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NOTCH PATHWAY IS ACTIVATED IN CELL CULTURE AND MOUSE MODELS OF MUTANT SOD1-RELATED FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS, WITH SUPPRESSION OF ITS ACTIVATION AS AN ADDITIONAL MECHANISM OF NEUROPROTECTION FOR LITHIUM AND VALPROATE

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Abstract—Amyotrophic lateral sclerosis (ALS) is an idiopathic and lethal neurodegenerative disease that currently has no effective treatment. A recent study found that the Notch signaling pathway was up-regulated in a TAR DNA-binding protein-43 (TDP-43) *Drosophila* model of ALS. Notch signaling acts as a master regulator in the central nervous system. However, the mechanisms by which Notch participates in the pathogenesis of ALS have not been completely elucidated. Recent studies have shown that the mood stabilizers lithium and valproic acid (VPA) are able to regulate Notch signaling. Our study sought to confirm the relationship between the Notch pathway and ALS and whether the Notch pathway contributes to the neuroprotective effects of lithium and VPA in ALS. We found that the Notch pathway was activated in *in vitro* and *in vivo* models of ALS, and suppression of Notch activation with a Notch signaling inhibitor, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT) and Notch1 siRNA significantly reduced neuronal apoptotic signaling, as evidenced by the up-regulation of Bcl-2 as well as the down-regulation of Bax and cytochrome c. We also found that lithium and VPA suppressed the Notch activation associated with the superoxide dismutase-1 (SOD1) mutation, and the combination of lithium and VPA produced a more robust effect than either agent alone. Our findings indicate that the Notch pathway plays a critical role in ALS, and the neuroprotective effects of lithium and VPA against mutant SOD1-mediated neuronal damage are at least partially dependent on their suppression of Notch activation.

Key words: ALS, lithium, VPA, Notch signaling pathway.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease. The majority of ALS cases are sporadic (sALS), while 10% are familial (fALS). Copper–zinc superoxide dismutase-1 (SOD1) gene mutations account for up to 20% of all fALS cases (Rosen et al., 1993). Excitotoxicity, oxidative stress, mitochondrial dysfunction, apoptosis, autophagy and dysfunction of signaling pathways are involved in the pathogenesis of the disease, although their roles are not fully understood (Wijesekera and Leigh, 2009).

Notch is a highly evolutionarily conserved pathway that controls cell fate decisions, migration, growth, synaptic plasticity and neuronal survival (Ables et al., 2011). The pathway consists of four Notch receptors (Notch1–4) and two types of ligands (Jagged1 and 2; Delta-like1, 3 and 4) (D'Souza et al., 2008). Upon ligand binding, Notch is rendered susceptible to proteolytic cleavages mediated by members of the disintegrin metalloproteinase (ADAM) family and the γ-secretase complex. The released Notch intracellular domain (NICD) translocates to the nucleus, where it binds to the Rbp-Jκ (recombination signal binding protein for immunoglobulin kappa J), co-activator mastermind-like-1 (Maml1) and forms an activator complex, leading to the transcription of its target gene the Hairy and Enhancer of split (Hes) and the related Hey genes. Abnormal activation of the Notch pathway is associated with several neurodegenerative diseases, including Down Syndrome, Alzheimer's and Pick's disease (Fischer et al., 2005; Nagarsheth et al., 2006; Lathia et al., 2008). A recent study revealed the deleterious effect of Notch activation in TAR DNA-binding protein-43 (TDP-43) transgenic flies (Zhan et al., 2013). However, the relationship between Notch and SOD1 G93A-induced neuronal toxicity requires further investigation.

