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# VITAMIN E IS ESSENTIAL FOR PURKINJE NEURON INTEGRITY

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**Abstract—α-Tocopherol (vitamin E) is an essential dietary antioxidant with important neuroprotective functions. α-Tocopherol deficiency manifests primarily in neurological pathologies, notably cerebellar dysfunctions such as spinocerebellar ataxia. To study the roles of α-tocopherol in the cerebellum, we used the α-tocopherol transfer protein for the murine version (*Ttpa*$^{-/-}$) mice which lack the α-tocopherol transfer protein (TTP) and are a faithful model of vitamin E deficiency and oxidative stress. When fed vitamin E-deficient diet, *Ttpa*$^{-/-}$ mice had un-detectable levels of α-tocopherol in plasma and several brain regions. Dietary supplementation with α-tocopherol normalized plasma levels of the vitamin, but only modestly increased its levels in the cerebellum and prefrontal cortex, indicating a critical function of brain TTP. Vitamin E deficiency caused an increase in cerebellar oxidative stress evidenced by increased protein nitrosylation, which was prevented by dietary supplementation with the vitamin. Concomitantly, vitamin E deficiency precipitated cellular atrophy and diminished dendritic branching of Purkinje neurons, the predominant output regulator of the cerebellar cortex. The anatomic decline induced by vitamin E deficiency was paralleled by behavioral deficits in motor coordination and cognitive functions that were normalized upon vitamin E supplementation. These observations underscore the essential role of vitamin E and TTP in maintaining CNS function, and support the notion that α-tocopherol supplementation may comprise an effective intervention in oxidative stress-related neurological disorders.** 

**Key words: vitamin E, tocopherol, Purkinje neuron, cerebellum, ataxia, oxidative stress.**

## INTRODUCTION

The term vitamin E refers to a family of plant-derived neutral lipids that function as lipid-soluble antioxidants by scavenging lipid peroxyl radicals generated in the cellular membranes. Of all naturally occurring members of the vitamin E family (tocopherols and tocotrienols), α-tocopherol is selectively enriched in blood and tissues of most vertebrates, whereas other 'vitamers' are excreted or catabolized (O'Byrne et al., 2000). This biochemical discrimination is achieved by the actions of the hepatic α-tocopherol transfer protein (TTP; Hosomi et al., 1997), responsible for the selective retention of α-tocopherol, and the vitamin E ω-hydroxylase CYP4F2 (cytochrome P450 family 4, subfamily F, polypeptide 2), which initiates catabolism of other vitamin E forms (Sontag and Parker, 2002, 2007). Adequate α-tocopherol status is considered essential for human health, and appropriate intake levels of the vitamin have been formalized by the Institutes of Medicine (Ford and Sowell, 1999; Ford et al., 2006). Despite the natural abundance of vitamin E in plant-based foods, a very significant number of individuals do not consume the recommended intake levels (Fulgoni et al., 2011a,b).

As a major lipid-soluble antioxidant, α-tocopherol is essential for all cells. However, the vitamin appears to be especially critical for CNS function. Thus, vitamin E deficiency presents primarily as neurological and neuromuscular disorders, specifically spinocerebellar ataxia (SCA) (Sokol, 1988, 1990; Gohil et al., 2010; Muller, 2010). Of special interest is familial ataxia with vitamin E deficiency (AVED, OMIM #277460; (Cavalier et al., 1998)), which results from heritable loss-of-function mutations in the gene encoding the human α-tocopherol transfer protein (*TTPA*), and also observed in the α-tocopherol transfer protein knock-out mouse model (*Ttpa*$^{-/-}$, Terasawa et al., 2000; Yokota et al., 2001). On the anatomic levels, prevailing lesions are characterized by axonopathy and neuromuscular injuries, with appearance of axonal swellings and reduced myelination (Southam et al., 1991). Massive deposition of lipid peroxidation products and lipofuscin aggregates are indicative of CNS oxidative stress. On the functional level, affected neurons display attenuated axonal transport, and altered mitochondrial respiratory control (Thomas et al., 1993; Cuddihy et al., 2008). Electrophysiological studies in vitamin E-deficient rodents indicated compromised somatosensory- and

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*Abbreviations:* 3-NT, 3-nitrotyrosine; AD, Alzheimer's disease; ANOVA, analysis of variance; AVED, ataxia with vitamin E deficiency; CS, conditioned stimulus; NPC, Niemann Pick disease type C; SCA, spinocerebellar ataxia; TBARS, thiobarbituric acid reactive substances; t-HODE, total hydroxyoctadecadienoic acid; TTP, α-tocopherol transfer protein; *Ttpa*, α-tocopherol transfer protein gene for the murine version; *TTPA*, α-tocopherol transfer protein gene for the human version; US, unconditioned stimulus.

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visual-evoked potentials (Goss-Sampson et al., 1988, 1990; Yokota et al., 2001). In accordance with the ataxic hallmark of vitamin E deficiency, mild cerebellar atrophy was reported in vitamin E-deficient patients (Sokol, 1988), and in one case, mild focal loss of cerebellar Purkinje neurons was observed (Yokota et al., 2000). Manifestation of the ataxic phenotype likely involves injury to cerebellar Purkinje neurons, since these cells are critical mediators of motor output that originates in the cerebellar cortex (Eccles et al., 1966a,d; Altman, 1972; Optican, 1998). In light of these observations, vitamin E supplementation has been utilized in a number of oxidative stress-related neurological disorders. Such intervention has proven noteworthy in human patients suffering from Alzheimer's disease (AD), Parkinson's disease and Down's syndrome (Sano et al., 1997; Buhmann et al., 2004; Sung et al., 2004; Liu et al., 2007; Perrone et al., 2007). Similarly, in a mouse model of Alzheimer disease, vitamin E delayed disease progression (Nishida et al., 2006), possibly by attenuating lipid peroxide-induced inhibition of beta amyloid clearance (Nishida et al., 2009).

