Longitudinal electrophysiological and biochemical studies in rats receiving different forms and intakes of vitamin E

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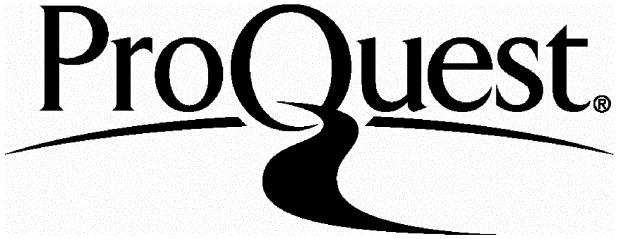

This thesis is dedicated to the memory of Dr Tony Kriss, one of my supervisors who died 3 days after my viva. Without his help in establishing the electrophysiological methods, this project and thesis would not have been possible.

Abstract

Vitamin E is a generic term for a number of different chemical compounds (tocopherols and tocotrienols), with α-tocopherol (αT) having the greatest biological activity. Natural RRR-αT is a single isomer, which has a greater biological activity (assessed by the rat foetal resorption assay) than synthetic all-rac-αT, which consists of a mixture of 8 isomers. A severe and chronic deficiency of αT causes a characteristic neurological syndrome in man and rats with similar electrophysiological abnormalities. The αT deficient rat is, therefore, an appropriate model for studying a deficiency in man. Electrophysiological techniques provide a simple, objective, non-invasive measure of nerve function and were used to a) determine the minimum requirements of αT necessary to prevent neural abnormalities, b) compare the biological activities of the different forms of αT in neural tissues and c) investigate the effect of repletion of αT deficient rats. Weanling animals were fed diets containing various amounts of either all-rac- or RRR-αT acetate and longitudinal electrophysiological studies (somatosensory evoked potentials, visual evoked potentials and electroretinogram) were carried out in the same animals at monthly intervals. After 14 months the rats were killed and concentrations of αT and malondialdehyde (a measure of lipid peroxidation) determined in neural and non-neural tissues by high performance liquid chromatography. Significant electrophysiological abnormalities were seen after 8 months of deficiency. A dietary intake of 1.0-1.25mg/kg all-rac- and 0.75 mg/kg RRR-αT acetate appeared to provide only marginal protection in preventing these abnormalities. The minimum requirement to prevent the development of all neural abnormalities is between 1.25 and 5mg/kg α-T acetate. These results indicated that the biological activity of all-rac-αT in neural tissues

was approximately 75% of that of RRR-α, which is similar to that previously shown using rat foetal resorption assays. The tissue αT concentrations showed the expected gradient with increasing dietary intake, with neural tissues tending to conserve αT. The concentration of free MDA was significantly increased in deficient compared to control animals in all tissues. The repleted group showed significant improvements in electrophysiological parameters compared to deficient rats after 6 weeks of repletion, indicating that repletion had halted neural degeneration. The results of this study are consistent with αT deficiency causing increased lipid peroxidation, leading to abnormal neural electrophysiology.

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Publications and presentations arising from the study

Publication

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Presentations

July 1999	Society for Free Radical Research (Europe) Summer Meeting. "Antioxidants, Adaptation and Ageing". Dresden **Neural function in vitamin E (α-tocopherol) deficient rats**. SM Hayton, A Kriss & DPR Muller
Mar 2000	11th European Fat-soluble Vitamins Group Meeting Toulouse **The minimum vitamin E requirements for the prevention of neural abnormalities in the rat**. SM Hayton, A Kriss & DPR Muller

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Abbreviations

ANOVA	analysis of variance
AVED	ataxia with vitamin E deficiency
BAEP	brainstem auditory evoked potentials
BSRF	brain stem reticular formation
C3	3rd cervical vertebra
CNS	central nervous system
CV	conduction velocity
ECG	electrocardiography
EEG	electroencephalography
EMG	electromyography
ERG	electroretinogram
FIVE	familial isolated vitamin E deficiency
GABA	γ-aminobutyric acid
HPLC	high performance liquid chromatography
i.m.	intra-muscular
i.p.	intra-peritoneal
L5	5th lumbar vertebra
LDL	low density lipoprotein
MDA	malondialdehyde
MTP	microsomal triglyceride transfer protein
NMDA	N-methyl-D-aspartate

ODFR	oxygen derived free radicals
QC	quality control
ROS	reactive oxygen species
RPE	retinal pigment epithelium
SEP	somatosensory evoked potential
TBARS	thiobarbituric acid reactive substances
VEP	visual evoked potential
VLDL	very low density lipoprotein

Chapter 1

Introduction

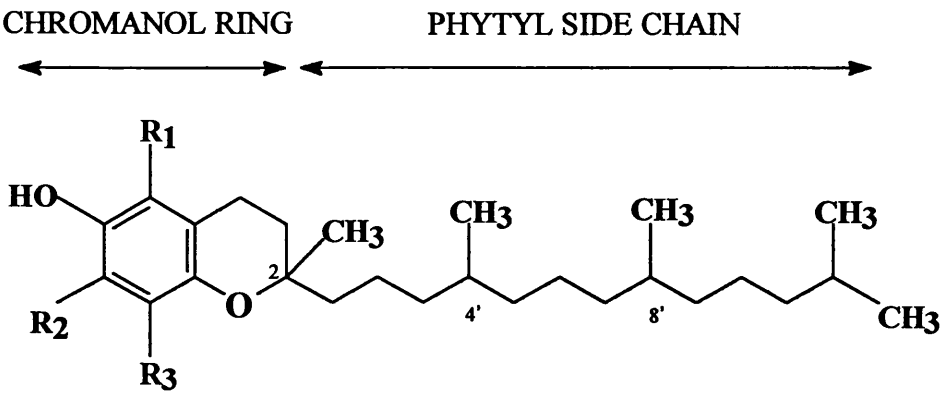
1.1 Early History of vitamin E

In 1922, Evans and Bishop discovered a dietary substance which was essential for normal reproduction in both male and female rats. Wheat germ oil and lettuce leaves were identified as good sources of this "antisterility vitamin" or "factor X". It was found to be fat soluble and was called vitamin E, since vitamins A to D had already been characterised (Sure, 1924). Evans and Burr (1925) saponified wheat germ oil and found that the unsaponifiable fraction had a very high concentration of vitamin E. Subsequent analysis of this fat-soluble fraction of wheat germ oil revealed that it had the chemical formula: $C_{29}H_{50}O_2$ (Evans, Emerson & Emerson, 1936). Evans named this substance tocopherol, from the Greek "tocos" meaning childbirth and "phero" meaning to bear; the suffix "ol" was chosen because the vitamin was an alcohol. Two years later, Fernholz determined the structural formula of α-tocopherol (Fernholz, 1938). In the same year, the α-isomer was chemically synthesised by Bergal *et al* (1938).

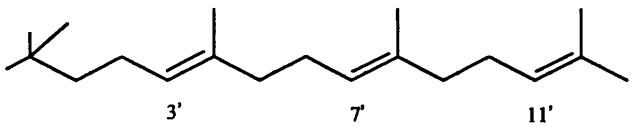
1.2 Structure of vitamin E

There are 8 naturally-occurring forms of vitamin E, grouped into two families which differ in the degree of saturation of the phytyl side chain (Figure 1.1). The compounds which have a saturated side chain are termed tocopherols (IUPAC-IUB Commission, 1974) and may be α, β, γ or δ forms, according to the number and position of the methyl groups on the chromanol ring (Figure 1.1). Tocotrienols have 3 double bonds

Figure 1.1 Structure of the tocopherols and tocotrienols

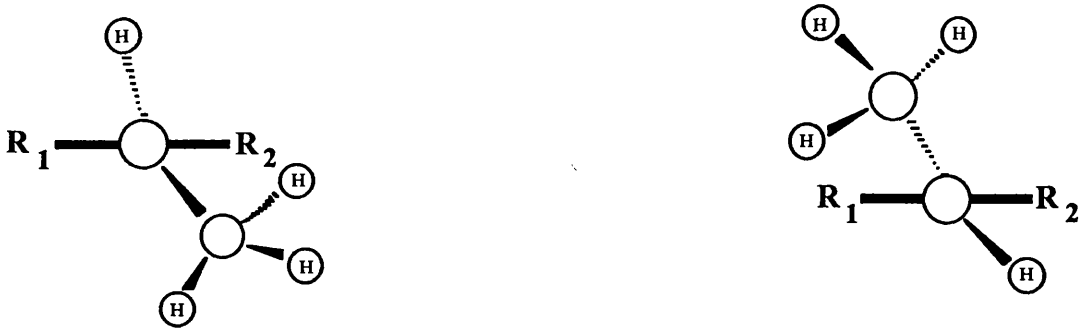


(TOCOTRIENOLS - As above but with the side chain below)



TOCOPHEROL	CHROMANOL -CH_3 GROUPS		
α	R_1	R_2	R_3
β	R_1		R_3
γ		R_2	R_3
δ			R_3

Figure 1.2 Diagram to illustrate the designation of R or S configuration to each chiral centre



○	Carbon atom
Ⓗ	Hydrogen atom
◢	Oriented away from page
⋰	Oriented into page
R_1	Chromanol ring plus phytyl side chain up to chiral centre
R_2	Remaining phytyl chain

in the side chain between carbons 3'-4', 7'-8', and 11'-12', and again may be α, β, γ or δ forms.

The tocopherols have 3 centres of asymmetry at carbons 2, 4' and 8'. This leads to 8 possible stereoisomers, described according to the spatial orientation of groups around each chiral centre (Figure 1.2). Each configuration has either **R** or **S** orientation, so the 8 isomers are:

RRR , RRS , RSR , SRR , RSS , SRS , SSR and **SSS.**

Synthetic forms of vitamin E comprise an equimolar mixture of all these isomers (racemic mixture), termed *all-rac*-tocopherol. However, the natural form shows the RRR configuration. Tocotrienols possess only one centre of asymmetry at carbon 2, as well as geometrical isomerism at carbons 3' and 7', again giving rise to 8 stereoisomers. Natural tocotrienols all have the (2R) 3'-*trans*,7'-*trans* configuration.

1.3 Biochemical action of vitamin E

The major function of tocopherol is as an antioxidant. It has been shown that it is the principal lipid soluble antioxidant in both human blood (Burton *et al*, 1983) and in various rat tissues (Cheeseman *et al*, 1988). It is therefore important for maintaining the structural integrity of membranes and other lipid-containing structures by preventing oxidative damage caused by oxygen derived free radicals.

The role of antioxidants in general and vitamin E in particular in protecting against the action of oxygen derived free radicals will now be briefly discussed.

