THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 41, pp. 34735–34740, October 14, 2005

ALS2/Alsin Regulates Rac-PAK Signaling and Neurite Outgrowth*[S]

Received for publication, June 7, 2005, and in revised form, July 25, 2005 Published, JBC Papers in Press, July 26, 2005, DOI 10.1074/jbc.M506216200

Elizabeth L. Tudor[‡§1], Michael S. Perkinton[‡§], Anja Schmidt[¶], Steven Ackerley[‡§], Janet Brownlees[‡§], Nicholas J. O. Jacobsen[‡§], Helen L. Byers[‖], Malcolm Ward[‖], Alan Hall[¶], P. Nigel Leigh[§], Christopher E. Shaw[§], Declan M. McLoughlin[**], and Christopher C. J. Miller[‡§2]

*From the Departments of [‡]Neuroscience and [§]Neurology and **Section of Old Age Psychiatry, Institute of Psychiatry, Kings College, London SE5 8AF, United Kingdom, [¶]Medical Research Council Laboratory for Molecular Cell Biology, University College, London WC1E 6BT, United Kingdom, and [‖]Proteome Sciences plc, Institute of Psychiatry, London SE5 8AF, United Kingdom*

Rac and its downstream effectors p21-activated kinase (PAK) family kinases regulate actin dynamics within growth cones to control neurite outgrowth during development. The activity of Rac is stimulated by guanine nucleotide exchange factors (GEFs) that promote GDP release and GTP binding. ALS2/Alsin is a recently described GEF that contains a central domain that is predicted to regulate the activities of Rac and/or Rho and Cdc42 activities. Mutations in *ALS2* cause some recessive familial forms of amyotrophic lateral sclerosis (ALS) but the function of ALS2 is poorly understood. Here we demonstrate that ALS2 is present within growth cones of neurons, in which it co-localizes with Rac. Furthermore, ALS2 stimulates Rac but not Rho or Cdc42 activities, and this induces a corresponding increase in PAK1 activity. Finally, we demonstrate that ALS2 promotes neurite outgrowth. Defects in these functions may therefore contribute to motor neuron demise in ALS.

Some forms of amyotrophic lateral sclerosis (ALS)[3] are familial and are passed through generations in autosomal dominant, recessive, or X-linked fashions (1, 2). Mutations in the gene encoding copper/zinc superoxide dismutase-1 (SOD1) cause some of these familial cases (3, 4), and recently, mutations in the *ALS2/Alsin* gene have been shown to cause some rare juvenile forms of ALS (5, 6). Mutations in ALS2 have also been linked to juvenile primary lateral sclerosis and infantile-onset ascending hereditary spastic paraplegia (7–9).

The structure of ALS2 predicts that it functions as a guanine nucleotide exchange factor (GEF). GEFs regulate the activity of members of the Ras superfamily of GTPases. These GTPases cycle between inactive (GDP-bound) and active (GTP-bound) conformational states, and GEFs stimulate GTP-binding so as to promote activation of the GTPase (10, 11). ALS2 contains three putative GEF domains: an amino-terminal domain that displays homology to the Ran GEF RCC1; a central region containing Dbl and pleckstrin homology (DH/PH) domains that are found in GEFs for Rho, Rac, and Cdc42; and a carboxyl-terminal vacuolar protein-sorting 9 (VPS9) domain that is found in GEFs for Rab5 (5, 6). Indeed, there is now evidence that ALS2 functions as a GEF, including one for Rab5 that is via its VPS9 domain (12–15).

The mechanisms by which mutant ALS2 induces disease are poorly understood. One possibility is that there are links between ALS2 and mutant SOD1 and that there are common pathways of toxicity (15, 16). However, the recessive nature and types of mutations in affected families strongly suggest that a loss of ALS2 function is the primary cause of disease. Indeed, at least some of the mutants generate unstable forms of the protein (17). A proper understanding of the molecular mechanisms by which mutant ALS2 induces motor neuron disease thus requires insight into ALS2 function. Here we demonstrate that ALS2 stimulates Rac1-p21-activated kinase (PAK) signaling and is involved in neurite outgrowth.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—A carboxyl-terminal myc-tagged human ALS2 cDNA was generated by PCR. Briefly, the 5′-end of *ALS2* was amplified by PCR from a human brain cDNA library and ligated to a 5′-truncated partial *ALS2* cDNA (clone KIAA1563) so as to create a full-length clone. A carboxyl-terminal myc tag was then added by PCR, and the tagged full-length cDNA was then cloned into pCIneo (Promega) as a NotI fragment. A mutant *ALS2* clone (ALS2ΔDH) in which the DH/PH GEF domain was disrupted was created by the deletion of sequences encoding the DH domain (residues 747–826). Sequences were deleted using an ExSite mutagenesis kit (Stratagene) with primers 5′-ACTCAGTTGATGGAAATACTGAATAC-3′ and 5′-ACACAGCTTGCTGAATCGGCTAGC-3′. *ALS2* phosphorylation sites were altered using a QuikChange Multi site-directed mutagenesis kit (Stratagene) and primers 5′-GCCAGCACTGCTCTCGCCCCCTCCACTGAAACC-3′ (S277A), 5′-GATTGTTGTCACAAGTTGCCCCCAGGCTCTTAAG-3′ (S492A), 5′-GAGGACAGTGGTTCTGGCCCCCCACATACAGTGG-3′ (T510A), 5′-GCCAGCACAGAGACGCTCCAGAAATATTGAGTCG-3′ (S1335A), and 5′-GATTCCCGATCTGAAGCACCAGAGCCAGGTTATG-3′ (S1464A). The DH/PH GEF domain (amino acids 681–1010) of ALS2 was generated by PCR and cloned as a BamHI-EcoRI fragment into pRK5myc. RhoA, Rac1, L61Rac1 (constitutively active Rac1), N17Rac1 (dominant-negative Rac1), Cdc42, and N39Rab5 (dominant-negative Rab5) were all expressed using pRK5myc variants. pCMV6myc-*PAK1* clone was obtained from Sashi Kesavapany (National Institutes of Health, Bethesda, MD). Vector pCIneoCAT expressing the *Escherichia coli* chloramphenicol acetyl transferase gene (18) was used as a control for

* This work was supported by grants from the Medical Research Council and Wellcome Trust (to C. C. J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbs.org) contains supplemental Figs. S1–S4.

[1] Supported by a Jim Tew Memorial Studentship and the UK Motor Neurone Disease Association.

