Molecular and Cellular Neuroscience 42 (2009) 134–141

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

P90 Ribosomal s6 kinase 2 negatively regulates axon growth in motoneurons

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ARTICLE INFO

Article history:
Received 2 December 2008
Revised 7 June 2009
Accepted 9 June 2009
Available online 22 June 2009

Keywords:
Rsk2
Motoneuron
Erk
Coffin–Lowry syndrome
Axon
Ribosomal s6 kinase

ABSTRACT

Mutations in *Ribosomal s6 kinase 2* (*Rsk2*) are associated with severe neuronal dysfunction in Coffin–Lowry syndrome (CLS) patients, flies and mice. So far, the mechanisms of how Rsk2 regulates development, maintenance and activity of neurons are not understood. We have investigated the consequences of Rsk2 deficiency in mouse spinal motoneurons. Survival of isolated Rsk2 deficient motoneurons is not reduced, but these cells grow significantly longer neurites. Conversely, overexpression of a constitutively active form of Rsk2 leads to reduced axon growth. Increased axon growth in Rsk2 deficient neurons was accompanied by higher Erk 1/2 phosphorylation, and the knockout phenotype could be rescued by pharmacological inhibition of MAPK/Erk kinase (Mek). These data indicate that Rsk2 negatively regulates axon elongation via the MAPK pathway. Thus, the functional defects observed in the nervous system of CLS patients and animal models with Rsk2 deficiency might be caused by dysregulated neurite growth rather than primary neurodegeneration.

Introduction

Rsk2 (90 kDa Ribosomal s6 kinase 2) is a growth factor activated serine/threonine kinase. It is a member of the Rsk family that includes 4 kinases in mammals. Rsks have two kinase domains: An N-terminal domain that activates the Rsk substrates and a C-terminal domain whose structure has been resolved recently (Malakhova et al., 2008) that is normally autoinhibitory for the N-terminal kinase domain and becomes active after docking of Erk1 and Erk2, and thus activates the N-terminal kinase domain.

Rsks are activated by Erk 1/2 and Erk 5 in response to growth factors, neurotransmitters, peptide hormones and other stimuli (Frödin and Gammeltoft, 1999; Hauge and Frödin, 2006; Ranganathan et al., 2006). Among known substrates of Rsk2 are cytosolic substrates like Glycogen Synthase Kinase 3β (Gsk3β) and Bcl-2-associated death promoter (Bad) as well as various nuclear substrates including ATF4, c-Fos, c-Jun, and Nur77 (Frödin and Gammeltoft, 1999). Rsk2 also acts on cAMP response element-binding protein (CREB) and Histone H3 (De Cesare et al., 1998; Sassone-Corsi et al., 1999). Rsk2 is widely expressed in the nervous system, in particular in brain regions important for learning and memory such as the hippocampus (Zeniou et al., 2002). It is believed to be the most important member of the Rsk family for regulation of synaptic plasticity. In fact, mutations of the *Rsk2* gene lead to Coffin–Lowry syndrome, an X-linked syndromic form of mental retardation with severe defects in learning and memory formation as well as motor defects such as abnormal startle responses, hyperexplexia, generalised congenital hypotonia and muscular hypodevelopment (Hanauer and Young, 2002; Ronce et al., 1999; Touraine et al., 2002; Trivier et al., 1996). Similar alterations in learning and memory are observed in mouse and *Drosophila* mutants that lack Rsk2. Mice with a targeted disruption of the *Rsk2* gene show impaired learning abilities and no (Poirier et al., 2007) or only mild signs (Dufresne et al., 2001) of impaired motor abilities. In *Drosophila*, there is only one *Rsk* gene that is highly homologous to the human *Rsk2* isoform (Wassarman et al., 1994). In a screen for mutants with learning deficits in the operant heat box paradigm, the *Rsk* mutant "ignorant" was isolated (Putz et al., 2004). Obvious motor defects were not observed, but specific tests were not performed. Despite cumulating knowledge on the behavioral consequences of Rsk2 deficiency in flies, mice and patients, little is known about the cellular effects of Rsk2 in neurons. Motor defects are among the prominent symptoms observed in CLS patients (Coffin, 2003), and Rsk2 is also expressed at relatively high levels in brain stem and spinal cord (Zeniou et al., 2002). We therefore studied the consequences of Rsk2 deficiency in mouse motoneurons on survival, axon and dendrite growth. These neurons can be easily identified and studied in highly enriched cultures *in vitro*, thus allowing analysis of signaling pathways responsible for neuronal dysfunction on a cell autonomous basis. We found that overexpression of a constitutively active form of Rsk2

