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RAS and downstream RAF-MEK and PI3K-AKT signaling in neuronal development, function and dysfunction

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Abstract

In postmitotic neurons, the activation of RAS family small GTPases regulates survival, growth and differentiation. Dysregulation of RAS or its major effector pathway, the cascade of RAF-, mitogen-activated and extracellular-signal regulated kinase kinases (MEK), and extracellular-signal regulated kinases (ERK) causes the Rasopathies, a group of neurodevelopmental disorders whose pathogenic mechanisms are the subject of intense research. I here summarize the functions of RAS – RAF – MEK – ERK signaling in neurons *in vivo*, and discuss perspectives for harnessing this pathway to enable novel treatments for nervous system injury, the Rasopathies, and possibly other neurological conditions.

Keywords

axon; MAP kinase; neuron; Rasopathies; regeneration; sensory function

Introduction

The RAS family small GTPases are critical to transmitting extracellular stimuli into neurons, regulating crucial cell functions such as survival, cytoskeletal structure, intracellular transport and gene expression patterns. Germline mutations affecting the RAS – RAF (RAF is an acronym for Rapidly Accelerated Fibrosarcoma) – MEK signaling cascade cause severe neurodevelopmental disorders referred to collectively as the Rasopathies. Understanding of the detailed functions of RAS signaling in the many parts of the nervous system under normal and disease conditions is growing rapidly. Together with the increasing availability of small molecule inhibitors and other methods of modulating intracellular signaling, the RAS – RAF – MEK pathway is poised to become a promising target for the development of novel treatments for congenital and neurodegenerative disorders as well as traumatic injuries to the nervous system.

RAS was first implicated in neuronal differentiation with the discovery that its activation in the neuroendocrine PC12 cells led to neurite outgrowth and the adoption of a neuronal phenotype (Bar-Sagi and Feramisco, 1985). In cultured embryonic sensory neurons, activated RAS similarly promoted axon extension, as well as survival in the absence of the

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crucial nerve growth factor (NGF) (Borasio *et al.*, 1989). Two distinct pathways downstream of RAS orchestrate the morphology of sensory axons *in vitro* – RAF signaling drives elongation while enhanced AKT signaling increases axon caliber and branching (Markus *et al.*, 2002). The major downstream signaling pathways triggered by RAS activation, namely the RAF – MEK – ERK and PI-3 kinase – AKT – mTOR cascades, have been extensively reviewed elsewhere (Reichardt, 2006; Castellano and Downward, 2011). RAS function in the pluripotency and differentiation of stem cells has been discussed previously (Chakrabarty and Heumann, 2008). In non-neuronal cells, RAS signaling controls cell proliferation. Overactivation of RAS signaling is a prominent cause of cancer. The current review focuses on the role of RAS signaling in development and function of postmitotic neurons *in vivo*, examining mouse models and their correlations with the symptoms caused by malfunction of RAS signaling in humans.

Neuronal RAS signaling *in vivo*

The RAS isoforms H-RAS, N-RAS and K-RAS are expressed ubiquitously with subtle variations between cell types and developmental stages. Only K-RAS is essential for mouse embryonic development (Johnson *et al.*, 1997). The specific functions of RAS activation are determined by its subcellular localization and the availability of regulatory as well as effector proteins. Mutant RAS proteins contribute to pathologies through inappropriate activation of their shared downstream effector pathways, the RAF-MAP kinase and PI3-kinase-AKT cascades. These mechanisms have been thoroughly studied for constitutively-activating RAS mutations in the context of cancer (see Giehl, 2005; Aksamitiene *et al.*, 2012; Charette *et al.*, 2014, and Nussinov *et al.*, 2015 for reviews), but far less is known about how RAS activity and RAS mutations affect the function of mature neurons. To begin to address these questions, Heumann *et al.* (2000) expressed the constitutively active H-RAS G12V in the postnatal nervous system under a synapsin-1 promoter ('synRAS' mice), revealing a plethora of effects, some expected and many surprising. The authors found hypertrophy of pyramidal neurons in cortex and hippocampus (also see Gartner *et al.*, 2004), where the transgene was strongly expressed. Constitutively elevated RAS signaling protected facial nucleus motor neurons from apoptosis after axonal lesion, as well as the tyrosine hydroxylase (TH)-positive neurons of the *substantia nigra* exposed to neurotoxins. The brains of synRAS neonates were resistant to oxygen neurotoxicity (Felderhoff-Mueser *et al.*, 2004). Taken together, these data suggest that increased intra-neuronal RAS signaling is generally neuroprotective and might be beneficial in the context of traumatic brain or spinal cord injury, stroke, Parkinson's disease, and possibly other neurodegenerative disorders.