1.3.1 Oxygen derived free radicals and antioxidants

Although molecular oxygen is essential for life, it can also be harmful. The damaging effects of oxygen result from the formation of chemical species which are collectively known as reactive oxygen species (ROS). These comprise oxygen containing compounds such as hydrogen peroxide (H_2O_2), ozone (O_3) and peroxynitrite ($ONOO^-$) as well as chemical species known as oxygen derived free radicals (ODFR). Halliwell and Gutteridge (1999) defined a free radical as "any species capable of independent existence that contains one or more unpaired electrons". Superoxide ($^{\bullet}O_2^-$), nitric oxide ($NO^{\bullet}$) and the hydroxyl radical ($^{\bullet}OH$) are examples of ODFR (Gershman *et al*, 1954). The presence of unpaired electrons causes free radicals to be paramagnetic (i.e. slightly attracted to a magnetic field) and highly reactive. They will attempt to abstract an electron from almost every type of molecule in cells.

By the above definition, molecular oxygen is itself a free radical since it contains two unpaired electrons. It is, however, relatively stable, because both the unpaired electrons have the same spin quantum number (i.e. they have parallel spins). For O_2 to accept a pair of electrons, both of these electrons must be of an antiparallel spin, whereas a pair of electrons in an atomic or molecular orbit does not meet this criterion as they have opposite spins (Halliwell & Gutteridge, 1984). Therefore, O_2 tends to

only accept electrons one at a time and reacts sluggishly with many non-radicals (Halliwell & Gutteridge, 1999).

Although hydrogen peroxide is not an ODFR it can, particularly in the presence of transition metals such as ferrous iron and copper, form the hydroxyl radical which is the most active ODFR. The reaction of hydrogen peroxide with ferrous iron (see below) is known as the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + {}^{\bullet}OH$$

The hydroxyl radical is very unstable and will react rapidly with almost every type of molecule found in cells (e.g. proteins, lipids, DNA) either by hydrogen abstraction, addition or electron transfer. These reactions produce another free radical, thereby leading to a chain reaction. It is therefore important that concentrations of hydrogen peroxide are kept to a minimum.

The body protects itself from the deleterious effects of ROS by a series of naturally-occurring antioxidants. An antioxidant is defined as "any substance that when present at low concentrations compared to those of an oxidizible substrate, significantly delays or inhibits oxidation of that substrate" (Halliwell & Gutteridge, 1999). The antioxidants found in biological systems function in a variety of different ways. They can act by removing reactive substrates, scavenging free radicals or by minimising the availability of pro-oxidants. The enzymes catalase and glutathione peroxidase (GPx) both remove hydrogen peroxide, thereby preventing the formation of free radicals.

Catalases contain a haem group and catalyse the following overall reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Glutathione peroxidase catalyses the reaction below, where the reducing equivalents are produced by reduced glutathione (GSH).

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

Superoxide dismutase (SOD) increases the rate of dismutation of the $^{\bullet}O_2^-$ to hydrogen peroxide:

$$^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

As well as enzymes, there are also a number of small molecules which act as antioxidants. These molecules, such as vitamin E (see section 1.3.2), ascorbate (vitamin C), glutathione and urate, are able to scavenge free radicals.

In health, there is a balance between the production of ODFR and their removal by antioxidants. Oxidative stress occurs when there is an imbalance, either as a result of an increased production of ODFR or decreased removal by antioxidants. Oxidative stress has been implicated in a number of clinical conditions including diabetes, atherosclerosis, Parkinson's disease, Alzheimer's disease, motor neurone disease and many cancers.

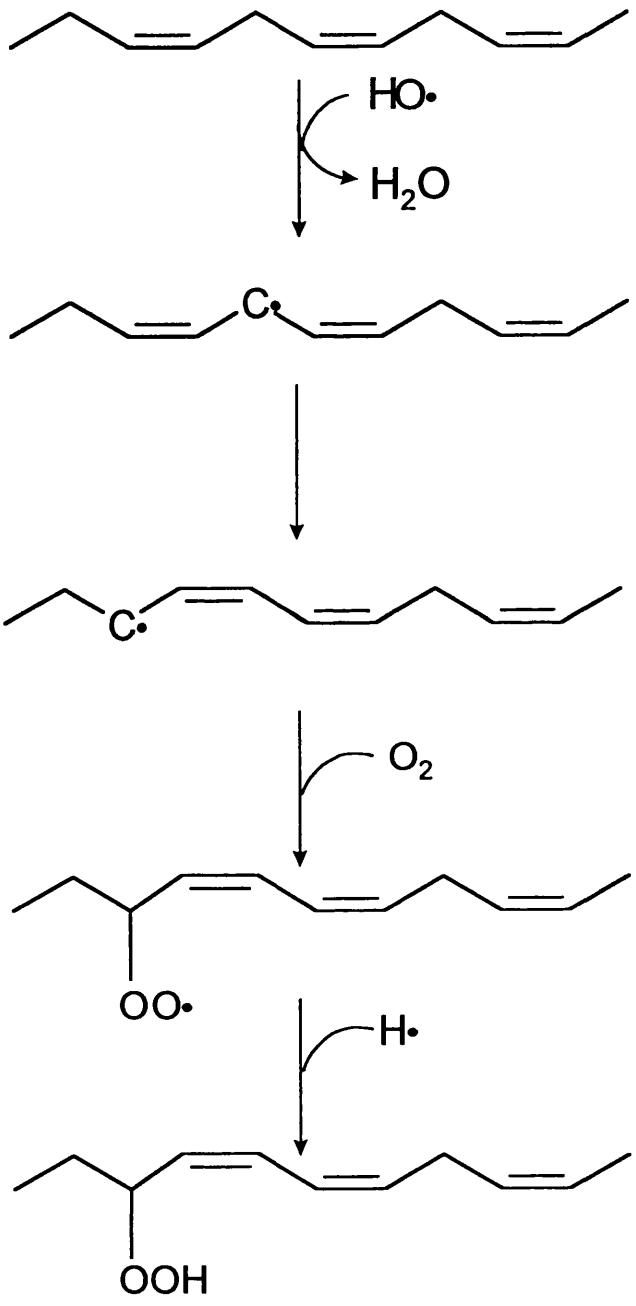
Although the above discussion concentrates on the deleterious effects of ODFR, some free radical producing reactions are essential to normal biological processes. These include the production of the superoxide radical ($^{\bullet}O_2^-$) during the "respiratory burst" of macrophages when they come into contact with foreign particles or immune complexes (Babior, 1978) and the detoxification of drugs in the cytochrome P450 system (Dormandy, 1983). The activity of the mitochondrial electron transport chains also yield $^{\bullet}O_2^-$ as some components, such as ubiquinone, leak electrons directly to O_2.

1.3.2 Vitamin E as an antioxidant

As mentioned above, vitamin E is the major lipid soluble antioxidant (Burton *et al*, 1983) and is important for the prevention of lipid peroxidation and the maintenance of membrane integrity and stability.

Lipid peroxidation (see Figure 1.3) can be broadly defined as the oxidative deterioration of fatty acids containing two or more carbon-carbon double bonds (i.e. polyunsaturated). The chain of reactions of lipid peroxidation is well-recognised and comprise three distinct stages: initiation, propagation and termination (Halliwell & Gutteridge, 1985). Lipid peroxidation is initiated when fatty acids are attacked by free radical species such as hydroxyl radicals ($^{\bullet}OH$), that are capable of abstracting a hydrogen atom from a methylene group ($-CH_2-$) adjacent to a carbon-carbon double bond. This results in the formation of a carbon centred free radical which undergoes molecular rearrangement and can react with molecular oxygen to produce lipid

Figure 1.3 Lipid peroxidation



INITIATION
Hydrogen abstraction

Molecular rearrangement

= Conjugated diene

Oxygen uptake

= lipid peroxyl free radical

PROPAGATION
Hydrogen abstraction from adjacent fatty acid

= Lipid hydroperoxide + lipid peroxyl radical

peroxyl free radicals. These peroxyl free radicals are sufficiently reactive to abstract further hydrogen atoms from adjacent polyunsaturated acyl chains, resulting in the formation of another lipid peroxyl free radical and a stable lipid hydroperoxide. This is referred to as the propagation stage, as this secondary radical can lead to further peroxidation. Termination occurs when either two radicals react to form stable products or a compound donates a hydrogen atom to the lipid peroxyl radical, again producing a non-radical product and therefore preventing further peroxidation.

Tocopherol (T-OH) acts by scavenging fatty acid peroxyl free radicals (L-OO$^{\bullet}$) and thereby terminating the chain reaction of lipid peroxidation. It donates the labile hydrogen atom of its phenol group to the lipid peroxyl radical, thereby forming a lipid hydroperoxide and a tocopheroxyl radical (T-O$^{\bullet}$) as follows:

$$\text{L-OO}^{\bullet} + \text{T-OH} \rightarrow \text{L-OOH} + \text{T-O}^{\bullet}$$

The tocopheroxyl radical is stabilized by delocalising the unpaired electron over the aromatic ring structure, and is not sufficiently reactive to abstract an hydrogen atom from other lipid molecules and thereby damage membranes. However, it can react with a second lipid peroxyl radical, to give inert products (Porter & Wagner, 1986), i.e.

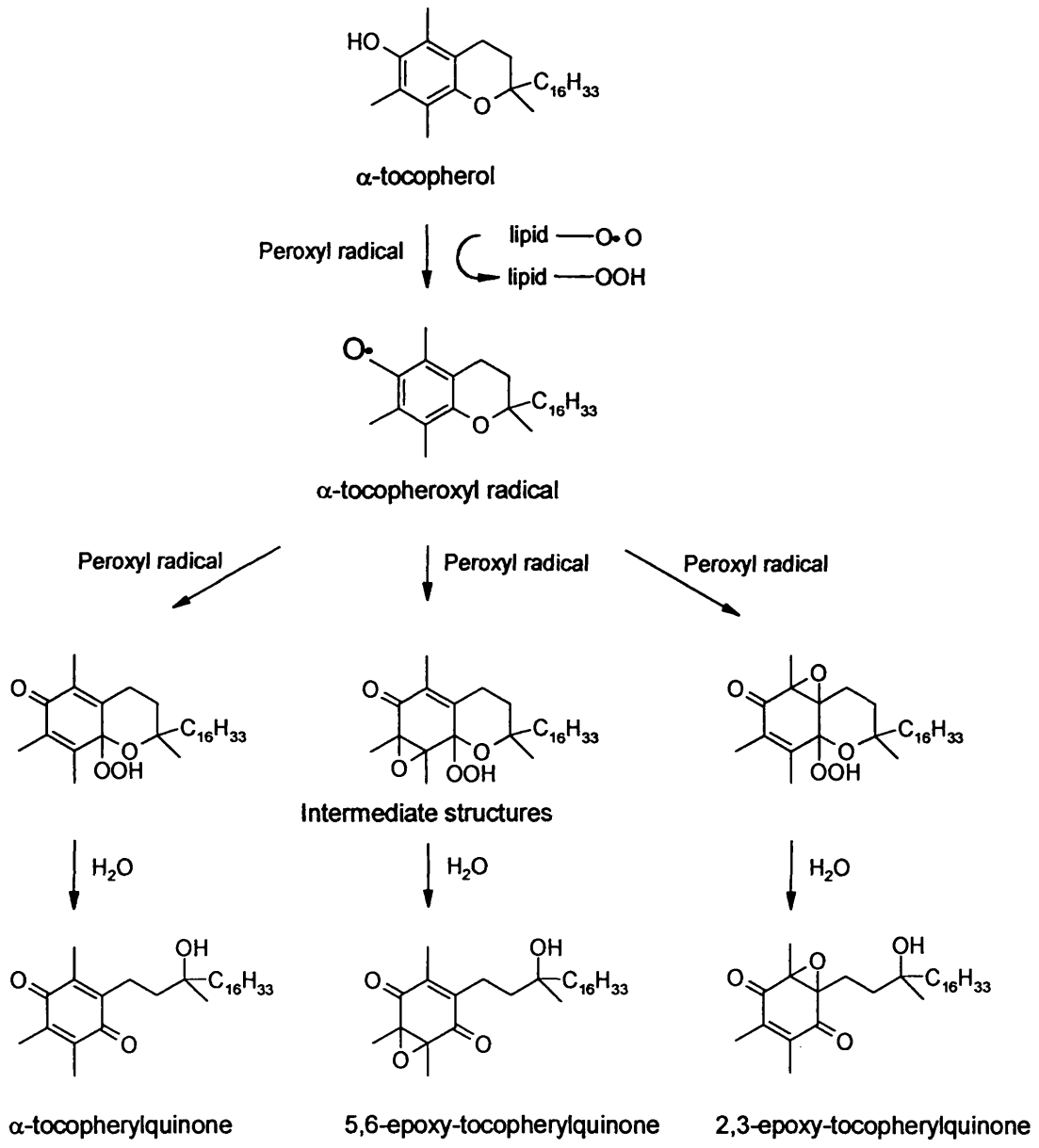
$$\text{L-OO}^{\bullet} + \text{T-O}^{\bullet} \rightarrow \text{Inert products}$$