Although the critical importance of vitamin E in neurological health has been recognized for over 50 years, little is known regarding the specific roles of α-tocopherol in the CNS, or the mechanisms by which it elicits its neuroprotective effects. To begin to address this issue, we characterized the neurological manifestation of vitamin E deficiency in the CNS of the *Ttpa*$^{-/-}$ mice, with specific emphasis on properties that can be prevented by high-level supplementation with α-tocopherol.

## EXPERIMENTAL PROCEDURES

### Mice and tissues

All animal work was performed according to the Institutional Animal Care and Use Committee (IACUC)-approved protocols at Case Western Reserve University. The *Ttpa*$^{-/-}$ (B6.129S4-Ttpa$^{tm1Far}$/J) mouse model was generated by targeted disruption in exons 1 and 2 of the *Ttpa* gene and was described earlier (Terasawa et al., 2000). For breeding, *Ttpa*$^{+/-}$ female mice were crossed with *Ttpa*$^{-/-}$ males and offspring genotype determined by PCR. At 4 weeks of age, *Ttpa*$^{+/+}$ mice were maintained on normal chow (34 mg α-tocopheryl acetate/kg diet), whereas, *Ttpa*$^{-/-}$ mice were placed on a vitamin E-deficient (no tocopherol) or vitamin E-supplemented diet (600 mg α-tocopheryl acetate/kg diet). Animals were sacrificed at 17 months of age, brain tissue excised, and either fixed in 3.7% paraformaldehyde for 24 h and paraffin-embedded, or flash frozen for use in biochemical analyses.

### Golgi-Cox staining

Freshly harvested sagittal-cut half brains were rinsed with phosphate-buffered saline (PBS), processed according to the Rapid GolgiStain protocol (FD Neuro Technologies, Columbia, MD, USA) and embedded in Tissue Freezing Media (Thermo-Fisher, Waltham, MA, USA). 150-μm-thick sections were cut using a vibratome (VT1000, Leica, Buffalo Grove, IL, USA), mounted onto gelatin-subbed slides (Thermo-Fisher, Waltham, MA, USA) and stained according to the kit protocol. Purkinje neurons that were spatially matched between the experimental groups at random cerebellar locations were chosen for analysis, and cells exhibiting obvious structural injury from sectioning were excluded. Purkinje neurons were visualized at 40× magnification using a Zeiss Axioplan microscope and traced utilizing Neurolucida neuron tracing software version 5 (mbf Bioscience, Williston, VT, USA). For each experimental group, a total of 12 neurons were assessed in three different animals.

### Lipid analysis

Lipids were extracted by a two-step separation process followed by silylation, as described in a previous report (Sontag and Parker, 2007). Extracted lipids were analyzed by GC–MS on a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5872 mass selective detector operated in selected ion mode. The GC–MS settings for analysis were as described in a previous report for long-chain metabolites (Sontag and Parker, 2007). α-Tocopherol and unesterified cholesterol concentrations were determined using internal deuterated standards.

### Measurement of total protein-bound 3-nitrotyrosine (3-NT)

The cerebellar protein-bound 3-NT was determined immunochemically as previously described (Sultana and Butterfield, 2008). Briefly, cerebella samples were homogenized in Laemmli buffer (0.125 M Trizma base, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol) and blotted onto nitrocellulose membrane using a vacuum-manifold apparatus. The membranes were incubated with 1:1000 dilution of rabbit anti-nitrotyrosine (Sigma-Aldrich, St. Louis, MO, USA) antibody followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody and visualization using Sigma fast tablet (BCIP/NBT; Sigma-Aldrich, St. Louis, MO, USA). Quantification of each sample was performed using Scion Image software.

### Morphological assessment of Purkinje neurons

Paraffin-embedded sagittal-cut brains were sectioned 25, 50, and 100 μm from the midline and stained with hematoxylin and eosin (H&E). The Purkinje neuron cell body area and number were determined in matched 500-μm regions of lobules III, V and VIII by tracing a region of interest (ROI) around the individual somas and calculating the area using ImageJ software. Histogram distribution were fit to a Gaussian function and used to quantitatively compare the frequency of the cell body area between the experimental groups. Three animals from each experimental group were analyzed.

### Rotarod

To assess motor coordination and balance mice were subjected to a Rotarod test. Each mouse performed in four trials on each of 4 consecutive days on a suspended accelerating Rotarod (Rotamex-5, Columbus Instruments, Columbus, OH, USA). For each run the time to falling off the rod (latency) and speed of the accelerating rod was recorded. Data from the final day of the testing paradigm are presented.

### Fear conditioning

To assess hippocampal and amygdala function in associative learning, mice were challenged with a fear-conditioning test, as previously described (Greco et al., 2010). Briefly, the testing paradigm included an initial habituation period on day 1 followed by contextual, altered context and cued testing on day 2 using a standardized fear-conditioning testing chamber equipped for sensing and recording freeze events (Med Associates, Burlington, VT, USA). A white noise of 80 dB for 30 s constitutes the Conditioned Stimulus (CS) that is followed by a 0.5-mA electric shock (Unconditioned Stimulus; US). The training day included repetitions of CS and US alterations followed by the animals' return to the home cage. On day 2 of the test, the number of contextual freeze events is recorded for a 5-min test period without stimuli. Once the testing chamber is 'redecorated' with different walls, floors and odor cues the mice are again recorded for freezing events in the absence of stimuli for 5 min. This arm of the test measures the animals' altered context fear response. During the final 3 min of the test, the number of freezes in the altered chamber in combination with the CS was determined to ascertain the cue-dependent fear conditioning.