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doi:10.1016/j.mcn.2009.06.006

results in reduced axon growth. In spinal motoneurons isolated from *Rsk2* knockout mice, axon growth is enhanced. This effect correlates with alterations in MAPK signaling. Thus Rsk2 appears as a physiological regulator of axon growth in developing motoneurons.

Results

Rsk2 promotes cell survival by phosphorylation and inactivation of Bad (Bonni et al., 1999; Hauge and Frödin, 2006), suggesting that it might play a role in regulation of neuronal survival. Therefore, we first investigated survival of motoneurons *in vivo* and *in vitro* in *Rsk2* null mutant mice. Since the *Rsk2* gene is localized on the X chromosome, we studied male $Rsk2^{y/-}$ and $Rsk2^{y/+}$ mice from litters obtained by crossing $Rsk2^{+/-}$ females with $Rsk2^{y/+}$ males. The number of neuronal cell bodies in the facial nucleus of two month old Rsk2 mutant mice was undistinguishable from controls (Fig. 1A). Similarly, the number of motoneurons in the lumbar spinal cord of two month old mutant mice did not differ between Rsk2 deficient and control mice (Fig. 1B). Moreover, the number of axons in the phrenic nerve was similar (Fig. 1C), indicating that the deficiency of Rsk2 has no influence on motoneuron survival *in vivo*.

Isolated motoneurons from E12 mouse embryos need neurotrophic factors for their survival. Because Rsks have been identified as a downstream kinase of TrkA in PC12 cells (Silverman et al., 2004) we tested whether the response to BDNF and CNTF was altered in Rsk2 deficient motoneurons after culturing them for seven days *in vitro*. Survival was not reduced in Rsk2 null motoneurons (Fig. 1D). Interestingly, cell death of $Rsk2^{y/-}$ motoneurons at seven days in culture in the absence of neurotrophic factors was reduced, indicating that Rsk2 might not normally act in a pathway activated by neurotrophic factors that leads to neuronal survival, but, in contrast, counteract survival mechanisms that are independent of neurotrophic factor signaling (Fig. 1D). This finding supports observations with PC12 cells and cortical neurons showing that suppression of Rsk2 by siRNA leads to increased Bad-phosphorylation that is considered neuroprotective (Clark et al., 2007).

As a next step, we investigated the distribution of Rsk2 within motoneurons (Figs. 2A and B). Because Rsk2 shares many structural elements with other members of this kinase family, we tested several different Rsk2 antibodies and used *Rsk2* knockout motoneurons as controls to investigate antibody specificity. Most antibodies also revealed a signal in $Rsk2^{y/-}$ cells (data not shown) and therefore were not further used in this study. One Rsk2 antibody (Alcorta et al., 1989) appeared specific and was therefore used for the subcellular localization studies (Fig. 2C). In motoneurons cultured for seven days, Rsk2 immunoreactivity was predominantly found in the cytoplasm and, much weaker, in the nucleus. There was also a strong signal in neurites and axonal growth cones. It colocalized with TAU and Map2 immunoreactivity (Figs. 2A and B). We also transfected a GFP-tagged Rsk2 protein into motoneurons by lentiviral gene transfer, and found a similar pattern of distribution (Fig. 2D).