Products of the above reaction include α-substituted tocopherones, which readily hydrolyse to tocopherylquinone, and epoxy tocopherones, which form epoxyquinones

on hydrolysis (Liebler, 1993), as shown in Figure 1.4. The tocopheryl radical can also form a dimer.

Since vitamin E is the major lipid soluble chain-breaking antioxidant present in membranes, Burton and Ingold (1986) concluded that it must be protected *in vivo*. There is evidence from *in vitro* studies that vitamin E can interact with the water soluble antioxidant ascorbate (vitamin C), reducing the tocopheroxyl radical back to the active tocopherol form (Niki *et al*, 1983; Burton & Ingold, 1986). However, evidence for a similar effect *in vivo* remains unconvincing. Chen and Thacker (1987) varied the vitamin E and/or vitamin C intake of rats and concluded that vitamin C may partially "spare" the degradative metabolism of vitamin E, only when vitamin E is present at low concentrations. This is in contrast to a similar study carried out with deuterated tocopherol in the guinea pig by Burton *et al* (1990), who concluded that any "sparing" effect was of negligible importance. It has been suggested that NAD(P)H dependent electron transport enzymes may have a role in the regeneration of tocopherol from the tocopheroxyl radical in mitochondrial (Maguire *et al*, 1989) and microsomal (Kagan *et al*, 1990) membranes. Kagan *et al* (1990a) have also reported that the antioxidant activity of ubiquinols results from their ability to reduce the tocopheroxyl radical, whereas their direct radical scavenging capacity appeared to be negligible in comparison to tocopherol.

Figure 1.4 Antioxidant reactions of α-tocopherol



1.3.3 Vitamin E as a membrane stabiliser

Tocopherol may also play a role in the stabilisation of membranes by physicochemical interaction with other membrane components. In 1973, Diplock and Lucy suggested that the tocopherol molecule is anchored within the membrane by means of a close association between its phytyl side chain and arachidonic acid fatty acid side chains of the membrane lipids. However, this theory is not now generally accepted since it has been calculated that there is approximately only one molecule of α-tocopherol for every 500 arachidonic acid molecules in the membrane (Buttriss & Diplock, 1988).

A series of experiments has been carried out using model membrane systems and cell culture (Maggio *et al*, 1977; Diplock *et al*, 1977; Giasuddin & Diplock, 1981) which showed vitamin E interacted with arachidonic acid, cholesterol, linoleic acid and its derivatives to produce a functional stability of membranes. It was further suggested that vitamin E may interact on a dynamic basis with more than one polyunsaturated phospholipid as opposed to a strict 1:1 molar ratio between the two (Maggio *et al*, 1977).

Gomez-Fernandez *et al* (1989) proposed that vitamin E is preferentially distributed into the most fluid domains within the membrane, which allows it to be associated with the most unsaturated fatty acid side chains. It is now known that α-tocopherol can also protect membranes from the damaging effects of phospholipases, especially phospholipase A, by interacting with the phospholipase hydrolysis products. Furthermore, it has been shown that α-tocopherol forms complexes with free fatty

acids and lysophospholipids, which reduces their membrane disordering effects (Kagan, 1989).

1.3.4 Other non-antioxidant functions of vitamin E

There is now evidence that vitamin E may have functions which are not related to its antioxidant activity, including effects on protein kinase C, gene expression and cell proliferation. It has been shown, for example, that α-tocopherol regulates protein kinase C (PKC) in several different cell types, including monocytes, macrophages, neutrophils, fibroblasts and mesangial cells (Chatelain *et al*, 1993; Tasino *et al*, 1995; Azzi & Stocker, 2000). PKC is an enzyme involved in signal transduction pathways within cells that govern events such as growth, differentiation and secretion. The inhibition of PKC by α-tocopherol in monocytes leads to the inhibition of phosphorylation and translocation of the cytosolic factor p47(phox) and to an impaired assembly of the NADPH-oxidase and of superoxide production (Cachia *et al*, 1998). α-Tocopherol also produces a significant decrease in monocyte interleukin-1β (IL-1β) release, via the inhibition of the 5-lipoxygenase pathway (Azzi & Stocker, 2000). β-tocopherol was found to have no effect on PKC or IL-1β, indicating that the mechanism involved is not related to the radical scavenging properties of the two molecules (Pryor *et al*, 1993). The α isoform of PKC has been shown to be the specific target of α-tocopherol, while the δ, ε, ζ and μ isoforms remained unaffected (Martiny *et al*, 1993).

α-Tocopherol has been shown to modulate the expression of genes such as those for α-tropomyosin (Azzi *et al*, 1998), liver collagen αI and α-tocopherol transfer protein (Azzi *et al*, 2001).

α-Tocopherol has also been shown to inhibit rat A7r5 smooth muscle cell proliferation, while β-tocopherol was ineffective (Azzi *et al*, 1995). Proliferation in a number of other cell lines has also been shown to be controlled by α-tocopherol, including rat and human aorta, mouse and human fibroblasts, human pigmented retinal epithelium and cancer cells (Azzi & Stocker, 2000). The gene for the smooth muscle cells scavenger receptor is down-regulated, at a transcriptional level, by α-tocopherol (but not β-tocopherol) (Azzi & Stocker, 2000).

1.4 Absorption of vitamin E and distribution throughout the body

The average human diet is rich in vitamin E, with nuts, seeds, oils, fruit, vegetables and dairy products providing good sources of the vitamin. The major tocopherol in nature is γ-tocopherol and therefore it is this form which predominates in the diet (Bieri & Evarts, 1973; Parker, 1989). Only 20% of the total tocopherol intake is in the form of α-tocopherol and yet α-tocopherol comprises approximately 90% of all tocopherol found in human tissues (Bieri & Prival, 1965). At first it was thought that the high percentage of α-tocopherol found in tissues was due to its more efficient absorption compared to γ-tocopherol. However, conflicting data have been reported. A number of studies have found that the absorption of γ-tocopherol is less efficient than that of

α-tocopherol (Pearson & Barnes, 1970; Handelman *et al*, 1985; Baker *et al*, 1986), whereas others have found no difference in the absorptions of α- and γ-tocopherol, but have concluded that γ-tocopherol disappears from tissues more rapidly than α-tocopherol (Peake & Bieri, 1977). Traber & Kayden (1989) performed a study which appears to have resolved the controversy. A mixture of 1g of α- and γ-tocopherols were fed with a meal to normal human volunteers and similar increases in plasma concentrations of both tocopherols were seen up to 12 hours after the oral load. By 24 hours, however, γ-tocopherol concentrations had decreased dramatically, whereas concentrations of α-tocopherol remained unchanged. These results suggest that both tocopherols are absorbed by the enterocyte and secreted into the bloodstream within chylomicrons to a similar extent but that thereafter their handling is different.

Using cynomolgus monkeys given a single dose of deuterated α- and γ-tocopherols, it was confirmed that both tocopherols were absorbed to similar extents but it was demonstrated that the liver secreted nascent VLDL preferentially enriched in α-tocopherol (Traber *et al*, 1990). This finding can be explained by the properties of a tocopherol binding protein, which has been described in both human and rat liver (Kaplowitz *et al*, 1989; Catignani & Bieri, 1977). The binding of tocopherol analogues to the rat protein has shown that [^{3}H]α-tocopherol could be displaced by α-tocopherol but not by trolox, which lacks the phytyl tail of α-tocopherol. γ-Tocopherol was only partially effective in displacing [^{3}H]α-tocopherol, and α-tocopheryl acetate and the quinone were completely ineffective (Catignani & Bieri, 1977). These findings suggest that the binding site of the protein recognises the methyl and hydroxyl groups on the

chromanol ring, as well as the stereochemistry of the 2 position, where the phytyl tail joins the ring.

As mentioned above, vitamin E is distributed throughout the body, mainly as α-tocopherol, and high concentrations are found in liver, nerves, adrenals, heart and uterus (Quaife *et al*, 1949; Wiss *et al*, 1962). It is transported in plasma by lipoproteins, particularly by low density lipoproteins (LDL) and very low density lipoproteins (VLDL), and a significant proportion is delivered to cells via the high affinity apolipoprotein B/E receptor (Traber *et al*, 1984). Relatively little is known about the metabolism of tocopherol. Two metabolites were characterised in the 1950s from the urine of rabbits (Simon *et al*, 1956a) and man (Simon *et al*, 1956b) given large oral doses of α-tocopherol. These compounds, also known as Simon's metabolites, were α-tocopheronic acid and α-tocopheronolactone and are formed following the opening of the hydrochromanol ring as a result of oxidation and subsequent metabolism (presumably β-oxidation) of the side chain. More recently, other urinary metabolites of α-tocopherol have been described, which have an intact chromanol ring structure but shortened side chain. These are the carboxyethyl-6-hydroxychromans or CEHCs (Schultz *et al*, 1995; Traber *et al*, 1998; Swanson *et al*, 1999) and the carboxymethylbutyl-6-hydroxychromans or CMBHCs (Pope *et al*, 2000). However, it is not clear whether the Simon metabolites are formed *in vivo* or produced artefactually during urine analysis (Schultz *et al*, 1995). There is also disagreement as to whether the metabolites are excreted as glucuronides (Swanson *et al*, 1999) or sulphates (Schultz *et al*, 1995).

