 h in culture (Fig. 19F) under the same condition as *B-raf*$^{+/-}$ or *B-raf*$^{+/+}$ motoneurons which were supported by both BDNF and CNTF (Fig. 19A,B,C,D,E). The motoneurons derived from *B-raf*$^{+/-}$ and *B-raf*$^{+/+}$ mice could survive for more than 5 d in the culture as observed in our previous investigations with isolated wild-type motoneurons from 14 day old mouse embryos (Wiese et al., 1999b). There was no difference in the survival rate of motoneurons from *B-raf*$^{+/-}$ and *B-raf*$^{+/+}$ mice. After 3 days in culture, CNTF, BDNF or GDNF could support almost 80% of cultured *B-raf*$^{+/-}$ and *B-raf*$^{+/+}$ motoneurons (Fig. 20). This result strongly indicates that B-Raf kinase is a key regulator of neuronal survival for motoneurons *in vitro* and probably also *in vivo*. Interestingly, our data also suggest that c-Raf-1, which is normally expressed in *B-raf* deficient motoneurons can not compensate for the lack of B-Raf during this late phase of development. This appears interesting, given that the expression of Islet-1 and p75^{NTR} which is thought to be upregulated by signals involving the Ras/Raf/MAPK pathway is only reduced but not absent in *B-raf*$^{-/-}$ motoneurons, suggestive of partial compensation during later developmental periods.

To proof that B-Raf but not c-Raf-1 is involved in mediating motoneuron survival after embryonic day 12, we have transfected an exogenous *B-raf* gene or a *c-raf-1* gene into the sensory neurons and motoneurons from E12.5 *B-raf*$^{-/-}$ mice. First data clearly show that B-Raf but not c-Raf-1 can rescue the survival of these sensory neurons (Fig. 28) and motoneurons in response to neurotrophic factors.

With regard to the mechanism of the anti-apoptotic effect of B-Raf-mediated signalling pathways, it is only known so far that PI3-kinase and Raf-kinases both are implicated in the suppression of apoptosis. Although the PI3-kinase/PKB/Akt pathway always appears to have a protective effect, Raf/MAPK pathway has been reported to have anti-apoptotic as well as pro-apoptotic effects. Moreover, these previous studies did not distinguish between the isoforms of Raf kinases but concentrated on c-Raf-1. The regulation of apoptosis by Raf kinases seem to be complex and highly dependent on cell type. Findings from one cell type may not hold far another, even if cells are closely related, perhaps depending on the degree of activation (Downward, 1998). In neuronal cells, the activation of the MAPK pathway protects cells from neurotrophic factor withdrawal induced apoptosis but the JNK pathway promotes cell death after its activation (Xia et al., 1995). Some recent reports also showed that Raf kinases have an anti-apoptotic function. For example, v-Raf can protect myeloid cells from apoptosis (Cleveland et al., 1994), possibly due to formation of direct complexes between Raf and Bcl-2 (Wang, et al., 1994). C-Raf-1 is recruited by Bcl-2 to the mitochondrial membrane (Wang et al., 1996a) then phosphorylates Bad and thus inhibits apoptosis. It has also been shown that c-Raf-1 acts together with Bcl-2 as an apoptosis suppressor in IL3-starved 32D.3 cells (Troppmair and Rapp, 1997). However, other studies have failed to find Raf interaction with Bcl-2 in fibroblasts (Olivier et al., 1997). Again, these studies did not distinguish between c-Raf-1 and B-Raf, a fact which might contribute to these apparently complicating results. It is not known whether B-Raf-mediated survival pathways in neurons involve Bcl-2. Interestingly, Erhardt et al. (1999) reported that overexpression of B-Raf in Rat-1 fibroblast cell lines leading to activation of the MEK/MAPK pathway inhibits apoptosis which is induced by growth factor withdrawal and even by PI3-kinase inhibition. This study showed that the activity of MEK is essential for cellular survival mediated by B-Raf overexpression, since either treatment with the specific MEK inhibitor or expression of a dominant inhibitory MEK mutant blocked the anti-

apoptotic activity of B-Raf. Moreover, the B-Raf/MEK/MAPK pathway confers protection against apoptosis at the level of cytosolic caspase activation, downstream of the release of cytochrome c from mitochondria. This suggests that B-Raf does not interfere with the release of cytochrome c after growth factor deprivation. Moreover, cytochrome c failed to induce caspase activation in cytosols prepared from B-Raf overexpressing Rat-1 cells. The authors of this study concluded that B-Raf/MEK/MAPK signalling may affect the expression or activation of caspase inhibitors such as IAPs (Erhardt et al., 1999). This fits perfectly with our observation that overexpression of ITA, a chicken homologue of mammalian cIAP-2 can inhibit cell death of primary sensory or sympathetic neurons, and that antisense ITA expression inhibits NGF-mediated survival of these primary neurons (Wiese et al., 1999a). Interestingly, both ITA in chick neurons (Wiese et al., 1999a) and cIAP-2 in neurons from rodents (C. Karch, personal communication) are strongly upregulated after addition of neurotrophic factors, indicating that the IAP molecules are important downstream effectors of neurotrophic factor-mediated survival pathways.

Taken together, our data indicate the B-Raf function can be partially compensated by c-Raf-1 in early motoneurons, which do not get dependent on neurotrophic factors for their survival, but that B-Raf then plays a specific role in mediating the survival effects of neurotrophic factors in motoneurons. This pathway probably involves the upregulation of IAP molecules, in response to neurotrophic factors, which then can act as inhibitors of specific caspases and then block apoptosis in these neurons, at least during a critical period of development when physiological cell death occurs. Future studies have to show whether disturbances of these signalling pathways are also involved postnatally under physiological conditions such as neurodegenerative disorders leading cell death of specific neuronal cell populations.

6. References

Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J. 1996. Human ICE/CED-3 protease nomenclature. Cell 87:171.

App H, Hazan R, Zilberstein A, Ullrich A, Schlessinger J, Rapp U. 1991. Epidermal growth factor (EGF) stimulates association and kinase activity of Raf-1 with the EGF receptor. Mol Cell Biol 11:913-919.

Arakawa Y, Sendtner M, Thoenen H. 1990. Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motoneurons in culture: Comparison with other neurotrophic factors and cytokines. J Neurosci 10: 3507-3515.

Attardi G, Schatz G. 1988. Biogenesis of mitochondria. Annu Rev Cell Biol 4:289-333.