### Statistics

Statistical analyses and graphing were done using GraphPad Prism6 (LaJolla, CA, USA). Student's *t*-test analysis was used to compare the dendritic branching in $Ttpa^{+/+}$ vs. $Ttpa^{-/-}$ on vitamin E-deficient diets (Fig. 4). A one-way analysis of variance (ANOVA) was used to test for mean differences in each experiment comparing $Ttpa^{+/+}$, $Ttpa^{-/-}$ −VE (vitamin E deficient), and $Ttpa^{-/-}$ +VE (vitamin E supplemented). The *p* value and *F* value are presented for each ANOVA in the figure legends (Figs. 1–3 and 5). Post-hoc comparisons using Tukey's procedure were used to determine significance between all three-group contrasts ($Ttpa^{+/+}$ vs. $Ttpa^{-/-}$ −VE, $Ttpa^{+/+}$ vs. $Ttpa^{-/-}$ +VE, $Ttpa^{-/-}$ −VE vs. $Ttpa^{-/-}$ +VE). In these analyses, the mean difference, 95% confidence intervals, and adjusted *p* values are provided in the figure legends.

## RESULTS

### Vitamin E levels are diminished in Ttpa-null mice

Fig. 1 shows the levels of α-tocopherol in plasma and cerebellar extracts from wild-type and TTP-null littermate mice. To eliminate variations resulting from changes in lipid profiles, we normalized vitamin E levels to unesterified cholesterol, and present these as mole ratios. As expected and reported earlier (Terasawa et al., 2000; Yokota et al., 2001) $Ttpa^{-/-}$ animals fed a supplemented diet exhibited the highest levels of circulating α-tocopherol, whereas vitamin E levels of $Ttpa^{-/-}$ animals fed vitamin E-deficient diet were below our limit of quantification (< 0.2 μM, Fig. 1A). Thus dietary supplementation with vitamin E completely normalizes plasma α-tocopherol levels of $Ttpa^{-/-}$ mice. In the cerebellum and prefrontal cortex, however, dietary supplementation with vitamin E increased α-tocopherol levels only modestly (Fig. 1B), despite the very high vitamin content of the diet (600 mg α-tocopherol/kg diet in normal rodent chow). These findings indicate that TTP functionally is essential for equilibrating vitamin E levels in the central nervous system.

### Vitamin E deficiency increases oxidative stress

In line with α-tocopherol's established antioxidant activity (Burton et al., 1983; Burton and Traber, 1990; Traber and Atkinson, 2007), vitamin E deficiency in $Ttpa^{-/-}$ mice is accompanied by significant increases in the levels of oxidative stress markers, such as thiobarbituric acid reactive substances (TBARS) (Yokota et al., 2001), F2-isoprostanes (Terasawa et al., 2000) and total hydroxyoctadecadienoic acids (t-HODEs) (Yoshida et al., 2010). To complement these signatures of oxidative stress, we investigated the levels of 3-NT. Modification of proteins by 3-NT is the outcome of nitrogen-generated reactive radical and has been shown to be elevated in AD patients and models (Butterfield et al., 2011). Indeed, data in Fig. 2 show that $Ttpa^{-/-}$ mice presented with a threefold increase in cerebellar 3-NT, indicating elevation of free radical levels under conditions of vitamin E deficiency. Supplementation of $Ttpa^{-/-}$ mice with vitamin E prevents the increase in 3-NT levels, albeit not completely; this indicates that brain-TTP is necessary to maintain α-tocopherol levels, and in turn control the accumulation of free radicals in the central nervous system.

### Vitamin E status affects the integrity of cerebellar Purkinje neurons

The cerebellar origin of vitamin E-deficiency-induced ataxia (Cavalier et al., 1998), the critical role of Purkinje neurons in projecting cerebellar cortex output (Eccles et al., 1966e; Optican, 1998) and the known sensitivity of cerebellar Purkinje neurons to oxidative stress (Chen et al., 2003; Kern and Jones, 2006) raise the possibility that integrity of Purkinje neurons is compromised in $Ttpa^{-/-}$ mice. To examine this issue, we undertook a comprehensive morphological analysis of the size, number and connectivity of cerebellar Purkinje neurons. First, we used computer-aided image analysis to assess the cell body size of individual Purkinje neurons in defined regions of the cerebellum (see Experimental procedures). Although the absolute number of Purkinje