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Abbreviations: ALS, amyotrophic lateral sclerosis; Cyt C, cytochrome c; DAPT, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester; EV, empty vector; fALS, familial amyotrophic lateral sclerosis; GSK3, glycogen synthase kinase 3; Hes1, the Hairy and Enhancer of split1; Maml1, mastermind-like-1; MTT, 3-(4,5-dimethyl thiozol-2-yl)-2,5-diphenyltetrazolium bromide; NICD, Notch intracellular domain; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time PCR; sALS, sporadic amyotrophic lateral sclerosis; SOD1, superoxide dismutase-1; TDP-43, TAR DNA-binding protein-43; VPA, valproic acid; WT, wild type.

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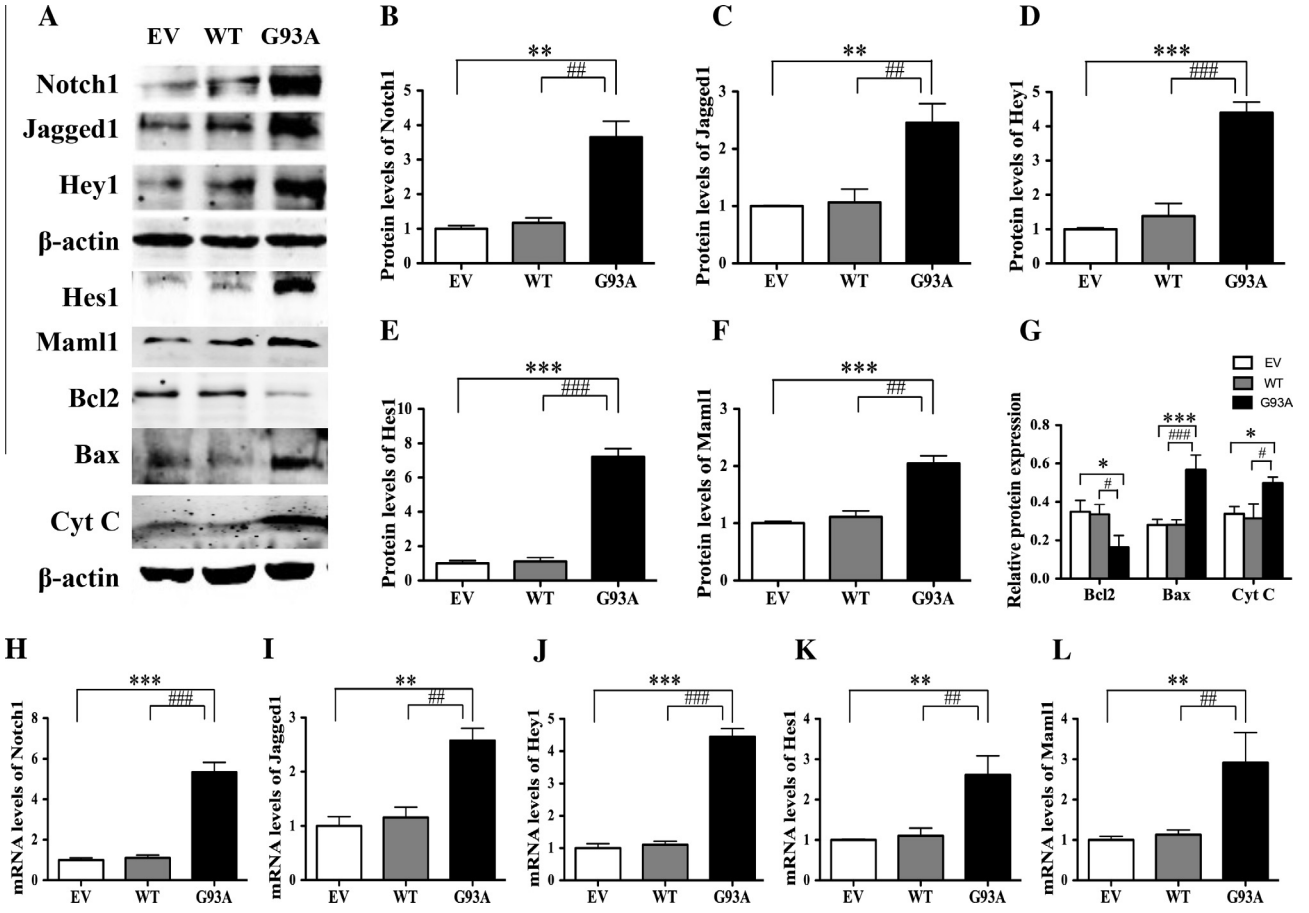