Survival signaling

In vitro, the loading of H-RAS G12V protein or RAS blocking antibodies into chick sensory and rat sympathetic neurons revealed a key role of RAS in peripheral neuron survival (Borasio *et al.*, 1989; Nobes *et al.*, 1996; Markus *et al.*, 1997). In sympathetic neurons, blocking of PI3 kinase signaling abolishes NGF- or BDNF-mediated survival (Kuruvilla *et al.*, 2000; Pierchala *et al.*, 2004; Atwal *et al.*, 2000). Insulin-like growth factor-I (IGF-I) similarly protects peripheral neurons from apoptosis by regulating PI3K/AKT pathway

effectors (Dudek *et al.*, 1997; Leininger *et al.*, 2004). In CNS neurons PI3-kinase–AKT signaling is important in regulating the neuronal survival and development of neocortex (Chan *et al.*, 2011). PI3K–AKT signaling also plays a protective role against diverse stresses in the mature CNS, for example against ethanol-induced neural apoptosis (de la Monte *et al.*, 2000) and oxidative stress (Tapodi *et al.*, 2005). The neuroprotective effects of PI3K–AKT signaling involve the blocking of a caspase-dependent pro-apoptotic mechanism, by triggering the expression of anti-apoptotic actors Bcl-xL and Bcl-2 (Leininger *et al.*, 2004; Anderton *et al.*, 2012) and inactivating multiple pro-apoptotic proteins including the BAD and Forkhead (FKHR) transcription factors (Orike *et al.*, 2001). Overall, neuronal hypertrophy and anti-apoptotic survival signaling as observed in the synRAS brain *in vivo*, have frequently been ascribed to PI3-kinase – AKT signaling. In the synRAS brain, however, MEK – ERK signaling was clearly activated, while no activation of the kinase AKT was detected (Heumann *et al.*, 2000).

Two groups have initially reported that RAF activity, in particular that of the major neuronal isoform B-RAF, is necessary and sufficient to mediate survival signaling in cultured embryonic sensory DRG neurons as well as sympathetic and motor neurons (Wiese *et al.*, 2001; Encinas *et al.*, 2008). *In vivo*, however, conditional ablation of B-RAF (Chen *et al.*, 2006a; Galabova-Kovacs *et al.*, 2008; Pfeiffer *et al.*, 2013) or deletion of both B- and C-RAF in neurons (Zhong *et al.*, 2007) did not lead to cell death of the targeted neurons, suggesting that RAF signaling is not a major mediator of survival signaling during embryonic development. The divergence between *in vitro* and *in vivo* results seems striking, however it should be noted that it has not yet been probed how neuronal RAF signaling would function under extremely stressful conditions, such as neurons presumably encounter in petri dishes. To add further controversy to the issue even *in vivo*, MAP kinase signaling downstream of RAF can be either pro-apoptotic (Luo *et al.*, 2007), or neuroprotective (Zhou *et al.*, 2005) in retinal ganglion cells stressed by either optic nerve injury or ocular hypertension, respectively. Either ERK1 or ERK2 are required for the survival *in vivo* of nociceptive sensory neurons in the DRG (O'Brien *et al.*, 2015), however, as the authors discuss, this effect is likely due to impaired axon growth and ensuing reduced access to trophic factors rather than a direct interaction of ERK signaling with the intracellular apoptotic machinery. Such indirect effects may account for pro-survival effects of RAF – MEK – ERK signaling in other neuronal populations as well. Alternatively it is possible that the effects of the RAF – MAP kinase signaling as well as the details of cell death mechanisms may vary substantially depending on neuron type, developmental age and environment, in ways that are as yet unknown.

Axon growth and regeneration

In vitro work from immortalized cell lines and cultured neurons has long implicated RAS signaling in developmental axon elongation. Elevation of RAF signaling is a far stronger driver of *in vitro* axon extension than elevated PI3 kinase signaling (Markus *et al.*, 2002; Snider *et al.*, 2002). In synRAS mice, where RAS activity remains elevated after axotomy *in vivo*, sprouting growth of facial nucleus motor axons was enhanced after facial motor nerve lesion, in addition to the neuroprotective effect (Makwana *et al.*, 2009). Conditional deletion of both B-RAF and C-RAF in the nervous system of mouse embryos resulted in sensory

axons' diminished arborization in the skin as well as stunted proprioceptor projections towards the ventral motor neurons in the spinal cord (Zhong *et al.*, 2007). Conversely, expression of the constitutively-active B-RAF^{V600E} from the endogenous *Braf* locus enabled strong elongation of developing nociceptive axons in embryos lacking NGF (O'Donovan *et al.*, 2014); NGF normally expressed in fetal skin acts as an essential target-derived factor for nociceptor survival and axon extension.