1.5 Biological activities of the tocopherols

Each of the different forms of vitamin E has a different biopotency, which reflects its antioxidant activity and bioavailability. The biological activity of vitamin E is traditionally determined by the rat fetal resorption-gestation assay (e.g. Bunyan *et al*, 1961; Leth & Sondergaard, 1977; Weiser & Vecchi, 1982). This assay exploits the fact that a deficiency of vitamin E in pregnant rat dams results in resorption of the fetus into the lining of the womb and hence a failure to produce live young. The fetal resorption-gestation assay measures the ability of the test compound, when administered to pregnant rats on a tocopherol deficient diet, to reverse this effect (Century & Horwitt, 1965). Table 1.1 shows the biological activities of the naturally-occurring forms of vitamin E, of synthetic (all-rac) α-tocopherol and the natural and synthetic forms of α-tocopheryl acetate. The activities of the different forms are compared to all-rac-α-tocopheryl acetate, which is given an activity of 1.00IU. It is commonly accepted that RRR-α-tocopheryl acetate is more active than all-rac-α-tocopheryl acetate by a factor of 1.36. In addition, the free tocopherol has a greater bioactivity (1.49IU) than the corresponding acetate ester, and each tocopherol has a higher activity than the corresponding tocotrienol. In laboratory experiments and in vitamin supplements, tocopherol acetate and succinate derivatives are often used, since these esters are more resistant to oxidation than the free alcohol. However, these derivatives are not active antioxidants, and no antioxidant activity is possible until the ester has been hydrolysed in the gut to α-tocopherol.

Table 1.1 Biological activities of the naturally-occuring forms of vitamin E, of synthetic (all-rac) α-tocopherol and of natural and synthetic α-tocopheryl acetate

Common name	Activity based on Rat Assay* IU/mg	Compared to RRR-α-tocopherol (%)
RRR-β-tocopherol	0.75	50
RRR-γ-tocopherol	0.15	10
RRR-δ-tocopherol	0.05	3
RRR-α-tocotrienol	0.75	50
RRR-β-tocotrienol	0.08	5
RRR-γ-tocotrienol	unknown	unknown
RRR-δ-tocotrienol	unknown	unknown
RRR-α-tocopherol	1.49	100
RRR-α-tocopheryl acetate	1.36	91
all-rac-α-tocopherol	1.10	74
all-rac-α-tocopheryl acetate	1.00	67

(* Source: Bunyan *et al*, 1961)

The *in vitro* antioxidant activity of the different forms of vitamin E does not account for their biopotencies. For example, γ-tocopherol has been shown to have a greater antioxidant activity than α-tocopherol in human endothelial cells in culture, whereas it is the α- form that has the greater activity as determined by *in vivo* rat fetal resorption assays (Tran & Chan, 1992). This difference is due to the fact that the *in vitro* antioxidant activity is determined by the chromanol ring alone, i.e. it is not influenced by the structure of the phytyl tail (Burton & Traber, 1990). *In vivo*, however, there are selective differences in the transport of different forms of vitamin E to and retention by tissues, which is partly determined by the structure of the phytyl tail and the properties of the hepatic tocopherol binding protein described above. Therefore, both antioxidant activity and bioavailability must be considered when determining biopotency.

It is important to note that only 12.5% of all-rac-α-tocopherol is the same as natural RRR-α-tocopherol, and each of the other seven unnatural stereoisomers has a lower activity in the resorption assay (Weiser & Vecchi, 1982). These differences arise primarily from the difference in chirality at carbon 2 (Ames, 1979; Weiser & Vecchi, 1981; Machlin *et al*, 1982). The four stereoisomers with the 2R configuration are generally more active than their corresponding 2S epimers (Ingold *et al*, 1987). Furthermore, single-dose experiments in which radioactively labeled RRR- and SRR-α-tocopherols were administered to rats (Weber *et al*, 1963, 1964) and chicks (Desai *et al*, 1965a, 1965b) showed that the uptake of the 2R stereoisomers into tissues was greater than for the 2S compounds, suggesting that this is the reason for their greater bioactivity. Ingold *et al* (1987) showed a preferential uptake of RRR- over SRR-α-tocopherol by all tissues, except the liver, when rats were fed deuterated

forms of the α-tocopherol acetates for several months. After faecal examination, they concluded that biodiscrimination begins in the gut with RRR- being more rapidly hydrolysed than SRR-α-tocopheryl acetate, as a result of the specificity of the active enzymes in the pancreatic juice or the bile salts necessary for hydrolysis to occur (Gallo-Tores, 1980; Lombardo *et al*, 1980). However, Weber *et al* (1964) showed that the 2S stereoisomers were absorbed more rapidly within the first half hour after dosing. A possible explanation to reconcile the findings of these two studies is that hydrolysis of the 2S isomers is faster but more susceptible to inhibition by free tocopherol (Ingold *et al*, 1987). As already discussed, the hepatic α-tocopherol transfer protein is responsible for the selective incorporation of RRR-α-tocopherol into VLDL (see section 1.4). The studies referred to above were performed on animals. However, it has been shown that humans also exhibit a substantial preference for uptake of RRR- over SRR-α-tocopherols into plasma (Traber *et al*, 1990).

As previously stated, α-tocopheryl acetate has a lower biological activity than the free alcohol. Burton *et al* (1988) performed studies to establish whether there were significant differences in the overall efficiency of absorption between the two compounds and whether this varied from species to species. No significant differences in the absorption of deuterated α-tocopherol and its acetate ester were found, when a mixture of the two compounds was given with a meal to five normal subjects. Different results were, however, obtained in the rat, depending on the way in which the vitamin was presented for absorption (Burton & Ingold, 1992). If the two forms were given together with vegetable oil, the plasma concentration of deuterated α-tocopherol from the free form was only 50% of that from the ester. However, when

given together with the standard animal diet, the plasma concentrations of the deuterated tocopherols were virtually identical. It is likely that increased oxidation and therefore destruction of free tocopherol took place in the intestine in the presence of the unsaturated fatty acids found in corn oil. This would result in less tocopherol being available for absorption.

1.6 Vitamin E in animals

After the initial recognition that a deficiency of vitamin E resulted in reproductive defects in the rat, it became clear that such a deficiency was responsible for a range of disorders within and between different animal species. There was also a marked degree of species specificity and a large diversity of tissue and organ functions affected by vitamin E deficiency (Mason & Horwitt, 1972). Paralysis, growth retardation, foetal resorption, testicular degeneration, uterine pigmentation and a myopathy have all been reported in the rat as a result of a deficiency of vitamin E (Ringsted, 1935; Martin & Moore, 1939; Mason, 1954). In chicks, however, the most common pathological states found are encephalomalacia, a cerebellar disorder with clinical signs of ataxia, spasms or paralysis (Pappenheimer & Goettsch, 1931), exudative diathesis (Dam & Glavind, 1939) and a myopathy (Dam *et al*, 1952). Guinea pigs and rabbits fed a semipurified diet developed nutritional muscular dystrophy (Pappenheimer & Goettsch, 1931), as did ducklings (Pappenheimer & Goettsch, 1934).

Considerable variability in the time course of the development of deficiency features and histopathological changes has been reported between species. For example,

histopathological changes were evident in rat muscle after 12 weeks on a vitamin E deficient diet, with paresis after 30 weeks (Machlin *et al*, 1977; Nelson *et al*, 1979), whereas the same abnormalities were evident in E-deficient rabbits after only 4 weeks (Chu *et al*, 1984). The most common feature of vitamin E deficiency in animals is now regarded to be an acute or chronic necrotising myopathy (Wasserman & Taylor, 1972), but a characteristic neuropathy has also been reported (see section 1.8.4).

1.7 Vitamin E in Man

Much controversy surrounded the role of vitamin E in human nutrition until the late 1950's and 1960's. This was because no clearly defined deficiency syndrome could be identified, due the difficulty in making "normal" man deficient of the vitamin. Many ill-founded claims were made: vitamin E was reported to improve the sex life, slow the ageing process, and even protect against the effects of ionising radiation generated by thermonuclear bombs. Such claims led cynics to dub the vitamin, "E for everything" and delayed its general acceptance as a compound having a genuine role in human nutrition.

In the last 40 years, evidence has accumulated that vitamin E has an important role in both disorders of the new-born and in the maintenance of normal neurological structure and function. The first disease state directly attributed to a deficiency of vitamin E was a previously unrecognised haemolytic anaemia and subcutaneous oedema in premature infants with low serum vitamin E levels, which could be

prevented by administering oral vitamin E (Oski & Barnes, 1967; Ritchie *et al*, 1968). This condition was found to be the result of insufficient vitamin E present in artificial milk formulas, which has since been rectified and the condition is not now seen. Supplementation with pharmacological doses of vitamin E has also been suggested to be of benefit in the management of certain disorders of the premature neonate, such as retinopathy of prematurity (Owens & Owens, 1949; Johnson *et al*, 1989) and intraventricular haemorrhage (Law *et al*, 1990) but this remains unproven and controversial.

The principal feature of severe and chronic vitamin E deficiency in man is a characteristic and progressive neurological syndrome (Muller *et al*, 1983; Harding, 1987; Muller & Goss-Sampson, 1990). The evidence that this neurological syndrome results from a severe and chronic deficiency of the vitamin will now be presented.

1.8 Vitamin E and neurological function

Vitamin E has an important role in the maintenance of normal neurological structure and function. Evidence for this role comes from four main sources -
1) patients with abetalipoproteinemia, 2) patients with other chronic disorders of fat malabsorption, 3) patients with a familial isolated deficiency of vitamin E without fat malabsorption, and 4) comparison of neuropathological findings in vitamin E deficient man and experimental animals. Each of these lines of evidence is considered below.

1.8.1 Abetalipoproteinemia

This condition, a rare inborn error of lipid metabolism with severe fat malabsorption, was first described by Bassen and Kornweig in 1950 and provided the first indication of a neurological role for vitamin E in man. The complete absence of low density (beta) lipoprotein from the plasma of such patients was independently demonstrated by three groups in 1960 (Lamy *et al*, 1960; Mabry *et al*, 1960; Salt *et al*, 1960). Gotto and his colleagues (1971) later showed that apolipoprotein B (apo B), an essential component of chylomicrons, very low density lipoproteins (VLDL) and low density lipoproteins (LDL), was undetectable in the plasma of patients with abetalipoproteinemia. The lack of apo B results in the absence of all these lipoproteins, which are involved in the absorption and transport of vitamin E. Chylomicrons are necessary for the transport of the vitamin out of and away from the enterocyte, and VLDL and LDL are major transporters of the vitamin in the circulation. Therefore, vitamin E is undetectable in the plasma of patients with abetalipoproteinemia from birth (Kayden *et al*, 1965; Muller *et al*, 1974) and such patients provide an ideal model for studying vitamin E deficiency in man.