[2] To whom correspondence should be addressed: Dept. of Neuroscience, P. O. Box P037, The Institute of Psychiatry, De Crespigny Park, Denmark Hill, London SE5 8AF, United Kingdom. Tel.: 44-2078480393; Fax: 44-2077080017; E-mail: chris.miller@iop.kcl.ac.uk.

[3] The abbreviations used are: ALS, amyotrophic lateral sclerosis; GEF, guanine nucleotide exchange factor; DIV, day(s) *in vitro*; GFP, green fluorescent protein; CAT, chloramphenicol acetyltransferase; PAK, p21-activated kinase; RBD, Rho-binding domain; PBD, p21-binding domain; SOD1, superoxide dismutase-1; DH, Dbl homology; PH, pleckstrin homology; GST, glutathione *S*-transferase; CHO, Chinese hamster ovary; TBS, Tris-HCl-buffered saline; VPS9, vacuolar protein-sorting 9; Cdk, cyclin-dependent kinase.

comparisons of the effects of ALS2 expression on GTPase and PAK1 activities and neurite outgrowth. Plasmid pEGFPC.1 was obtained from Clontech.

Antibodies—Sequences encoding residues 452–668 of ALS2 were amplified by PCR using primers 5′-GCGGAATTCGAACAGGTTAAACAGGAATCAATGC-3′ and 5′-GCGGAATTCTCCAAGCTTACTACAGGAGAGAAG-3′ and cloned into pGEX5X1 as an EcoRI fragment. Glutathione *S*-transferase (GST)-ALS2-(452–668) was expressed in *E. coli* BL21-CodonPlus(DE3)RIL, purified essentially according to the manufacturer's instructions (Amersham Biosciences), and used to immunize rabbits. The ALS2 antibody was affinity-purified against antigen prior to use. Antibodies to PAK1 and to myc tags (antibody 9B11) were obtained from New England Biolabs; Rac, Rho(A,B,C), and Cdc42 antibodies were from Upstate; Rab5 antibody was from BD Biosciences; α-tubulin antibody (DM1A) was from Sigma; AlexaFluor 568-phalloidin was from Molecular Probes.

Cell Culture and Transfection—CHO cells were grown in HAM's F-12 medium containing 10% (v/v) fetal bovine serum supplemented with 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). Cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Primary cortical and hippocampal neurons were obtained from embryonic day 18 rat embryos and cultured on glass coverslips coated with poly-D-lysine in 12-well plates (Falcon) in neurobasal medium and B27 supplement (Invitrogen) containing 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. Neurons were transfected using Lipofectamine 2000 (Invitrogen) as described previously (19).

SDS-PAGE and Immunoblotting—Samples were processed for SDS-PAGE by the addition of SDS-PAGE sample buffer and heating immediately in a boiling water bath. Proteins were separated on 10% (w/v) acrylamide gels and transferred to Protran nitrocellulose membranes (Schleicher & Schuell) using a Bio-Rad TransBlot system. Following blocking and probing with primary antibodies, the blots were washed and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit Ig (Amersham Biosciences) and developed using an enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer's instructions.

GTPase and PAK1 Assays—Cellular Rho, Rac, and Cdc42 activities were assayed using commercially available kits essentially according to the manufacturer's instructions (Upstate Biotechnology). Briefly, CHO cells were co-transfected with RhoA, Rac1, or Cdc42 in combination with ALS2 plasmids or vector encoding CAT (pCIneoCAT) as a negative control. Cells were harvested into ice-cold lysis buffer (composed of 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM NaF, 1 mM sodium orthovanadate, and complete protease inhibitor mixture (Roche Applied Science)) 24 h posttransfection and following 16 h of serum starvation. Active (GTP-bound) Rho, Rac, and Cdc42 were captured on GST-bait beads. Active Rho was captured using GST-rhotekin Rho-binding domain (GST-RBD) and active Rac and Cdc42 captured using GST-PAK1 p21-binding domain (GST-PBD). Captured Rho, Rac, and Cdc42 were detected on immunoblots, and the relative amounts were quantified by pixel densitometry using a Bio-Rad GS710 imaging densitometer and Quantity 1 software as described previously (20).

PAK1 activities in transfected CHO cells were assayed essentially as described previously by us for other kinases (21, 22). Briefly, cells were co-transfected with PAK1, Rac, and ALS2, ALS2ΔDH, or vector encoding CAT as a negative control. For a positive control, cells were co-transfected with PAK1 and a constitutively active Rac (L61Rac1). Cells were harvested into ice-cold lysis buffer (composed of 20 mM HEPES (pH 7.4),

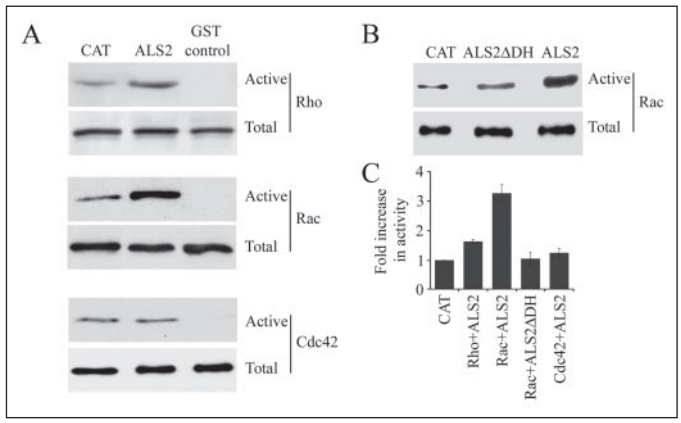

FIGURE 1. **ALS2 stimulates Rac activity.** Rho, Rac, and Cdc42 activation assays were conducted in CHO cells transfected with Rho, Rac, or Cdc42, + ALS2 or control vector pCIneoCAT (*CAT*). *A*, representative assays for the three GTPases. Active Rho was pulled down from the lysates using GST-RBD, and active Rac and Cdc42 were pulled down with GST-PBD. The bound Rho, Rac, and Cdc42 were then detected by immunoblotting (*Active*). The amounts of Rho, Rac, and Cdc42 transfected into the cells were also detected by immunoblotting so as to demonstrate equal transfection efficiencies (*Total*). GST alone did not bind any GTPase in the transfected cells. *B*, disruption of the ALS2 Rac GEF domain (in ALS2ΔDH) abrogates the stimulatory effect on Rac activity. *C*, histogram of fold increases in GTPase activity that were obtained from four separate experiments for each GTPase. *Error bars* are S.E. One-way analysis of variance tests showed that ALS2 significantly increased Rac activity by 3.3-fold ($p < 0.001$) but not Rho or Cdc42 activities.