Avruch J, Zhang XF, Kyriakis JM. 1994. Raf meets Ras: completing the framework of a signal transduction pathway. Trends Biochem Sci 19:279-283.

Baccarini M, Sabatini DM, App H, Rapp UR, Stanley ER. 1990. Colony stimulating factor-1 (CSF-1) stimulates temperature dependent phosphorylation and activation of the RAF-1 proto-oncogene product. EMBO J 9:3649-3657.

Baffy G, Miyashati T, Williamson JR, Reed JC. 1993. Apoptosis induced by withdrawal of interleukin-3 (IL-3) dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncogene production. J Biol Chem 268:6511-6519.

Barnier JV, Papin C, Eychene A, Lecoq O, Calothy G. 1995. The mouse B-raf gene encodes multiple protein isoforms with tissue-specific expression. J Biol Chem 270:23381-23389.

Becker E, Soler RM, Yuste VJ, Gin E, Sanz-Rodriguez C, Egea J, Martin-Zanca D, Comela JX. 1998. Development of survival responsiveness to brain-derived neurotrophic factor,

neurotrophin 3 and neurotrophin 4/5, but not to nerve growth factor, in cultured motoneurons from chick embryo spinal cord. J Neurosci 18:7903-7911.

Bernardi P, Petronilli V. 1996. The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. J Bioenerg Biomembr 28:129-136.

Blackshear PJ, Haupt DM, App H, Rapp UR. 1990. Insulin activates the Raf-1 protein kinase. J Biol Chem 265:12131-12134.

Bonner TI, Kerby SB, Sutrave P, Gunnell MA, Mark G, Rapp UR. 1985. Structure and biological activity of human homologs of the raf/mil oncogene. Mol Cell Biol 5:1400-1407.

Bonner TI, Oppermann H, Seeburg P, Kerby SB, Rapp UR. 1986. The complete coding sequence of the human c-*raf*-1 protooncogene and the corresponding structure of the c-*raf*-1 gene. Nucl Acids Res 14:1009-1015.

Borasio GD, Markus A, Wittinghofer A, Barde YA, Heumann R. 1993. Involvement of ras p21 in neurotrophin-induced response of sensory, but not sympathetic neurons. J Cell Biol 121: 665-672.

Boulikas T. 1993. Nuclear localization signals (NLS). Crit Rev Eukaryot Gene Expr 3:193-227.

Boulton TG, Cobb MH. 1991. Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptid antibodies. Cell Regul 2:357-371.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD. 1991. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65:663-675.

Brambilla R, Gnesutta N, Minichiello L, White G, Roylance AJ, Herron CE, Ramsey M, Wolfer DP, Cestari V, Rossi-Arnaud C, Grant SG, Chapman PF, Lipp HP, Sturani E, Klein R.

1997. A role for the Ras signalling pathway in synaptic transmission and long-term memory. Nature 390:281-286.

Brennscheidt U, Riedel D, Kolch W, Bonifer R, Brach MA, Ahlers A, Mertelsmann RH, Herrmann F. 1994. Raf-1 is a necessary component of the mitogenic response of the human megakaryoblastic leukemia cell line MO7 to human stem cell factor, granulocyte-macrophage colony-stimulating factor, interleukin 3, and interleukin 9. Cell Growth Differ 5:367-372.

Bump NJ, Hackett M, Hugunin M, Seshagiri S, Brady K, Chen P, Ferenz C, Franklin S, Ghayur T. 1995. Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. Science 269:1885-1888.

Carroll MP, May WS. 1994. Protein kinase C-mediated serine phosphorylation directly activates Raf-1 in murine hematopoietic cells. J Biol Chem 269:1249-1256.

Casaccia Bonnefil P, Carter BD, Dobrowsky RT, Chao MV. 1996. Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. Nature 383:716-719.

Castedo M, Hirsch T, Susin SA, Zamzami N, Marchetti P, Macho A, Kroemer G. 1996. Squential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. J Immunol 157:512-521.

Catling AD, Reuter CWN, Cox ME, Parson SJ, Weber MJ. 1994. Partial purification of a mitogen-activated protein kinase activator from bovine brain-Identification as B-Raf or a B-Raf-associated activity. J Biol Chem 269:30014-30021.

Cleveland JL, Troppmair J, Packham G, Askew DS, Lloyd P, Gonzalez-Garcia M, Nunez G, Ihle JN, Rapp UR. 1994. v-raf suppresses apoptosis and promotes growth of interleukin-3-dependent myeloid cells. Oncogene 9:2217-2226.

Crowder RJ, Freeman RS. 1998. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci 18:2933-2943.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91:231-241.

Daum G, Eisenmanntappe I, Fries HW, Troppmair J, Rapp UR. 1994. The ins and outs of Raf kinases. Trends Biochem Sci 19:474-480.

Davey F, Davies AM. 1998. TrkB signalling inhibits p75-mediated apoptosis indicated by nerve growth factor in embryonic proprioceptive neurons. Curr Biol 8:915-918.

Davis RJ. 1993. The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 268:14553-14556.

Dechant G, Barde Y-A. 1997. Signalling through the neurotrophin receptor $p75^{NTR}$. Current Opinion in Neurobiol 7:413-418.

de Long D, Prins FA, Mason DY, Reed JC, van Ommen GB, Kluin PM. 1994. Subcellular localization of the bcl-2 protein in malignant and normal lymphoid cells. Cancer Res 54:256-260.

Del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. 1997. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 278:687-689.

Deveraux QL, Takahashi R, Salvesen GS, Reed JC. 1997. X-linked IAP is a direct inhibitor of cell-death proteases. Nature 388:300-304.

Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula M, Alnemri ES, Salvesen GS, Reed JC. 1998. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 17:2215-2223.

Digby MR, Kimpton WG, York JJ, Connick TE, Lowenthal JW. 1996. ITA, a vertebrate homologue of IAP that is expressed in T lymphocytes. DNA Cell Biol 11:981-988.

Distelhorst CW, Lam M, McCormick TS. 1996. Bcl-2 inhibits hydrogen peoxide-induced ER Ca^{2+} pool depletion. Oncogene 12:2051-2055.

Dobrowsky RT, Jenkins GM, Hannun YA. 1995. Neurotrophins induce sphingomyelin hydrolysis: modulation by co-expression of p75 with Trk receptors. J Biol Chem 270:22135-22142.

Downward J. 1998. Ras signalling and apoptosis. Curr Biol 8:49-54.

