Mutations in the extracellular signal-regulated kinase (ERK) pathway, particularly in the mitogen-activated protein kinase/ERK kinase (MEK) activator B-Raf, are associated with human tumorigenesis and genetic disorders. Hence, B-Raf is a prime target for molecule-based therapies, and understanding its essential biological functions is crucial for their success. B-Raf is expressed preferentially in cells of neuronal origin. Here, we show that in mice, conditional ablation of B-Raf in neuronal precursors leads to severe dysmyelination, defective oligodendrocyte differentiation, and reduced ERK activation in brain. Both B-Raf ablation and chemical inhibition of MEK impair oligodendrocyte differentiation in vitro. In glial cell cultures, we find B-Raf in a complex with MEK, Raf-1, and kinase suppressor of Ras. In B-Raf-deficient cells, more Raf-1 is recruited to MEK, yet MEK/ERK phosphorylation is impaired. These data define B-Raf as the rate-limiting MEK/ERK activator in oligodendrocyte differentiation and myelination and have implications for the design and use of Raf inhibitors.

Introduction

The Raf kinases (A-Raf, B-Raf, and Raf-1) relay extracellular signals to the MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) kinase (MEK) signaling module. Among the three Raf kinases, B-Raf binds best to MEK and has the highest basal MEK kinase activity. Growth factor–stimulated ERK activation is reduced (~60%) in cells lacking B-Raf but not in A-Raf– or Raf-1–deficient cells (Wojnowski et al., 2000; Huser et al., 2001; Mikula et al., 2001; Mercer et al., 2002; Pritchard et al., 2004). Finally, Raf kinases from lower organisms (lin-45 in Caenorhabditis elegans and D-Raf in Drosophila melanogaster) are more similar to B-Raf than to A-Raf or Raf-1 (Wellbrock et al., 2004). All these observations hint at B-Raf as the essential mammalian MEK kinase, whereas A-Raf and Raf-1 may have a subordinate role or have diverged to perform other functions.

B-Raf is mutated in a high percentage of certain human cancers (Davies et al., 2002) and congenital progressing conditions like cardio-facio-cutaneous syndrome (Rodriguez-Viciana et al., 2006). This discovery has generated huge industrial interests and inhibitors are currently being tested in preclinical and clinical trials. However, our fundamental understanding of B-Raf biology is still scanty, and embryonic lethality associated with germline B-Raf ablation (Wojnowski et al., 1997; Galabova-Kovacs et al., 2006) has precluded its investigation. B-Raf is highly expressed in cells of neuronal origin (Wojnowski et al., 2000) and reportedly plays a prosurvival role in cultured primary embryonic neurons (Wiese et al., 2001). Therefore, the analysis of animals in which B-Raf is selectively ablated in neuronal precursors leads to growth retardation, disorders of hypothalamic-pituitary function, and premature death. b-raf ablation does not interfere with neurogenesis or neuron survival, and the only molecular defect investigated to date is the reduced expression of the glial cell line–derived neurotrophic factor receptor Ret in dorsal root
ganglion neurons at postnatal stages, a rather mild phenotype. Concomitant elimination of b-raf and c-raf-1 strongly reduced axon growth in vitro and cutaneous axon terminal arborization in vivo, which suggests that Raf-1 can compensate for the loss of B-Raf function in this system (Zhong et al., 2007).

We show that mice with epiblast-restricted (Mox2cre; b-raf f/f) and neuronal precursor–restricted b-raf ablation (b-raf f/f neu) develop progressive loss of coordination, tremors, and severe muscular weakness and die around postnatal day 21 (P21).

In line with this phenotype, we detect a major defect in central nervous system (CNS) myelination and reduced oligodendrocyte differentiation. B-Raf knockout (KO) oligodendrocytes in situ and in culture fail to activate MEK/ERK and to differentiate, despite the increased recruitment of Raf-1 to MEK in KO cells. Blunting ERK activation in wild-type (WT) oligodendrocytes with a MEK inhibitor similarly impairs differentiation. Together, the data indicate an essential role of B-Raf and its downstream effector ERK in oligodendrocyte differentiation and myelination.