2 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, and complete protease inhibitor mixture (Roche Applied Science)), and following preclearing of the samples, PAK1 was isolated by immunoprecipitation. *In vitro* kinase assays were performed at 30 °C in 25 mM HEPES, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenylphosphate, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol, 0.26 MBq [λ-^{32}P]ATP, 20 μM ATP, and 5 μg of myelin basic protein as substrate in a final volume of 30 μl. The reactions were terminated after 20 min (which pilot studies had demonstrated was within the linear range of PAK1 activity) by the addition of SDS-PAGE sample buffer and heating in a boiling water bath, and the samples were separated by SDS-PAGE. The gels were stained with Coomassie Blue, and a radioactive signal was detected using Fujix BAS1000 phosphorimaging with a BAS reader and TINA 2.07 software and by autoradiography. A proportion of the reaction was also probed for immunoprecipitated PAK1 on immunoblots so as to demonstrate equal amounts of kinase in the different transfections.

Immunofluorescence Studies and Neurite Length Measurements—Neurons grown on coverslips were fixed in 4% (w/v) paraformaldehyde in Tris-HCl-buffered saline (TBS) for 20 min, permeabilized in 0.1% (w/v) Triton X-100 in TBS for 10 min, blocked with 5% (v/v) goat serum/0.2% (w/v) Tween 20 in TBS for 1 h, and then probed with primary antibodies diluted in blocking solution. Primary antibodies were then detected using goat anti-mouse or goat anti-rabbit Ig coupled to AlexaFluor 350 or AlexaFluor 546 (Molecular Probes), and the samples were mounted in Vectashield (Vector Laboratories).

For analyses of the effects of ALS2 on neurite outgrowth, 2 DIV cortical neurons were co-transfected with plasmid pEGFPC.1 (Clontech) expressing enhanced green fluorescent protein (GFP) plus experimental or control plasmids. GFP was used to determine cell shape because it has been shown to distribute uniformly throughout neurons and has been used as a marker for neuronal cell shape in numerous studies (e.g. Refs. 23 and 24). Experimental and control plasmids included ALS2, ALS2ΔDH, dominant-negative N17Rac and N39Rab5, and vector expressing pCIneoCAT, which was used to balance transfections so that all cells received the same numbers and amounts of plasmid. All of the GFP-expressing cells were immunostained for co-transfected protein to

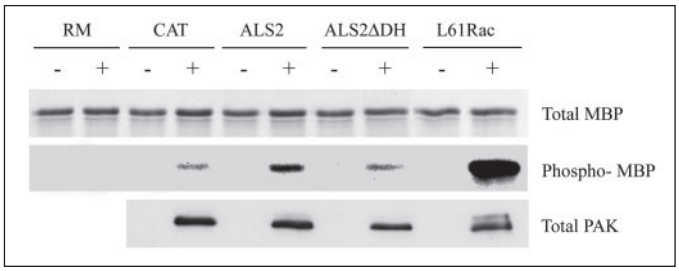

FIGURE 2. **ALS2 stimulates PAK1 activity.** PAK1 *in vitro* kinase assays were performed with CHO cells co-transfected with PAK1, Rac1, and ALS2, ALS2ΔDH, or vector pCIneoCAT (*CAT*). For a positive control, cells were co-transfected with a constitutively active Rac1 (*L61Rac*). − and + refer to the absence or presence of PAK1 immunoprecipitating antibody in the reactions; reaction mix (*RM*) contains no immunoprecipitation sample. The *top panel* shows the Coomassie-stained gel with myelin basic protein substrate (*MBP*), and the *middle panel* shows the corresponding autoradiograph of the samples. Also shown are levels of PAK1 (*bottom panel*) in the samples. The assays were repeated an additional three times, and one-way analysis of variance tests revealed that the stimulatory effect of ALS2 and L61Rac1 on PAK1 activity was significant ($p < 0.01$).

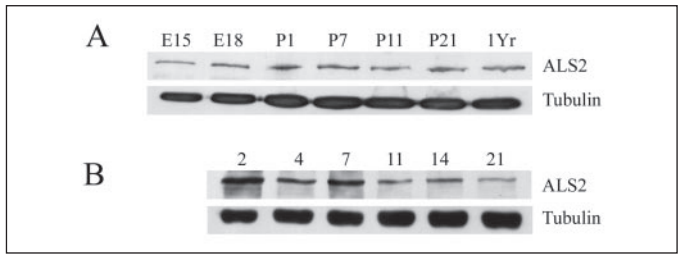

FIGURE 3. **Expression of ALS2 in brain and cultured rat cortical neurons.** *A*, an immunoblot to demonstrate developmental expression of ALS2 in mouse brain from embryonic day 15 (*E15*) to postnatal (*P*) 1 year. *B*, a similar immunoblot of rat cortical neurons cultured for 2–21 DIV as indicated. The samples were also probed for tubulin to demonstrate equal protein loadings.

confirm expression. Only healthy cells as judged by morphology (including nuclear staining with Hoechst 33258 (Sigma) to confirm that nuclei had a nonapoptotic appearance) were analyzed. Cells were analyzed 24 h later; the analysis included counting the numbers and measuring the lengths of axons and dendrites. However, only the longest neurite in each cell was used in the comparisons, which is similar to the methods used in numerous other studies on the effects of signaling cascades on neurite outgrowth (*e.g.* Refs. 25 and 26). Cells were analyzed without knowledge of the transfected plasmids.

Conventional images were captured using a Zeiss Axioscop microscope and charge-coupled device camera (Princeton Instruments), and confocal images were captured using a Zeiss LSM 510 META confocal microscope. Images for neurite outgrowth experiments were analyzed using MetaMorph image analysis software. Neurite lengths were determined as the distance from the edge of the cell body to the growth cone tip.

Mass Spectrometric Sequencing of ALS2—ALS2 was isolated by immunoprecipitation and sequenced to identify phosphorylation sites, essentially as described by us for other proteins (18, 27). Briefly, ALS2 was immunoprecipitated from transfected CHO cells by use of the myc tag and then isolated by excision of bands after SDS-PAGE. Bands were reduced, alkylated, and digested with trypsin, chymotrypsin, or Asp-N (Roche Applied Science), and peptides were extracted with two wash cycles of 50 mM NH_4HCO_3 and acetonitrile and then lyophilized and resuspended in 20 μl of 50 mM NH_4HCO_3.

