Suppression of MAP4K4 Signaling Ameliorates Motor Neuron Degeneration in Amyotrophic Lateral Sclerosis-Molecular Studies Toward New Therapeutics

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ABSTRACT: Amyotrophic lateral sclerosis (ALS), the most common motor neuron (MN) disease of adults, is characterized by the degeneration of upper MNs in the motor cortex and lower MNs in the brain stem and spinal cord. Our recent work suggests that a MAP kinase family member, MAP4K4 (mitogen-activated protein kinase kinase kinase kinase 4), regulates MN degeneration in ALS. Activation of MAP4K4 occurs prior to MN death and inhibition of MAP4K4 improves neurite integrity and neuronal viability in a cell autonomous manner. The mechanism through which MAP4K4 reduction specifically modulates MN viability can be attributed to the attenuation of the c-Jun apoptotic pathway, as well as to the activation of FoxO1-mediated autophagy that reduces the accumulation of protein aggregates. We additionally show the feasibility of MAP4K4 as a drug target using a MAP4K4-specific inhibitor, which improves the survival of both primary and induced pluripotent stem cell (iPSC)-derived MNs. Our studies are thus far the first to highlight a MAP4K4-initiated signaling cascade that contributes to MN degeneration in ALS, providing a new mechanism underlying MN death in disease and a druggable target for new therapeutics. We propose exciting future directions and unexplored avenues based upon this work.

KEYWORDS: amyotrophic lateral sclerosis (ALS), MAP4K4, induced pluripotent stem cell (iPSC), motor neuron, survival, apoptosis, autophagy, neurite

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Amyotrophic lateral sclerosis (ALS) is a devastating and fatal neurological disorder involving the rapid injury and death of both upper and lower motor neurons (MNs).[1] The deterioration of motor function as a result of progressive neuronal death presents as weakness and atrophy in one of the upper limbs, lower limbs, or bulbar muscles responsible for speech, and continues until paralysis, respiratory failure, and death 2-5 years after the initial clinical manifestations.[1] Despite the health and economic burden, as well as massive research efforts, treatment options remain limited, with only 2 FDA approved drugs for ALS-Riluzole (Rilutek) and Edaravone (Radicava), both of which improve life expectancy only modestly.[2]

Effective drug discovery for ALS has been hindered by the inaccessibility of affected tissue, the lack of clinically predictive models, and the etiological complexity of this disease. The ability to biopsy and study affected post mortem spinal cord tissue is limited, and animal models of disease have consistently failed to predict efficacy in clinical trials.[3] Progress in the stem cell field, particularly the development of induced pluripotent stem cell (iPSC) technologies and differentiation methodologies, has improved opportunities to study diseased human neuronal populations for drug screening and mechanistic studies. However, even iPSC-based approaches must accommodate the genetically complex nature of the disease. For example, mutations such as those in the genes encoding superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43 (TDP-43), fused in sarcoma (FUS), and C9orf72 can cause familial forms of the disease, but these variants account for only 10% of all cases. The remaining approximately 90% of ALS cases arise sporadically, with unknown and/or complex pathological drivers. Currently, there are numerous proposed pathogenic initiators, including dysregulated protein homeostasis, perturbations in DNA/RNA-binding proteins and RNA processing, excitotoxicity, and oxidative stress, to name only a few.[1] Such diverse mechanisms contributing to the development and progression of ALS add to the already described challenges in therapeutic discovery. It additionally raises the question of whether scientists and clinicians should target genetic forms of disease with personalized medicine or rather target points of convergence shared by all or at least many forms of disease.[4]

To address this question, our group previously performed a high-throughput, small molecule screen to identify compounds that prevent MN death,[5] arguably the last convergent phenotype among all ALS patients. This screen identified a multikinase inhibitor, kenpaullone, as being able to significantly improve both mouse and human ALS MN viability, regardless of genetic mutation. To understand the mechanism behind kenpaullone's protective activity, we identified two potential cellular targets, GSK3β and MAP4K4. GSK3β had previously been implicated in neuronal survival; yet, surprisingly, multiple GSK3β inhibitors did not consistently improve neuronal survival to the extent that kenpaullone did. This led us to hypothesize that the pronounced survival effect of kenpaullone was through additional molecular activities, potentially the

inhibition of MAP4K4, providing the impetus and rationale for our current work.

MAP4K4 (mitogen-activated protein kinase kinase kinase kinase 4) is a member of the sterile 20 protein (Ste20p) kinase family and acts functionally as a serine threonine kinase. MAP4K4 is expressed ubiquitously in all tissue types, with enhanced expression in the testes and brain, and has known involvement in numerous cellular and disease processes.[6] Recently, MAP4K4 inhibition has been shown to improve the survival of mouse sensory ganglion neurons after nerve growth factor (NGF) withdrawal.[7] However, little work had been done to investigate the role of this protein in MNs or in a specific neurodegenerative disease context.