Most excitingly, expression of the kinase-activated B-RAF in retinal ganglion cells (RGC) enabled substantial regeneration of retinofugal axons after optic nerve injury. This effect was dependent on the MEK kinases, the classical direct effectors of RAS – RAF signaling in the canonical RAF – MEK – ERK cascade (O'Donovan *et al.*, 2014; Zhong, 2015).

Growth-related intracellular mechanisms are generally downregulated in the maturing nervous system (Harel and Strittmatter, 2006; Sun and He, 2010; Moore and Goldberg, 2011), presumably to enable stability of its adult structure and circuitry. Enhanced expression of phosphatases dampening MAP kinase signaling during maturation may be one cause of the reduced growth competency in adult neurons (Finelli *et al.*, 2013a). This downregulation affects both major signaling cascades downstream from RAS, the RAF – MEK – ERK and the PI3 kinase – AKT – mTOR pathways. The latter has also been shown to enhance axonal regeneration, in both the optic nerve (Park *et al.*, 2008) and in the lesioned corticospinal tract (Liu *et al.*, 2010). These authors activated PI3 kinase signaling by ablation of its cell-intrinsic antagonist, the phospholipid phosphatase PTEN. Combined activation of B-RAF and PI3 kinase can drive post-injury axon elongation in the optic nerve synergistically to achieve a more-than-additive effect (O'Donovan *et al.*, 2014).

While these recent results in mouse models of CNS injury are promising, their translation into clinical application will not be straightforward. RAS and B-RAF are well-known as proto-oncogenes and PTEN is an anti-oncogene. B-RAF activating mutations are frequently found in pediatric and adult astrocytomas and gliomas (Dahiya *et al.*, 2014). Prolonged and especially combined activation of B-RAF and inhibition of PTEN would therefore present immediate safety concerns (Dankort *et al.*, 2009). One way to minimize the danger of uncontrolled growth could be to limit activation to postmitotic neurons by stringent spatio-temporal control. A remote but more promising option would be to identify the specific downstream effectors of RAS – RAF and RAS – PI3 kinase signaling that execute axon growth and regeneration processes in neurons without also having mitogenic effects in non-neuronal cell types. Potential candidate genes could be the MEK1/2 kinases, or transcription factors such as Serum Response Factor 1 (SRF1) and the Early Growth Response protein (EGR) family transcription factors, which are required for neurite formation in embryonic neurons or PC12 cells, respectively (Wickramasinghe *et al.*, 2008; Levkovitz *et al.*, 2001). In the peripheral nervous system axon lesion triggers retrograde, importin- and vimentin-dependent transport of phosphorylated Erk1/2 from the axon to the cell body. This transport is crucial for the phosphorylation of the ETS family transcription factor Elk1, and for the ensuing axon regenerative response (Hanz *et al.*, 2003; Perlson *et al.*, 2005; Mar *et al.*, 2015). The efficacy of all these downstream signaling pathways in promoting CNS axon regeneration *in vivo* remains to be tested.

Apart from transcription factors, RAF – MEK – ERK signaling can also affect epigenetic control of gene expression. The histone deacetylase HDAC6 is phosphorylated by Erk1/2, and cell migration triggered by RAS – RAF – ERK signaling requires HDAC6 (Williams *et al.*, 2013). The effects of this phosphorylation *in vivo* or in the nervous system have not yet been defined, but would certainly be very interesting to know. The removal of acetyl modifications on histones by HDACs generally results in reduced DNA transcription. Axon growth and regeneration are transcription-dependent processes, and consequently treatment with HDAC inhibitors can enhance axon growth as well as peripheral axon regeneration (Rivieccio *et al.*, 2009; Tang, 2014), and even the regeneration of ascending sensory fibers in the spinal cord following dorsal column transection (Finelli *et al.*, 2013b; reviewed in Zhong and Zou, 2014).