Peptide digests were analyzed by on-line liquid chromatography tandem mass spectrometry. Peptides were ionized by electrospray ionization using a Z-spray source fitted to a QTof-micro mass spectrometer (Micromass UK). The instrument was set to run in automated switching mode, selecting precursor ions based on their intensity and charge state, for sequencing by collision-induced fragmentation. The tandem mass spectrometry analyses were conducted using collision energy profiles that were chosen based on the mass/charge (m/z) and the charge state of the peptide and optimized for phosphorylated peptides.

The mass spectral data were processed into peak lists containing the m/z value of each precursor ion and the corresponding fragment ion m/z values and intensities. Data were searched against a custom-built data base containing the full-length sequence of ALS2 using the Mascot searching algorithm (Matrix Science). Peptides and phosphopeptides of ALS2 were identified as described previously (18, 27).

Statistical Analyses—Statistical significance for GTPase and PAK1 activities and for neurite outgrowth was determined using one-way analysis of variance followed by Tukey post-hoc tests for pairwise comparison. Differences were considered significant at $p < 0.05$.

RESULTS

ALS2 Stimulates Rac1-PAK1 Signaling—ALS2 contains a central DH/PH domain that shows homology to GEFs that regulate Rho, Rac, and Cdc42 GTPases. To determine whether ALS2 activates these GTPases, we utilized *in vivo* pull-down assays to monitor the activities of RhoA, Rac1, and Cdc42 GTPases in transfected CHO cells. Active (GTP-bound) Rho binds to rhotekin, whereas active Rac and Cdc42 both bind PAK1. GST-RBD and GST-PBD "baits" can thus be used to isolate GTP-bound RhoA, Rac1, and Cdc42 from experimentally manipulated cells; the amounts of these GTPases detected on immunoblots correlates with their activities (28, 29).

Co-transfection of cells with ALS2 induced a significant (3.3-fold) increase in the amount of Rac1 but not Cdc42 pulled down by GST-PBD bait. Although a small increase in the amount of Rho pulled down by GST-RBD bait was also observed in ALS2-co-transfected cells, this was not statistically significant (Fig. 1*A*). To confirm that the stimulatory effect on Rac activity was indeed mediated by the DH/PH domain, we created an ALS2 mutant (Fig. 1*B*, *ALS2ΔDH*) in which the majority of the catalytic DH domain (residues 747–826) was deleted. This mutant did not activate Rac (Fig. 1*B*). However, the isolated DH/PH GEF domain (residues 681–1010) similarly did not activate Rac1 in these assays, which suggests other regions of ALS2 may be necessary for control of its Rac GEF function (supplemental data, Fig. S1). Thus, ALS2 stimulates Rac1 activity, and this stimulation requires the full-length ALS2 holoprotein. Recently, others have also shown that ALS2 stimulates Rac activity (13, 15).

Immediate downstream targets for Rac include members of the PAK family of serine/threonine kinases (30). PAK1 is a major neuronal member of the PAK family. We therefore inquired whether ALS2 also stimulated PAK1 activity. *In vitro* PAK1 assays were performed from CHO cells co-transfected with PAK1 ± ALS2. These experiments revealed that ALS2 stimulated PAK1 activity but that this stimulation was lost in cells co-transfected with the nonfunctional mutant ALS2ΔDH, which indicates that this activity is dependent on a functional DH/PH (Rac GEF) domain (Fig. 2). Thus, ALS2 functions as a GEF to regulate Rac1-PAK1 signaling.

ALS2 Is Present in Neuronal Growth Cones and Co-localizes with Rac—Rho, Rac, and Cdc42 GTPases play a major role in organizing the cytoskeleton and in particular the actin cytoskeleton (11, 30). In developing neurons, Rho, Rac, and Cdc42 are present in growth cones of axons and dendrites, in which they regulate actin dynamics to control neurite outgrowth. In some paradigms, Rac and Cdc42 promote neurite outgrowth, whereas Rho inhibits it (30–34). PAK1 is also present within the growth cone where it too can function in neurite outgrowth, although its precise role may depend upon the stage and type of neuron (30). We therefore studied developmental expression and also the subcellular distribution of ALS2 in rat cortical and hippocampal neurons. Immunoblots of mouse brains aged from embryonic day 15 (Fig. 3A,

