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Valproic acid ameliorates *C. elegans* dopaminergic neurodegeneration with implications for ERK-MAPK signaling

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HIGHLIGHTS

- The anti-epileptic drug, valproic acid, exhibits neuroprotective activity in an animal model of Parkinson's disease.
- Valproic acid attenuates dopaminergic neurodegeneration associated with human α-synuclein overproduction in *C. elegans*.
- Neuroprotection by valproic acid is mediated through ERK-MAPK signaling in vivo.

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ABSTRACT

Parkinson's disease (PD) is a currently incurable neurodegenerative disorder that affects the aging population. The loss of dopaminergic neurons in the substantia nigra is one of the pathological features of PD. The precise causes of PD remain unresolved but evidence supports both environmental and genetic contributions. Current efforts for the treatment of PD are directed toward the discovery of compounds that show promise in impeding age-dependent neurodegeneration in PD patients. Alpha-synuclein (α-Syn) is a human protein that is mutated in specific populations of patients with familial PD. Overexpression of α-Syn in animal models of PD replicates key symptoms of PD, including neurodegeneration. Here, we use the nematode *Caenorhabditis elegans* as a model system, whereby α-Syn toxicity causes dopaminergic neurodegeneration, to test the capacity of valproic acid (VA) to protect neurons. The results of our study showed that treatment of nematodes with moderate concentrations of VA significantly protects dopaminergic neurons against α-Syn toxicity. Consistent with previously established knowledge related to the mechanistic action of VA in the cell, we showed through genetic analysis that the neuroprotection conferred by VA is inhibited by cell-specific depletion of the *C. elegans* ortholog of the MAP extracellular signal-regulated kinase (ERK), MPK-1, in the dopaminergic neurons. These findings suggest that VA may exert its neuroprotective effect via ERK-MAPK, or alternately could act with MAPK signaling to additively provide dopaminergic neuroprotection.

The degeneration of dopaminergic neurons in Parkinson's disease (PD) patients represents one of the pathological features of PD. Risk factors for PD include both environmental and genetic factors [7]. The gene encoding the protein alpha-synuclein (α-Syn) is a well-studied genetic risk factor. α-Syn is a major constituent of the Lewy bodies found in PD patients, and is considered to play a key role in the pathogenesis of PD. For instance, it was found that point mutations as well as multiplication of the α-Syn locus cause familial forms of PD [24,25]. Similarly, overexpression of α-Syn in mice and other model organisms mimic symptomatic features of PD, including accumulation of misfolded proteins, cellular toxicity, and neurodegeneration [26]. Current efforts for the treatment of PD are aimed at identifying compounds that exhibit potency in ameliorating age-dependent neurodegeneration.

Valproic acid (VA) is an FDA approved compound that is normally prescribed for the treatment of epilepsy and bipolar disorder. Studies have shown that VA affects GABA transmission, voltage gated Na+ channels, T-type calcium channels, and histone deacetylases (HDACs) [4]. However, several studies revealed that VA can also activate ERK-MAPK both in vivo and in vitro. This action of VA,

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through ERK-MAPK, could have both neuroprotective and positive growth effects on neurons [4,12]. For example, it has been reported that application of VA resulted not only in neuroprotection, but regeneration of injured retinal ganglion cells. This neuroprotective/regenerative effect was accompanied by prolonged activation of phosphorylated ERK 1/2 [1], suggesting that ERK-MAPK mediates both the neuroprotective and neuroregenerative effect of VA. In two other independent studies, VA was also shown to promote neurite growth through ERK [30], and positively affect cortical neuron growth and hippocampal neurogenesis in adult mice, also through the ERK pathway. As such, it was postulated that VA plays a role in promoting neurotrophic factors that positively regulate neuronal growth and maintenance to counteract neuronal cell death [9].

The neuroprotective effect of VA has also been documented in select models of PD. In one study, it was observed that chronic dietary administration of VA reduced dopaminergic cell death in neurodegenerative rats that were treated with rotenone [20]. In another model developed by the same group, VA was shown to confer neuroprotection in the degenerating brain cells of rats that were previously injected with the toxin 6-hydroxydopamine (6-OHDA) [21]. In a mouse model of PD, VA protected the nigrostriatal dopamine system against the toxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) [15]. These findings support the hypothesis that VA may have a neuroprotective effect on the dopaminergic neurons. Although such studies using acute neurotoxins have been insightful, it remains to be known whether or not VA can protect dopaminergic neurons impacted by the overproduction of α-Syn. Moreover, it has not been demonstrated if VA can exert its protective effect on the dopaminergic neurons through ERK-MAPK pathway.