Results

Ablation of B-Raf in the epiblast and in neuronal precursors causes severe neuromuscular defects

b-raf was inactivated by Cre-loxP-mediated deletion of exon 11, which encodes the kinase domain (Chen et al., 2006). Deletion leads to a shift in the open reading frame and completely abrogates B-Raf protein expression (Fig. 1, C–D). Epiblast-restricted ablation in the 129/Sv background yielded live offspring. Mox2cre; b-raf f/f were indistinguishable from littermate controls at birth but showed growth retardation starting around P10. This phenotype was followed by loss of coordination, the onset of tremors, ataxia, and muscle weakness (at P15). P10–21, B-Raf–deficient animals suspended by the tail clasped their limbs to their trunks in a dystonic fashion, a diagnostic sign of neurological impairment (Fig. 1 A). After P18, the Mox2cre; b-raf f/f mice deteriorated rapidly, showing increasing difficulties in ambulating and finally in breathing (Videos 1–3, available at http://www.jcb.org/cgi/content/full/jcb.200709069/DC1).

Spleen size was markedly decreased (unpublished data), likely because of the previously reported essential role of B-Raf in B cell development (Brummer et al., 2002). With the exception of the latter, all phenotypes were phenocopied in NestinCre;b-raf f/f (b-raf ΔΔ neu) animals, which express the Cre recombinase in CNS neural precursor cells that give rise to both neurons and glia (Tronche et al., 1999; Haigh et al., 2003). NestinCre mediated efficient recombination in brain and spinal cord as early as embryonic day 11.5 (Fig. S1). Complete conversion of the b-raf Δ allele to the b-raf ΔΔ allele was evident in brain and spinal cord (not depicted) but not in other tissues derived from b-raf ΔΔ neu mice (Fig. 1 B). Accordingly, B-Raf could not be detected by immunoblotting in b-raf ΔΔ neu brain (Fig. 1, C and D), spinal cord (Fig. 1 D), and glial cell cultures derived from P0 animals (see Fig. 6, A–C). In B-Raf–deficient brains, A-Raf expression was unchanged, whereas Raf-1 was slightly up-regulated (Fig. 1 D). Thus, the pathology (growth retardation, muscle weakness, tremors, and ataxia) observed in Mox2cre; b-raf f/f was caused by a defect of neural precursor cells. Histological examination revealed severe atrophy of skeletal muscle fibers (Fig. S2 C) but axon retraction/regeneration was not detected, and both the morphology and innervation of the neuromuscular junctions were normal in the b-raf ΔΔ neu mice (Fig. S2 D).

Macroscopically and histologically, the brains of Mox2cre; b-raf f/f and b-raf ΔΔ neu did not show gross anomalies. P18 B-Raf–deficient brains were slightly smaller than those of WT littermates and the molecular layer of their brain cortex was thinner (Fig. S2, A and B; Zhong et al., 2007). In the cerebellum, B-Raf ablation caused a slight accumulation of granular cells at the cerebellar surface, which could be caused by a delay in postnatal migration or a mild increase in precursor proliferation (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200709069/DC1). These defects were observed with a 100% penetrance.