Additional functions beyond histone deacetylation have been ascribed to a number of HDACs. Several members of the Class II HDACs (HDACs 4, 5, 6, 7, 9, 10) can move between nucleus and cytoplasm in response to extracellular signals. HDAC6 has been shown to de-acetylate alpha-tubulin and cortactin (Hubbert *et al.*, 2002; Zhang *et al.*, 2007) among other substrates, thus increasing cytoskeletal plasticity, which would be important to growth cone formation after axonal lesion as well as to axonal growth itself. In the periphery, HDAC5 is induced after sciatic nerve injury and promotes axon regeneration via tubulin deacetylation (Cho and Cavalli, 2012; Cho *et al.*, 2013). For a comprehensive review of HDAC functions in the development and regeneration of the nervous system, see Cho and Cavalli (2014).

RAF – MEK – ERK signaling in sensory neuron specification and function

Sensory neurons are born as part of the neural crest, immigrate into the peripheral ganglia, there undergo a phase of stringent selection with only about 20-25% survive, and then diversify into multiple subgroups over the weeks of later fetal development and the early postnatal phase up to age three weeks. The process of sensory neuron subtype differentiation is orchestrated by the transient expression of a small number of known transcription factors and receptors (see Liu and Ma, 2011, for a review). Curiously, the intracellular signaling mechanisms that link receptors with nuclear effectors and cause the induction and suppression of transcription factors along precise timelines have been little researched. Given that tyrosine kinase receptors including TrkA, TrkB, TrkC and Ret play crucial roles in sensory neuron diversification, it seems likely that RAS – RAF – MEK signaling would be involved.

The majority of surviving young TrkA-positive sensory neurons develop into nociceptors (pain-sensing neurons); smaller proportions of neural crest-derived neurons become thermoreceptors and mechanoreceptors. The nociceptors themselves differentiate into subtypes expressing distinct combinations of receptors and channels and transmitting frequently overlapping types of painful stimuli such as noxious heat, noxious cold, and noxious pressure. The small group of pruriceptors (itch-sensing neurons) emerge from the pool of nociceptor precursors that express both TrkA and the transcription factor Runx1 (Chen *et al.*, 2006b; Kramer *et al.*, 2006; Zhong *et al.*, 2006). RAF kinase signaling activates the transcription complex Runx1/CBF β in DRG neurons (Zhong *et al.*, 2007; Huang *et al.*,

2015), and postnatal *in vivo* activation of B-RAF in nociceptors selectively induces expression of the two most prominent pruriceptor markers, Mas-related G protein-coupled receptor 3A (Mrgpr3A) and gastrin-releasing peptide (GRP) (Zhao *et al.*, 2013). As a result, hybrid neurons expressing both noci- and pruriceptors emerge, and nociceptors are transformed into functional pruriceptors that cause a severe chronic itching phenotype in the mice. It has not yet been shown directly, however, whether RAF – MEK signaling is involved in the initial specification of pruriceptors.

Pain sensation and transmission also involves MEK – ERK signaling. Intradermal or intramuscular injection of NGF causes persistent pain and allodynia in rodents and humans. In two rat models of neuropathic pain, heat and mechanical allodynia behaviors were attenuated by selective inhibition of ERK1/2 but not ERK5 (Matsuoka and Yang, 2012). Conversely, painful conditions are associated with increased phosphorylation of ERK1/2 at different levels of the pain processing pathway, i.e. the dorsal root ganglia (DRG) and spinal cord (Ji *et al.*, 2009). O'Brien *et al.* (2015) identified ERK2 as a crucial mediator of cold nociception in the Nav1.8-expressing subpopulation of nociceptive sensory neurons. In Rasopathies patients, whose conditions are caused by various germline mutations that affect RAS downstream signaling, chronic pain is a common symptom (Vegunta *et al.*, 2015).

The Rasopathies

A group of distinct but related congenital neurodevelopmental syndromes, the 'Rasopathies', also referred to as neuro-cardio-facio-cutaneous syndromes (NCFCs), have been linked to malfunction of RAS signaling and the expansive signaling network it triggers. The Rasopathies affect about 1 in 1000-2000 newborns and are caused by mutations in regulatory proteins of the RAS–RAF–MEK signaling pathway (Rauen, 2013; Myers *et al.*, 2014; Frye, 2015; Jindal *et al.*, 2015). In addition to cardiac defects and dysmorphic facial features, patients frequently present with growth retardation and a variety of neurological, cognitive, behavioral and/or motor coordination problems (Roberts *et al.*, 2006; Yoon *et al.*, 2007). The cardiac and craniofacial issues of Rasopathies patients can to a large extent be attributed to abnormal development of non-neuronal cell types derived from the neural crest. This concept has been buttressed by a set of mouse models where B-RAF, C-RAF, MEK1/2, ERK1/2 or the transcription factor SRF were partially or completely inactivated in neural crest, recapitulating in severe form the craniofacial and cardiac abnormalities seen in Rasopathies patients (Newbern *et al.*, 2008).

