Please cite this article in press as: Zhao J et al. The identification of a novel isoform of EphA4 and ITS expression in SOD1^{G93A} mice. Neuroscience (2017), http://dx.doi.org/10.1016/j.neuroscience.2017.01.038

Neuroscience xxx (2017) xxx–xxx

THE IDENTIFICATION OF A NOVEL ISOFORM OF EPHA4 AND ITS EXPRESSION IN SOD1^{G93A} MICE

JING ZHAO, [a] **ANDREW W. BOYD** [b,c] **AND PERRY F. BARTLETT** [a]*

[a] *Queensland Brain Institute, University of Queensland, Qld 4072, Australia*

[b] *School of Medicine, University of Queensland, Qld 4072, Australia*

[c] *QIMR Berghofer Medical Research Institute, Qld 4006, Australia*

Abstract—Amyotrophic lateral sclerosis (ALS) is characterized by the degeneration of motor neurons, leading to progressive muscle atrophy and fatal paralysis. Mutations in more than 20 genes, including full-length *EphA4* (*EphA4-FL*), have been implicated in this pathogenesis. The present study aimed to identify novel isoforms of EphA4-FL and to investigate the expression of EphA4-FL and its isoforms in the superoxide dismutase 1 (SOD1) mutant mouse model of ALS. Two novel transcripts were verified in mouse and humans. In transfected cells, both transcripts could be translated into proteins, which respectively contained the N- and C-termini of EphA4-FL, referred as EphA4-N and EphA4-C. EphA4-N, which was expressed on the surface of transfected cells, was shown to act as a dominant negative receptor by significantly suppressing the activation of EphA4-FL *in vitro*. The expression of both *EphA4-FL* and *EphA4-N* was significantly higher in the nervous tissue of SOD1^{G93A} compared to wild-type mice suggesting that both forms are modulated during the disease process. © **2017 Published by Elsevier Ltd on behalf of IBRO.**

Key words: EphA4 receptor, isoforms, SOD1^{G93A} mice, amyotrophic lateral sclerosis.

INTRODUCTION

The Eph receptor family, one of the largest families of receptor tyrosine kinases, includes 16 members in vertebrates. It is composed of ephrin type-A receptor (EphA) and ephrin type-B receptor (EphB) subgroups categorized on the basis of extracellular region sequence similarity and affinity for binding ephrins (ligands). Members of the EphA subgroup, of which there are 10, bind the five GPI-anchored ephrin A ligands, whereas the six EphB molecules bind the three transmembrane (TM) ephrin B ligands (Flanagan and Vanderhaeghen, 1998). EphA4 is distinguished by its ability to bind with both ephrinA and ephrinB ligands (Bowden et al., 2009). Its structure is highly conserved between species; for example, human and mouse EphA4 share about 98.58% amino acid sequence identity (Nelersa et al., 2012). EphA4 has been shown to play a vital role in promoting axonal regeneration, neurogenesis, synaptogenesis and angiogenesis during developmental and adult stages (Dottori et al., 1998; Cheng et al., 2002; Kullander et al., 2003; Klein, 2004; Ho et al., 2009; Khodosevich et al., 2011). Most recently, EphA4 has also been implicated in amyotrophic lateral sclerosis (ALS) in animal models and in humans (Van Hoecke et al., 2012).

ALS is an adult-onset, neuromuscular disease that is characterized by the degeneration of both the upper and the lower motor neurons, leading to progressive muscle atrophy and fatal paralysis. In approximately 90% of people with ALS, the disease is sporadic, while in the remainder, it is familial. ALS is a multi-factorial disease, with more than 20 genes implicated in its pathogenesis, such as the *Cu/Zn superoxide dismutase1* (*SOD1*) gene (Rosen et al., 1993), *TARDBP* (Sreedharan et al., 2008) and chromosome 9 open reading frame 72 (*C9orf72*) (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Van Hoecke and colleagues (Van Hoecke et al., 2012) first reported that the *EphA4* gene has a role in ALS, demonstrating that lower levels of expression of *EphA4* mRNA in total blood samples correlated with later disease onset and prolonged disease progression in ALS patients. They also demonstrated that reducing the level of EphA4 in SOD1^{G93A} mice significantly improved motor performance and survival, and that administration of a pharmacological blocker of EphA4 to SOD1^{G93A} rats delayed disease onset. This study elegantly revealed that, although variations in *EphA4* do not directly cause ALS, altering its level of expression or activation could affect disease progression, making it an attractive target for ALS therapies.