Ablation of B-Raf in neuronal precursors causes CNS dysmyelination

The mild anomaly in cerebellar development may contribute to the ataxic behavior of the b-raf ΔΔ neu mice (Grusser-Cornehls
and Baurle, 2001). Overall, however, the neurological symptoms of the b-raf\(^{\Delta}\Delta\alpha_{\text{neu}}\) mice and their onset on P14 are reminiscent of those observed in myelin mutants occurring naturally, like shiverer or jimpy (Sturrock, 1980; Popko et al., 1987; Nave, 1994), or generated by gene targeting (Olig1 null mice; Xin et al., 2005), which suggests a defect in myelination. Indeed, at P18, significantly fewer myelinated fibers could be observed in different areas of P18 b-raf\(^{\Delta}\Delta\alpha_{\text{neu}}\) brains (Fig. 2, A–C; Fig. S4 A; and Fig. S5, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200709069/DC1), and immunoblotting confirmed a reduction in the total amount of myelin basic protein (MBP; Figs. 2 D and S4, B and C). In the spinal cord and peripheral nerves, MBP staining was very strong and the differences between WT and b-raf\(^{\Delta}\Delta\alpha_{\text{neu}}\) were not as clear (unpublished data). Immunohistochemical analysis and quantitative immunoblotting of P0–18 brains showed that myelination was reduced significantly in b-raf\(^{\Delta}\Delta\alpha_{\text{neu}}\) animals throughout postnatal development (Fig. S4 D). The decrease in MBP expression was also evident at the mRNA level (Fig. S4 E). In contrast to MBP, the expression of other major myelin components (MAG, MOG, and PLP/DM20) was unchanged. Transmission electron microscopy of selected CNS areas showed that B-Raf ablation did not prevent axon recognition and ensheathment; however, the number of unmyelinated fibers in the optic nerve, corpus callosum, and, less obviously, in the spinal cord (Fig. 3 and not depicted) of b-raf\(^{\Delta}\Delta\alpha_{\text{neu}}\) mice was clearly increased. In addition, the g ratio (the numerical ratio between the diameter of the axon proper and the outer diameter of the myelinated fiber) was higher than in WT CNS. Signs of gliosis were observed in the optic nerve but we did not observe axonal swelling or degeneration, fragmented organelles, myelin debris, or macrophage infiltration. In addition, the total number of axons in WT and b-raf\(^{\Delta}\Delta\alpha_{\text{neu}}\) optic nerves did not differ. All these data are consistent with a delay in CNS myelination (dysmyelination) rather than with demyelination.

Oligodendrocytes are the myelin-forming macroglia of the vertebrate CNS. They originate mostly from ventral but also dorsal sources in the spinal cord and forebrain (Richardson et al., 2006). Oligodendrocyte precursors with proliferative capacity express the NG2 proteoglycan and PDGF receptor \(\alpha\) (PDGFR\(\alpha\)) and migrate from their sites of origin to their final position in the white matter, where terminal differentiation starts at the end of embryogenesis. Terminal differentiation is characterized by loss of NG2 and PDGFR\(\alpha\), the up-regulation of the cytoskeletal marker \(\beta\)IV-tubulin (expressed by premyelinating, early myelinating, and myelinating oligodendrocytes; Terada et al., 2005), and the induction of myelin genes like MBP. At P18, B-Raf–deficient brains contained more undifferentiated PDGFR\(\alpha^{+}\) oligodendrocytes than WT brains in all regions investigated (Fig. 4 A and Fig. S5, A, D). In parallel, the amount of \(\beta\)IV-tubulin\(^{+}\) cells was reduced. Together with the reduced expression of MBP and the dysmyelination observed in B-Raf KO CNS, this suggested that B-Raf ablation caused a delay in oligodendrocyte differentiation. To confirm this, we established oligodendrocyte precursor cultures (Zezula et al., 2001) from WT and B-Raf KO brains. The initial purity (88 ± 12% NG2\(^{+}\) cells) and the total number of cells present in these cultures were comparable. After differentiation, however, B-Raf KO cultures contained significantly higher numbers of NG2\(^{+}\) prooligodendrocytes, whereas the amount of pre-myelinating oligodendrocytes (NG2\(^{+}\)/O4\(^{+}\) with radial processes and limited arborization, and O4\(^{+}\) cells with a complex process network) was correspondingly decreased (Fig. 4 B). MBP\(^{+}\) cells were hardly present in B-Raf–deficient cultures and the few detected expressed much less MBP than WT cells (Fig. 4 C). WT oligodendrocyte precursor cultures differentiating in the presence of the MEK inhibitor U0126 also failed to produce MBP\(^{+}\) cells (Fig. 4 C). This shows a causal link between ERK phosphorylation and oligodendrocyte differentiation and suggests that B-Raf is required for efficient ERK phosphorylation in this system.
At P18, ERK phosphorylation was readily detectable by immunohistochemistry in various areas of the WT brain, including cortex, cerebellum, and hypothalamus (Fig. 5B and not depicted). In particular, a strong positive signal was observed in the neuropil of the forebrain and cerebellar cortical plate, which consists of axons, dendrites and glial processes, and in the Purkinje cells of the cerebellar cortex. In the forebrain cortex, the pERK signal was more rarely associated with cell bodies, and if it was, it was mainly observed in cells featuring the small heterochromatic nuclei typical of glial cells. pERK was dramatically reduced in all cells and areas of b-rafΔ/Δ neu brains (Fig. 5B).