Dozier C, Ansieau S, Ferreira E, Coll J, Stehelin D. 1991. An alternatively spliced c-mil/raf mRNA is predominantly expressed in chicken muscular tissues and conserved among vertebrate species. Oncogene 6:1307-1311.

Ellis HM, Horvitz HR. 1986. Genetic control of programmed cell death in the nematode C. elegans. Cell 44:817-829.

Emoto Y, Manome Y, Meinhardt G, Kisaki H, Kharbanda S, Robertson M, Ghayur T, Wong WW, Kamen R, Weichselbaum R. 1995. Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells. EMBO J 14:6148-6156.

English JD, Sweatt JD. 1997. A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. J Biol Chem 272:19103-19106.

Erhardt P, Troppmair J, Rapp UR, Cooper GM. 1995. Differential regulation of Raf-1 and B-Raf and Ras-dependent activation of mitogen-activated protein kinase by cyclic AMP in PC12 cells. Mol Cell Biol 15:5524-5530.

Erhardt P, Schremser EJ, Cooper GM. 1999. B-Raf inhibits programmed cell death downstream of cytochrome c release from mitochondria by activating the MEK/Erk pathway. Mol Cell Biol 19:5308-5315.

Ericson J, Thor S, Edlund T, Jessell TM, Yamada T. 1992. Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1. Science 256:1555-1560.

Ernfors P, Henschen A, Olson, L, Persson H. 1989. Expression of nerve growth factor mRNA is developmentally regulated and increased after axotomy in rat spinal cord motoneurons. Neuron 2:1605-1613.

Evans MJ, Scarpulla RC. 1988. The human somatic cytochrome c gene: two classes of processed pseudogenes demarcate a period of rapid molecular evolution. Proc Natl Acad Sci USA 85:9625-9629.

Fabian JR, Daar IO, Morrison DK. 1993. Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. Mol Cell Biol 13:7170-7179.

Faleiro L, Kobayashi R, Fearnhead H, Lazebnik Y. 1997. Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. EMBO J 16:2271-2281.

Fernandes-Alnemri TG, Litwack G, Alnemri ES. 1994. CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein CED-3 and mammalian interleukin-1β converting enzyme. J Biol Chem 269:30761-30764.

Fiore RS, Bayer VE, Pelech SL Posada J, Cooper JA, Baraban JM. 1993. p42 mitogen-activated protein kinase in brain: prominent localization in neuronal cell bodies and dendrites. Neuroscience 55:463-472.

Frade JM, Rodriguez-Tebar A, Barde Y-A. 1996. Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature 383:166-168.

Frade JM. 2000. Unscheduled re-entry into the cell cycle induced by NGF precedes cell death in nascent retinal neurons. J Cell Sci 113:1139-1148.

Gajewski TF, Thompson CB. 1996. Apoptosis meets signal transduction: Elimination of a Bad influence. Cell 87:589-592.

Galaktionov K, Jessus C, Beach D. 1995. Raf1 interaction with Cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation. Gene Dev 9:1046-1058.

Green DR, Reed J. 1998. Mitochondria and apoptosis. Science 281:1309-1312.

Gonzales DH, Neupert W. 1990. Biogenesis of mitochondrial c-type cytochromes. J Bioenerg Biomembr 22:753-768.

Hagemann C, Rapp UR. 1999. Isotype-specific functions of Raf kinases. Exp Cell Res 253: 34-46.

Hall A. 1994. A biochemical function for ras-at last. Science 264:1413-1414.

Han Z, Hendrickson EA, Bremner TA, Wyche JH. 1997. A sequential two-step mechanism for the production of the mature p17:p12 form of caspase-3 in vitro. J Biol Chem 272:13432-13436.

Haycock JW, Ahn NG, Cobb MH, Krebs EG. 1992. ERK1 and ERK2, two microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 in situ. Proc Natl Acad Sci USA 89:2365-2369.

Heidecker G, Cleveland JL, Beck T, Huleihel M, Kolch W, Storm SM, Rapp UR. 1989. In: Colburn NH, editor. Gene and signal transduction in multistage carcinogenesis. New York: Marcel Dekker, Inc. p 339-373.

Henderson CE, Camu W, Mettling C, Gouin A, Poulsen K, Karihaloo M, Rullamas J, Evans T, Mc Mahon S, Armanini MP, Berkemeier L, Phillips HS, Rosenthal A. 1993. Neurotrophins promote motor neuron survival and are present in embryonic limb bud. Nature 363:266-270.

Hengartner MO, Ellis RE, Horvitz HR. 1992. *Caenorhabditis elegans* gene *ced*-9 protects cells from programmed cell death. Nature 356:494-499.

Hengartner MO, Horvitz HR. 1994. *C. elegans* cell survival gene *ced-9* encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell 76:665-676.

Hughes RA, Sendtner M, Thoenen H. 1993. Members of several gene families influence survival of rat motoneurons in vitro and in vivo. J Neurosci Res 36:663-671.

Huleihel M, Goldsborough M, Cleveland JL, Gunnell M, Bonner T, Rapp UR. 1986. Characterization of murine A-raf, a new oncogene related to the v-raf oncogene. Mol Cell Biol 6:2655-2662.

Ikawa S, Fukui M, Ueyama Y, Tamaoki N, Yamamoto T, Toyoshima K. 1988. B-raf, a new member of the raf family, is activated by DNA rearrangement. Mol Cell Biol 8:2651-2654.

Jaiswal RK, Weissinger E, Kolch W, Landreth GE. 1996. Nerve growth factor-mediated activation of the mitogen-activated protein (MAP) kinase cascade involves a signalling complex containing B-Raf and HSP90. J Biol Chem 271:23626-23629.

Jovanovic JN, Benfenati F, Siow YL, Sihra TS, Sanghera JS, Pelech SL, Greengard P, Czernik AJ. 1996. Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions. Proc Natl Acad Sci USA 93:3679-3683.

Kaplan DR, Miller FD. 1997. Signal transduction by the neurotrophin receptors. Curr Opin Cell Biol 9:213-221.

Kayalar C, Ord T, Testa MP, Zhong LT, Bredesen DE. 1996. Cleavage of actin by interleukin 1 beta-converting enzyme to reverse DNase I inhibition. Proc Natl Acad Sci USA 93:2234-2238.

Kerr JFR, Wyllie AH, Currie AR. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239-257.

Kharbanda S, Pandey P, Schofield L, Israels S, Roncinske R, Yoshida K, Bharti A, Yuan ZM, Saxena S, Weichselbaum R, Nalin C, Kufe D. 1997. Role of Bcl-X_L as an inhibitor of cytosolic

cytochrome c accumulation in DNA damage-induced apoptosis. Proc Natl Acad Sci USA 94:6939-6942.