Our current work explored this uninvestigated role and expanded upon our previous results, testing the hypothesis that suppression of activated MAP4K4 signaling alone is sufficient to confer MN protection. We first utilized a trophic factor (TF) withdrawal system to accelerate degeneration of MNs derived from mouse and human pluripotent stem cells. We found that lowering TF levels not only increased MN death, but increased levels of phosphorylated MAP4K4 (p-MAP4K4) and downstream effectors c-Jun N terminal kinase (JNK) and c-Jun.[6] We additionally observed higher levels of p-MAP4K4, JNK, and c-Jun in ALS MNs in TF-containing medium compared with wild-type MNs, suggesting that with both exogenous stress (TF removal) and endogenous stress (ALS-causing mutations), the MAP4K4-JNK-c-Jun death signaling pathway is activated. Remarkably, when we suppressed MAP4K4, with independent specific siRNAs or shRNAs, the levels of activated p-JNK and p-c-Jun were significantly diminished and MN survival substantially increased in both mouse and human ALS MNs. In addition, the reduction of MAP4K4 protected against neuritic degeneration, an early symptom of neuronal disorders including ALS, that generally precedes cell death. We found that neurite length and number were maintained when MAP4K4 was suppressed in TF withdrawal conditions. Moreover, the ability of MAP4K4 inhibition to prevent cell death and maintain neuritic structure was cell autonomous, as evaluated using purified MN cultures. These results support the hypothesis that the MAP4K4-JNK-c-Jun signaling cascade is upregulated in stressed MNs prior to death, and that suppression of MAP4K4 alone is sufficient to promote MN survival.

We next investigated the mechanism through which MAP4K4 inhibition protects MNs. Given that JNK and c-Jun are known downstream effectors of MAP4K4 that are activated in MN degeneration and apoptosis, we further investigated these proteins. There are three types of JNKs that differ in expression and function—JNK1 and JNK2 are expressed ubiquitously whereas JNK3 is primarily expressed in the central nervous system (CNS).[8] To determine which JNK is important in MAP4K4-mediated MN degeneration, siRNAs to each of the JNKs were transfected into ALS MN cultures subjected to TF withdrawal. Surprisingly, knockdown of JNK3, but not JNK1 or JNK2, enhanced MN viability. To verify that MAP4K4 signals through JNK3 to confer MN protection, a classical complementation test was performed, where we simultaneously reduced expression of both MAP4K4 and JNK3. We observed no additive increase in survival in MAP4K4-JNK3 double knockdown conditions compared with reduction of either MAP4K4 or JNK3 alone, suggesting that MAP4K4 and JNK3 act in the same signaling cascade to confer MN death. We next investigated the role of c-Jun, a transcription factor downstream of JNK known to drive apoptosis in some neuronal cell types.[9] We found that knockdown of c-Jun ameliorated TF withdrawal induced MN death, and that inhibition of MAP4K4 decreased active phosphorylated c-Jun levels, and additionally reduced the levels of cleaved caspase 3 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). These data cumulatively support the hypothesis that MAP4K4 inhibition attenuates JNK-c-Jun-mediated neuronal apoptosis.

An interesting observation throughout this work was that ALS MNs exhibited enhanced death compared with control MNs, and MAP4K4 knockdown had an even greater ability to rescue ALS MN death. This led us to hypothesize that there may be another mechanism, in addition to the attenuation of JNK-c-Jun apoptosis, through which MAP4K4 reduction promotes survival of ALS MNs. To explore this possibility, we extended the culture of ALS MNs containing the SOD1 mutation G93A to allow for the accumulation of mutant SOD1. We observed a significant decrease in mutant SOD1 levels in MAP4K4 reduced conditions compared with controls, suggesting that reducing mutant SOD1 levels may be a disease-specific MAP4K4 protective mechanism. Given that the MAPK-JNK signaling pathway is a known regulator of autophagy,[10] a pathway essential for degrading misfolded proteins, we reasoned that the reduction of mutant SOD1 by MAP4K4 suppression might be due to the activation of autophagy. Two crucial proteins govern the process of autophagy-p62, a specific autophagy substrate, and LC3B-II, a conjugated protein recruited to autophagosomal membranes. During autophagy, LC3B-II undergoes a recycling process, such that high levels of LC3B-II can be associated with either autophagy activation or the impairment of autophagosome turnover.[11] To evaluate autophagy activity following MAP4K4 suppression, we blocked autophagasome turnover by employing a lysosomal protease inhibitor (NH_4Cl), which results in an accumulation of LC3B-II. MAP4K4 suppression in NH_4Cl treated MNs resulted in an additional LC3B-II increase, suggesting activation of the autophagy pathway by MAP4K4 inhibition. In addition, we observed decreased p62 amounts in MNs treated with MAP4K4 siRNAs, providing further support for this conclusion. We next investigated the mechanism through which MAP4K4 activates autophagy. Previous literature suggested a role for mTOR and FoxO1 in this process.[12,13]