B-Raf is required for MEK/ERK activation in oligodendrocyte-enriched cultures and in oligodendrocytes in vivo

To assess whether B-Raf was required for MEK/ERK activation in cells of the oligodendrocytic lineage, we stimulated oligodendrocyte-enriched primary mixed glial cultures with PDGF, which reportedly regulates oligodendrocyte development (Fruttiger et al., 1999) and activates ERK in differentiating oligodendrocytes (Bhat and Zhang, 1996). PDGF-induced phosphorylation of MEK and ERK was impaired in the B-Raf–deficient cells (Fig. 6A). In contrast, B-Raf ablation had hardly any effect on basal and PDGF-induced MEK/ERK phosphorylation in oligodendrocyte-depleted glial cell cultures (≥90% astrocytes; Fig. 6B), which supports the idea that oligodendrocytes are the glial cell type in which ERK phosphorylation is most affected by the lack of B-Raf. A decrease in MEK/ERK phosphorylation was also apparent in B-Raf–deficient, oligodendrocyte-enriched cultures treated with other stimuli involved in oligodendrocyte differentiation, namely FGF (Kessaris et al., 2001).
Finally, to confirm that B-Raf is the ERK activator in differentiating oligodendrocytes in vivo, we colabeled P18 brain sections with antibodies against pERK and βIV-tubulin. On the basis of their morphology (Zhang, 2001), pERK+ cells were classified as premyelinating oligodendrocytes, featuring processes with a low degree of arborization (Fig. 6 F, left) or more elaborate process networks (middle); and mature myelinating cells extending their processes toward myelin sheets (Fig. 6 F, right). In the latter case, a very strong pERK signal was associated with the myelinated fibers. ERK phosphorylation was undetectable in βIV-tubulin+ cells from b-rafΔ/Δneu brains (Fig. 6 F).

Discussion

Our data allow several important conclusions. The fact that the deletion of b-raf in neuronal precursors almost completely phenocopies the deletion in the embryo proper (with the exception of the reduced spleen) implies that in vivo, at least during the first 3 wk of life, B-Raf is not absolutely required for proliferation, survival, or differentiation of other cell types. As has been previously suggested (Zhong et al., 2007), the lack of major
modular response analysis has recently confirmed that ERKs negatively feed back on MEKs (Santos et al., 2007). If this were true in the developing brain in vivo, less sustained ERK activity could explain the stronger MEK phosphorylation.

Be that as it may, in postnatal brain development, the highest levels of ERK phosphorylation were observed in coincidence with the increased expression of B-Raf and with the onset and progress of myelination, which was critically dependent on B-Raf. B-Raf–deficient oligodendrocyte precursors show impaired MEK/ERK activation and differentiation in vitro and in vivo, and chemical inhibition of MEK prevents the development of MBP-expressing cells in WT cultures (Figs. 4 and 6), demonstrating a causal relationship between ERK activation and terminal oligodendrocyte differentiation. The latter result is in agreement with previous reports demonstrating that FGF-induced ERK activation cooperates with Sonic hedgehog to promote oligodendrocyte maturation in culture (Kessaris et al., 2004) and that MEK/ERK inhibition reduces MBP production by basal forebrain oligodendrocytes (Du et al., 2006).

