Effects on Neural Function of Repleting Vitamin E–Deficient Rats With α-Tocopherol

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INTRODUCTION

Vitamin E (α-tocopherol) is the major lipid soluble chain-breaking antioxidant in vivo and a severe and prolonged deficiency of the vitamin can result in a progressive neurological syndrome in both humans (Harding 1987; Muller and Goss-Sampson 1990; Muller et al. 1983) and animals (Machlin et al. 1977; Nelson et al. 1981). In humans, the clinical features of severe vitamin E deficiency include areflexia, ataxia, loss of position sense (proprioception), loss of vibration sense, abnormal feet (pes cavus), curvature of the spine (kyphoscoliosis), abnormal eye movements (ophthalmoplegia), a pigmentary retinopathy, and generalized muscle weakness (Muller et al. 1983). Vitamin E–deficient rats show similar clinical features including ataxia and muscle weakness (Goss-Sampson et al. 1990; Hayton et al. 2003; Machlin et al. 1977). The neuropathology is similar in vitamin E–deficient humans, monkeys, and rats, consisting of axonal degeneration in the posterior columns and the gracile and cuneate nuclei, together with a selective loss of large caliber myelinated axons in the spinal cord with peripheral nerves less affected (Nelson et al. 1981). These changes constitute a primary “dying-back” axonopathy with secondary demyelination, which principally affects the primary sensory axons (Thomas et al. 1984; Wichman et al. 1985). We have also shown that electrophysiological abnormalities of neural and visual function including somatosensory-evoked potentials (SEPs), flash electroretinograms (ERGs), and visual-evoked potentials (VEPs) are similar in vitamin E–deficient humans and rats (Goss-Sampson et al. 1990; Hayton et al. 2003).

The effects of repleting vitamin E–deficient human subjects with the vitamin have been studied in patients with abetalipoproteinemia (Azizi et al. 1978; Bishara et al. 1982; Brin et al. 1986; Fagan and Taylor 1987; Muller et al. 1977, 1983), chronic cholestasis (Álvarez et al. 1983; Guggenheim et al. 1982; Perlmuter et al. 1987; Sokol et al. 1985, 1993), cystic fibrosis (Elias et al. 1981), multiple ileal resection (Harding et al. 1982; Howard et al. 1982), and ataxia with vitamin E deficiency (AVED) (Harding et al. 1985; Sokol et al. 1988). The progression of the neurological changes associated with these disorders has been halted or, in some cases, reversed after appropriate vitamin E therapy.

There have, however, been few studies in animals to study the effects of repletion with vitamin E on the neurological signs of deficiency. Nelson et al. (1981) maintained seven rhesus monkeys on a deficient diet for 30 mo, after which time progressive central and peripheral nervous system lesions had developed. Two of the animals were repleted with oral RRR-α-tocopheryl acetate (100 mg) for 2 mo, after which the muscle weakness and anemia that were present resolved, and there was an apparent cessation of axonal degeneration (Nelson et al. 1981).

Visual function in repleted rats was studied by Goss-Sampson et al. (1998) using electrophysiological parameters. Rats were maintained on a vitamin E–deficient diet for 30 wk before being changed to the same diet to which vitamin E (α-tocopheryl acetate) had been added for a further 22 wk. After 1 yr, the deficient group exhibited the neurological syndrome characteristic of chronic vitamin E deficiency, whereas none of the repleted rats developed the syndrome. After 20–24 wk of deficiency, electrophysiological measures of visual function became abnormal, but repletion with α-tocopheryl acetate did not correct any of the electrophysiological abnormalities.

The authors are not aware of any studies in vitamin E–deficient rats that have investigated the effects of repletion on central and peripheral nerves. In this study, electrophysiological parameters, which provide objective, sensitive, and non-invasive indicators of neural and visual function, were measured in control and vitamin E–deficient rats and a third group fed a vitamin E–deficient diet until...
neurological abnormalities became evident and then changed to a diet containing 36 mg/kg all-rac-α-tocopheryl acetate (repleted group).