Abetalipoproteinemia is caused by a mutation in the gene for the microsomal triglyceride transfer protein (MTP) (Wetterau *et al*, 1992; Sharp *et al*, 1993), which is located on chromosome 4 (Narcisi *et al*, 1995). MTP is a soluble protein present in the lumen of microsomes isolated from the liver and intestine, which mediates the transport of triglycerides, cholesteryl ester and phosphatidyl choline between membranes (Wetterau & Zilversmit, 1985 and 1986). In this way, MTP plays an important role in the assembly of VLDL in the liver and chylomicrons in the intestine.

Other features of the condition include acanthocytosis (spiky red cells), also present from birth, and an atypical retinitis pigmentosa and severe ataxic neuropathy which usually develop during the second decade of life. The neurological syndrome is characterised by ataxia, areflexia, proprioceptive loss (loss of position sense), loss of vibration sense, abnormal feet (pes cavus), curvature of the spine (scoliosis), abnormal eye movements, a pigmentary retinopathy and a general muscle weakness. Treating these patients with very large oral doses of α-tocopherol (100mg/kg/day, compared to a normal requirement of a total of 10-30mg/day) has resulted in definite clinical benefit. If started sufficiently early, vitamin E supplementation can prevent the development of all the neurological and retinal features. If supplementation is commenced after the development of the neurological signs and symptoms, progression is invariably halted and in some cases reversed (Muller *et al*, 1977; Azizi *et al*, 1978; Muller & Lloyd, 1982).

1.8.2 Other chronic disorders of fat malabsorption

There are a number of conditions other than abetalipoproteinemia in which a severe deficiency of vitamin E is associated with chronic fat malabsorption. The vitamin is highly hydrophobic, requiring an adequate supply of bile salts to facilitate solubilisation and absorption (Harries & Muller, 1971). Therefore a pronounced deficiency occurs when the bile salt concentration in the intestinal lumen is reduced, e.g. biliary atresia (Muller *et al*, 1974). There are many published reports relating a severe deficiency of vitamin E in children with cholestatic liver disease to a neurological syndrome almost identical to abetalipoproteinemia (Elias *et al*, 1981; Rosenblum *et al*, 1981;

Guggenheim *et al*, 1983; Muller *et al*, 1983; Sokol *et al*, 1983). To overcome the problems of solubilization, it is generally necessary to administer either intra-muscular injections of tocopherol or provide oral vitamin E in the form of α-tocopheryl polyethylene glycol-1000 succinate (TPGS), which is water soluble and does not require bile salts for solubilisation (Sokol *et al*, 1987; 1993). Improvement in neurological function following treatment with vitamin E has been reported in children with cholestasis (Elias *et al*, 1981; Guggenheim *et al*, 1983; Sokol *et al*, 1985).

The same neurological disorder has also been reported as a result of severe vitamin E deficiency associated with cystic fibrosis (Umetsu *et al*, 1980; Elias *et al*, 1981) and in patients who have had large amounts of the small bowel surgically removed (multiple ileal resections) (Harding *et al*, 1982; Nakajima *et al*, 1987). The neurological features in these patients have also been shown to be alleviated by appropriate vitamin E therapy (Harding *et al*, 1982).

1.8.3 Ataxia with vitamin E deficiency

A small number of patients have been described who have low serum vitamin E concentrations and the associated neurological abnormalities, but show no evidence of general fat malabsorption (Burck *et al*, 1981; Laplante *et al*, 1984; Harding *et al*, 1985; Yokota *et al*, 1987; Sokol *et al*, 1988). This disorder was initially termed familial isolated vitamin E deficiency (FIVE), but more recently has been re-named ataxia with vitamin E deficency (AVED). Vitamin E therapy has again been shown to either prevent deterioration or improve neurological status (e.g. Sokol *et al*, 1988). Using deuterated tocopherol, Traber *et al* (1990) showed that the absorption of

tocopherol was normal in patients with AVED but that there was a rapid subsequent decline in plasma concentrations compared with controls. They suggested that the deficiency was due to the absence of the liver tocopherol-binding protein necessary to incorporate α-tocopherol into VLDL and for its secretion into the circulation (see also section 1.4).

It has been subsequently shown that AVED is an autosomal recessive disorder caused by a mutation in a gene on chromosome 8, which results in the lack of the functional hepatic-binding protein for α-tocopherol (Ouahchi *et al*, 1995; Gotoda *et al*, 1995). The fact that these patients develop the characteristic neurological syndrome without any generalised fat malabsorption strengthens the causal link between vitamin E deficiency and the observed neurological sequelae.

1.8.4 Comparative neurological studies

The characteristic neuropathological features in vitamin E deficient humans with abetalipoproteinemia and cholestasis (Kayden *et al*, 1965; Muller *et al*, 1974; Rosenblum *et al*, 1981) are very similar to those observed in vitamin E deficient experimental animals, such as rats (Einarson *et al*, 1952; Machlin *et al*, 1977; Towfighi, 1981) and monkeys (Nelson *et al*, 1981). Axons in the posterior column nuclei are found to be dystrophic, especially those in the gracile nuclei of rat and man, whereas the cuneate nuclei are more affected in the monkey. A selective loss of large calibre myelinated sensory axons from the posterior columns of the spinal cord is also seen, particularly in the rostral segments. There is also a selective loss of large calibre

myelinated sensory axons in the peripheral nervous system, confined to the most distal segments of peripheral axons e.g. of the ulnar (Rosenblum *et al*, 1981) and sural (Wichman *et al*, 1985) nerves. Severe degeneration has been reported to occur at neuromuscular junctions i.e. end-plates and cutaneous sensory capsules of the hind paw of vitamin E deficient rats (Towfighi, 1981). Disorganised neurofilaments, tubulovesicular bodies and abnormal vesicles are seen in affected axons using electron microscopy. These observations indicate a distal or dying back neuropathy (Southam *et al*, 1991).

There is some evidence that the neuropathology is due to a primary axonopathy, possibly resulting from peroxidative damage of cell membrane lipids, with secondary demyelination (Nelson *et al*, 1981; Thomas *et al*, 1984; Wichman *et al*, 1985). The pathogenesis of this axonopathy is similar in both the central and peripheral nervous systems, but with greater central involvement. It is not known why there is this difference in axonal loss. It has been suggested that central axons are more vulnerable to vitamin E deficient induced damage (Nelson *et al*, 1981). Another possibility is that central and peripheral axons are both equally susceptible, but the peripheral nervous system has a greater regenerative capability (Wujek & Lasek, 1983).

It has also been suggested that mitochondria may be particularly susceptible to peroxidation since their membrane lipids are highly polyunsaturated (Molenaar *et al*, 1972). Functional impairment of axonal mitochondria could lead to abnormalities in fast anterograde and retrograde transport along the axons, which are energy-dependent processes, and thus to defective "turnaround" (Muller & Goss-Sampson, 1990) - see also page 55. This would result in the accumulation of organelles which

may "plug off" the terminal axons, leading to their isolation from the cell body and ultimately degeneration. The process could then spread in a "dying back" manner. This hypothesis could explain the role of vitamin E in axons and is supported by two studies. Firstly, muscle mitochondria from vitamin E deficient rats showed significant decreases in the activities of the respiratory chain complexes I and IV, a reduction in the respiratory control ratio (indicative of membrane damage) and increased membrane fluidity (Thomas *et al*, 1993). Secondly, MacEvilly and Muller (1996) have used fractionation studies of myelinated nerves to show that the organelles of the axon, including the mitochondria, are particularly susceptible to oxidative stress during severe and chronic vitamin E deficiency.

There have been few reports describing the effect of vitamin E repletion on the neuropathological lesions found in experimental animals. Two monkeys, which had been on a vitamin E deficient diet for 30 to 33 months, were given oral α-tocopheryl acetate for 2 months by Nelson *et al* (1981) but no changes in the nervous system indicative of regeneration were observed. Such changes may have become evident if the repletion period had been longer, since regeneration proceeds slowly in peripheral nerves, and only to a limited extent, if at all, in the more centrally directed areas of the posterior columns.

1.9 Electrophysiological studies

1.9.1 Studies in man

Nerve

A number of electrophysiological studies of neurological function have been carried out in patients with abetalipoproteinemia (Muller *et al*, 1977; Miller *et al*, 1980; Lowry *et al*, 1984; Wichman *et al*, 1985; Brin *et al*, 1986), cholestatic liver disease (Alvarez *et al*, 1983; Werlin *et al*, 1985; Landrieu *et al*, 1985) and AVED (Laplante *et al*, 1984; Harding *et al*, 1985; Sokol *et al*, 1988). These studies showed that central conduction was more severely affected than peripheral, and that abnormalities of sensory function were more common than those of motor. A marked delay in central conduction as measured by somatosensory evoked potentials (SEPs) was seen, whereas conduction velocities of motor and peripheral sensory nerves were generally within normal limits or only slightly reduced. There was, however, a decrease in the amplitude of the sensory nerve action potential, which tended to be more pronounced in the distal than the proximal segments of the tibial and sural nerves (Wichman *et al*, 1985). Brainstem auditory evoked potentials (BAEPs) were found to be consistently normal in patients with the above mentioned disorders, whereas one study has reported abnormal BAEPs in patients with cystic fibrosis (Vaisman *et al*, 1996). Improvements in sensory and motor conduction, normalization of SEPs and an increase in sensory action potentials have been recorded in patients, following repletion with vitamin E (Muller *et* al, 1977; Harding *et* al, 1982; Landrieu *et al*, 1985; Brin *et al*, 1986).

The electrophysiological findings associated with vitamin E deficiency are what would be expected from a distal dying-back axonal neuropathy, caused by primary damage to the axon of the neuron followed by secondary demyelination. The axon depends upon the continuous delivery of materials from the cell body, since it lacks the ribosomes necessary to synthesise proteins (Griffin & Watson, 1988). This intra-axonal transport can be fast or slow. In the anterograde direction (i.e. away from the cell body), the slow system conveys the bulk of the the axoplasmic constituents, including cytoskeletal elements, and the fast system carries small vesicles and other particulate organelles. Material that has descended the axon is transported back to the cell body for reprocessing, packaged in lysosomes, by the retrograde tansport system (Tsukita & Ishikawa, 1980; Bisby & Bulge, 1977). This process is known as 'turnaround'. Alterations in slow transport are reflected in changes in axonal calibre, which influences conduction velocity, excitability and the extent of myelination (Friede & Samorajski, 1971; Griffin *et al*, 1982). Sustained impairment of fast axonal transport, as studied in acrylamide neuropathy, leads to degeneration of the distal axon (Sahenk & Mendell, 1981). The reduction in the number of axons would be expected to result in a decrease in conduction volume, and therefore amplitude, of the action potentials but would not affect the velocity of the response.