Khwaja A, Rodriguez-Viciana P, Wennström S, Warne PH, Downward J. 1997. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J 16:2783-2793.

Kim CN, Wang X, Huang Y, Ibrado AM, Liu L, Fang G, Bhalla K. 1997. Overexpression of Bcl-X(L) inhibits Ara-C-induced miotchondrial loss of cytochrome c and other perturbations that activate the molecular cascade of apoptosis. Cancer Res 57:3115-3120.

Klein R, Silos-Santiago I, Smeyne RJ, Lira SA, Brambilla R, Bryant S, Zhang L, Snider WD, Barbacid M. 1994. Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. Nature 368:249-251.

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275:1132-1136.

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR. 1993. Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature 364:249-252.

Kornhause JM, Greenberg ME. 1997. A kinase to remember: Dual roles for MAP kinase in long-term memory. Neuron 18:839-842.

Kou WL, Abe M, Rhee J, Eves EM, McCarthy SA, Yan MH, Templeton DJ, McMahon M, Rosner MR. 1996. Raf, but not MEK or ERK, is sufficient for differentiation of hippocampal neuronal cells. Mol Cell Biol 16:1458-1470.

Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC. 1993. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res 53:4701-4714.

Kroemer G. 1997. The proto-oncogene Bcl2 and its role in regulating apoptosis. Nat Med 3:614-620.

Kroemer G, Petit PX, Zamzami N, Vayssiere JL, Mignotte B. 1995. The biochemistry of apoptosis. FASEB J 9:1277-1287.

Kroemer G, Zamzami N, Susin SA. 1997. Mitochondrial control of apoptosis. Immunol Taday 18:44-51.

Kucera J, Ernfors P, Walro J, Jaenisch R. 1995. Reduction in the number of spinal motor neurons in neurotrophin-3-deficient mice. Neuroscience 69:321-330.

Kyriakis JM, App H, Zhang X, Banerjee P, Brautigan DL, Rapp UR, Avruch J. 1992. *raf*-1 kinase activates MAP kinase-kinase. Nature 358:417-421.

Kyriakis JM, Force TL, Rapp UR, Bonventre JV, Avruch J. 1993. Mitogen regulation of c-*raf*-1 protein kinase activity toward mitogen-activated protein kinase-kinase. J Biol Chem 268:16009-16019.

Lam M, Dubyak G, Chen L, Nunez G, Miesfeld RL, Distelhorst CW. 1994. Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca^{2+} fluxes. Proc Natl Acad Sci USA 91:6569-6573.

Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC. 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 371:346-347.

Lazebnik YA, Takahashi A, Moir RD, Goldman RD, Poirier GG, Kaufmann SH, Earnshaw WC. 1995. Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution. Proc Natl Acad Sci USA 92:9042-9046.

Leevers SJ, Paterson HF, Marshall CJ. 1994. Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature 369:411-414.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479-489.

Li P, Wood K, Mamon H, Haser W, Roberts TM. 1991. Raf-1: a kinase currently without a cause but not lacking in effects. Cell 64:479-482.

Lissoos TW, Beno DW, Davis BH. 1993. 1,25-Dihydroxyvitamin D3 activates Raf kinase and Raf perinuclear translocation via a protein kinase C-dependent pathway. J Biol Chem 268:25132-25138.

Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, Farahani R, McLean M, Ikeda JE, MacKenzie A, Korneluk RG. 1996. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 379:349-353.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86:147-157.

Lu D, Yang H, Raizada MK. 1998. AT1 receptor-mediated nuclear translocation of Raf-1 in brain neurons. J Neurochem 70:424-427.

Majewski M, Nieborowska-Skorska M, Salomoni P, Slupianek A, Reiss K, Trotta R, Calabretta B, Skorski T. 1999. Activation of mitochondrial Raf-1 is involved in the antiapoptotic effects of Akt. Cancer Res 59:2815-2819.

Marais R, Light Y, Paterson HF, Marshall CJ. 1995. Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. EMBO J 14:3136-3145.

Marais R, Marshall CJ. 1996. Control of the ERK MAP kinase cascade by Ras and Raf. Cancer Surv 27:101-125.

Marais R, Light Y, Paterson HF, Mason CS, Marshall CJ. 1997. Differential regulation of Raf-1, A-Raf and B-Raf by oncogenic ras and tyrosine kinases. J Biol Chem 272:4378-4383.

Marchetti P, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A, Haeffner A, Hirsch F, Geuskens M, Kroemer G. 1996. Mitochondrial permeability transition is a central coordinating event of apoptosis. J Exp Med 184:1155-1160.

Marin MC, Fernandez A, Bick RJ, Brisbay S, Buja LM, Snuggs M, McConkey DJ, von Eschenbach AC, Keating MJ, McDonnell TJ. 1996. Apoptosis suppression by bcl-2 is corrected with the regulation of nuclear and cytosolic Ca^{2+} . Oncogene 12:2259-2266.

Mark CE, Rapp UR. 1984. Primary structure of v-raf: relatedness to the src family of oncogenes. Science 224:285-289.

Marshall CJ. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179-185.

Mashima T, Naito M, Fujita N, Noguchi K, Tsuruo T. 1995. Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16-induced U937 apoptosis. Biochem Biophys Res Commun 217:1185-1192.

Marte BM, Downward J. 1997. PKB/Akt: connecting PI3-kinase to cell survival and beyond. Trends Biochem Sci 22:355-358.

Marte BM, Rodriguez-Viciana P, Wennström S, Warne PH, Downward J. 1997. PI 3-kinase and PKB/Akt act as an effector pathway for R-Ras. Curr Biol 7:63-70.

Martins LM, Kottke T, Mesner PW, Basi GS, Sinha S, Frigon N, Tatar E, Tung JS, Bryant K, Takahashi A, Svingen PA, Madden BJ, McCormick DJ, Earnshaw WC, Kaufmann SH. 1997. Activation of multiple interleukin-1β converting enzyme homologues in cytosol and nuclei of HL-60 cells during etoposide-induced apoptosis. J Biol Chem. 272:7421-7430.

Matsubara M, Kusubata M, Ishiguro K, Uchida T, Titani K, Taniguchi H. 1996. Site-specific phosphorylation of synapsin I by mitogen-activated protein kinase and Cdk5 and its effects on physiological functions. J Biol Chem 271:21108-21113.