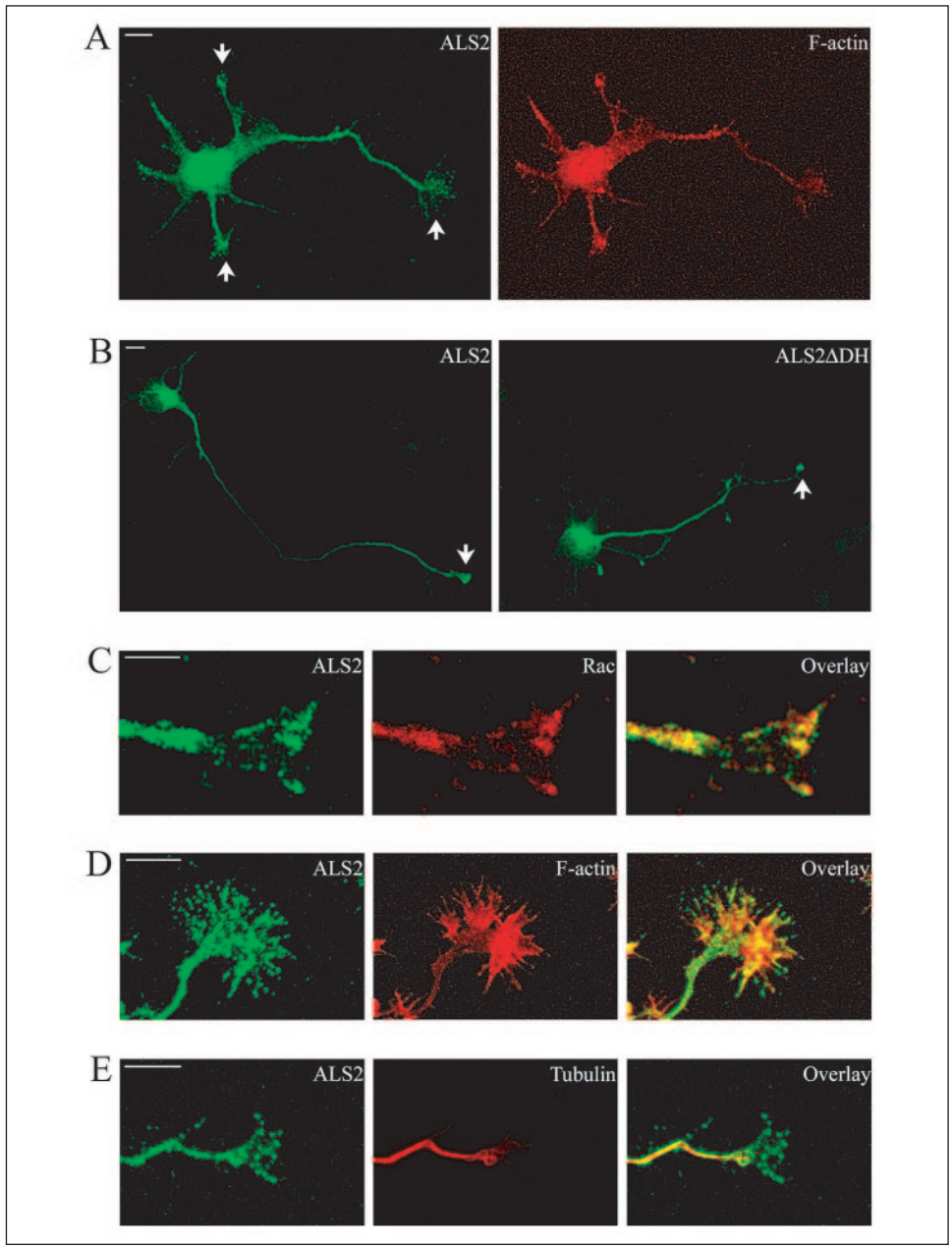

FIGURE 4. **ALS2 is present in the growth cone and co-localizes with Rac.** *A*, endogenous ALS2 and F-actin in 2 DIV rat hippocampal neurons; note the presence of ALS2 in growth cones (*arrows*). *B*, localization of transfected ALS2 and ALS2ΔDH in cortical neurons; again both are present in cell bodies but also in the growth cone (*arrows*). *C–E*, images of ALS2 and Rac (*C*), F-actin (*D*), and tubulin (*E*) in growth cones as indicated. Images in *A*, *B*, *D*, and *E* are confocal Z projections; *C* shows a confocal slice. *Scale bars*, 10 μm (*A* and *B*) and 5 μm (*C–E*).

E15) to 1 year revealed the presence of similar levels of ALS2 that migrated as a single major species of ~180 kDa. This 180-kDa species co-migrated with ALS2 in transfected CHO cells and corresponds to the predicted molecular mass of ALS2. Others have also reported that ALS2 expression does not change markedly in the rodent brain after embryonic day 10 (35). Similar immunoblots of rat cortical neurons also revealed the presence of ALS2; however, there was a small but consistent decrease in ALS2 levels in cultures that were >11 days old (Fig. 3*B*).

Application of the ALS2 antibody to 2 DIV rat hippocampal and cortical neurons produced prominent staining within cell bodies; labeling of punctate structures within neurites was also detected (Fig. 4). These results are similar to those described by others in 7 DIV rat hippocampal neurons (13). However, in the 2–3 DIV neurons, we also detected labeling within growth cones of both axons and dendrites (Fig. 4*A*); Rac and actin are known to be enriched within growth cones. We also studied the subcellular distributions of transfected ALS2 and ALS2ΔDH by use of the myc tags, and these too localized to cell bodies, axons, and dendrites, and also within growth cones, in ways that were not noticeably different from that of endogenous ALS2 (Fig. 4*B*). Transfected ALS2 therefore appears to localize to the appropriate cellular compartments in which endogenous ALS2 resides.

Growth cones are made up of two domains: the central domain and the peripheral domain. The central domain contains microtubules, whereas the peripheral domain is actin-rich, containing the most motile structures, the lamellipodia and filopodia. Co-staining for ALS2 with tubulin or actin revealed that ALS2 was present throughout the whole growth cone (Fig. 4, *C–E*). We also performed double labeling for ALS2 and Rac1, and this revealed a close overlap in the distributions of the two proteins in growth cones (Fig. 4*C*). These results complement the biochemical studies that demonstrate that ALS2 is a Rac GEF, and together they suggest that ALS2 may function in growth cone motility and neurite outgrowth.

ALS2 Stimulates Neurite Outgrowth—A number of GEFs that stimulate the activities of Rac, Rho, and Cdc42 GTPases have been shown to

regulate neurite outgrowth (23, 24, 26, 36, 37). We therefore examined the effect of overexpression of ALS2 on the development of axons and dendrites in rat cortical neurons. To do so, we transfected 2 DIV neurons with ALS2 or control vectors and analyzed neurite outgrowth in the cells 24 h later.

The numbers of neurites (neurite tip number per cell) were not affected by overexpression of ALS2 or ALS2ΔDH (supplemental data, Fig. S2). However, ALS2 significantly increased (by 1.5-fold) the length of the longest neurite (Fig. 5). This level of stimulation in neurite outgrowth is similar to that observed by other Rac GEFs (23). By contrast, overexpression of ALS2ΔDH had no effect on neurite outgrowth (Fig. 5). To determine, in addition, that the ALS2-mediated stimulation of neurite outgrowth involves Rac signaling, we compared neurite outgrowth in cells co-transfected with ALS2 and either a dominant-negative Rac (N17Rac) or a dominant-negative Rab5 (N39Rab5). ALS2 stimulates both Rac activity via its central DH/PH domain and Rab5 activity via its VPS9 domain (Refs. 12–15 and see above). This approach, involving dominant-negative GTPases to dissect out pathways by which other GEFs stimulate neurite outgrowth, has been utilized successfully in many other studies (e.g. Refs. 23 and 26). Co-transfection of ALS2 with N17Rac but not N39Rab5 abrogated the stimulatory effect of ALS2 on neurite outgrowth (Fig. 5). Thus, ALS2 promotes neurite outgrowth in rat cortical neurons, and this involves its Rac but not its Rab5 GEF activity.

Expression of N17Rac alone had no effect on neurite outgrowth, which is consistent with a number of other reports (23, 38). However, Rac has also been shown to both promote and inhibit neurite outgrowth, and it is likely that such conflicting results are due to the different types of neurons, ages, and culture conditions used for experimentation (*e.g.* Refs. 39–42). Nevertheless, our findings that N17Rac blocks the effect of ALS2 but has no effect on neurite outgrowth alone is similar to that seen in studies with other Rac GEFs (23, 26).