Our laboratory had previously reported that overexpression of α-Syn in the dopaminergic neurons of *Caenorhabditis elegans* causes age-dependent neurodegeneration [3]. Despite its vast anatomical difference from humans, the *C. elegans* nervous system possesses important cellular and molecular features of mammalian neurons, which include conserved neurotransmitter systems (dopamine, GABA, acetylcholine, serotonin, etc.), receptors, axon guidance molecules, ion channels, and synaptic features. Moreover, the *C. elegans* genome contains homologs of many human genes including those that have been implicated in PD and other neurodegenerative diseases. Using this model system, we set out to test the hypothesis that VA may protect dopaminergic neurons of *C. elegans* against α-Syn toxicity via an ERK-MAPK-dependent mechanism.

We investigated this hypothesis using a combination of pharmacology and cell-specific RNA interference technology (RNAi). For the cell-specific RNAi experiments, the *C. elegans* ortholog of ERK-MAPK, MPK-1, and an upstream regulator MEK-2, were depleted in the dopaminergic neurons in the presence of overexpressed α-Syn. The cell-specific RNAi strain used in this study was created by introducing a *sid-1* loss-of-function mutation in conjunction with SID-1 genomic DNA UA195 [*sid-1(pk3321)*; *baIn33* (P*dat-1*::*sid-1*, P*myo-2*::mCherry)] into animals expressing α-Syn in the dopaminergic neurons UA44 [*baIn11*(P*dat-1*:: α-syn, P*dat-1*::GFP)]. The resulting strain UA196 [*sid-1(pk3321)*; *baIn11*; *baIn33*], renders only the dopaminergic neurons susceptible to the effect of RNAi [10]. Using this strain, we monitored the loss of the dopaminergic neurons of *C. elegans* in adult animals. In this experimental paradigm, animals were treated with or without VA at various drug concentrations. All animals were cultured according to standard worm maintenance procedures [2]. Molten nematode growth medium (NGM) was used to dissolve VA inside conical flasks at 55–60 °C. Three independent mixtures were made containing 1 mM, 2 mM, and 3 mM final concentrations. These mixtures were then poured into 60 mm Petri dishes and incubated at room temperature overnight. On the next day plates were seeded with bacteria (strain OP50), and incubated at 37 °C. Gravid adults were

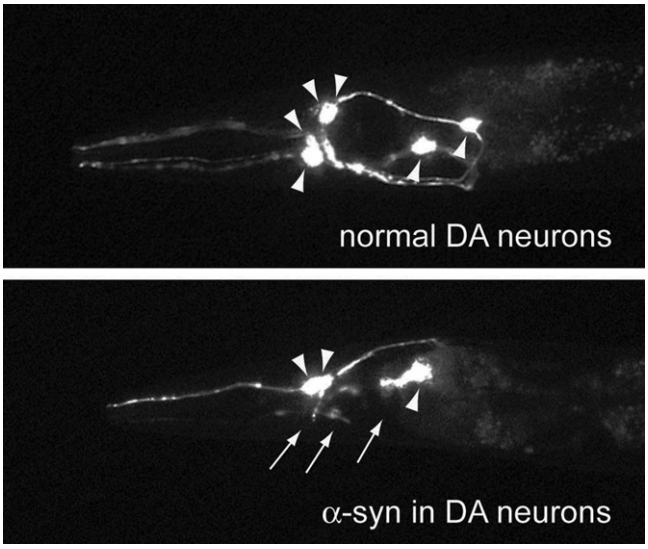

Fig. 1. Dopaminergic neurons (DA) of *C. elegans* in day-7 adult animals. (A) The six anterior DA neurons remain intact in the absence of α-synuclein (α-Syn). (B) Anterior neurons undergoing neurodegeneration when α-Syn is overexpressed. Arrowheads indicate the cell bodies, and arrows depict the dying processes.

then transferred onto the plates to lay embryos and removed after 24 h. The offspring were analyzed at day 7 post-embryonically because significant neurodegeneration is reproducibly observed in populations at this time [9]. At day 7, 88.9% of α-syn animals displayed neurodegeneration in the dopaminergic neurons (11.1% animals with WT neurons) (Figs. 1 and 2).