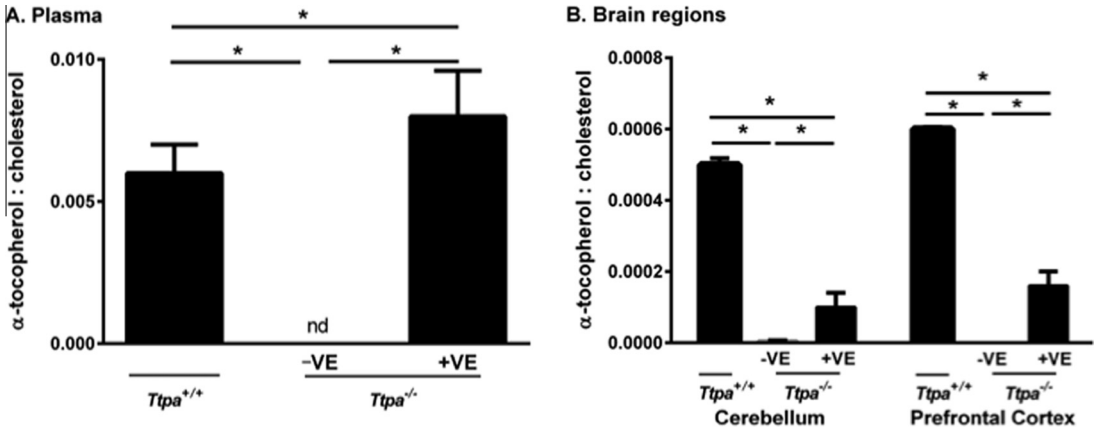


**Fig. 1.** α-Tocopherol status in plasma (A) and brain regions (B) of experimental models. α-Tocopherol was assayed by GC–MS and normalized to unesterified cholesterol content of the same sample −VE: vitamin E-deficient diet; +VE: vitamin E-supplemented diet; CX- prefrontal cortex and CB – cerebellum; *nd* indicates 'non-detectable'. Error bars reflect the standard deviation of the mean. $n = 4$–5 animals per group. Asterisks indicate statistically significant differences of $p < 0.05$ per one-way ANOVA and Tukey's post hoc analysis. Plasma ANOVA: [$F$ (2, 8) = 53.07, $p = 0.000024$]. CX ANOVA: [$F$ (2, 12) = 885.1, $p \leqslant 0.0001$] CB ANOVA: [$F$ (2, 12) = 537, $p \leqslant 0.0001$]. Tukey's post hoc: Plasma: $Ttpa^{+/+}$ vs. −VE (Mean difference = 0.0054, 95% CI [0.0033, 0.0075], adjusted $p = 0.0002$), $Ttpa^{+/+}$ vs. +VE (Mean difference = −0.0025, 95% CI [−0.0048, −0.0002], adjusted $p = 0.03$), −VE vs. +VE (Mean difference = −0.0079, 95% CI [−0.0101, −0.0056], adjusted $p < 0.0001$); CX: $Ttpa^{+/+}$ vs. −VE (Mean difference = 0.0006, 95% CI [0.00056, 0.00064], adjusted $p < 0.0001$), $Ttpa^{+/+}$ vs. +VE (Mean difference = 0.00044, 95% CI 0.00040, 0.00048], adjusted $p < 0.0001$), −VE vs. +VE (Mean difference = −0.00016, 95% CI [−0.000199, −0.000121], adjusted $p < 0.0001$); CB: $Ttpa^{+/+}$ vs. −VE (Mean difference = 0.000495, 95% CI [0.000452, 0.000538], adjusted $p < 0.0001$), $Ttpa^{+/+}$ vs. +VE (Mean difference = −0.0004, 95% CI [0.00036, 0.00044], adjusted $p < 0.0001$), −VE vs. +VE (Mean difference = −0.000095, 95% CI [−0.000138, −0.000052], adjusted $p = 0.0002$).

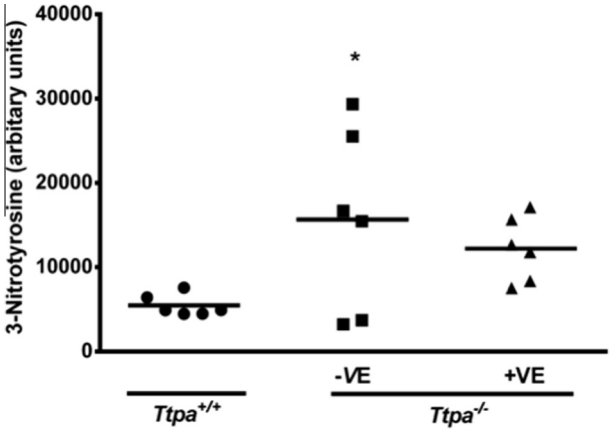


**Fig. 2.** Oxidative stress is prevented with vitamin E supplementation. 3-Nitrotyrosine (3-NT) levels in cerebella lysates from each experimental group ($Ttpa^{+/+}$; $Ttpa^{-/-}$ −VE: vitamin E-deficient diet; $Ttpa^{-/-}$ +VE: vitamin E-supplemented diet).were measured by slot blot analysis method and quantified as described in Experimental procedures. Data are represented in a scatter plot, in which the horizontal line designates the mean value. Asterisks indicate a statistically significant difference between the $Ttpa^{-/-}$ mice and wild-type littermates, as determined by a one-way ANOVA [$F$ (2, 15) = 3.68, $p = 0.0499$] and Tukey's multiple comparisons post hoc analysis $Ttpa^{+/+}$ vs. −VE (Mean difference = −10,228, 95% CI [−20,175, −280.1], adjusted $p = 0.04$); $n = 6$ animals.

neurons was not different between the experimental groups (data not shown), individual cell body area was drastically different (Fig. 3). Accordingly, the average cell body size of the vitamin E-depleted $Ttpa^{-/-}$ animals was markedly reduced by 25% compared to the $Ttpa^{+/+}$ mice. Long-term supplementation with vitamin E completely prevented this atrophy (mean cell body size: $Ttpa^{+/+}$: 803 ± 268 μm$^2$; $Ttpa^{-/-}$ vitamin E deficient: 619 ± 189 μm$^2$; $Ttpa^{-/-}$ vitamin E supplemented: 852 ± 282 μm$^2$; ANOVA: [$F$ (2, 1405) = 106, $p \leqslant 0.0001$]. Tukey's post hoc analysis $Ttpa^{+/+}$ vs. −VE (Mean difference = 183, 95% CI [144, 223], adjusted $p < 0.0001$), $Ttpa^{+/+}$ vs. +VE (Mean difference = 51.3, 95% CI [13.5, 89.0], adjusted $p = 0.0042$), −VE vs. +VE (Mean difference = 235, 95% CI [195, 274], adjusted $p < 0.0001$) (Fig. 3C). These findings indicate that vitamin E deficiency contributes to marked atrophy of the Purkinje neurons, and that this fate was prevented by high-dose, long-term supplementation with α-tocopherol. Apparently, the physiological benefit of supplementation was achieved despite the modest increase in cerebellar α-tocopherol levels (Fig. 1B) and minor improvement of oxidative stress (Fig. 2).