ALS2 Is a Phosphoprotein—To begin to understand the upstream mechanisms that regulate ALS2 activity, we sequenced the protein to identify phosphorylation sites. A number of GEFs are known to be phosphorylated, and this can regulate their activities; this includes roles in neurite outgrowth (e.g. Refs. 43 and 44). ALS2 is a particularly low abundance protein (17), and so to obtain sufficient protein for sequencing, we isolated it from transfected CHO cells. Using a combination of trypsin, chymotrypsin, and Asp-N protease digestion, we obtained 81% sequence coverage (supplemental data, Fig. S3). Serines 277, 492, 1335, and 1464 and threonine 510 were all unambiguously identified as phosphorylation sites; a number of other phosphopeptides were also detected, although the responsible residues could not be identified. The identified residues all precede a proline making them candidates for phosphorylation by proline-directed kinases such as those of the mitogen-activated protein kinase superfamily, the cyclin-dependent kinases (Cdks) and glycogen synthase kinase-3α/β. To inquire whether phosphorylation of these sites influenced ALS2 regulation of Rac activity, we constructed mutants in which serine/threonine residues were altered to alanine to preclude phosphorylation, but these mutants had no discernible effect on ALS2 Rac activity, PAK1 activity, or neurite outgrowth (supplemental data, Fig. S4).

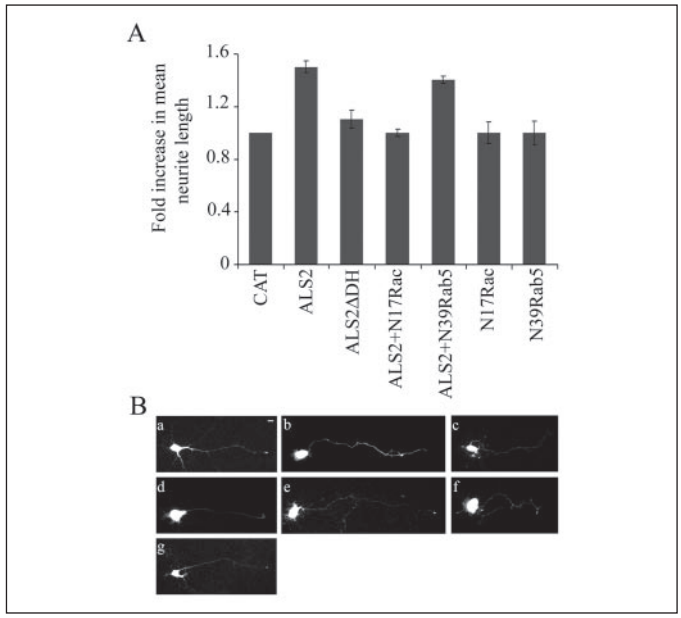

FIGURE 5. **ALS2 promotes neurite outgrowth in cultured rat cortical neurons.** 2 DIV rat cortical neurons were transfected with GFP + control or experimental plasmids, and the length of the longest neurite was measured using GFP as a marker. Transfections were balanced with CAT plasmid so that all of the cells received the same number and amount of plasmids. *A*, histogram of mean neurite length for each transfection condition as indicated. Data were obtained from 40–50 cells per transfection, and the experiments were repeated at least three times. ALS2 but not ALS2ΔDH stimulates neurite outgrowth by 1.5-fold ($p < 0.001$), and this effect is lost upon co-transfection with N17Rac but not N39Rab5. *B*, representative images of cells in the different transfections: *a*, GFP+CAT; *b*, GFP+ALS2; *c*, GFP+ALS2ΔDH; *d*, GFP+ALS2+N17Rac; *e*, GFP+ALS2+N39Rab5; *f*, GFP+N17Rac; *g*, GFP+N39Rab5. *Scale bar*, 10 μm.

DISCUSSION

Rho, Rac, and Cdc42 are key regulators in the development of axons and dendrites. They achieve this by transducing upstream signaling cues to regulate actin dynamics within the growth cone (11, 30–34). The activities of Rho, Rac, and Cdc42 are regulated in a variety of fashions including activation by GEFs that stimulate GTP binding and inhibition by GTPase activating proteins that promote GTP release (10). A number of GEFs have now been shown to be involved in the development of axons and dendrites (23, 24, 26, 36, 37).

ALS2 is a recently described protein that contains three potential GEF domains (5, 6). The carboxyl-terminal, VPS9 homology domain has been shown to act as a GEF for Rab5; Rab5 is an essential regulator of endocytosis and endosome biogenesis (12, 13). The central region of ALS2 contains Dbl homology and pleckstrin homology (DH/PH) domains and thus resembles GEFs for Rho, Rac, and Cdc42. Immediate downstream effectors of Rac include the PAK family kinases, and Rac1-PAK1 signaling is known to modulate actin dynamics so as to regulate the growth of both axons and dendrites (30, 32). We show here that ALS2 is present within growth cones of both axons and dendrites and that it acts as a GEF for Rac1 to stimulate PAK1 activity and neurite outgrowth. The DH/PH GEF domain is essential for this signaling because its disruption abrogates the effect on both Rac1 and PAK1 activities and on the ability of ALS2 to promote neurite outgrowth.

We also demonstrate that ALS2 is a phosphoprotein and report the identification of five cellular phosphorylation sites. All of these are Ser/Thr-Pro motifs, which makes them targets for proline-directed kinases such as members of the stress-activated protein kinase family (c-Jun amino-terminal kinases and p38) and Cdks. Interestingly, aberrant activation of both p38 and cdk5 (a neuronal Cdk) are seen in ALS and mutant SOD1 transgenic models of ALS, and this activation may be part of mutant SOD1 toxicity (45–51). However, mutation of these sites did not noticeably alter the effect of ALS2 on Rac activity. Whether phosphorylation regulates other ALS2 GEF (Rab5 or Ran) activities will be addressed in future studies and when the full complement of ALS2 phosphorylation sites have been identified.