Fig. 1. The expression of Notch1, Jagged1, Hey1, Hes1, Maml1, Bax and cytochrome c increased, while the expression of Bcl2 decreased in human SOD1 G93A-overexpressing NSC34 cells. NSC34 cells were stably transfected with empty vector (EV), wild type human SOD1 (WT), or mutant human SOD1 G93A (G93A). (A) Western blotting showed that protein levels of Notch1, Jagged1, Hey1, Hes1, Maml1, Bax and cytochrome c were elevated while Bcl2 protein was decreased in NSC34 cells transfected with G93A compared with EV and WT cells. (B)–(G) Quantification of Notch1, Jagged1, Hey1, Hes1, Maml1, Bcl2, Bax and cytochrome c protein levels, with β-actin as a loading control. (H)–(L) mRNA levels of Notch1, Jagged1, Hey1, Hes1 and Maml1 in NSC34 cells transfected with EV, WT or G93A were determined by qRT-PCR. The protein and mRNA levels of Notch1, Jagged1, Hey1, Hes1 and Maml1 increased significantly in G93A cells compared with EV and WT cells. The levels of Bax and cytochrome c protein increased but Bcl2 decreased in G93A cells. Data are shown as the mean ± standard deviation. Values were normalized to β-actin and were expressed as relative level compared to the control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the result of the EV group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ when compared with the result of the WT group.

Lithium and valproic acid (VPA), which are primarily used to treat bipolar disorder, have a neuroprotective effect in neurologic diseases such as stroke, traumatic brain injury, Huntington's disease, Alzheimer's disease, ALS and Fragile X Syndrome (Chiu et al., 2011, 2013; King et al., 2014). Fornai et al. (2008a) first reported the therapeutic efficacy of lithium in both ALS patients and in the SOD1 G93A mouse model of ALS. Moreover, previous work conducted in our laboratory has shown that combined treatment with lithium and VPA delayed disease onset, reduced neurological deficits and prolonged the lifespan in the SOD1 G93A mouse model of ALS (Feng et al., 2008). Interestingly, lithium and VPA in combination significantly increased survival and exhibited a neuroprotective effect in ALS patients (Boll et al., 2014). However, the mechanisms underlying the synergistic therapeutic effect of lithium and VPA are not fully understood. Recent studies showed that lithium modulates the Notch signaling pathway in NIH-3T3, HEK-293T and neuroblastoma 2a cells (Foltz et al., 2002; Espinosa et al., 2003). Furthermore, VPA modulates the Notch signaling pathway in a variety of cancers (Stockhausen et al., 2005; Greenblatt et al., 2007, 2008). Treatment with VPA reduced levels of Notch1 within the hippocampus (Umka et al., 2010). Whether Notch signaling is involved in the neuroprotective effects of lithium and VPA in ALS remains to be discovered.

In the current study, we examined the role of Notch1, its ligand Jagged1, the co-activator Maml1 and the targets Hey1 and Hes1 in *in vitro* and *in vivo* models of ALS. We also investigated the combined effects of lithium and VPA on the Notch signaling pathway in the SOD1 G93A background.

EXPERIMENTAL PROCEDURES

Cell culture and treatment

The NSC34 cell line (Cedarlane Laboratories, Vancouver, Canada), a hybrid cell line of mouse neuroblastoma and embryonic spinal motor neurons that is stably transfected with mutant human SOD1 G93A (G93A), was chosen as a cell model of ALS, with empty vector