Next we examined the arborization of Purkinje neurons in the various experimental groups. Visual inspection of the Golgi-stained cerebellar sections indicated that dendritic branching of Purkinje neurons was diminished in the vitamin E-deficient $Ttpa^{-/-}$ animals compared to the $Ttpa^{+/+}$ mice (Fig. 4A, B). The variation in arborization between animals from the different experimental groups was confirmed by dedicated computer-aided tracing software (Neurolucida) which analyzes the dendritic branching of Purkinje neurons in three dimensions, (as shown in Fig. 4C, D). Sholl analysis (Fig. 4E), the gold standard (Sholl, 1953) for quantitative evaluation of neuritic arborization, showed that dendrites in the vitamin E-deficient $Ttpa^{-/-}$ animals presented with less intersections (mean branching distance from the cell body was ∼70 and ∼130 μm for the vitamin E-deficient

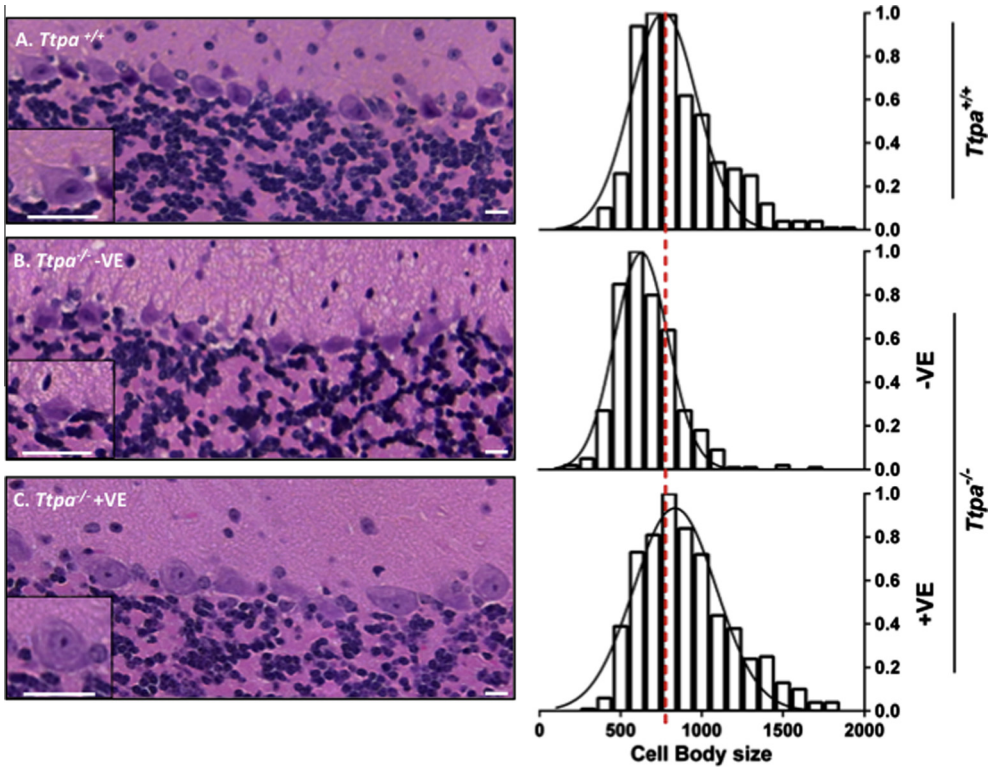


**Fig. 3.** Vitamin E is required for Purkinje neuron integrity. Cerebella of the indicated experimental groups ($Ttpa^{+/+}$; $Ttpa^{-/-}$ −VE: vitamin E-deficient diet; $Ttpa^{-/-}$ +VE: vitamin E-supplemented diet) were fixed and embedded as described in Experimental procedures. Thin sections (5 μm) were stained with hematoxylin and eosin and microscopic images of the Purkinje neuron layer were obtained. Left panels: representative images from sections of each of the indicated experimental groups (representative Purkinje neuron cell body is shown in the inset for each image; scale bar = 50 μm). Right panels: frequency distribution histograms of Purkinje neuron cell body size in each genotype, measured using ImageJ software. Size analysis was performed on 425 Purkinje neurons from three different animals for each experimental group. Solid line shows fit of the experimental data (bars) to a Gaussian distribution. Red-dotted line compares the amplitude for each experimental group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and wild-type mice, respectively), and a narrower distribution profile (area under the curve (AUC) 1391 and 2756 for the vitamin E-deficient $Ttpa^{-/-}$ and wild-type mice, respectively). These anatomic differences suggested that vitamin E deficiency reduced the ability of Purkinje neurons to synapse with climbing fibers of the inferior olive and the parallel fibers of the granule cells (Eccles et al., 1966c,d,e; Optican, 1998). Moreover, the absolute number of intersections in $Ttpa^{-/-}$ vitamin E-deficient mice was significantly reduced (30%) compared to the control animals (Fig. 4F). Collectively, our results suggest that vitamin E is essential for maintaining the morphological and functional integrity of the cells that are critical regulators of motor coordination.

### Altered behavior in Vitamin E-deficient mice

To evaluate the functional consequences of the morphological changes described above, we assessed the motor coordination ability of mice in the different experimental groups. The ability of a trained mouse to remain on a rotating rod at increasing speeds is an accepted measure of cerebellar-controlled motor coordination (Dunham and Miya, 1957; Jones and Roberts, 1968). We found that the vitamin E deficiency severely compromised performance in this assay. Specifically, the average time to fall (latency) for the vitamin E-depleted $Ttpa^{-/-}$ mice was approximately threefold shorter than that exhibited by the wild-type animals (Fig. 5A). Importantly, dietary supplementation with α-tocopherol completely prevented the motor coordination deficit of the $Ttpa^{-/-}$ mice. This indicated that the modest increase in cerebellar α-tocopherol content (30% of the levels in the wild-type animals; Fig. 5B) was sufficient for providing protection against some functional deficits. Taken together, these observations suggest that adequate vitamin E status is essential for anatomic integrity and physiological function of the Purkinje neurons, essential regulators of motor coordination.