Nine disease-causing mutations in *ALS2* have been described in nine different autosomal recessive kindreds. All of the affected individuals

are homozygous for the mutation and develop a slowly progressive, ascending upper motor neuron disorder that presents with a lower limb spasticity and can have onset in infancy, childhood, or adolescence. All of the mutations result in premature translational termination and truncation of the full-length native protein (5–9). The neuropathology has not been described, but in a clinically similar genetic disorder, hereditary spastic paraplegia due to mutations in SPG4, upper motor neurons projecting into the corticospinal tract develop a dying-back axonopathy. The recessive nature of ALS2 and truncation mutations suggest that the disorder is caused by a loss of normal ALS2 function, but the function of ALS2 is unclear and the precise mechanisms by which this leads to clinically selective motor neuron degeneration are unknown.

One possibility is that the loss of ALS2 Rab5 GEF function perturbs membrane trafficking so as to induce disease. Indeed, disruptions to membrane trafficking and the Golgi are seen in mutant SOD1 transgenic mice models of ALS, and recently mutations in the vesicle-trafficking protein VAPB have been shown to cause late onset spinal muscular atrophy and ALS (52, 53). There may even be mechanisms linking mutant SOD1 and ALS2 forms of ALS (15, 16).

Another possibility, and one that is supported by the findings reported here, is that the loss of ALS2 Rac1 GEF function compromises proper development of motor neurons making them more susceptible to later toxic insults. Indeed, upper motor neurons are the largest in the central nervous system with the longest axons, and so any defect in axonal growth induced by the loss of ALS2 function is likely to be most severe in these cells. However, none of the above hypotheses are mutually exclusive. Whatever the mechanisms by which mutations in ALS2 induce disease, a proper understanding of ALS2 function is likely to assist in unraveling the aberrant molecular processes by which motor neurons die in ALS.

Acknowledgments—We thank the Kazusa DNA Research Institute (Japan) for the gift of partial ALS2 cDNA, Alison Stevenson and Boris Rogelj for tissue samples, and Gerald Finnerty for advice on confocal imaging.