However, we observed only an increase of FoxO1 following MAP4K4 knockdown, with no change in mTOR levels, suggesting that FoxO1 may be the key intermediate through which MAP4K4 activates autophagy. Additional work showing that FoxO1 inhibition reduced the rescue effect achieved with MAP4K4 suppression further supports the role of FoxO1 in MAP4K4-mediated autophagy activation and MN survival.

Considering the unmet need for new ALS therapeutics, it was critical that we evaluate the potential for MAP4K4 as a viable ALS drug target by testing a selective inhibitor, MAP4K4i, in human MNs.[14] This was particularly important given the notable failure of 2 compounds, olesoxime and dexpramipexole, neither of which had ever been tested on any type of human MN, in phase III clinical trials.[15] In this "preclinical test," MAP4K4i significantly and dose dependently improved the survival of all types of MNs tested, including ALS MNs with various genetic mutations ($SOD1^{L144F}$, $TDP43^{G298S}$, and $TDP43^{M377V}$). Furthermore, MAP4K4i improved survival of MNs treated with tunicamycin or thapsigargin, activators of endoplasmic reticulum (ER) stress and the unfolded protein response, thought to be important in ALS. Since genetic and pharmacological reduction of MAP4K4 activity promoted survival of ALS MNs in a variety of pathological conditions, our results strongly support the notion that MAP4K4 may be a viable target for developing ALS therapeutics. Exciting, important questions, however do remain from our work, of which we propose a few:

1. What is the effect of MAP4K4 reduction in vivo and in other MN/neurodegenerative diseases?

Our data, as well as work by other groups,[7] suggest that MAP4K4 inhibition is protective to neurons broadly, not just to ALS MNs in stressed conditions. It will be worthwhile to test whether MAP4K4 reduction can confer benefit in other MN diseases such as Spinal Muscular Atrophy (SMA), as well as to other neurological disorders. Particularly, it will be interesting to evaluate whether MAP4K4 inhibition can affect TDP-43 protein misfolding and aggregation given that TDP-43 aggregation is observed in almost all ALS cases. Moreover, it will be important to demonstrate that inhibiting MAP4K4 in vivo enacts a similar benefit as that seen in vitro. Pharmacodynamic parameters, including CNS penetration, need to be established and optimized for this compound class. Nonetheless, we predict that targeting MAP4K4 will confer a delay in deterioration of neuromuscular function and an increase in survival in ALS mouse models.

2. What activates MAP4K4?

We report that MAP4K4 and its downstream targets are involved in neurodegeneration, but it is still unclear how MAP4K4 is activated in stressed MNs. Inferences from other model systems and homologous MAPKs provide some clues, suggesting that TNFα stimulation via kinase receptor activation and/or interaction with GTPases may be potential ways in which MAP4K4 is activated.[6] Identifying and targeting this upstream effector will be important and may serve therapeutic benefit in addition to MAP4K4 inhibition.

3. Can MAP4K4 suppression function in a cell non-autonomous mechanism?

We show that MAP4K4 reduction works autonomously to promote MN viability. However, cell non-autonomous mechanisms are known to play a role in neuronal health and neurological diseases.[1] It will be interesting to determine whether MAP4K4 is activated in non-neuronal populations (i.e., astrocytes) in stress and disease, and whether MAP4K4 inhibition in these cells can confer neuronal protection. Given the protective effect of MAP4K4 suppression in heterogenous populations, as well as with other neuronal types, we hypothesize that suppression of MAP4K4 in non-neuronal cells will result in a survival effect.

4. Can MAP4K4 inhibition reduce cell death mechanisms in addition to apoptosis?

Our work shows that apoptosis is significantly attenuated in MAP4K4 reduced conditions. Given that other modes of cell death have been implicated in ALS, namely necrosis and necroptosis,[1] it will be informative to determine whether MAP4K4 inhibition modulates both types of death or whether it is apoptosis specific.

In summary, while critical questions remain, our findings support the claim that MAP4K4 mediates MN degeneration in ALS, and that pharmacological reduction of MAP4K4 signaling may be a promising new therapeutic avenue to pursue.

Author Contributions

MEW, CW and LLR conceived and wrote the manuscript.

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