Although, vitamin E deficiency manifests primarily in cerebellar disorders, α-tocopherol is maintained at considerable levels in other regions of the CNS (Vatassery et al., 2006, 2007; Gohil et al., 2008). To investigate the possible roles of vitamin E in other brain regions, we subjected mice from the different experimental groups to the fear-conditioning tests, generally accepted to report on functionality of the hippocampus and amygdala (Buccafusco, 2001). We did not observe any significant differences among the experimental groups in the context-dependent or altered-context-dependent results (data not shown), suggesting that hippocampal functioning is not affected

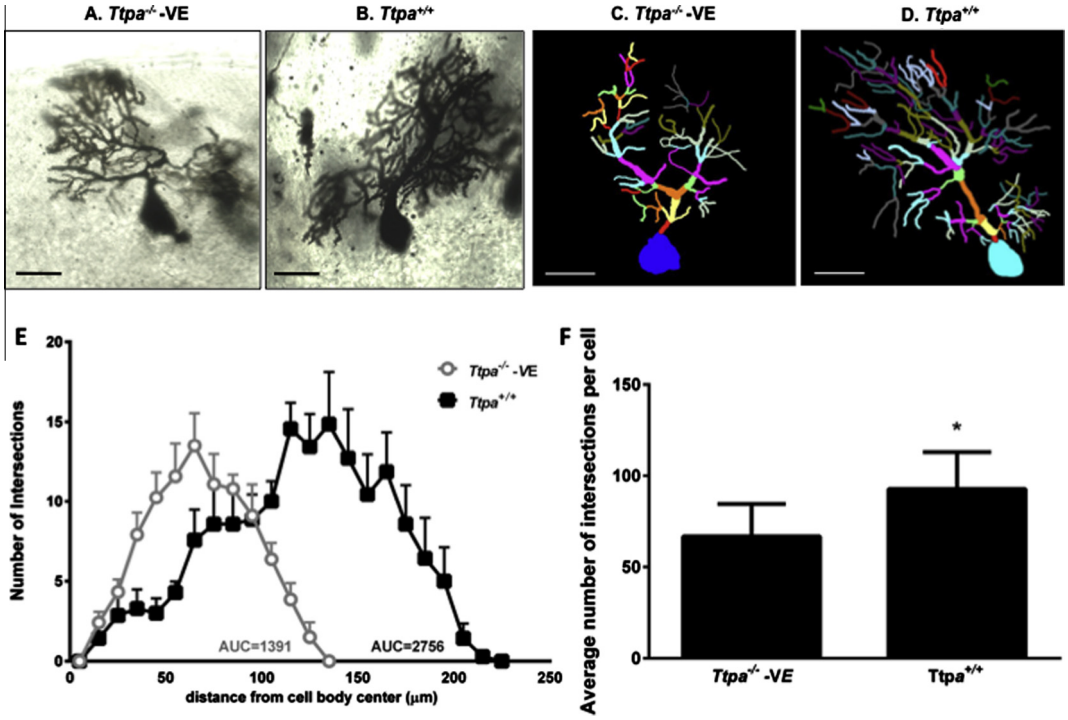


**Fig. 4.** Vitamin E deficiency disrupts dendritic branching of Purkinje neurons. Cerebella from the indicated experimental groups ($Ttpa^{+/+}$; $Ttpa^{-/-}$ −VE: vitamin E-deficient diet) were excised and stained with Golgi-Cox reagents. Micrographs of representative Purkinje neurons from vitamin E-deficient $Ttpa^{-/-}$ and wild-type mice are shown in panels (A) and (B), respectively. Panels (C) and (D) show computer-generated (Neurolucida software) three-dimensional tracings of Golgi-Cox stained Purkinje neurons from $Ttpa^{-/-}$ vitamin E-deficient and $Ttpa^{+/+}$ mice, respectively. (E) Scholl profile of Neurolucida-generated data, showing the number of intersections in each 5-μm concentric circle from the middle of the Purkinje neuron cell body. AUC: area under curve. (F) The average number of intersections per Purkinje neuron in $Ttpa^{-/-}$ vitamin E-deficient and $Ttpa^{+/+}$ mice. Twelve Purkinje neurons were analyzed from three animals of each genotype. All scale bars = 50 μm. In panels (E) and (F) the error bars reflect the standard deviation of the mean. Asterisks indicate statistical significance as determined by Student's *t*-test; $p < 0.05$ compared to the wild-type mice.