Thus, in the oligodendrocyte lineage, B-Raf is the critical ERK activator. This is underscored by the inability of Raf-1 to compensate for B-Raf ablation, notwithstanding its binding to MEK in B-Raf–deficient oligodendrocytes (complex III; Fig. 6 G). The molecular basis for this may be simply that the higher intrinsic kinase activity of B-Raf is directly required for MEK activation in these cells (complex I; Fig. 6 G); alternatively, the presence of B-Raf may be required to regulate the kinase activity of Raf-1 in the context of the recently described B-Raf–Raf-1 heterodimer, which represent the Raf kinase with the highest activity (Garnett et al., 2005; Rushworth et al., 2006). In the

changes in the cellularity and architecture of the nervous system itself further implies that, in the context of the whole animal, B-Raf is not essential for the proliferation or survival of neurons. Previous studies have predicted a role for B-Raf in neuronal survival (Wiese et al., 2001), differentiation (Marshall, 1995; Kao et al., 2001), and axon growth (Markus et al., 2002). With the caveat that the level of analysis presented in this paper is not sufficient to draw conclusions on subtle differences in neuronal differentiation, the simplest explanation for the absence of major defects is that the in vivo environment provides a variety of signals that promote neuronal survival and sustains differentiation independently of B-Raf, possibly via other Raf kinases. In line with this hypothesis, the marginal defects in dorsal root ganglia differentiation caused by ablation of b-raf alone become progressively more dramatic when one or both c-raf-1 alleles are eliminated (Zhong et al., 2007). Compound tissue-restricted KOs of the Raf genes will be required to circumvent redundancy and clarify the biological role of Raf in neuronal tissues.

In brain lysates, MEK and ERK phosphorylation are not synchronized. MEK phosphorylation is highest at early stages of postnatal development, when B-Raf is hardly expressed, whereas ERK phosphorylation coincides with B-Raf expression and is evident at later stages. One trivial but not likely explanation is that MEK and ERK may not be expressed concomitantly in the same cell types during early postnatal development. Alternatively, if MEK and ERK are expressed concomitantly, the discrepancy between MEK and ERK phosphorylation could be explained by a stronger negative feedback (e.g., phosphatase activity) targeting ERK at these early stages. In PC12 cells,
latter case (complex II; Fig. 6 G), the physical presence of B-Raf may be more important than its kinase activity (Garnett et al., 2005). Consistent with the latter hypothesis, mice expressing a kinase-dead form of B-Raf do not have a neurological phenotype (Pritchard, C., personal communication).

In oligodendrocyte-enriched cultures, B-Raf promotes the recruitment of KSR1 to the Raf-MEK-ERK module (Fig. 6 C and complex I and II in Fig. 6 E). Currently, KSR is thought to bind constitutively to MEK/ERK and present it to Raf, thereby both insulating and enhancing ERK signaling. Our data instead imply that B-Raf regulates the scaffold function of KSR. It is conceivable that B-Raf binds to KSR1 and brings it to the MEK/ERK module in the context of a B-Raf–KSR heterodimer. Alternatively, B-Raf may activate KSR, enabling it to bind to MEK1. Considering the strong similarity between Raf-1 and KSR, activation may occur allosterically, in analogy to the recently described activation of Raf-1 by B-Raf (Garnett et al., 2005). Finally, the association of KSR with the MEK/ERK module might be regulated by KSR phosphorylation, mediated directly or indirectly by B-Raf.