**METHODS**

Details of the animals, diets, anesthesia, and electrophysiological techniques have been previously described in detail (Hayton et al. 1999, 2003).

**Animals**

In summary, weaning (21 ± 7 days), male, Wistar rats were obtained from B and K Universal, and housed in groups of three in standard solid-bottomed cages. The animals were kept at a constant temperature of 21 ± 1°C, relative humidity of 55 ± 5%, and a 12/12-h light/dark cycle. Fresh drinking water was provided ad libitum, and each cage received 90 g pelleted experimental diet per day (i.e., ~30 g/rat/day). Forty-five rats were randomly allocated to one of three groups. One group of rats (group D, \( n = 15 \)) received a synthetic vitamin E–deficient diet (Machlin/Draper-HLR 814; Dyets), while another group (\( n = 15 \)) received the same diet to which 5 mg/kg RRR-α-tocopheryl acetate was added (group C, controls). This concentration was found to be sufficient to prevent the neurological changes associated with vitamin E deficiency (Hayton and Muller 2004; Hayton et al. 2003) A third group of animals (\( n = 15 \)) were fed the deficient diet for 38 wk (termed group R– during this time) and were changed to a diet containing 36 mg/kg all-rac-α-tocopheryl acetate for 20 wk (termed group R+). The rats were weighed weekly.

**Anesthesia**

Before the electrophysiological studies, the rats were anesthetized with a combination of fentanyl/fluanisone (Hypnorm, Janssen, UK) and midazolam (Hynovel, Roche, UK) as described previously (Hayton et al. 1999). One part Hypnorm (fentanyl 0.315 mg/ml; fluanisone 10 mg/ml) was mixed in the same syringe with two parts sterile water and administered intraperitoneally at a dose of 2.7 ml/kg. Buprenorphine (Temgesic, Reckitt and Coleman Products) was injected intramuscularly at 0.1 ml/kg to aid recovery. This anesthetic regimen has minimal and reproducible effects on evoked potentials (Hayton et al. 1999). The depth of anesthesia was regularly assessed by testing responses to pedal and palpebral (corneal) stimulation. Rectal temperature was maintained throughout between 35 and 37°C using a heat-lamp. Each rat was given 3 ml saline ip to combat fluid loss during the procedure.

**Electrophysiology**

Because in previous studies (Hayton and Muller 2004; Hayton et al. 2003) significant abnormalities of electrophysiological function were not seen until 24 wk of deficiency, the recordings in this study were made at monthly intervals (i.e., every 4 wk) between 20 and 58 wk. Twelve rats from each group were tested at each time-point. Where possible, these were the same rats on each occasion. However, some rats were lost because of anesthetic complications (\( n = 2 \)), deterioration caused by vitamin E deficiency (\( n = 2 \)), or health problems unrelated to vitamin E status (\( n = 3 \)). All recordings were completed within 20 min of the induction of anesthesia.

Subdermal needle electrodes were used to stimulate peripheral nerves and record the SEPs, as previously described (Hayton et al. 1999). After electrical stimulation of the tibial nerve at the right ankle, the lower limb SEPs were recorded from an electrode inserted between the fifth and sixth lumbar vertebrae (peripheral) and one inserted over the contralateral somatosensory cortex (central). These active electrodes were referred to electrodes inserted at lumbar vertebra 2 and at the caudal tip of the frontal bone (snout), respectively. An earth electrode was inserted into the left hind foot. A constant current stimulus (1–2 mA) of 0.1-ms duration was delivered at the rate of 3/α, with responses averaged over a 30-ms period (including 1.5-ms prestimulus interval). The amplifier bandwidth was 1 Hz to 3 kHz. One hundred twenty-eight responses were averaged for each run, and runs were replicated four times.

A Medelec Sensor was used to average the responses, which were transferred to a computer and stored on disk until subsequent analysis. The latencies and peak-to-peak amplitudes of the ERG b-wave and VEP onset (1st significant deviation from baseline) responses and the conduction velocities (calculated from the latency of the 1st major positive peak and the distance between the stimulating and recording electrodes) and baseline-to-peak amplitudes of the SEPs from each group of rats were compared.