REFERENCES

1. Figlewicz, D. A., and Orrell, R. W. (2003) *Amyotroph. Lateral Scler. Other Motor Neuron Disord.* **4,** 225–231
2. Bruijn, L. I., Miller, T. M., and Cleveland, D. W. (2004) *Annu. Rev. Neurosci.* **27,** 723–749
3. Deng, H. X., Hentati, A., Tainer, J. A., Iqbal, Z., Cayabyab, A., Hung, W. Y., Getzoff, E. D., Hu, P., Herzfeldt, B., Roos, R. P., Warner, C., Deng, G., Soriano, E., Smyth, C., Parge, H. E., Ahmed, A., Roses, A. D., Hallewell, R. A., Pericakvance, M. A., and Siddique, T. (1993) *Science* **261,** 1047–1051
4. Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J. P., Deng, H. X., and Brown, R. (1993) *Nature* **362,** 59–62
5. Hadano, S., Hand, C. K., Osuga, H., Yanagisawa, Y., Otomo, A., Devon, R. S., Miyamoto, N., Showguchi-Miyata, J., Okada, Y., Singaraja, R., Figlewicz, D. A., Kwiatkowski, T., Hosler, B. A., Sagie, T., Skaug, J., Nasir, J., Brown, R. H., Jr., Scherer, S. W., Rouleau, G. A., Hayden, M. R., and Ikeda, J. E. (2001) *Nat. Genet.* **29,** 166–173
6. Yang, Y., Hentati, A., Deng, H. X., Dabbagh, O., Sasaki, T., Hirano, M., Hung, W. Y., Ouahchi, K., Yan, J. H., Azim, A. C., Cole, N., Gascon, G., Yagmour, A., Ben-Hamida, M., Pericak-Vance, M., Hentati, F., and Siddique, T. (2001) *Nat. Genet.* **29,** 160–165
7. Gros-Louis, F., Meijer, I. A., Hand, C. K., Dube, M. P., MacGregor, D. L., Seni, M. H., Devon, R. S., Hayden, M. R., Andermann, F., Andermann, E., and Rouleau, G. A. (2003) *Ann. Neurol.* **53,** 144–145
8. Eymard-Pierre, E., Lesca, G., Dollet, S., Santorelli, F. M., di Capua, M., Bertini, E., and Boespflug-Tanguy, O. (2002) *Am. J. Hum. Genet.* **71,** 518–527
9. Devon, R. S., Helm, J. R., Rouleau, G. A., Leitner, Y., Lerman-Sagie, T., Lev, D., and Hayden, M. R. (2003) *Clin. Genet.* **64,** 210–215
10. Schmidt, A., and Hall, A. (2002) *Genes Dev.* **16,** 1587–1609
11. Etienne-Manneville, S., and Hall, A. (2002) *Nature* **420,** 629–635
12. Otomo, A., Hadano, S., Okada, T., Mizumura, H., Kunita, R., Nishijima, H., Showguchi-Miyata, J., Yanagisawa, Y., Kohiki, E., Suga, E., Yasuda, M., Osuga, H., Nishimoto, T., Narumiya, S., and Ikeda, J. E. (2003) *Hum. Mol. Genet.* **12,** 1671–1687
13. Topp, J. D., Gray, N. W., Gerard, R. D., and Horazdovsky, B. F. (2004) *J. Biol. Chem.*
14. Kunita, R., Otomo, A., Mizumura, H., Suzuki, K., Showguchi-Miyata, J., Yanagisawa, Y., Hadano, S., and Ikeda, J. E. (2004) *J. Biol. Chem.* **279,** 38625–38635
15. Kanekura, K., Hashimoto, Y., Kita, Y., Sasabe, J., Aiso, S., Nishimoto, I., and Matsuoka, M. (2005) *J. Biol. Chem.* **280,** 4532–4543
16. Kanekura, K., Hashimoto, Y., Niikura, T., Aiso, S., Matsuoka, M., and Nishimoto, I. (2004) *J. Biol. Chem.*
17. Yamanaka, K., Vande Velde, C., Eymard-Pierre, E., Bertini, E., Boespflug-Tanguy, O., and Cleveland, D. W. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 16041–16046
18. Perkinton, M. S., Standen, C. L., Lau, K. F., Kesavapany, S., Byers, H. L., Ward, M., McLoughlin, D. M., and Miller, C. C. (2004) *J. Biol. Chem.* **279,** 22084–22091
19. Brownlees, J., Ackerley, S., Grierson, A. J., Jacobsen, N. J., Shea, K., Anderton, B. H., Leigh, P. N., Shaw, C. E., and Miller, C. C. (2002) *Hum. Mol. Genet.* **11,** 2837–2844
20. Lee, J. H., Lau, K. F., Perkinton, M. S., Standen, C. L., Rogelj, B., Falinska, A., McLoughlin, D. M., and Miller, C. C. (2004) *J. Biol. Chem.* **279,** 49099–49104
21. Guidato, S., McLoughlin, D., Grierson, A. J., and Miller, C. C. J. (1998) *J. Neurochem.* **70,** 492–500
22. Brownlees, J., Yates, A., Bajaj, N. P., Davis, D., Anderton, B. H., Leigh, P. N., Shaw, C. E., and Miller, C. C. J. (2000) *J. Cell Sci.* **113,** 401–407
23. Bryan, B., Kumar, V., Stafford, L. J., Cai, Y., Wu, G., and Liu, M. (2004) *J. Biol. Chem.* **279,** 45824–45832
24. May, V., Schiller, M. R., Eipper, B. A., and Mains, R. E. (2002) *J. Neurosci.* **22,** 6980–6990
25. Nikolic, M., Dudek, H., Kwon, Y. T., Ramos, Y. F. M., and Tsai, L. H. (1996) *Genes Dev.* **10,** 816–825
26. Penzes, P., Johnson, R. C., Kambampati, V., Mains, R. E., and Eipper, B. A. (2001) *J. Neurosci.* **21,** 8426–8434
27. Standen, C. L., Perkinton, M. S., Byers, H. L., Kesavapany, S., Lau, K. F., Ward, M., McLoughlin, D., and Miller, C. C. (2003) *Mol. Cell. Neurosci.* **24,** 851–857
28. Benard, V., Bohl, B. P., and Bokoch, G. M. (1999) *J. Biol. Chem.* **274,** 13198–131204
29. Ren, X. D., Kiosses, W. B., and Schwartz, M. A. (1999) *EMBO J.* **18,** 578–585
30. Nikolic, M. (2002) *Int. J. Biochem. Cell Biol.* **34,** 731–745
31. Dickson, B. J. (2001) *Curr. Opin. Neurobiol.* **11,** 103–110
32. Lundquist, E. A. (2003) *Curr. Opin. Neurobiol.* **13,** 384–390
33. Threadgill, R., Bobb, K., and Ghosh, A. (1997) *Neuron* **19,** 625–634
34. Li, Z., Van Aelst, L., and Cline, H. T. (2000) *Nat. Neurosci.* **3,** 217–225
35. Devon, R. S., Schwab, C., Topp, J. D., Orban, P. C., Yang, Y. Z., Pape, T. D., Helm, J. R., Davidson, T. L., Rogers, D. A., Gros-Louis, F., Rouleau, G., Horazdovsky, B. F., Leavitt, B. R., and Hayden, M. R. (2005) *Neurobiol. Dis.* **18,** 243–257
36. Estrach, S., Schmidt, S., Diriong, S., Penna, A., Blangy, A., Fort, P., and Debant, A. (2002) *Curr. Biol.* **12,** 307–312
37. Leeuwen, F. N., Kain, H. E., Kammen, R. A., Michiels, F., Kranenburg, O. W., and Collard, J. G. (1997) *J. Cell Biol.* **139,** 797–807
38. Arakawa, Y., Bito, H., Furuyashiki, T., Tsuji, T., Takemoto-Kimura, S., Kimura, K., Nozaki, K., Hashimoto, N., and Narumiya, S. (2003) *J. Cell Biol.* **161,** 381–391
39. Luo, L., Liao, Y. J., Jan, L. Y., and Jan, Y. N. (1994) *Genes Dev.* **8,** 1787–1802
40. Ruchhoeft, M. L., Ohnuma, S., McNeill, L., Holt, C. E., and Harris, W. A. (1999) *J. Neurosci.* **19,** 8454–8463
41. Jin, Z., and Strittmatter, S. M. (1997) *J. Neurosci.* **17,** 6256–6263
42. Kuhn, T. B., Brown, M. D., Wilcox, C. L., Raper, J. A., and Bamburg, J. R. (1999) *J. Neurosci.* **19,** 1965–1975
43. Sahin, M., Greer, P. L., Lin, M. Z., Poucher, H., Eberhart, J., Schmidt, S., Wright, T. M., Shamah, S. M., O'Connell, S., Cowan, C. W., Hu, L., Goldberg, J. L., Debant, A., Corfas, G., Krull, C. E., and Greenberg, M. E. (2005) *Neuron* **46,** 191–204
44. Tybulewicz, V. (2005) *Curr. Opin. Immunol.* **17,** 267–274
45. Ackerley, S., Grierson, A. J., Banner, S., Perkinton, M. S., Brownlees, J., Byers, H. L., Ward, M., Thornhill, P., Hussain, K., Waby, J. S., Anderton, B. H., Cooper, J. D., Dingwall, C., Leigh, P. N., Shaw, C. E., and Miller, C. C. J. (2004) *Mol. Cell. Neurosci.* **26,** 354–364
46. Nguyen, M. D., Lariviere, R. C., and Julien, J. P. (2001) *Neuron* **30,** 135–147
47. Tortarolo, M., Veglianese, P., Calvaresi, N., Botturi, A., Rossi, C., Giorgini, A., Migheli, A., and Bendotti, C. (2003) *Mol. Cell. Neurosci.* **23,** 180–192
48. Hu, J. H., Chernoff, K., Pelech, S., and Krieger, C. (2003) *J. Neurochem.* **85,** 422–431
49. Hu, J. H., Zhang, H., Wagey, R., Krieger, C., and Pelech, S. L. (2003) *J. Neurochem.* **85,** 432–442
50. Raoul, C., Estevez, A., Nishimune, H., Cleveland, D., deLapeyriere, O., Henderson, C., Haase, G., and Pettmann, B. (2002) *Neuron* **35,** 1067–1083
51. Zhu, S., Stavrovskaya, I. G., Drozda, M., Kim, B. Y., Ona, V., Li, M., Sarang, S., Liu, A. S., Hartley, D. M., Wu du, C., Gullans, S., Ferrante, R. J., Przedborski, S., Kristal, B. S., and Friedlander, R. M. (2002) *Nature* **417,** 74–78
52. Mourelatos, Z., Gonatas, N. K., Stieber, A., Gurney, M. E., and Dal Canto, M. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 5472–5477
53. Nishimura, A. L., Mitne-Neto, M., Silva, H. C., Richieri-Costa, A., Middleton, S., Cascio, D., Kok, F., Oliveira, J. R., Gillingwater, T., Webb, J., Skehel, P., and Zatz, M. (2004) *Am. J. Hum. Genet.* **75,** 822–831