Axonal atrophy correlates with a decrease both in the numbers of cytoskeletal neurofilaments and in the delivery of neurofilament constituents by slow transport (Griffin & Watson, 1988). Experimental models have shown that impairment of slow intra-axonal transport leads to neurofilament accumulation and axonal enlargement proximal to the site of impairment, and to downstream distal axonal atrophy (Griffin & Watson, 1988). Axonal atrophy leads to secondary demyelination, which has been

studied using experimental models, such as axotomy (Dyck, 1984; Hoffman *et al*, 1984) and β,β'-iminodipropionitrile (IDPN) neuropathy (Baba *et al*, 1983). It was found that axonal enlargement stimulated one cycle of Schwann cell division and hence an increase in the amount of myelin in the sheaths, and this was then followed by a relative decrease in calibre. At this stage the amount of myelin was excessive for axonal calibre, and remodelling of myelin (i.e. demyelination) occurred. Repetition of this sequence resulted in major onion bulb formation (Griffin & Price, 1981). The myelin sheath of normal myelinated nerve fibres is segmental, and electrical activity is restricted to the narrow spaces between the segments, the nodes of Ranvier (Thomas *et al*, 1984). The effect of this arrangement is that ionic activity leaps from one node to the next in series, resulting in conduction velocities of 20-70ms^{-1}, depending on fibre diameter and myelin sheath thickness. Loss of one or more myelin segments (i.e. demyelination), as seen in chronic vitamin E deficiency, results in a conduction block but the axon subsequently develops the capacity to sustain continuous conduction, in a manner comparable to that seen in normal non-myelinated fibres (Bostock & Sears, 1978; Sears & Bostock, 1981). This change is reflected in a significant decrease in nerve conduction velocities.

Eye

The effects of vitamin E deficiency on visual function have been investigated in man, by measuring the response of the retina (ERG) and visual cortex (VEP) to flash stimulation. Abnormalities in the electroretinogram (ERG) have been reported as a result of a prolonged vitamin E deficiency, followed by changes in the visual evoked potential (VEP) (Alvarez *et al*, 1983). The first sign of abnormality observed in

children with chronic cholestasis was a decrease in the amplitude of the ERG 'b' wave, which suggested that vitamin E deficiency involved primarily the periphery of the retina (Alvarez *et al*, 1983). The retina has a plentiful supply of oxygen, an abundance of mitochondria and an unusually high rate of oxidative metabolism (Handelman & Dratz, 1986). For these reasons, it is likely to be particularly vulnerable to a deficiency of vitamin E and thereby to the deleterious effects of ODFRs. The outer segments of the rod photoreceptor cells are especially susceptible to lipid peroxidation as more than 65% of the membrane fatty acids are polyunsaturated (Handelman & Dratz, 1986). Goss-Sampson *et al* (1998) put forward the hypothesis that excessive lipid peroxidation led to the loss of long chain PUFA from the photoreceptors which resulted in changes in the membrane micro-environment and, consequently, affected the membrane bound proteins involved in ion transport. These changes, in turn, could alter phototransduction, by altering the light induced movement of rhodopsin within the membrane (Goss-Sampson *et al*, 1998).

Abnormalities in VEPs have been reported in patients with cholestasis (Alvarez *et al*, 1983) and abetalipoproteinemia (Lowry *et al*, 1983; Brin *et al*, 1986). It has been suggested that these abnormalities are secondary to retinal dysfunction (Goss-Sampson *et al*, 1998), but delayed VEP latencies have been reported in patients with neurological abnormalities and a normal ERG (Muller *et al*, 1983). VEPs represent electrical activity within the visual cortex. Vitamin E deficiency would, therefore, be expected to have similar effects on these visual cortical nerves as on other nerves within the CNS, i.e. demyelination and a reduction in the total number of axons.

1.9.2 Studies in animals

Only a limited number of electrophysiological studies in vitamin E deficient animals have been published. A study of motor conduction in the sciatic nerve of vitamin E deficient and control rabbits reported a pronounced weakness and paresis, with a significant slowing of motor nerve conduction in the sciatic nerve of the deficient animals (Ernst, 1986). From this study, it was concluded that vitamin E deficiency produced a neurogenic as well as a myogenic lesion. Sensory studies, carried out in vitamin E deficient and control rats by Bradley *et al* (1986), failed to observe any differences between the two groups in either central or peripheral sensory conduction, to the level of the brainstem.

A detailed study was performed on 40 to 42 week vitamin E deficient and control rats by Goss-Sampson *et al* (1988a). No differences were found between the two groups in the waveforms or conduction velocities of the SEPs recorded at the lumbar (L5) area i.e. responses from the peripheral (sciatic and tibial) nerves, which was similar to that reported by Bradley *et al* (1986). However, a significant increase in the latencies of the cortical response and a significant decrease in the central conduction velocities from the lumbar region to the somatosensory cortex, were reported in the vitamin E deficient rats compared with controls. The vitamin E deficient animals also produced generally less well-defined cortical SEP waveforms than the control group, indicating a possible defect in sensory processing between the spinal cord and the somatosensory cortex.

A longitudinal electrophysiological study has also been carried out in the rat (Goss-Sampson *et al*, 1990). This study reported a significant slowing of central conduction after 8 months of deficiency, whereas changes in peripheral conduction only became apparent after 11 months. They were, however, unable to show significant differences in the brainstem auditory evoked potentials or peripheral sensory motor responses between the vitamin E deficient and control rats over the one year period. The results of these studies are consistent with the neuropathology following chronic vitamin E deficiency in both humans and rats.

Abnormalities have been found in the electromyogram (EMG) of vitamin E deficient rats (Goss-Sampson *et al*, 1988a), suggesting an ongoing process of chronic partial denervation. This contrasts with the histological studies of vitamin E deficient rat muscles, which show a predominantly necrotising myopathy. Together, therefore, the findings suggest that both myopathic and neuropathic processes are occurring.

The effects of vitamin E deficiency on visual parameters were investigated by Goss-Sampson *et al* (1991a, 1998) in rats. They reported significant increases in the latencies and decreases in the amplitudes of the ERGs in the deficient animals compared with controls. Visual evoked potentials (VEPs) were also investigated. Significant differences in latencies but not amplitudes were noted between the deficient and control groups. These electrophysiological results suggested a causal relationship between vitamin E deficiency and deterioration of visual parameters.

1.10 Aims of the present study

In man, neural tissues appear to be most severely affected by a deficiency of vitamin E. Because both rat and man develop a similar neurological syndrome, the vitamin E deficient rat is accepted as an appropriate model for studying and assessing neurological function in man.

In this study objective measures of neural and visual function were made, using non-invasive electrophysiological techniques. The parameters were measured longitudinally in the same animals at monthly intervals for 14 months. The principal aims of the study were to

a) define the minimum requirements of vitamin necessary to prevent the neurological features of deficiency
b) compare the biological activities of all-rac- and RRR-α-tocopheryl acetate in neural tissues
c) investigate the effects of repleting deficient animals with vitamin E.

At the end of the study, concentrations of α-tocopherol and malondialdehyde (a marker of lipid peroxidation) were determined in a number of neural and non-neural tissues. This enabled correlations to be made between the electrophysiological and biochemical parameters.

Chapter 2

Materials & Methods

2.1 Animals

Weanling male Wistar rats (mean age +/- 1 SD of 21 +/- 7 days) were obtained from B & K Universal Ltd, UK. They were housed in groups of three in standard solid-bottomed cages, and kept in a room with controlled constant temperature (21 +/- 1°C) and relative humidity (55 +/- 5%). Fresh drinking water was provided *ad libitum* and each cage received 90g pelleted experimental diet per day (i.e. approximately 30g/rat/day). The animals were weighed weekly.

The animals were kept in accordance with Home Office guidelines, and all experimental procedures were carried out under appropriate Home Office project and personal licences.

2.2 Experimental diet

2.2.1 Introduction

Initially, vitamin E deficient diets were produced 'in house', but commercial preparations have become available during the last 15 years, which are cheaper and more convenient to use. The majority of these diets use either vitamin free casein or torula yeast as the main protein source, and stripped lard or corn-oil as the major fat source (Machlin *et al*, 1977; Vatassery *et al*, 1984). Most of these diets were presented as a sticky powder or slurry, which can easily fall through the bars of the food-hopper and become lost among the sawdust on the cage floor. For this reason,

the present study used pelleted diets which were supplied by Dyets Inc. (Pennsylvania, USA); this company having previously supplied defined vitamin E diets to our department.

2.2.2 Experimental diet

A synthetic vitamin E deficient diet based on a Machlin modified Draper diet (HLR 814) was used, the composition of which is given in Table 2.1. The composition of the salt and vitamin mixes, used in the diet preparation, are shown in Tables 2.2 and 2.3 respectively. Various levels of vitamin E were added to the deficient diet and these were distinguished by the addition of coloured dyes (see sections 4.1 and 5.1). The diets from both studies were analysed by high performance liquid chromatography following saponification and the concentrations of vitamin E were found to be generally close to the nominal values (mean of 10 diets 82%; range 51-107%).

Table 2.1 Composition of the vitamin E deficient diet supplied by Dyets Inc.

Ingredient	Grams/kg
Vitamin free casein	200.00
Dextrose	653.10
Salt mix #200650	40.00
Vitamin mix #302362	5.00
Choline bitartrate	1.81
Tocopherol stripped lard*	100.00

*Stabilised with 0.02% (w/v) butylated hydroxyanisole

Table 2.2 Composition of salt mix #200650*

Ingredient	Grams/kg
Calcium carbonate	163.56
Calcium phosphate, dibasic	355.56
Cupric carbonate	0.08
Ferric citrate, USP	16.00
Magnesium carbonate	40.89
Manganese sulphate H_20	1.38
Potassium citrate	236.53
Potaasium iodide	0.04
Potassium phosphate, dibasic	77.33
Sodium chloride	108.19
Zinc carbonate	0.44

*Draper *et al* (1964)

Table 2.3 Composition of Vitamin mix #302362*

Ingredient	Grams/kg
Vitamin A acetate (500 000 IU/g)	10.00
Vitamin D2 (500 000 IU/g)	0.80
Menadione $NaHSO_3$	0.32
Niacin	5.00
Calcium pantothenate	2.00
Riboflavin	1.00
Thiamin HCL	2.00
Pyridoxine HCL	1.00
Folic acid	0.20
Biotin	0.02
Vitamin B12 (0.1% w/v)	20.00
Dextrose	957.66

2.3 Electrophysiological measurements

2.3.1 Anaesthesia

Anaesthetic agents act on the nervous system and could potentially affect the electrophysiological parameters measured in this study. A pilot study was, therefore, undertaken to find an agent which had minimal and consistent effects. Four different anaesthetics were compared (see Chapter 3 for details), and it was concluded that fentanyl/fluanisone-midazolam was the most appropriate for longitudinal electrophysiological recordings.

The rats were anaesthetised with a combination of fentanyl/fluanisone (Hypnorm, Janssen, UK) and midazolam (Hypnovel, Roche, UK). One part Hypnorm (fentanyl-0.315mg/ml; fluanisone-10mg/ml) plus one part Hypnovel (5mg/ml) were mixed in the same syringe with two parts sterile water and administered by intraperitoneal (i.p.) injection at a dose of 2.7ml/kg. The depth of anaesthesia was regularly assessed by the reflex response to pedal and palpebral (corneal) stimulation. A heated blanket was used to warm the body and maintain rectal temperature between 35 and 37°C throughout the procedure.