There is increasing evidence that alternative transcripts are involved in genetic diseases, including some neurological diseases, such as schizophrenia and

*Corresponding author at: Queensland Brain Institute, The University of Queensland, Brisbane, Queensland 4072, Australia
E-mail address: p.bartlett@uq.edu.au (P. F. Bartlett).

Abbreviations: ALS, amyotrophic lateral sclerosis; BCA, bicinchoninic acid; CHO, Chinese hamster ovary; DAPI, 4′,6-diamidino-2-phenylin dole; EphA, ephrin type-A receptor; EphA4-FL, full-length EphA4; EphB, ephrin type-B receptor; ESTs, expressed sequence tag sequences; FBS, fetal bovine serum; HEK-293T, human embryonic kidney; KO, knockout; NMD, nonsense-mediated decay; P, postnatal day; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline and 0.02% Tween 20; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; RIPA, radio immunoprecipitation assay; RPMI, Roswell Park Memorial Institute; RT-PCR, reverse transcription polymerase chain reaction; SOD1, Cu/Zn superoxide dismutase1; TM, transmembrane; WGA, wheat germ agglutinin; WT, wild-type.

http://dx.doi.org/10.1016/j.neuroscience.2017.01.038
0306-4522/

ALS (Gagliardi et al., 2012; Feng and Xie, 2013). In ALS, in particular, it has been shown that alteration of the RNA profile occurs from transcription, through to post-transcriptional regulation, and finally to protein non-coding RNA. In the case of EphA4, little is known about its post-transcriptional modification, which is a common and principal process resulting in alternative transcripts. However, EphA7, another EphA receptor similar to EphA4, has alternative transcripts in both mouse and human, and the truncated proteins produced from these alternative transcripts affect the function of full-length EphA7. In mouse, expression of a TM protein lacking the kinase domain results in a switch from cellular repulsion to adhesion (Holmberg et al., 2000). In man, the soluble isoform of EphA7 acts as an inhibitor of kinase function by heterodimerizing with full-length, membrane-bound Eph receptors (Oricchio et al., 2011). Therefore, the aims of the present study were twofold: (1) to identify novel alternative transcripts of EphA4 and (2) to investigate the expression of EphA4 and its novel transcripts in the SOD1^{G93A} mouse model of ALS.

EXPERIMENTAL PROCEDURES

Animals

Adult C57BL/6J mice were used as wild-type (WT) controls. EphA4 knockout (KO) mice were used to determine the existence of novel isoforms and have been described previously (Dottori et al., 1998). The SOD1^{G93A} mouse model of ALS was used to investigate the involvement of full-length *EphA4* (*EphA4-FL*) and its alternative transcripts in ALS (Gurney et al., 1994). Based on the pathogenesis in SOD1^{G93A} mice (Vinsant et al., 2013), the presymptomatic stage was defined as postnatal day (P) 35. SOD1^{G93A} mice were immediately euthanized if they showed any of the following signs (also defined as the survival end-point): loss of the righting reflex (unable to right within 30 s of being placed on their back), excessive weight loss (greater than 20% of the highest body weight), or complete paralysis of any hind-limb that rendered the animal incapable of reaching food and water (Weydt et al., 2003). The end-point of life is usually around P150. These mice were sourced from the Jackson Laboratory.

The total number of mice used was 42. All animals were housed in groups of 4 or 5 and experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, with ethical approval from the University of Queensland Animal Ethics Committee.