Matsuda S, Kosako H, Takenaka K, Moriyama K, Sakai H, Akiyama T, Gotoh Y, Nishida E. 1992. Xenopus MAP kinase activator: identification and function as a key intermediate in the phosphorylation cascade. EMBO J 11:973-982.

Masu Y, Wolf E, Holtmann B, Sendtner M, Brem G, Thoenen H. 1993. Disruption of the CNTF gene results in motor neuron degeneration. Nature 365:27-32.

McCormick F. 1993. Signal transduction. How receptors turn Ras on. Nature 363:15-6.

Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, Peter ME. 1997. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J 16:2794-2804.

Michaelidis TM, Sendtner M, Cooper JD, Airaksinen M, Holtmann B, Meyer M, Thoenen H. 1996. Inactivation of the bcl-2 gene results in progressive degeneration of motoneurons, sensory and sympathetic neurons during early postnatal development. Neuron 17:75-89.

Mihaly A, Oravecz T, Olah Z, Rapp UR. 1991. Immunohistochemical localization of raf protein kinase in dendritic spines and spine apparatuses of the rat cerebral cortex. Brain Res 547:309-314.

Mihaly A, Endresz V, Oravecz T, Rapp UR, Kuhnt U. 1993. Immunohistochemical detection of raf protein kinase in cerebral cortical areas of adult guinea pigs and rats. Brain Res 627:225-238.

Mihaly A, Priestley JV, Molnar E. 1996. Expression of raf serine/threonine protein kinases in the cell bodies of primary sensory neurons of the adult rat. Cell Tissue Res 285:261-271.

Mihaly A, Rapp UR. 1993. Neuronal expression of the *raf* protooncogene in the spinal cord of adult guinea pigs. Acta Histochem 94:189-196.

Mihaly A, Rapp UR. 1994. Expression of the *raf* protooncogene in glial cells of the adult rat cerebral cortex, brain stem and spinal cord. Acta Histochem 96:155-164.

Miller FD. 1994. NGF and neuronal gene expression. Prog Brain Res 103:23-33.

Miyata Y, Kashihara Y, Homma S, Kuno M. 1986. Effects of nerve growth factor on the survival and synaptic function of Ia sensory neurons axotomized in neonatal rats. J Neurosci 6:2021-2018.

Moelling K, Heimann B, Beimling P, Rapp UR, Sander T. 1984. Serine- and threonine-specific protein kinase activities of purified gag-mil and gag-raf proteins. Nature 312:558-561.

Monaghan P, Robertson D, Amos TAS, Dyer MJS, Mason DY, Greaves MF. 1992. Ultrastructural localization of bcl-2 protein. J Hist Cytochem 40:1819-1825.

Moodie SA, Paris MJ, Kolch W, Wolfman A. 1994. Association of MEK1 with $p21^{ras}$. GMPPNP is dependent on B-Raf. Mol Cell Biol 14:7153-7162.

Morice C, Nothias F, König S, Vernier P, Baccarini M, Vincent JD, Barnier JV. 1999. Raf-1 and B-Raf proteins have similar regional distributions but differential subcellular localization in adult rat brain. Eur J Neurosci 11:1995-2006.

Morishima-Kawashima M, Kosik KS. 1996. The pool of MAP kinase associated with microtubules is small but constitutively active. Mol Biol Cell 7:893-905.

Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong SL, Ng SC, Fesik SW. 1996. X-ray and NMR structure of human Bcl-X_L: a inhibitor of programmed cell death. Nature 381:335-341.

Murphy AN, Bredesen DE, Cortopassi G, Wang E, Fiskum G. 1996. Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. Proc Natl Acad Sci USA 93:9893-9898.

Naumann U, Hoffmeyer A, Flory E, Rapp UR. 1996. Raf protein serine/threonine kinases. In: Protein phosphorylation. VCH, Weinheim.

Newmeyer DD, Farschon DM, Reed JC. 1994. Cell-free apoptosis in *Xenopus* egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. Cell 79:353-364.

Nicholson WD, Ali A, Thornberry NA, Vailancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376:37-43.

Nicholson WD, Thornberry NA. 1997. Caspases: killer proteases. Trends Biochem Sci 22:299-306.

Ohmichi M, Pang L, Decker SJ, Saltiel AR. 1992. Nerve growth factor stimulates the activities of the raf-1 and the mitogen-activated protein kinases via the trk protooncogene. J Biol Chem 267:14604-14610.

Olah Z, Komoly S, Nagashima N, Joo F, Rapp UR, Anderson WB. 1991. Cerebral ischemia induces transient intracellular redistribution and intranuclear translocation of the raf proto-oncogene product in hippocampal pyramidal cells. Exp Brain Res 84:403-410.

Olivier R, Otter I, Monney L, Wartmann M, Borner C. 1997. Bcl-2 does not require Raf kinase activity for its death-protective function. Biochem J 324:75-83.

Oppenheim RW, Qin-Wei Y, Prevette D, Yan Q. 1992. Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. Nature 373:344-346.

Oppenheim RW. 1996. Neurotrophic survival molecules for motoneurons: an embarrassment of riches. Neuron 17:195-197.

Ortiz J, Harris HW, Guitart X, Terwilliger RZ, Haycock JW, Nestler EJ. 1995. Extracellular signal-regulated protein kinases (ERKs) and ERK kinase (MEK) in brain: regional distribution and regulation by chronic morphine. J Neurosci 15:1285-1297.

Pages G, Lenormand P, L'Allemain G, Chambard JC, Meloche S, Pouyssegur J. 1993. Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. Proc Natl Acad Sci USA 90:8319-8323.

Pastorino JG, Simbula G, Yamamoto K, Glascott PAJ, Rothman RJ, Farber JL. 1996. The cytotoxicity of tumor necrosis factor depends on induction of the mitochondrial permeability transition. J Biol Chem 271:29792-29798.

Pelech SL, Sanghera JS. 1992. MAP kinases: charting the regulatory pathways. Science 257:1355-1356.

Pelech SL, Sanghera JS. 1992. Mitogen activated protein kinase: versitle transducers for cell signalling. TIBS 17:233-238.

Pfaff SL, Mendelsohn M, Stewart CL, Edlund T, Jessell TM. 1996. Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. Cell 84:309-320.

Pritchard CA, Samuels ML, Bosch E, McMahon M. 1995. Conditionally oncogenic forms of the A-Raf and B-Raf protein kinases display different biological and biochemical properties in NIH 3T3 cells. Mol Cell Biol 15:6430-6442.