To score dopaminergic neurons, animals were placed in a 3 mM solution of levamisole and mounted with a coverslip on a 2% agarose pad. Animals were examined with a Nikon Eclipse E800

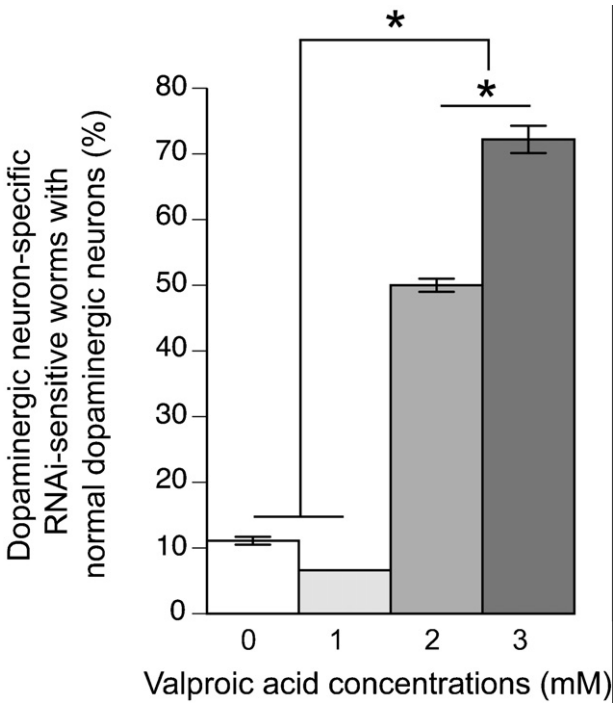

Fig. 2. Treatment of α-Syn animals with valproic acid (VA) is neuroprotective. Dopaminergic neuroprotection of α-Syn-expressing animals was commensurate with VA concentration, where 1 mM was not protective, while 2 mM and 3 mM both displayed significant neuroprotection when compared to animals treated with vehicle alone. 3 mM VA also provided significantly more neuroprotection than 2 mM VA. $P < 0.05$; One-way ANOVA with a Bonferroni post hoc analysis.