Both soluble and axonal signals could stimulate B-Raf activation and oligodendrocyte differentiation in vivo. Candidates are FGF, PDGF, and ErbB receptor ligands, including the membrane-tethered neuregulin-1 type III, which has been implicated as a major axonal signal regulating myelination in Schwann cells (Nave and Salzer, 2006). Whatever the signals in vivo may be, the impaired differentiation observed in \( b^{raf}\)-null oligodendrocyte cultures suggests that B-Raf is either an essential effector of the crucial signal or a point of convergence of multiple factors promoting oligodendrocyte differentiation.
Histological examination shows that ERK phosphorylation is strongly reduced from P15 on in the brain of $b$-raf$^{1/1\Delta neu}$ mice, and we couldn’t identify any specific cell type or location that would be spared. With the exception of Purkinje cells, pERK staining was seldom associated with the soma of neurons but was rather found in smaller cells with heterochromatic nuclei characteristic of glia. Specifically, pERK was clearly detectable in premyelinating and myelinating oligodendrocytes of WT but not $b$-raf$^{1/1\Delta neu}$ brains (Figs. 5 and 6). Given the extensive cross-talk between glia and neurons, it is possible that the lack of ERK phosphorylation in B-Raf–deficient neurons may not, or not exclusively, be cell autonomous but rather due to the lack of support by the oligodendrocytes. It is, for instance, conceivable that the defect may result from the decreased action potentials generated as a consequence of delayed/decreased myelination.

Moreover, other oligodendrocyte functions may impact on neurons. Among these are the secretion of factors that promote neuronal survival (Sherman and Brophy, 2005) and the recently reported physical connection between NG2+ glial progenitors present in different brain regions with surrounding neurons via direct synapses. Particularly relevant to the topic of this work, the NG2+ cells of the cerebellum are directly innervated by the climbing fibers from the inferior olive (Lin et al., 2005) and actually share climbing fibers with the surrounding Purkinje cells. Therefore, they are ideally positioned to modulate Purkinje cells excitation by climbing fibers and cerebellar functions (Lin et al., 2005).

The involvement of the macroglia is being increasingly recognized in an expanding number of neurological diseases, including Alzheimer’s disease and schizophrenia (Rowitch, 2004; Georgieva et al., 2006). We have shown that B-Raf ablation and the consequent impairment of ERK activation in oligodendrocytes lead to delayed differentiation and dysmyelination and are ultimately incompatible with extraterine life. With the development of safe B-Raf inhibitors for clinical use in mind, it will be important to assess whether B-Raf is essential in this lineage during early development exclusively or whether its ablation later in life will lead to harmful disturbances of the macroglia including demyelination.

Materials and methods
Mice
Generation of mice carrying floxed $b$-raf alleles ($b$-raf$^{fl}$) has been described previously (Chen et al., 2006). $b$-raf$^{fl}$ mice were maintained on a 129/Sv background and crossed to transgenic mice expressing Cre under the control of the Nestin promoter (Tronche et al., 1999) for neuronal precursor-restricted ablation. Animal care was in accordance with the guidelines of the Max F. Perutz Laboratories.

PCR analysis of offspring and tissues
Tail and tissue DNA preparation and b-raf PCR were performed as described previously (Galabova-Kovacs et al., 2006).

Histology, immunohistochemistry, and electron microscopy
Unless stated otherwise, serial sections of 4% paraformaldehyde-fixed and paraffin-embedded tissues were cut at 3-μm thickness and placed on precoated slides (0.5% VECTABOND reagent; Vector Laboratories) and routinely stained with hematoxylin and eosin.

The following primary antibodies were used for immunohistochemistry: α-ERK (Cell Signaling Technology), α-IVIV-V (Sigma-Aldrich), α-MBP (Santa Cruz Biotechnology, Inc.), α-PDGFRα (Thermo Fisher Scientific), or unrelated, isotype-matched controls. Immunocomplexes were visualized using appropriate secondary antibodies conjugated with Alexa 488 or 594 (Invitrogen), the ultraviolet-sensitive avidin biotinylated enzyme complex staining kit (ABC; Vector Laboratories), or the EnVision peroxidase system (Dako) according to the manufacturer’s instructions, followed by incubation with 0.01% diaminobenzidine (Sigma-Aldrich). Epifluorescence was performed at room temperature using Immersion 518 (Carl Zeiss, Inc.) as an imaging medium and a microscope (AxioImager M2; both from Carl Zeiss, Inc.) equipped with a Plan Neofluar 40× 1.30 oil objective (Carl Zeiss, Inc.) and a charge-coupled device (CCD) camera (Spot2; Diagnostic Instruments, Inc.). Images were acquired using the MetaVue 5.06 software (MDS Analytical Technologies). Light microscopy was performed at room temperature using Immersion 518 as an imaging medium and a microscope (AxioImager M2) equipped with a CCD camera (AxioCam Mrc5; both from Carl Zeiss, Inc.). Images were acquired using the Axiosview 4.6 software (Carl Zeiss, Inc.).