Pritchard CA, Bolin L, Slattery R, Murray R, McMahon M. 1996. Post-natal lethality and neurological and gastrointestinal defects in mice with targeted disruption of the A-Raf protein kinase gene. Curr Biol 6:614-617.

Prouty SM, Maroo A, Maucher C, Mischak H, Kolch W, Sedivy JM. 1998. Studies of perinuclear and nuclear translocation of the Raf-1 protein in rodent fibroblasts. Biochimica et Biophysica Acta 1402:6-16.

Rapp UR, Cleveland JL, Bonner TI, Storm SM. 1988a. The raf oncogenes. In: Reddy EP, Skalka AM, Curran T, editors. The oncogene handbook. Amsterdam: Elsevier Press. p 213-253.

Rapp UR, Heidecker G, Huleihel M, Cleveland JL, Choi WC, Pawson T, Ihle JN, Anderson WB. 1988b. Raf family serine/threonine protein kinases in mitogen signal transduction. Cold Spring Harb Symp Quant Biol 53:173-184.

Rapp UR. 1991. Role of Raf-1 serine/threonine protein kinase in growth factor signal transduction. Oncogene 6:495-500.

Reed JC. 1994. Bcl-2 and the regulation of programmed cell death. J Cell Biol 124:1-6.

Reed JC. 1997a. Cytochrome c: can't live with it-can't live without it. Cell 91:559-562.

Reed JC. 1997b. Double identity for proteins of the Bcl-2 family. Nature:387:773-776.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J. 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370:527-532.

Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, Moelling K, Yancopoulos GD, Glass DJ. 1999. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. Science 286:1738-1741.

Roy N, Mahadevan MS, McLean M. 1995. The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. Cell 80:167-178.

Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. 1997. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors specific caspases. EMBO J 16:6914-6925.

Schendal S, Montal M, Reed JC. 1998. Bcl-2 family proteins as ion-channels. Cell Death Diff 5: 372-380.

Schlenstedt G. 1996. Protein import into the nucleus. FEBS Lett 389:75-79.

Schlessinger J. 1993. How receptor tyrosine kinases activate Ras. Trends Biochem Sci 18:273-275.

Sendtner M, Gnahn H, Wakade A, Thoenen H. 1988. Is activation of the Na+K+ pump necessary for NGF-mediated neuronal survival. J Neurosci 8:458-462.

Sendtner M, Holtmann B, Kolbeck R, Thoenen H, Barde Y-A. 1992. Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. Nature 360:757-758.

Sendtner M, Carroll P, Holtmann B, Hughes RA, Thoenen H. 1994. Ciliary neurotrophic factor. J Neurobiol 25:1436-1453.

Sendtner M, Götz R, Holtmann B, Thoenen H. 1997. Endogenous ciliary neurotrophic factor is a lesion factor for axotomized motoneurons in adult mice. J Neurosci 17:6999-7006.
Sendtner M, Pei G, Beck M, Schweizer U, Wiese S. 2000. Developmental motoneuron cell death and neurotrophic factors. Cell Tissue Res In press.

Shaham S, Horvitz HR. 1996a. An alternatively spliced *C. elegans ced-4* RNA encodes a novel cell death inhibitor. Cell 86:201-208.

Shaham S, Horvitz HR. 1996b. Developing *Caenorhabditis elegans* neurons may contain both cell-death protective and killer activities. Genes Dev 10:578-591.

Simons M, Beinroth S, Gleichmann M, Liston P, Korneluk RG, MacKenzie AE, Bahr M, Klockgether T, Robertson GS, Weller M, Schulz JB. 1999. Adenovirus-mediated gene transfer of inhibitors of apoptosis protein delays apoptosis in cerebellar granule neurons. J Neurochem 72:292-301.

Sozeri O, Vollmer K, Liyanage M, Frith D, Kour G, Mark GE, Stabel S. 1992. Activation of the c-Raf protein kinase by protein kinase C phosphorylation. Oncogene 7:2259-2262.

Stephens RM. 1992. 95-Kilodalton B-raf serine/threonine kinase: identification of the protein and its major autophosphorylation site. Mol Cell Biol 12:3733-3742.

Stokoe D, Macdonald SG, Cadwallader K, Symons M, Hancock JF. 1994. Activation of Raf as a result of recruitment to the plasma membrane. Science 264:1463-1467.

Storm SM, Cleveland JL, Rapp UR. 1990. Expression of raf family proto-oncogenes in normal mouse tissues. Oncogene 5:345-351.

Sugden PH, Clerk A. 1997. Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. Cell Signal 9:337-351.

Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, Daugas E, Geuskens M, Kroemer G. 1996. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J Exp Med 184:1331-1341.

Szebeni A, Herrera JE, Olson MO. 1995. Interaction of nucleolar protein B23 with peptides related to nuclear localization signals. Biochemistry 34:8037-8042.

Takahashi A, Hirata H, Yonehara S, Imai Y, Lee KK, Moyer RW, Turner PC, Mesner PW, Okazaki T, Sawai H, Kishi S, Yamamoto K, Okuma M, Sasada M. 1997. Affinity labeling displays the stepwise activation of ICE-related proteases by Fas, staurosporine, and CrmA-sensitive caspase-8. Oncogene 14:2741-2752.

Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS, Reed JC. 1998. A single BIR domain of XIAP sufficient for inhibiting caspases. J Biol Chem 273:7787-7790.

Tewari M, Quan L, O'Rourke K, Desnoyers S, Zeng Z, Beidler, DR, Poirier GG, Salvesen GS, Dixit VM. 1995. Yama/CPP32β, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81:801-809.

Thompson CB. 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267:1456-1462.

Troppmair J, Bruder JT, Munoz H, Lloyd PA, Kyriakis J, Banerjee P, Avruch J, Rapp UR. 1994. Mitogen-activated protein kinase/extracellular signal-regulated protein kinase activation by oncogenes, serum, and 12-O-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation. J Biol Chem 269:7030-7035.

Troppmair J, Rapp UR. 1997. Apoptosis regulation by Raf, Bcl-2 and R-Ras. In: Müller-Hermelink HK, Neumann HG, Dekant W, editions. Results in Cancer Research Vol. 143, Risk and Progression Factors in Carcinogenesis. Springer-Verlag, Berlin/Heidelberg.

Tsuchida T, Ensini M, Morton SB, Baldassare M, Edlund T, Jessell TM, Pfaff SL. 1994. Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. Cell 79:957-970.