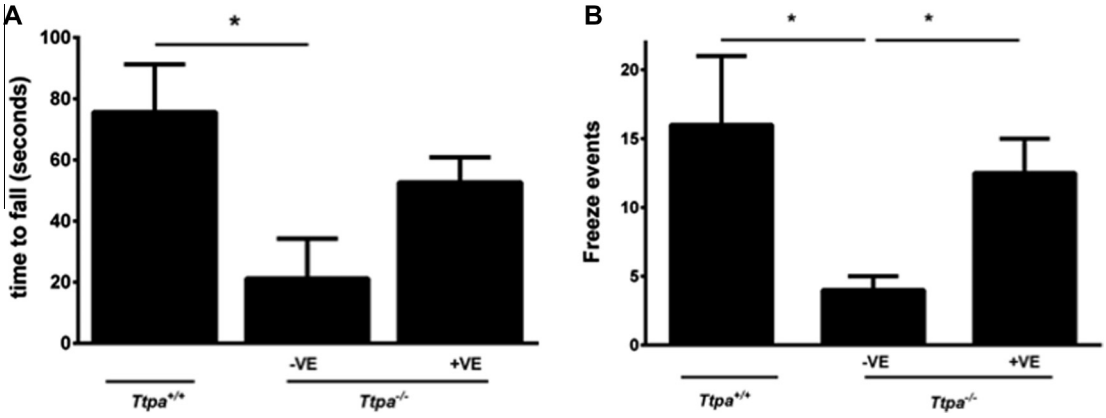


**Fig. 5.** Vitamin E is essential for motor coordination and for cognitive function. Mice from each experimental group ($Ttpa^{+/+}$; $Ttpa^{-/-}$ −VE: vitamin E-deficient diet; $Ttpa^{-/-}$ +VE: vitamin E-supplemented diet) were subjected to behavioral testing. (A) Rotarod testing was carried out after 3 days of training runs. Shorter time to latency reflects diminished motor coordination. Asterisks indicate a statistically significant difference between the $Ttpa^{-/-}$ mice and wild-type littermates, as determined by a one-way ANOVA [$F$ (2, 7) = 4.77, $p < 0.049$] and Tukey's multiple comparisons post hoc analysis (Mean difference = 54.4, 95% CI [2.27, 106.5], adjusted $p = 0.04$) (B) Cue-dependent fear conditioning was performed after a 24-h period of habituation and training. A decrease in freeze events reflects compromised memory formation. Asterisks indicate statistically significant differences of $p < 0.05$ per one-way ANOVA and Tukey's post hoc analysis between the vitamin E-deficient animals and the other experimental groups. ANOVA: [$F$ (2, 6) = 10.63, $p = 0.011$]. Tukey's post hoc: $Ttpa^{+/+}$ vs. −VE (Mean difference = 12, 95% CI [3.79, 20.2], adjusted $p = 0.0099$), −VE vs. +VE (Mean difference = −8.5, 95% CI [−16.71, −0.286], adjusted $p = 0.044$). Error bars reflect the standard deviation of the mean. Analyses of three to five animals per experimental group.

by our experimental treatments. However, the vitamin E-depleted *Ttpa*$^{-/-}$ animals exhibited a profound (ca. fourfold) deficit in the cue-dependent fear-conditioning test (Fig. 5B). Dietary supplementation with α-tocopherol prevented the cognitive deficit. These results support the notion that adequate vitamin E status is important in proper amygdala function and emotive learning.

## DISCUSSION

It has been recognized for more than 50 years that adequate levels of vitamin E are critical for maintaining neurological health. The most striking evidence for this requirement is the pathological manifestations of the familial syndrome AVED in affected humans (Ouahchi et al., 1995; Cavalier et al., 1998). These individuals present with low or non-detectable levels of plasma α-tocopherol and progressive SCA, sometimes accompanied by retinitis pigmentosis, proprioception and areflexia (Harding, 1987; Goss-Sampson et al., 1988; Sokol, 1990; Cavalier et al., 1998). Importantly, pathologic progression can be halted in affected individuals by high-dose supplementation with α-tocopherol (Harding et al., 1985; Matsuya et al., 1994; Amiel et al., 1995; Yokota et al., 1996; Cavalier et al., 1998). Similar findings were established in genetic and dietary models of vitamin E deficiency in mice (Yokota et al., 2001), rats (Einarson, 1953; Goss-Sampson et al., 1988), monkeys (Dinning and Day, 1957) and horses (Mayhew et al., 1987; Aleman, 2011).

Vitamin E deficiency-induced neurodegeneration is characterized by axonopathy, demyelination, spheroid formation and defective retrograde transport, prominently affecting the sensory neurons and more specifically the dorsal root ganglion cells (Goss-Sampson et al., 1988; Southam et al., 1991). Importantly, our understanding of the cellular and molecular origins of the cerebellar defects observed in vitamin E-deficient animals and humans has been extremely limited. In line with vitamin E's established function as an antioxidant, increased levels of oxidative stress markers have been described in the vitamin E-depleted *Ttpa*$^{-/-}$ mice. Specifically, altered levels of TBARS, 4-hydroxy-2-nonenal (4-HNE) adducts, total iso-prostaglandin F2α (IsoPGF$_{2\alpha}$) and t-HODE and glutathione were demonstrated in the cerebellum, cortex, and spinal cord of these mice as compared to vitamin E-sufficient animals (Yokota et al., 2001; Gohil et al., 2010; Yoshida et al., 2010). These changes are thought to cause specific cellular dysfunctions such as defective retrograde axonal transport (Southam et al., 1991) and impaired mitochondrial function in affected neurons (Thomas et al., 1993; Pillai et al., 1994), which eventually lead to axonal degeneration (Koeppen, 2005). However, the specific nature and site(s) of neurological injury have not been reported. We therefore examined the anatomic and functional integrity of the cerebellum during vitamin E deficiency. As a model system we utilized a genetic mouse model of AVED in which expression of the TTP protein is disrupted (Terasawa et al., 2000; Jishage et al., 2001). These animals display systemic vitamin E deficiency, and present late-onset ataxic symptoms similar to affected humans (Yokota et al., 2001). To correlate the biochemical, anatomical and functional deficits that accompany vitamin E deficiency, we terminated our experiments at the age of 17 months, when overt ataxic symptoms were obvious.