All electrophysiological recordings were completed within 30 minutes from the induction of surgical anaesthesia (i.e. after the loss of corneal and pedal withdrawal reflexes). At the end of the test procedure, buprenorphine (Vetergesic, Reckitt & Coleman Products Ltd., UK) was injected to reverse the action of fentanyl, and thereby aid recovery of the rat.

2.3.2 Somatosensory testing

2.3.2.1 Introduction

The somatosensory system incorporates the senses to touch, pressure, position, movement, vibration, pain and temperature. The electrical responses recorded from the central nervous system following sensory stimulation are known as somatosensory evoked potentials (SEPs). These responses can provide an index of the conduction efficiency of the sensory pathways. The nervous system can be divided into the central system (CNS) incorporating brain and spinal cord, and the peripheral system incorporating the nerves of the body which connect to the CNS. By measuring lumbar SEPs (recorded at the level of the lumbar spine following lower limb stimulation), peripheral conduction through the tibial and sciatic nerves can be assessed. Peripheral conduction can also be investigated by recording cervical SEPs (recorded at the level of the cervical spine) following distal medial nerve (i.e. upper limb) stimulation. Central conduction, following stimulation of the upper or lower limbs, can be assessed by measuring the latencies of cortical SEPs (which are recorded over the somatosensory cortex). Other types of evoked potentials commonly studied are those produced in response to visual (VEP) or auditory (AEP) stimulation.

Sensory nerve conduction is usually measured orthodromically, i.e. in the direction of normal conduction, by stimulating distally and recording proximally. The potentials are evoked by distal electrical stimulation using a needle or a surface electrode. The elicited response following the electrical stimulus is recorded with a needle or a surface electrode, placed proximally along the sensory pathway from the point of stimulation. Needle electrodes tend to be used more often than surface electrodes in

experimental studies, since the former can be positioned more accurately and also record larger responses with better signal-to-noise ratios.

Evoked potentials are characterised by a series of peaks or waves, which are identified by their polarity, number in sequence, latency, amplitude, wave shape and surface distribution. Electrical stimulation of a nerve causes ion channels in the cell membrane to open, allowing sodium ions to flow into the cell and potassium ions to flow out. These changes in the intra- and extra-cellular ion concentrations cause a localised area of depolarisation, which is transmitted along the nerve as a wave. The recording electrode reflects the extracellular ionic flow (i.e. potential change) when the wave of depolarisation passes in proximity to it. This potential change is relatively small, 5mV or less, and can be obscured by electrical 'noise' from endogenous (ECG or EEG activities) or exogenous (50 or 100Hz electrical supplies and radio frequencies) sources. Up to 1000 responses may need to be recorded, amplified and computer averaged, in order to reliably isolate the actual nerve potential. This averaging method is based on the principle that stimulus-linked events will enhance as successive responses are added and averaged, while randomly occurring activity will tend to cancel out.

2.3.2.2 Recording equipment and data handling

A Medelec Sensor neurophysiological system (Medelec Ltd, UK) was used to deliver the stimulus for SEP measurements and average the responses. A Grass (PS22) photic stimulator (Grass Instruments, MA, USA) generated flashes for visual testing. The

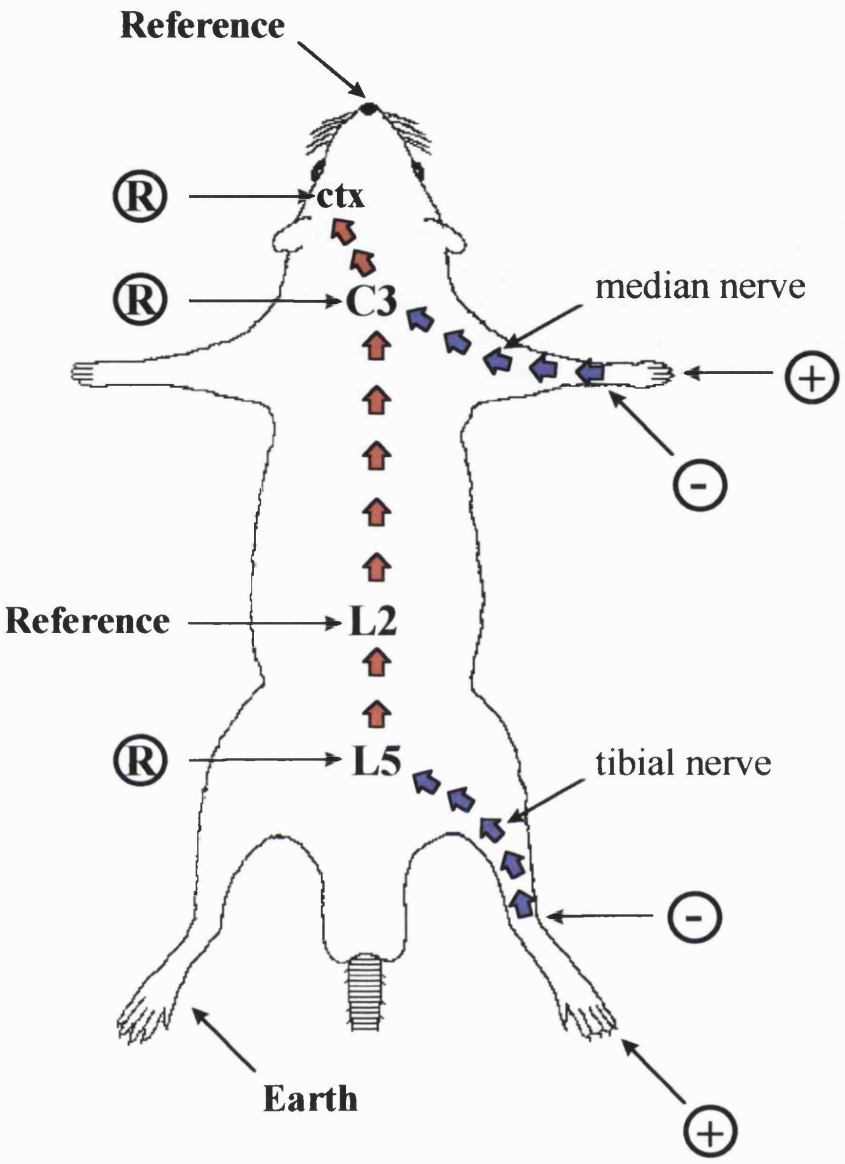
evoked potentials were amplified and averaged using the ER94a Sensor and responses were then transferred to a computer and stored on disk for further analysis.

2.3.2.3 SEP recordings following lower limb stimulation

Two stainless steel sub-dermal needle electrodes (Medelec Ltd, UK) were used to stimulate the tibial nerve distally, one (negative) inserted into the right ankle and one (positive) inserted into the right hindpaw (see Figure 2.1). The nerve was stimulated by a constant current of 1-1.5mA (to produce a maximal response) and 0.1ms duration delivered at $3s^{-1}$; 128 sweeps of 30ms duration were averaged for each run, and runs were repeated 4 times (the mean of these 4 runs was used for subsequent analysis). The lumbar evoked potentials were recorded from an electrode inserted between the 5^{th} and 6^{th} lumbar vertebrae (L5), which was referred to an electrode at L2. The cortical evoked potentials were recorded from an electrode inserted subcutaneously at the scalp over the contralateral somatosensory cortex, referred to an electrode inserted at the snout. An earth reference electrode was inserted into the left hindpaw. All electrodes were inserted to a depth of 1cm.

The neurophysiological convention whereby a downward deflection in the evoked potential represents a positivity, and an upward deflection represents a negativity, was used in the presentation of these results. Following stimulation of the tibial nerve at the tarsus, a consistent triphasic response was recorded at the lumbar region of L5 from all the rats in the different groups (Figure 2.2). This response was characterised by a prominent negative peak (LI), which represents a combined sciatic nerve and dorsal root response, followed by a large positive peak (LII) which corresponds to the early spinal response, including activation of the ventral horns (Magladery *et al*, 1951;

Figure 2.1 Electrode positions for SEP recordings



Peripheral pathways

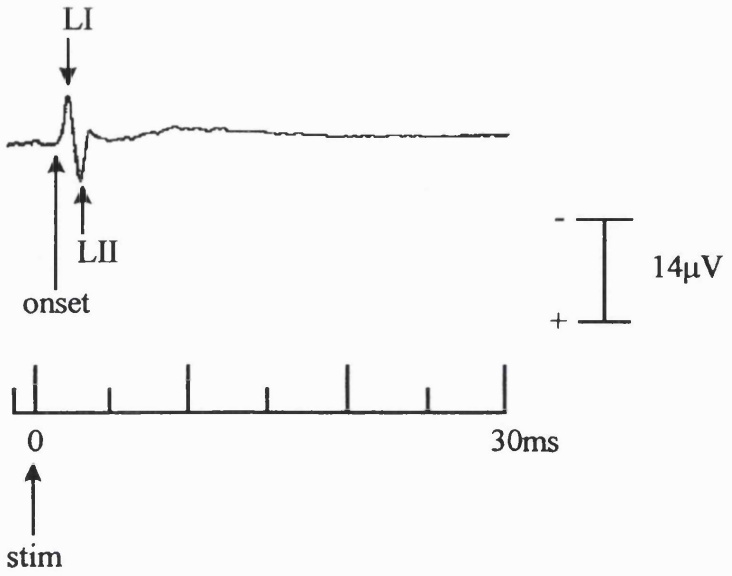
Central pathways

Recording electrode

Stimulating electrodes

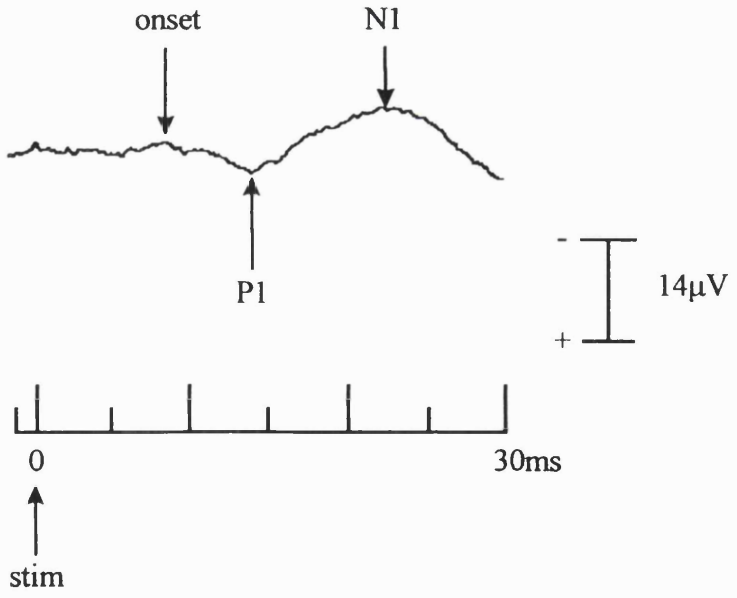
ctx Somatosensory cortex

Figure 2.2 Lumbar SEP waveform



LI peak represents combined sciatic and dorsal root response, and LII corresponds to the early spinal response, including activation of the ventral horns.