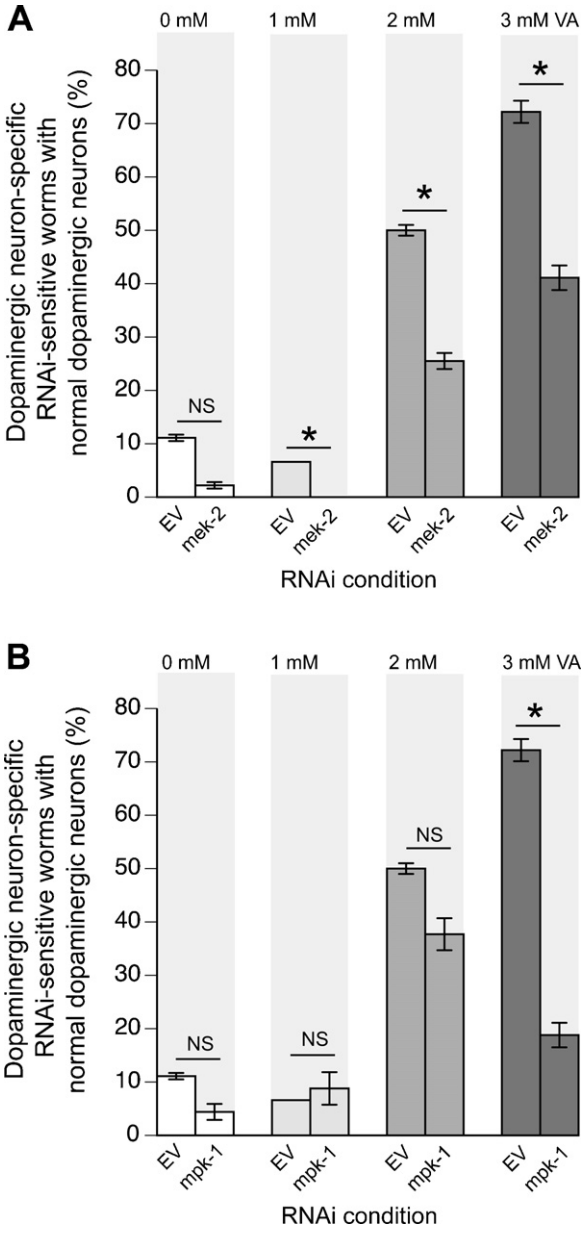

Fig. 3. VA neuroprotection of α-Syn-overexpression in DA neurons is dependent on the ERK-MAPK signaling pathway. (A) Cell-specific RNAi depletion of *mek-2* negates the dopaminergic neuroprotection provided by VA at all concentrations of examined compared to empty vector (EV) control, where neuroprotection is still observed at 1, 2, and 3 mM VA. (B) Similar to A, *mpk-1* depletion reverses neuroprotection of VA in dopaminergic neurons at 3 mM VA compared to EV control. $P < 0.05$; Student's *t*-test.

epifluorescence microscope at 400× magnification. The six anterior dopaminergic neurons of *C. elegans* were scored in our experiments. An animal with normal neurons is defined as having all intact dopaminergic neurons (i.e. cell bodies and processes). An animal with degenerating neurons is defined as having at least one missing neuron within that individual (i.e. missing cell body or dendrite). Statistical analyses were performed using either one-way ANOVA followed by the Bonferroni post hoc analysis for multiple comparisons (Fig. 2) or Student's *t*-test when comparing two sets of data/experiment involving control and treated animals (RNAi with VA treatments), as described in Fig. 3. The mean values for all data sets were shown with standard deviations, where a *p*-value of less than 0.05 is considered significant. In total, 90 animals were examined, per experimental condition.

Our results showed that VA is significantly neuroprotective against α-Syn neurodegeneration at 2 mM and 3 mM concentrations in day-7 adult animals. In untreated animals, only 11.1% of animals expressing α-Syn exhibit normal dopaminergic neurons (Fig. 2). When animals were treated with 2 mM of VA, the percentage of the population displaying the normal complement of dopamine neurons rose significantly to approximately 50%. There was an even greater increase when animals were treated with 3 mM VA, as 72% of these animals displayed normal dopaminergic neurons at day 7 of development (Fig. 2). There was a significant difference between the population of animals with normal neurons following treatment with 2 mM and 3 mM VA. In contrast, no significant protection was observed at 1 mM (Fig. 2). We could not analyze animals treated with 4 mM VA (or higher) because 4 mM VA slowed the growth of animals to such an extent that at day 7 of development we were unable to obtain sufficient quantities of animals for analysis while, at the lower concentrations, there was an abundance of animals. Moreover, the size of the surviving animals treated with 4 mM VA was significantly smaller than the non-treated animals, signifying a developmental delay. However, our results suggest that VA can protect the dopaminergic neurons of *C. elegans* against α-Syn toxicity at 2 mM and 3 mM concentrations.

We proceeded to ask whether VA protected the *C. elegans* dopaminergic neurons via ERK-MAPK. To answer this question we used cell-specific RNAi to deplete ERK-MAPK in the dopaminergic neurons. Until recently, it was difficult to achieve dopaminergic RNAi knockdown of target genes in *C. elegans*. However, new methods for neuronal-sensitive RNAi allow for selective knockdown of target genes in subsets of neurons. For example, the impact of candidates knocked down by RNAi can be examined exclusively in the dopaminergic neurons in our $P_{dat\text{-}1}$::α-syn + $P_{dat\text{-}1}$::GFP strain {UA196 [*sid-1(pk3321)*; P*dat-1*::α-syn, P*dat-1*::GFP; P*dat-1*::*sid-1*, P*myo-2*::mCherry]}. Worms expressing GFP without α-syn in the dopaminergic neurons {UA202 [*sid-1(pk3321)*; P*dat-1*::GFP; P*dat-1*::*sid-1*, P*myo-2*::mCherry]} act as a control strain [10].