Multiple neurodegenerative diseases are characterized by deterioration of Purkinje neurons and ataxia, such as ataxia-telangiectasia (A-T) (Paula-Barbosa et al., 1983; Gatti and Vinters, 1985), Friedreich ataxia (Simon et al., 2004) and Niemann Pick Type C disease (NPC) (Yu and Lieberman, 2013). Our findings demonstrate that prolonged vitamin E deficiency causes marked damage to cerebellar Purkinje neurons. Specifically, we observed atrophy of the cell bodies (Fig. 3) and diminished dendritic branching (Fig. 4) of these cells. To our knowledge, this is the first detailed illustration of morphological aberrations observed in the Purkinje neurons during vitamin E deficiency. Since Purkinje neurons are key regulators of motor coordination of the cerebellar cortex (Eccles et al., 1966a,b,d,e; Optican, 1998; Womack and Khodakhah, 2003), the anatomic deterioration of these cells likely contributes to the ataxic manifestations of vitamin E deficiency. Since our studies focused only on cerebellar Purkinje neurons, we cannot rule out the possibility that the primary injury occurred at other site(s), rendering pathological consequences described here to be secondary in nature. Also, we cannot conclusively determine the involvement of other cell-types in vitamin E deficiency-induced cerebellar damage. For example, our findings may reflect the outcome of demyelination or sensory neuropathy that cause compromise Purkinje neuron integrity. Multiple studies demonstrated that vitamin E deficiency negatively affects the composition, transcriptome profile and mitochondrial function in myelin (Enrione et al., 1999; Yokota et al., 2001; Gohil et al., 2004; Podratz et al., 2004; Hyland et al., 2006; Cuddihy et al., 2008). Similarly, oligodendrocytes dysfunction is known to contribute to Purkinje neuron degeneration in NPC disease and multiple sclerosis (MS) (Ko et al., 2005; Yu and Lieberman, 2013). Lastly, axonal dystrophy in sensory tracts has been reported in AVED patients (Sung et al., 1981; Harding, 1982, 1985), in cystic fibrosis patients, in secondary vitamin E deficiency in humans (Geller et al., 1977), and in rodent models of vitamin E deficiency (Sung et al., 1981; Southam et al., 1991; Yokota et al., 2001). Thus, despite the data that link vitamin E deficiency to Purkinje neuron degeneration, the detailed process that underlies disease etiology awaits further investigation.

We note that structural and functional damage to the cerebellum was evident at a relatively late stage of deficiency (17 months) as compared to published reports of the onset of oxidative stress in the same deficiency model. Reduction in cerebellar glutathione was reported to occur after 5 months (Gohil et al., 2008) and increased plasma lipid peroxidation was obvious at 6 months (Yoshida et al., 2010). These observations

indicate that oxidative stress preceded the pathological changes in Purkinje neurons and the ataxic phenotype, and support a cause-and-effect relationship between oxidative stress and cerebellar damage. Moreover, our findings suggest a modest increase in vitamin E in the cerebellum can sufficiently decrease levels of free radical generation enough to elicit beneficial outcomes.

Inherited ataxias, such as Friedreich's ataxia and SCA-6 (Koeppen, 2005, 2011) share with vitamin E deficiency not only deterioration of Purkinje neurons, but also a dramatic elevation in the levels of oxidative stress markers (Pandolfo, 2008) and low-rate of correct diagnosis. The efficacy of vitamin E supplementation in preventing neurological decline reported here (Figs. 2 and 4) and elsewhere (Ren et al., 2006; Gohil et al., 2008, 2010; Nishida et al., 2009) indicates that supplementation with α-tocopherol may compromise a valuable therapeutic approach. In support of this notion a limited trial reported that intervention with vitamin E and coenzyme Q led to significant improvements in standardized ataxia scores in Friedreich's ataxia patients (Hart et al., 2005). Careful examination of similar interventions in the treatment of other oxidative stress-related neuropathologies will provide critically valuable information.

It is interesting to note that although the dose of α-tocopherol used here normalized plasma levels of the vitamin (Fig. 1A), it had only a modest effect on concentrations of the vitamin in the cerebellum and prefrontal cortex (< 30% of the levels in un-supplemented wild-type animals, Fig. 1B). This observation indicates that TTP is essential for 'communicating' between two separate pools of vitamin E: one in the circulation and the other in the CNS. Indeed, in cases of the heritable mutations in *TTPA*, extremely high doses of vitamin E (up to 2 g α-tocopherol/day) are necessary to achieve symptomatic effect (Gohil et al., 2010). An additional important conclusion from these results is that in cases of TTP dysfunction (e.g. in AVED patients or in carriers of common polymorphic variants in the TTP promoter; (Wright et al., 2009; Ulatowski et al., 2012), plasma concentrations of α-tocopherol levels *do not* reflect vitamin E status in the CNS. It is therefore critically important to examine the utility of CNS-localized markers for diagnostic assessment, such as concentrations of α-tocopherol or oxidative stress markers in the cerebrospinal fluid (Hensley et al., 2011).

In addition to the established deficits in motor coordination that accompany vitamin E deficiency, we observed that *Ttpa*$^{-/-}$ mice exhibit a cognitive deficit, manifesting in reduced response to the fear-conditioning test, specifically in the altered environment cue-dependent challenge (Fig. 5B). These results indicate that vitamin E deficiency caused a functional compromise of the amygdala region of the brain, thought to control memory formation and emotive responses. Since accurate measurements of α-tocopherol levels in the amygdala are technically challenging, we measure the vitamin's level in the prefrontal cortex, which modulates amygdalar emotive stimuli. Levels of α-tocopherol in this region in deficient *Ttpa*$^{-/-}$ mice were below our detection level (Fig. 1B).

A number of groups reported that the levels of vitamin E vary among different regions of the brain (Vatassery et al., 2006, 2007; Gohil et al., 2008). These observations indicate that selected micro-environments in the CNS regulate α-tocopherol's level independently of the cerebellar vitamin pool. These isolated pools of the antioxidant may be meaningful in disease conditions, and underscore the need to understand the function and transport of vitamin E and TTP throughout the CNS.

## CONCLUSION

Taken together, our findings extend previous observations regarding the critical roles of α-tocopherol in the CNS, and provide new insights into the requirement for vitamin E in supporting the functions of cerebellar Purkinje neurons and the amygdala. Ongoing work on the transport of vitamin E within the CNS will reveal the molecular mechanisms that underlie these phenomena.

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