For transmission electron microscopy, tissue was collected and fixed in 3% glutaraldehyde in 0.1 M Sorenson phosphate buffer, pH 7.4, at 4°C. After fixation, specimens were contrasted with osmium tetroxide and embedded in epoxy resin. Ultrathin sections were cut at 70 nm, stained with alkaline-lead citrate and methanolic uranyl acetate, and viewed with a transmission electron microscope (EM 900; Carl Zeiss, Inc.).

Cell culture
Mixed glial cell cultures were obtained from newborn animals (P0), plated in NM15 media (DME containing 15% fetal calf serum) for at least 7 d. Oligodendrocyte precursors were obtained by differential shaking of mixed glial cultures. Immediately after shaking, progenitors were plated on poly-c-lysine-coated dishes in NM15 medium. Differentiation was induced 24 h later by changing the medium to differentiation medium (DME supplemented with 0.5% FCS, 25 μg/ml insulin, 5 ng/ml selenium, 50 μg/ml transferrin, and 20 ng/ml triiodothyronine; all from Sigma-Aldrich) and was assessed by immunofluorescence, staining the cells with α-NG2 (Millipore), α-O4 (R&D Systems), and α-MBP (Santa Cruz Biotechnology, Inc.) followed by Alexa 488– or 594–conjugated secondary antibodies (Invitrogen). The overall purity of the cultures used in the experiments shown in Figs. 4 and 6 was between 80 and 90%, as assessed by immunofluorescence using a combination of antibodies against GFAP, NG2, O4, MBP, pan-tubulin, and pIV-V (Sigma-Aldrich). For biochemistry, oligodendrocyte-enriched cultures were generated by plating the mixed glial cell cultures in differentiation medium supplemented with 10 ng/ml rat recombinant ciliary neurotrophic factor for 2 d to allow oligodendrocyte precursors to differentiate. Oligodendrocyte-depleted cultures were obtained after repeated differential shaking of mixed glial cell cultures to get rid of oligodendrocyte precursors and consisted of α-90% astrocytes.

Immunoblot analysis and immunoprecipitation
Preparation of brain lysates has been described previously (Morice et al., 1999). MEK1 immunoprecipitates were prepared using antibodies from Transduction Laboratories and washed four times in brain lysate buffer. Immunoprecipitates and lysates were subjected to immunoblotting as described previously (Nikula et al., 2001). The antibodies used were α-CtC19 (against a C terminus epitope), H14.5 (against an N-terminal epitope), α-Raf C19, actin, MBP (Santa Cruz Biotechnology, Inc.), Raf-1, MEK1, MEK2, KSR1, panERK (Transduction Laboratories), pMEK1/2, p44/42 MAPK, EGR1 (Cell Signaling Technology), pIV-V (Sigma-Aldira), and tubulin (Sigma-Aldira).

Online supplemental material
Fig. S1 shows the NestinCre activity pattern in an embryonic day 11.5 embryo. Figs S2 and S3 show the morphological defects of b-raf-deficient mouse brain and cerebellum. Fig. S4 shows the myelination defect of b-raf+/Δ neu throughout postnatal development. Fig. S5 shows hypomyelination and supernumerary oligodendrocyte precursor in different regions of b-raf+/Δ neu brains. Videos 1–3 show the neurological defects of b-raf+/Δ neu mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200709069/DC1.

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