Reverse transcriptase PCR

A reverse transcription polymerase chain reaction (RT-PCR) was used to verify the existence of novel alternative transcripts of *EphA4-FL* in mouse and human tissue samples. RNA samples from the left hemisphere and whole spinal cord of mice were extracted using TRIzol Reagent (Invitrogen) (WT, $n = 3$; SOD1^{G93A}, $n = 3$). DNA was removed using the DNA-free Kit (Life Technologies). The quality of samples was assessed using 2100 Bioanalyzer Nano Chips (Agilent Technologies), and the quantity estimated using a Qubit RNA BR Assay Kit (Life Technologies). One microgram of RNA was then reverse transcribed to cDNA using SuperScript III, as per the manufacturer's protocol (Invitrogen). Healthy human cDNA panel were bought from Invitrogen. All primers are listed in Table 1.

Cloning of *EphA4-N* and *EphA4-C*

Based on the sequences of alternative transcripts, the possible protein isoforms were predicted to contain either the N- or C-terminal region of the EphA4-FL protein. We therefore referred to them as *EphA4-N* and *EphA4-C*. To determine if *EphA4-N* and *EphA4-C* produced mature protein, their open reading frames (ORFs) were cloned into the pCMV-Tag 1 vector and transiently transfected into human embryonic kidney (HEK) 293T cells using the FuGENE® 6 transfection reagent (Promega). Transfected HEK-293T cells were grown in humidified 5% CO_2 at 37 °C in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies). Cells were collected 48 h after the transfection for western blot analysis.

To illustrate the cellular localization of EphA4-N and its effect of the EphA4-FL activation, the ORF of EphA4-N was cloned into the pmCherry-N1 vector and fused in frame with a mCherry fluorescent reporter. Using the same transfecting method as described above, these DNA plasmids were transfected into Chinese hamster ovary (CHO) cells that were already stably transfected with mouse EphA4-FL (nucleotides 55–3024; NM_007936.3) (referred to as CHO-FL cells) (Spanevello et al., 2013). These transfected CHO-FL cells were collected 48 h later and then these transfected CHO-FL cells went through sorting procedure to isolate those cells expressing a high level of mCherry using flow cytometry, as well as the mCherry-positive gate was set relative to the basal fluorescence levels obtained from non-transfected CHO-FL cells, which have been consistent throughout the whole project (BD influx sorter; BD Bioscience). These mCherry-positive CHO-FL cells were cultured for another 7–14 days, and then went through the same sorting procedure again. After around five rounds of sorting process, the percentage of mCherry-

Table 1. Specific sets of primers were used for RT-PCR.

mEphA4-N	Forward 5′-TGCTGGCTACGGAGACTTCA-3′
	Reverse 5′-GTGCATGCAGAGTCCAGACT-3′
mEphA4-C	Forward 5′-TGAGGCAGAAGCTTGGCTTG-3′
	Reverse 5′-GTACCCTTCCTCGATGGCTT-3′
mPgk1	Forward 5′-CGGAGGCCCGGCATTCTG-3′
	Reverse 5′-AGCAGCCTTGATCCTTTGGTTG-3′
hEphA4-N	Forward 5′-GCAGCTGGCTATGGAGACTT-3′
	Reverse 5′-GAGGAAACTTGGGATGCAGA-3′
hEphA4-C	Forward 5′-AGGCATAAGCTTGGCTTGTT-3′
	Reverse 5′-AGAAAGAAGCCACCCAGGTT-3′
hPgk1	Forward 5′-GTGTGGGGCGGTAGTGTG-3′
	Reverse 5′-TTGGGACAGCAGCCTTAATC-3′

Please cite this article in press as: Zhao J et al. The identification of a novel isoform of EphA4 and ITS expression in SOD1^{G93A} mice. Neuroscience (2017), http://dx.doi.org/10.1016/j.neuroscience.2017.01.038