An initial exploratory analysis consisted of comparing differences in electrophysiological measurements (latencies and amplitudes) between the three groups at each time-point using t-tests. The medians and interquartile ranges for the three groups are presented in the figures, with significant differences highlighted by symbols. This analysis, although standard, does not account for the repeated nature of the data (multiple measures per rat at different time-points), and hence multilevel models (Goldstein 2005) were used to compare within rat trajectories over time. By incorporating two levels (level 2: rat; level 1: time of measurement for that rat), these models took into account the correlations between measurements within rats and corrected for between rat variability at the start of the study. To normalize the data, peripheral SEP amplitude, central SEP amplitude, ERG latency, ERG amplitude, and VEP latency were logged before analysis. Estimates were obtained of the average change per month that could be attributed to vitamin E deficiency and of the extent to which repletion of group R– at 38 wk modified this. All estimates are presented with 95% CIs.

The experiments were performed under appropriate personal and project licenses issued by the Home Office and following local ethical approval.

**RESULTS**

**Growth and physical condition**

The rats were weighed weekly, and the mean monthly weights of the three groups are shown in Fig. 1. For the first 16 wk, the animals in all the groups gained weight rapidly. The rate of gain then slowed in the control group (C), whereas there was no further increase in the mean weight of the deficient group (D). The repleted group (R–) also stopped gaining weight after week 16 and was significantly lighter than the control group after 20 wk [means, 429 ± 12.6 and 478 ± 17.6 (SE) g, respectively; \( P < 0.05 \)]. The repleted group was placed on the vitamin E diet (36 mg/kg diet) after 38 wk (R+) and became significantly heavier than the deficient group after 44 wk (529 ± 19.4 and 415 ± 15.6 g, respectively; \( P < 0.001 \)). By week 52 of the study, the mean weight of the repleted group was similar to that of the control group (619 ± 22.6 and 616 ± 25.3 g, respectively).
After 16 wk on the tocopherol-deficient diet, the general condition of the rats in groups D and R− began to deteriorate. Their coats became greasy and the fur became matted. This was accompanied by an increase in porphyrin secretion, which gave the fur a pink tinge. The first signs of neurological dysfunction became apparent in group D after 43 wk, when four rats presented with an abnormal gait and slightly impaired balance. During the remaining 15 wk of the study, all the rats in group D continued to deteriorate and had severely abnormal gait, balance, and marked muscle wastage by 58 wk. In group R−, abnormal gait and impaired balance was noted in three rats after 45 wk. The condition of two of these three animals did not deteriorate further, but that of the third continued to worsen. None of the remaining rats in groups R+ or C displayed any signs of neural dysfunction or muscle wastage at any point during the study.

**Electrophysiology**

Table 1 shows the average difference per month in the electrophysiological measures between groups D, R−, R+, and C.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Group D [Average Difference From Controls (C) per Month]</th>
<th>Group R− [Average Difference From Controls (C) per Month]</th>
<th>Group R+ [Average Difference From Controls (C) From Month 10–14]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central SEP CV, ms⁻¹</td>
<td>−0.847 (−1.016, −0.678)</td>
<td>−0.828 (−1.098, −0.558)</td>
<td>0.529 (0.262, 0.796)</td>
</tr>
<tr>
<td>VEP amplitude, μV</td>
<td>−0.85 (−1.4, −0.26)</td>
<td>−0.44 (−1.26, −0.37)</td>
<td>0.145 (−0.680, 0.390)</td>
</tr>
<tr>
<td></td>
<td>*P = 0.004</td>
<td>*P = 0.285</td>
<td>*P = 0.595</td>
</tr>
<tr>
<td>Logged values*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral SEP amplitude, μV</td>
<td>0.971 (0.962, 0.981)</td>
<td>0.964 (0.947, 0.981)</td>
<td>1.028 (1.010, 1.047)</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
<td>*P = 0.019</td>
</tr>
<tr>
<td>Central SEP amplitude, μV</td>
<td>0.966 (0.952, 0.979)</td>
<td>0.942 (0.920, 0.964)</td>
<td>1.039 (1.015, 1.063)</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
<td>*P = 0.0015</td>
</tr>
<tr>
<td>ERG latency, ms</td>
<td>1.016 (1.012, 1.020)</td>
<td>1.008 (1.002, 1.014)</td>
<td>0.994 (0.990, 0.998)</td>
</tr>
<tr>
<td></td>
<td>*P = 0.007</td>
<td>*P = 0.007</td>
<td>*P = 0.005</td>
</tr>
<tr>
<td>ERG amplitude, μV</td>
<td>0.835 (0.794, 0.879)</td>
<td>0.914 (0.850, 0.983)</td>
<td>1.053 (0.997, 1.113)</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P = 0.015</td>
<td>*P = 0.063</td>
</tr>
<tr>
<td>VEP latency, ms</td>
<td>1.014 (1.010, 1.018)</td>
<td>1.011 (1.005, 1.017)</td>
<td>0.994 (0.990, 0.998)</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
<td>*P = 0.003</td>
</tr>
</tbody>
</table>