75% of patients diagnosed with cardio-facio-cutaneous (CFC) syndrome, one of the Rasopathies, carry mutations in the *Braf* gene, and 25% carry mutations in either the MEK1 or the MEK2 genes (Rauen, 2012; <http://www.ncbi.nlm.nih.gov/books/NBK1186/>). Interestingly, both gain- and loss-of-function mutations in the *Braf* gene can cause CFC syndrome. The kinase-activating Braf Q257R point mutation accounts for approximately 20% of CFC cases. In a zebrafish model, the expression of CFC-associated B-RAF, MEK1 or MEK2 point mutation alleles all caused a similar disruption of gastrulation, independent of whether the mutations were considered kinase gain-of-function (12 alleles, including all MEK1/2), loss-of-function (one B-RAF allele, the G596V mutation), or the effect on kinase activity was unknown (four B-RAF mutations) (Anastasaki *et al.*, 2009). The pathogenic

mechanism is therefore unlikely to rely on the mutant kinases' specific kinase activity. Nevertheless, all CFC-related alleles caused elevated ERK activation, and sustained partial inhibition of MEK – ERK signaling with a small molecule inhibitor administered at low dose ameliorated the phenotype substantially for all tested alleles (Anastasaki *et al.*, 2012). These data indicate that causal treatments for Rasopathies may be possible, and also raise the intriguing possibility that MEK and ERK signaling may involve molecular interactions beyond their kinase activities. Confirmation of these findings in mammalian models is urgent.

Loss- and gain-of-function mouse models for CFC syndrome have been generated over the last few years. Gain-of-function was achieved by low-level expression of the strongly kinase-activated B-RAF^{V600E} (Urosevic *et al.*, 2011), or by expression of the activating mutation B-RAF^{Q241R}, the mouse equivalent of the human B-RAF^{Q257R} found in CFC patients (Moriya *et al.*, 2015). Loss-of-function mice were generated by genetic inactivation of B-RAF in essentially all neurons (Zhong *et al.*, 2007; Galabova-Kovacs *et al.*, 2008). All these mouse models exhibit growth retardation, cardiac defects, reduced lifespan, and some level of craniofacial or cutaneous abnormalities. Neuron-specific inactivation of B-RAF causes severe growth retardation from age 10 days onwards, with low levels of circulating growth hormone (GH) but high levels of GH in anterior pituitary neuroendocrine cells, indicating a malfunction of vesicle release mechanisms (Zhong *et al.*, 2007). Reduced hippocampal LTP has also been reported in B-RAF loss-of-function mice (Chen *et al.*, 2006a); which may reflect the cognitive problems often associated with CFC syndrome and the Rasopathies in general. Along similar lines, cortex-specific ablation of ERK2 in a mouse model of DiGeorge syndrome and autism altered the excitability and network activity of cortical neurons; the mice displayed strong anxiety-like behaviors and a reluctance to explore novel environments (Pucilowska *et al.*, 2012). Combined loss of ERK1 and ERK2 activity in the forebrain led to profound depletion of neuronal precursor cells peri- and postnatally in the dentate gyrus of the hippocampus (Vithayathil *et al.*, 2015). As dentate gyrus neurogenesis is implicated in memory formation (Jessberger *et al.*, 2009), reduced neurogenesis might well contribute to cognitive impairments in Rasopathies patients.

Summary

RAS signaling and that of its major downstream cascade consisting of B-RAF/C-RAF, MEK1/2 and ERK1/2 affect the nervous system development in manifold ways. This pathway is crucial to normal function, its dysregulation underlies complex diseases, and it may be harnessed to enable neuroprotection and axon regeneration. As yet our methods to manipulate RAS–RAF – MEK – ERK signaling are too crude to allow therapeutical application. As more refined tools such as the highly selective ERK inhibitors used to ameliorate neuropathic pain in rats (Matsuoka and Yang, 2012) and gastrulation phenotypes in the zebrafish model (Anastasaki *et al.*, 2012) become available, the RAS – to – ERK cascade will become a target for novel therapies to counteract axonal degeneration, enable functional recovery after traumatic injury, and restore cognitive function in conditions such as the Rasopathies.

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