Because of the high abundance of Rsk2 in neurites, we investigated the effect of Rsk2 deficiency on neurite elongation in isolated motoneurons. Surprisingly, the axons of Rsk2 deficient motoneurons that were cultured with BDNF and CNTF were longer than those from WT littermate control neurons. The difference was significant both for the longest axon and for all axonal branches (Figs. 3A and C). Also the number of branches per motoneuron was increased in Rsk2 deficient

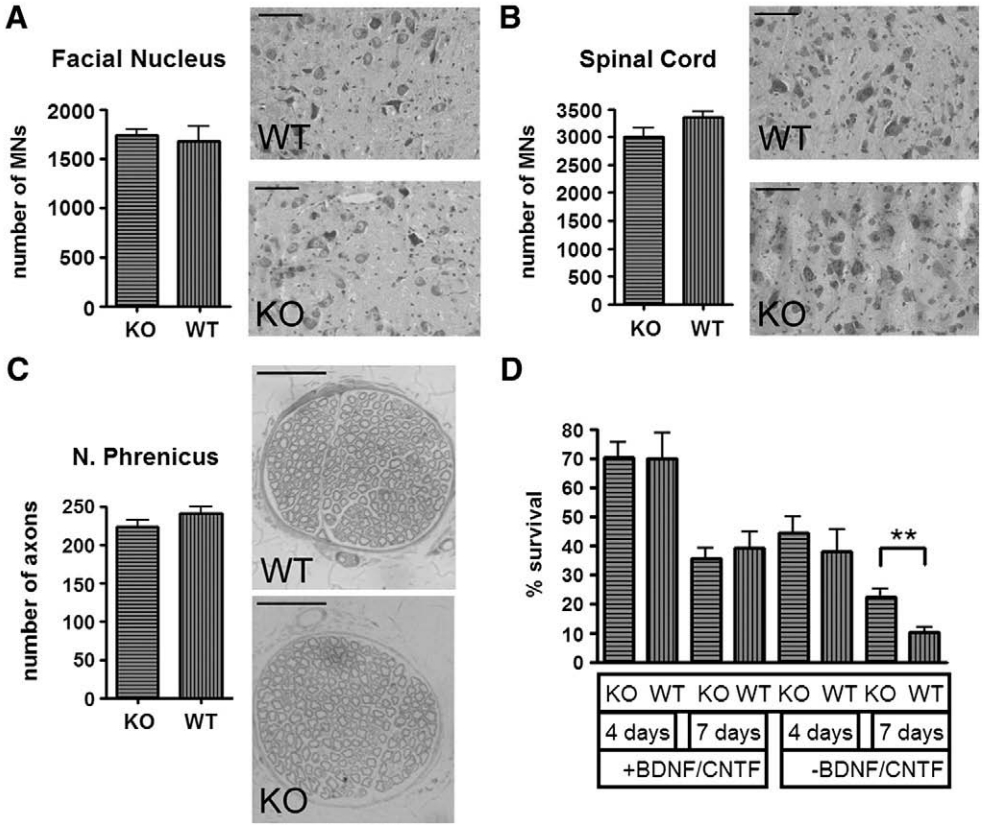

Fig. 1. (A and B) The number of motoneuron cell bodies in the facial nucleus (A; $Rsk2^{y/-}$: 2900 ± 99, $N = 8$; WT: 2796 ± 246, $N = 8$; $p > 0.05$) and spinal cord (B; $Rsk2^{y/-}$: 2997 ± 169, $N = 4$; WT: 3360 ± 101, $N = 4$; $p > 0.05$) of two month old Rsk KO and WT littermate control mice is similar. Representative cross-sections through the facial nucleus (A) and spinal cord (B), bar = 100 μm. (C) Number of axons in the phrenic nerve of two month old mice was not different between KO and WT ($Rsk2^{y/-}$: 223.3 ± 8.3, $N = 3$; WT: 240.3 ± 8.6, $N = 3$; $p > 0.05$). Cross-section through the phrenic nerve, bar = 50 μm. (E) Survival of motoneurons *in vitro* grown for four and seven days with BDNF and CNTF was not different for KO and WT (after four days: $Rsk2^{y/-}$ 70.14 ± 5.28%, $N = 14$; WT 69.71 ± 8.97%, $N = 7$; after seven days: $Rsk2^{y/-}$ 35.61 ± 3.36%, $N = 18$; WT 39.00 ± 5.50%, $N = 11$; N = number of embryos). Without neurotrophic factors, significantly less KO neurons died at 7 DIV ($Rsk2^{y/-}$ 22.21 ± 2.91, $N = 14$; WT 10.29 ± 1.49, $N = 7$; $p < 0.01$). Results represent the mean ± SEM.