Figures in parentheses are 95% CIs. *P < 0.05 was considered to be significant. *For logged values, the exponentiated coefficients are given and hence these are the multiplicative differences. For example, each month group D peripheral SEP amplitudes were reduced on average by 0.971 compared with the control group (95% CI, 0.962, 0.981), so that after 3 mo they were 0.971³ = 0.915 or 91.5% of the average level seen in the controls (or 8.5% lower). SEP, somatosensory evoked potentials; VEP, visual-evoked potentials; ERG, flash electroretinograms.
animals compared with controls ($P < 0.001$ for both groups; Table 1). Group R+ showed a significant per month increase in central CV ($P < 0.001$). There were no significant differences in peripheral CV between any of the groups at any time-point during the study (data not shown).

SEP amplitudes

The median amplitudes of the peripheral SEPs are presented in Fig. 3A. Group C maintained an approximately constant amplitude throughout the study, whereas the amplitudes of groups D and R− fell significantly compared with controls ($P < 0.001$ for both groups). After changing to a diet containing vitamin E, the peripheral SEP amplitudes of group R+ increased ($P = 0.019$).

Figure 3B shows the median amplitudes of the central SEPs. Group C maintained an approximately constant SEP amplitude throughout the study, whereas the responses of groups D and R− decreased significantly in amplitude from 20 wk ($P < 0.001$ for both groups). Group R+ rats showed a significant increase in central SEP amplitudes ($P = 0.0015$).

ERG latency

The median ERG latencies recorded from the rats in the three groups are shown in Fig. 4A. Groups D and R− latencies were significantly increased per month compared with group C ($P < 0.001$ and $P = 0.007$, respectively). The repleted animals (group R+) showed a significant decrease in ERG latency per month ($P = 0.003$).

ERG amplitudes

Figure 4B shows the median ERG amplitudes recorded in groups C, D, and R. ERG amplitudes decreased significantly per month in groups D and R− ($P < 0.001$ and $P = 0.015$, respectively). The changes in the ERG amplitudes in group R+ were not significant ($P = 0.063$).

### DISCUSSION

The deficient rats (group D) showed a similar pattern of growth and physical changes (poor coat condition, muscle...
wasting, and ataxia) to that reported previously (Goss-Sampson et al. 1988, 1990; Machlin et al. 1977; Towfighi 1981). Group R grew similarly to group D until repleted with vitamin E (R/H11001). Repletion with 36 mg/kg all-rac-
H9251-tocopheryl acetate resulted in rapid weight gain as previously described (Goss-Sampson et al. 1998) and halted the progression of the neurological changes in two of the three rats in which they were observed. In contrast to the deficient animals, neurological signs did not develop in the remainder of the group R rats.