Turner BC, Tonks NK, Rapp UR, Reed JC. 1993. Interleukin 2 regulates Raf-1 kinase activity through a tyrosine phosphorylation-dependent mechanism in a T-cell line. Proc Natl Acad Sci USA 90:5544-5548.

Vance BA, Zacharchuk CM, Segal DM. 1996. Recombinant mouse Bcl-2(1-203). Two domains connected by a long protease-sensitive linker. J Biol Chem 271:30811-30815.

van der Zee CEEM, Ross GM, Riopelle RJ, Hagg T. 1996. Survival of cholinergic forebrain neurons in developing $p75^{NGFR}$-deficient mice. Science 274:1729-1732.

Vaux DL, Strasser A. 1996. The molecular biology of apoptosis. Proc Natl Acad Sci USA 93:2239-2244.

Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJS. 1997. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap- dependent pathway. Cell 89:73-82.

Wang HG, Miyashita T, Takayama S, Sato T, Torigoe T, Krajewski S, Tanaka S, Hovey L, Troppmair J, Rapp UR. 1994. Apoptosis regulation by interaction of Bcl-2 protein and Raf-1 kinase. Oncogene 9:2751-2756.

Wang HG, Rapp UR, Reed JC. 1996a. Bcl-2 targets the protein kinase Raf-1 to mitochondria. Cell 87:629-638.

Wang HG, Takayama S, Rapp UR, Reed JC. 1996b. Bcl-2 interacting protein, Bag-1, binds to and activates the kinase Raf-1. Proc Natl Acad Sci USA 93:7063-7068.

Wang X, Pai JT, Wiedenfeld EA, Medina JC, Slaughter CA, Goldstein JL, Brown MS. 1995. Purification of an interleukin-1β converting enzyme-related cysteine protease that cleaves sterol regulatory element-binding proteins between the leucine zipper and transmembrane domains. J Biol Chem 270:18044-18050.

Wang X, Zelenski NG, Yang J, Sakai J, Brown MS, Goldstein JL. 1996. Cleavage of sterol regulatory element binding proteins (SREBPs) by CPP32 during apoptosis. EMBO J 15:1012-1020.

Wiese S, Digby MR, Gunnersen JM, Gotz R, Pei G, Holtmann B, Lowenthal J, Sendtner M. 1999a. The anti-apoptotic protein ITA is essential for NGF-mediated survival of embryonic chick neurons. Nat Neurosci 11:978-983

Wiese S, Metzger F, Holtmann B, Sendtner M. 1999b. The role of p75NTR in modulating neurotrophin survival effects in developing motoneurons. Eur J Neurosci 11:1668-1676.

Williams NG, Roberts TM. 1994. Signal transduction pathways involving the Raf proto-oncogene. Cancer Metast Rev 13:105-116.

Wojnowski L, Zimmer AM, Beck TW, Hahn H, Bernal R, Rapp UR, Zimmer A. 1997. Endothelial apoptosis in Braf-deficient mice. Nat Genet 16:293-297.

Wojnowski L, Stancato LF, Zimmer AM, Hahn H, Beck TW, Larner AC, Rapp UR, Zimmer A. 1998. Craf-1 protein kinase is essential for mouse development. Mech Dev 76:141-149.

Wu J, Harrison JK, Vincent LA, Haystead C, Haystead TA, Michel H, Hunt DF, Lynch KR, Sturgill TW. 1993. Molecular structure of a protein-tyrosine/threonine kinase activating p42

mitogen-activated protein (MAP) kinase: MAP kinase kinase. Proc Natl Acad Sci USA 90:173-177.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326-1331.

Xue D, Horvitz RH. 1995. Inhibition of the *Caenorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. Nature 377:248-251.

Yamamori B, Kuroda S, Shimizu K, Fukui K, Ohtsuka T, Takai Y. 1995. Purification of a Ras-dependent mitogen-activated protein kinase from bovine brain cytosol and its identification as a complex of B-Raf and 14-3-3 proteins. J Biol Chem 270:11723-11726.

Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X. 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275:1129-1132.

Yao RJ, Cooper GM. 1995. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 267:2003-2006.

Yeo TT, Chua-Couzens J, Butcher LL, Bredesen DE, Cooper JD, Valletta JS, Mobley WC, Longo FM. 1997. Absence of $p75^{NTR}$ causes increased basal forebrain cholinergic neuron size, choline acetyltrasnferase activity, and target innervation. J Neurosci 17:7594-7605.

Yuan JY, Horvitz HR. 1990. Genetics mosaic analysis of *ced-3* and *ced-4*, two genes that control programmed cell death in the nematode *C. elegans*. Dev Biol 138:33-41.

Yuan JY, Shaham S, Ledoux S, Ellis HM, Horvitz HR. 1993. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1β-converting enzyme. Cell 75:641-652.

Zamzami N, Brenner C, Marzo I, Susin SA, Kroemer G. 1998. Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins. Oncogene 16:2265-2282.

Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Petit PX, Mignotte B, Kroemer G. 1995a. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med 182:367-377.

Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX, Kroemer G. 1995b. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death *in vivo*. J Exp Med 181:1661-1672.

Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey L, Castedo M, Kroemer G. 1996. Mitochondrial control of nuclear apoptosis. J Exp Med 183:1533-1544.

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X. Cell 87:619-628.

Zimmermann S, Moelling K. 1999. Phosphorylation and regulation of Raf by Akt (protein kinase B). Science 286:1741-1744.

Zoratti M, Szabo I. 1995. The mitochondrial permeability transition. Biochem Biophys Acta 1241:139-176.

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. 1997. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90:405-413.

Zörnig M, Busch G, Beneke R, Gulbins E, Lang F, Ma A, Korsmeyer S, Moroy T. 1995. Survival and death of prelymphomatous B-cells from N-myc/bcl-2 double transgenic mice correlates with the regulation of intracellular Ca_{2+} fluxes. Oncogene 11:2165-2174.