In *C. elegans*, the ortholog of ERK is encoded by the gene *mpk-1* [16,17,27]. The direct upstream regulator of MPK-1 is MEK-2, which is a MAP2 kinase [5,22,28]. RNAi bacterial clones for *mek-2* and *mpk-1* were obtained from a comprehensive *C. elegans* RNAi library (MRC Cambridge) [14]. RNAi experiments were performed by growing RNAi bacteria (HT115) on agar plates containing 0.25% beta-lactose [19]. Gravid adult animals, UA196 α-Syn RNAi strain (described above), were transferred onto the RNAi plates and allowed to lay embryos for 24 h. The offspring from these parental animals were left on the plates to eat HT115 bacteria containing the specified RNAi clones and were analyzed for neurodegeneration at day 7. The results of our RNAi experiments showed that depletion of *mek-2* negates the protective effect of VA on the dopaminergic neurons of adult animals at all concentrations of VA tested (1, 2 and 3 mM) compared to empty vector (EV) control (Fig. 3A). In contrast, dopaminergic-selective RNAi against *mpk-1* abolished neuroprotection only at the 3 mM VA concentration in comparison to the EV control (Fig. 3B). The difference in responses between *mek-2* (RNAi) and *mpk-1* (RNAi) could possibly have occurred because of differential levels of *mpk-1* and *mek-2* transcripts when worms are treated with different concentrations of VA. However, given that knockdown of both genes reverses the neuroprotective effect of VA compared to EV at one dose of VA implies that VA requires the presence of endogenous levels of MEK-2 and MPK-1 in order to protect the dopaminergic neurons from α-Syn toxicity. We therefore concluded that VA attenuates dopaminergic neurodegeneration via ERK-MAPK. However, we do not rule out the possibility that VA and ERK-MAPK may also act additively to protect the dopaminergic neurons.

What are the possible mechanisms and implications of our findings in relation to neuroprotection and PD? Treatment of neurons

with VA can significantly enhance or prolong the phosphorylation of ERK-MAPK [1,9]. Such prolonged phosphorylation can lead to an increase in the amount of neurotrophic factors mediating positive neuronal growth through an unknown mechanism [9]. However, it has also been suggested that activation of p-ERK-MAPK by VA can impact downstream transcription factors resulting in altered nuclear gene expression and enhanced cell survival [6]. Interestingly, our data showed that VA significantly protected the dopaminergic neurons from the toxicity caused by α-Syn overexpression. Moreover, we showed that independent RNAi knockdowns of *mek-2* and *mpk-1* abolished the neuroprotective effect of VA on the dopaminergic neurons. This finding implicates ERK-MAPK as a plausible pathway mediating the neuroprotective effect of VA in the *C. elegans* dopaminergic neurons. Despite its pleiotropic roles in cells and tissues, ERK-MAPK is also an affirmed downstream component of D2 dopamine receptor signaling in the mammalian brain [29]. Such a role asserts that ERK-MAPK additionally plays a vital function in the regulation of various aspects of dopamine signaling and homeostasis. Coincidently, there is strong evidence that ERK-MAPK physically associates with α-Syn [13,23], suggesting its potential role in PD. Moreover, transient overexpression of α-Syn in neuro2A cells suppressed the function of ERK-MAPK, resulting in decreased cell viability [13]. Such in vitro findings corroborate our findings in vivo, all of which affirm the notion that downregulation of ERK-MAPK mediates α-Syn induced cellular toxicity and neurodegeneration. In addition, studies using other cellular models of PD have also offered supporting evidence regarding the role of ERK-MAPK in cell viability. For instance, in one study it was reported that activation of ERK-MAPK is necessary for protection of dopaminergic cells against rotenone toxicity [11]. In another study, it was revealed that rapid activation of ERK-MAPK promoted the survival of dopaminergic neurons in the presence of 6-OHDA [18]. These findings corroborate the potential neuroprotective role of ERK-MAPK in the cell, and suggest that administration of VA may serve to enhance the neuroprotective function of this pathway. It is also important to note that cytoplasmic accumulation of phosphorylated ERK-MAPK has been detected in the substantia nigra of PD and Dementia Lewy Body (DLB) patients [8,31], suggesting the potential link of this pathway to PD and other common Lewy Body diseases. Our finding that VA can ameliorate dopaminergic neurodegeneration via ERK-MAPK not only confirms VA as a neuroprotective compound, but also implicates ERK-MAPK signaling as the plausible molecular target of VA in the protection of the dopaminergic neurons.

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