In a study of eight patients with abetalipoproteinemia, the five given vitamin E from the age of 16 mo did not develop any neurological signs, and the condition of the remaining three older children either improved or did not deteriorate further (Muller et al. 1983). Similar results have been reported by others (Azizi et al. 1978; Kane and Havel 2001). Vitamin E therapy also halted or reversed the progression of neurological abnormalities in patients with chronic cholestatic liver disease (Alvarez et al. 1983; Guggenheim et al. 1982; Perlmutter et al. 1987; Sokol et al. 1985, 1993), cystic fibrosis (Elias et al. 1981), multiple intestinal resections (Harding et al. 1982; Howard et al. 1982), and AVED (Harding et al. 1985; Sokol et al. 1988).

The electrophysiological results in group R+ were, as expected, very similar to those recorded in the deficient animals (group D). After repletion with vitamin E, with the exception of the VEP amplitudes, group R+ electrophysiological parameters tended to diverge from group D and become more like group C (controls).

The SEP results in group R+ obtained in this study (i.e., an increase in central CV and peripheral and central amplitudes) agree with the findings of several studies of α-tocopherol repletion in deficient humans. Five patients with abetalipoproteinemia were re-examined after ≥3 yr of oral vitamin E and A therapy, and four were found to have improved sensory CV (Brin et al. 1986). The condition of none of the patients deteriorated, and CV returned to normal in two. Fagan and Taylor (1987) reported a normal peripheral SEP but frequently an abnormal central response in five children with abetalipoproteinemia. The central SEP remained stable in three of the patients during the 4-yr study period, whereas fluctuations were noted in the other two. Guggenheim et al. (1982) reported SEP abnormalities in four children with chronic cholestatic liver disease. A decreased CV was recorded in the median nerve of one patient and sural nerve potentials were unobtainable. Definite improvements in neurological function were

![FIG. 4. A: median latencies (±interquartile ranges) of electroretinograms in the 3 groups after stimulation with bright white light (n = 12). B: median amplitudes (±interquartile ranges) of electroretinograms in the 3 groups after stimulation with bright white light (n = 12).](http://jn.physiology.org/)

![FIG. 5. A: median latencies (±interquartile ranges) of visual evoked potentials in the 3 groups after stimulation with bright white light (n = 12). B: median amplitudes (±interquartile ranges) of visual evoked potentials in the 3 groups after stimulation with bright white light (n = 12).](http://jn.physiology.org/)
reported after treatment with either oral or intramuscular \( \alpha \)-tocopherol (Guggenheim et al. 1982). A low-amplitude of the sural nerve compound sensory action potential (CSAP) in six of seven children with vitamin E deficiency, associated with chronic cholestasis, was reported by Sokol et al. (1985). After 10–12 mo of vitamin E repletion, the CSAP amplitudes increased in the four of the five patients who showed clinical neurologic improvement.

In this study, repletion of deficient (group R\(^{-} \)) rats with 36 mg/kg all-rac-\( \alpha \)-tocopheryl acetate halted the deterioration or improved most of the visual electrophysiological parameters. The median ERG and VEP latencies decreased and ERG amplitudes increased in response to repletion. Repletion of vitamin E–deficient humans produced similar results. Muller et al. (1977) reported a halt in the retinal changes of a patient with abetalipoproteinemia after vitamin E therapy. Serial ERG studies in eight patients with abetalipoproteinemia showed no progression after treatment with vitamins E and A for 2–6 yr. Bishara et al. (1982) and Brin et al. (1986) similarly reported no change in serial ERG and VEP in eight patients with abetalipoproteinemia receiving vitamin E. Partial improvement in ocularmotor function was recorded in 4 of 11 cholestatic children, given intramuscular injections of vitamin E after developing neurologic signs (Alvarez et al. 1983). Cynamon et al. (1988), however, did not find a correlation between VEP latency and vitamin E status in 17 patients with chronic cholestasis. A patient has been reported with multiple intestinal resections who developed a visual field defect with pigmentary degeneration of the retina and an abnormal ERG despite having a normal serum vitamin A concentration. After 2 yr of “aggressive” vitamin E treatment, there was objective improvement of his visual fields and ERG (Howard et al. 1982).