7. Abbreviations

A	adenosine
AIF	apoptosis-inducing factor
AMP	adenosine monophosphate
ANT	adenosine nucleotide translocation
Apaf-1	apoptotic protease activating factor-1
Apaf-2	apoptotic protease activating factor-2
Apaf-3	apoptotic protease activating factor-3
BDNF	brain-derived neurotrophic factor
BH	Bcl-2 homology
BIR	baculovirus iap repeat
bp	base pair
C	cytosine

°C	degrees Celsius
cAMP	cyclic AMP
CARD	caspase recruitment domain
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CO_2	carbon dioxide
Cy2	carboxymethyl indocyanine-2
Cy3	carboxymethyl indocyanine-3
d	day
Da	dalton
DD	death domain
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
E	embryonic day
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
et al	and others

FITC	fluorescein isothiocyanate
g	gram
G	guanosine
GDNF	glial-derived neurotrophic factor
GDP	guanosine diphosphate
GEF	GDP-GTP exchange factor
GTP	guanosine triphosphate
h	hour
HA	haemagglutinin
HBSS	Hank´s balanced salt solution
IAP	inhibitor of apoptosis
ICE	interleukin-1β-converting enzyme
Ig	immunoglobulin
ITA	inhibitor of T cell apoptosis
JNK	Jun amino-terminal kinase
k	kilo
l	liter
m	milli
M	molar
MAPK	mitogen-activated protein kinase
MEK	MAPK and ERK kinase
min	minute
mRNA	messenger ribonucleic acid
n	nano
NAIP	neuronal apoptotic inhibitory protein
NGF	nerve growth factor
NT3	neurotrophin 3
NT4/5	neurotrophin 4/5
p	pico
P	postnatal day
$p75^{NTR}$	low-affinity neurotrophin receptor
PARP	poly(ADP-ribose) polymerase
PBS	phosphate-buffered saline

PC12 cells	pheochromocytoma PC12 cells
PCD	programmed cell death
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI3-kinase	phosphatidylinositol 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PS	phosphatidylserine
PT	mitochondrial permeability transition
R	receptor
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
SCG	sympathetic chain ganglia
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
Shh	Sonic hedgehog
SREBP	sterol-regulatory element-binding protein
T	thymidine
TBS	Tris-buffered saline
TGT	Tris-buffered saline with gelatine and Triton
Tris	tris (hydroxymethyl) aminomethane
v/v	volume per volume
WT	wild-type
w/v	weight per volume
XIAP	X-chromosome-linked IAP
µ	micro
$\Delta\Psi_m$	mitochondrial transmembrane potential

8. Zusammenfassung

Die Vermittlung von wachstumsfördernden und entwicklungsspezifischen Signalen von aktivierten Zelloberflächenrezeptoren führt zur Initiation von Transkriptionsprogrammen im Zellkern, die durch das koodinierte Zusammenwirken von intrazellulären Signalproteinen synergistisch gesteuert werden. Der Ras/Raf/MEK/MAPK-Weg steuert die Expression von Genen für die physiologische Regulation der Zellproliferation, Differenzierung und Apoptose. Innerhalb dieser Signalkaskade stellen die Serin/Threonin Kinasen der Raf Familie eine zentrale Zwischenstufe dar, die Verbindungen zu vielen anderen Signaltransduktionswegen herstellt.

Um die Funktionen der verschiedenen Raf-Kinasen in Motoneuronen während der Entwicklung aufzuklären, wurden die Expression, Verteilung und subzelluläre Lokalisation der Raf-Isoformen in spinalen Motoneuronen und im Nucleus Fazialis der Maus während der Embryonalentwicklung und postnatal untersucht. Desweiteren haben wir die intrazelluläre Umverteilung der Raf-Moleküle in isolierten Motoneuronen von 13 oder 14 Tage alten Mäusembryonen untersucht. Um die Rolle der Raf-Kinasen nach Zugabe oder Entzug von neurotrophen Faktoren bei Motoneuronen zu untersuchen, analysierten wir die Überlebens-und Differenzierungseffekte von neurotrophen Faktoren bei Motoneuronen von *B-raf* oder *c-raf-1* defizienten Mäusen.

Wir zeigen in dieser Arbeit, daß alle drei Raf-Kinasen in spinalen Motoneuronen der Mäuse exprimiert sind. Ihre Expression steigt während der Zeit des natürlich auftretenden Zelltods. An Schnitten von embryonalem und postnatalem Rückenmark exprimieren Motoneurone ausschließlich B-Raf and c-Raf-1, aber nicht A-Raf. Raf-Kinasen sind offensichtlich an

Mitochondrien lokalisiert. In isolierten Motoneuronen findet man B-Raf und c-Raf-1, Immunreaktivität vor allem im perinukleären Bereich, aber auch im Zellkern, vor allem nach Aktivierung durch Zugabe von CNTF und BDNF *in vitro*. Wir haben gefunden, daß die Translokation von c-Raf-1 vom Zytosol in den Nukleus von Motoneuronen nach Aktivierung durch neurotrophe Faktoren ein spezifischer Vorgang ist.

Als zentralen Befund dieser Arbeit beobachteten wir, daß Motoneurone von *B-raf*$^{-/-}$, aber nicht von *c-raf-1*$^{-/-}$, Embryonen nicht lebensfähig sind, auch nicht in Gegenwart von CNTF oder anderer neurotropher Faktoren. Dies bedeutet, daß B-Raf und nicht c-Raf-1, welches noch immer in *B-raf* defizienten Motoneuronen präsent ist, eine entscheidende Rolle als Vermitter des Überlebenseffektes von neurotrophen Faktoren spielt. Um zu beweisen, daß B-Raf hierbei eine essentielle Komponente darstellt, haben wir in *B-raf* defizienten sensorischen und Motoneuronen B-Raf durch Transfektion exprimiert. Erfolgreich mit *B-raf* Plasmid transfizierte *B-raf*$^{-/-}$ sensorische und Motoneurone zeigten dieselbe Überlebensfähigkeit in Gegenwart von neurotrophen Faktoren wie primäre Neurone von Wildtyp-Mäusen.

Diese Arbeit zeigt daher, daß Raf-Kinasen wichtige Funktionen in Motoneuronen der Maus haben. Die Aktivierung von Raf-Kinasen *in vitro* führt zur Änderung ihrer subzellulären Verteilung, vor allem von c-Raf-1 vom Zytoplasma in den Kern. Diese Umverteilung von c-Raf-1 ist jedoch nicht notwendig für den Überlebenseffekt von neurotrophen Faktoren, vor allem, wenn man in Betracht zieht, daß *B-raf* defiziente sensorische und Motoneuronen trotz der Gegenwart von c-Raf-1 nicht überleben. Wir nehmen an, daß die nukleäre Translokation von c-Raf-1 eine direkte Rolle bei der transkriptionellen Regulation durch neurotrophe Faktoren spielt. Die Indentifizierung von c-Raf-1 regulierten Zielgenen und von durch diese beeinflussten zellulären Funktionen ist eine Aufgabe für die Zukunft.