This study has shown for the first time that repletion of vitamin E–deficient rats halted the progressive deterioration of visual function and resulted in some improvement. One other study of visual function in vitamin E–repleted rats has been carried out by Goss-Sampson et al. (1998), in which VEPs and ERG were recorded during 30 wk of deficiency followed by 20 wk of repletion. The animals were found to have normal retinal vitamin E and A concentrations, but the electrophysiological parameters continued to deteriorate throughout the period of repletion. A possible explanation for the different results between the two studies is the size of the animals at the time of repletion. In this study, the mean weight of the repleted group was 433 g, whereas in the study of Goss-Sampson et al. (1998), the rats weighed ~500 g when repleted. The heavier animals would have contained extra fat deposits, which in the absence of vitamin E could have led to increased lipid peroxidation and neural degeneration. The degeneration therefore probably occurred earlier in the study of Goss-Sampson et al. and might explain why visual function continued to deteriorate.

These animal studies do not provide any information as to whether the developing neurological system is particularly at risk from a deficiency of vitamin E. However, a few adults have been reported who have developed a deficiency of vitamin E after massive intestinal resection and who went onto develop the typical neurological sequelae ~10 yr after the onset of the gastrointestinal symptoms (Harding et al. 1982; Howard et al. 1982). These observations therefore suggest that the mature neurological system is also at risk from a deficiency of this vitamin.

The underlying mechanism(s) for the neural sequelae of severe and chronic vitamin E deficiency remain unknown. \( \alpha \)-Tocopherol is the major lipid soluble chain breaking antioxidant in vivo and is able to protect biological membranes from oxidative damage (i.e., lipid peroxidation) caused by oxygen derived free radicals (Burton et al. 1983). There are a number of lines of evidence to support an antioxidant mechanism for the action of vitamin E in neural tissues. For example, we have shown that concentrations of malondialdehyde (a measure of lipid peroxidation) is significantly increased in neural tissues from vitamin E–deficient animals compared with controls (Hayton and Muller 2004; MacEvilly and Muller 1996). In addition Nelson (1987) has shown that the characteristic neuropathology of vitamin E deficiency in the rat can be prevented by the addition of synthetic antioxidants such as ethoxyquin and promethazine. Further evidence was provided by Southam et al. (1991) who reported that the addition of excess peroxidation substrate in the form of polyunsaturated fat, markedly accelerated the rate of development of the neurological syndrome in vitamin E–deficient rats. Southam et al. (1991) suggested that the neural abnormalities could result from damage to mitochondria and other intra-axonal membranous structures. The membranes of mitochondria and the smooth endoplasmic reticulum contain a high proportion of polyunsaturated fatty acid chains and may well therefore be more susceptible to damage during vitamin E deficiency. In addition, there is a continuous production of oxygen-derived free radicals in mitochondria as a result of oxidative phosphorylation. A disturbance of the axonal mitochondria could lead to the reported abnormalities in fast retrograde transport (Southam et al. 1991), which in turn could result in the characteristic “dying back” axonal neuropathy. Support for this hypothesis comes from two studies. First, it has been shown from fractionation studies of myelinated nerves that the organelles of the axon, including the mitochondria, are particularly susceptible to oxidative stress during severe and chronic vitamin E deficiency (MacEvilly and Muller 1996). Second, muscle mitochondria from vitamin E–deficient rats showed significant decreases in the activities of complexes I and IV of the respiratory chain, a reduction in the respiratory control ratio (indicative of membrane damage), and increased membrane fluidity (Thomas et al. 1993).

There is, however, increasing evidence that \( \alpha \)-tocopherol may have other more specific functions, with the regulation of a number of genes being reported (Azizi and Stocker 2000; Brigelius-Flohe et al. 2002; Gohil et al. 2003; Landes et al. 2003). Gene expression could be modulated either directly by the presence or absence of \( \alpha \)-tocopherol and/or mediated by an increased concentration of reactive oxygen species resulting from oxidative stress. Studies of global gene expression in the cortex of rats from this study are currently underway to gain a greater understanding of the mechanism of action of \( \alpha \)-tocopherol in the nervous system. Whatever the precise mechanism(s) involved, it is clear from both human and these animal studies that repletion with vitamin E can halt the progression and sometimes result in significant improvement of both the neural signs and symptoms of vitamin E deficiency.

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