Figure 2.3 Cortical SEP waveform



P1 represents the first major positive peak and N1 the major negative peak.

Wietholter & Hulser, 1985). The 'onset' represents the time when the wave of depolarisation is first detected by the recording electrode at L5, typically 1.3-1.5ms after stimulation at the tarsus. Peripheral conduction velocities (pCV) were derived from the distance (mm) between the stimulating and recording electrodes (measured using calipers) and the time taken (ms) for the response (LI peak) to be picked up at the spine. The peak-to-peak amplitude in μV between the LI and LII components was used as a measure of the amplitude of the lumbar (L5) response.

A typical cortical SEP, recorded over the contralateral somatosensory cortex in response to tibial nerve stimulation, is shown in Figure 2.3. The SEP consisted of an initial positive peak (P1) followed by one of negative polarity (N1). The central conduction velocity (cCV) in metres/second was calculated using the following formula,

$$cCV = \frac{\text{distance between electrodes at L5 and cortex (mm)}}{\text{cortical onset latency - L5 onset latency - 2 (ms)}}$$

where the cortical onset latency is the time when the wave of depolarisation was first picked up at the scalp and the L5 onset latency is the same as described previously. Two milliseconds were subtracted to take account of synaptic delays at the gracile nucleus and the ventral lateral nucleus of the thalamus (Goss-Sampson *et al*, 1988).

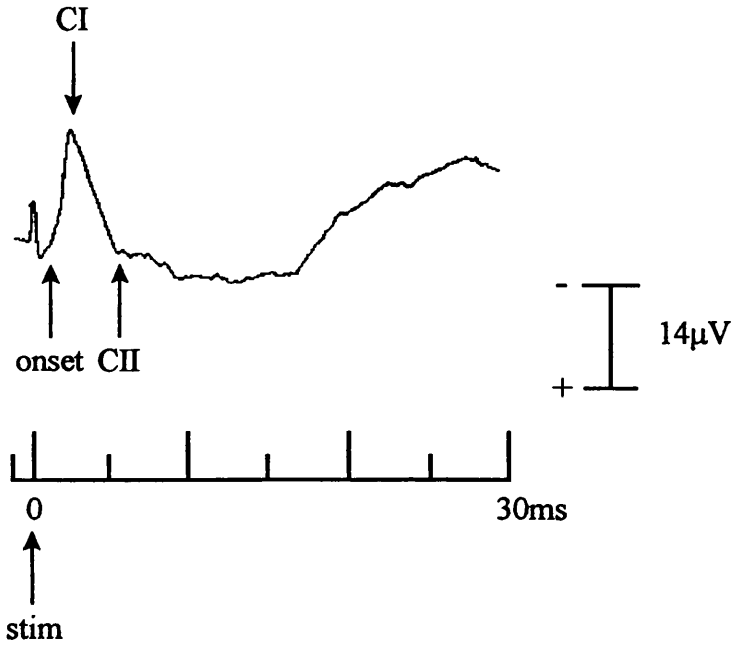
Cortical SEP amplitude was determined by calculating the peak-to-peak difference in μV between the onset and P1 components.

2.3.2.4 SEP recordings following upper limb stimulation

The median nerve was stimulated using a needle electrode inserted at the right wrist. An electrode inserted at the right forepaw (positive) acted as a reference to the stimulating electrode (see Figure 2.1). Cervical SEPs were recorded at the 3rd cervical vertebra (C3), and cortical SEPs at the scalp (as for the lower limb). Both of these recording electrodes were referred to a sub-dermal electrode at the snout. As before, an earth electrode was inserted into the left hindpaw. 128 sweeps of 30ms duration were averaged for each run, and runs were repeated 4 times.

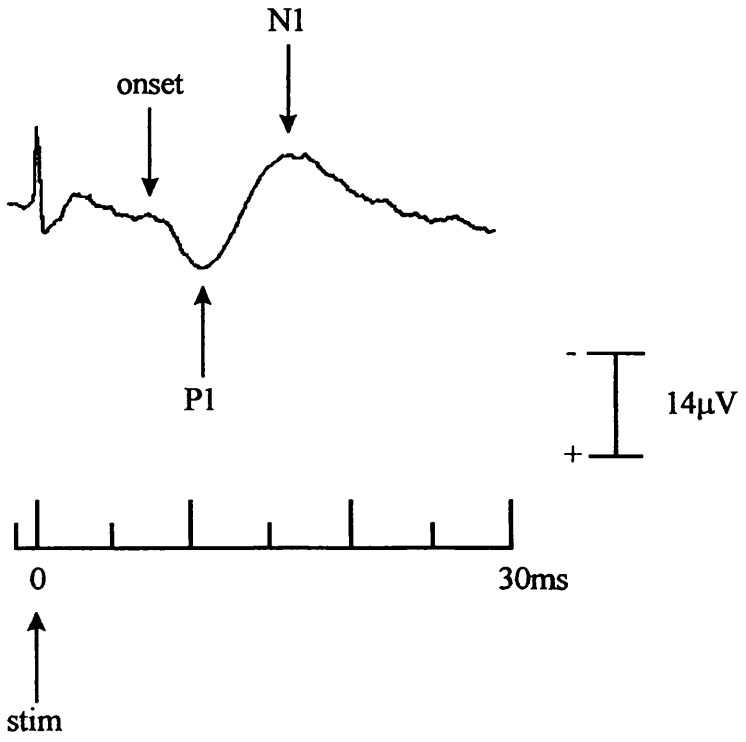
Figure 2.4 shows a typical cervical response - characterised by a large negative peak (CI) followed by a minor positive peak (CII). Peripheral CV in metres/second was determined by dividing the distance in millimetres between the stimulating and recording (C3) electrodes by the latency of the CI peak in milliseconds. The peak-to-peak difference in μV between the CI and CII components was taken as a measure of the SEP amplitude. The cortical response (Figure 2.5) had the same morphology to that obtained after lower limb nerve stimulation, but the latencies of all the components were shorter. Central CV and amplitude were calculated from this response in the same way as for the lower limb responses (see above).

Figure 2.4 Cervical (C3) SEP waveform



The 'onset' represents the time when the wave of depolarisation is first detected by the recording electrode at the 3rd cervical verterbra, CI the first major negative peak and CII the first positive peak.

Figure 2.5 Cortical SEP waveform



2.3.2.5 Reproducibility

To investigate the reproducibility of the effects of fentanyl/fluanisone-midazolam on SEPs, a group of 12 male adult Wistar rats (250-320g) were studied longitudinally for 4 successive months from 4-7 months of age. The animals were housed in groups of 3 in standard solid floor cages and fed a standard rat diet (rat maintenance diet RM1, Special Diet Servies, UK). The rats were provided with food and water *ad libitum*.

Peripheral and central SEPs were recorded in response to both upper and lower limb stimulation and the mean onset latencies compared (see Table 2.4). As can be seen, the responses were highly reproducible over time and there were no statistically significant differences between the monthly recordings.

Table 2.4 Onset latencies (ms) of somatosensory evoked potentials recorded in adult rats during anaesthesia with fentanyl/fluanisone-midazolam on four successive months (n=12)

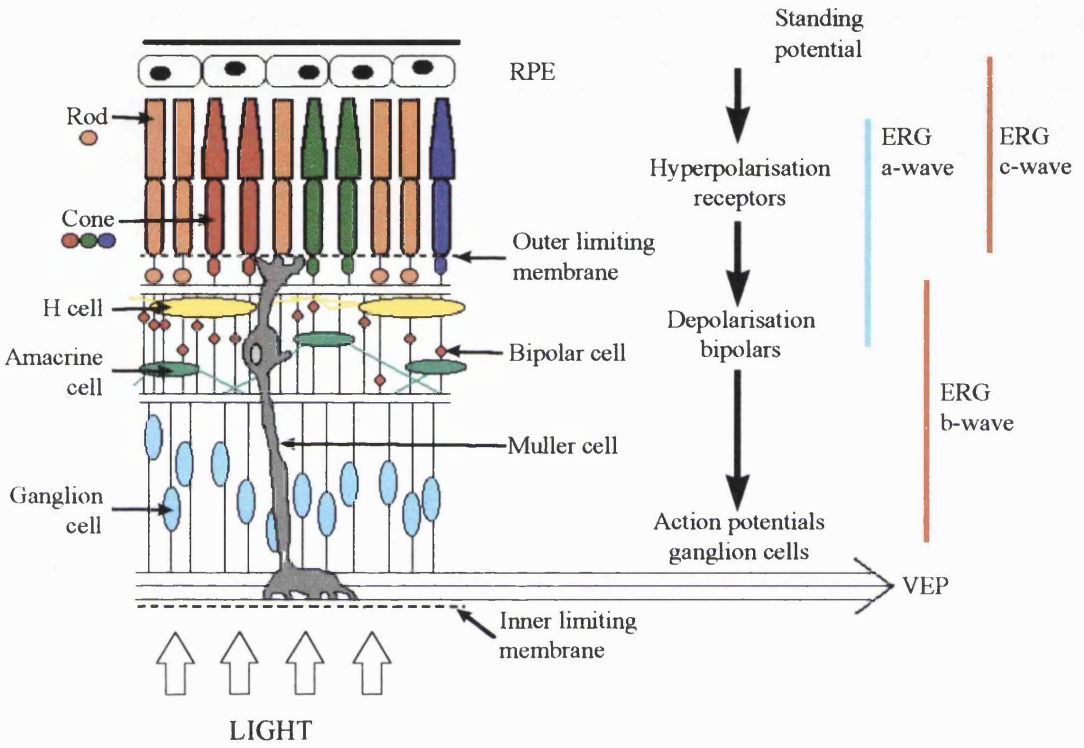
	AGE (months)							
	4		5		6		7	
Latency (ms)	mean	(SD)	mean	(SD)	mean	(SD)	mean	(SD)
Forelimb SEPs								
C3	1.38	(0.11)	1.36	(0.13)	1.39	(0.09)	1.40	(0.10)
cortex	3.69	(0.58)	3.67	(0.35)	3.70	(0.42)	3.57	(0.25)
Hindlimb SEPs								
L5	1.29	(0.09)	1.22	(0.08)	1.27	(0.04)	1.25	(0.11)
cortex	7.19	(0.54)	7.16	(0.78)	7.72	(0.78)	7.22	(0.89)

2.3.3 Visual testing

2.3.3.1 Introduction

The electrical responses of the eye to flash stimuli can be used to investigate neural events in the retina and visual cortex. The retina is the thin layer of neural tissue that lines the back of the eye and is responsible for the conversion of stimulation by light into neural excitations. Partial processing of the sensory information occurs at the retina and at the lateral geniculate nucleus, before the signal is relayed on to the visual cortex of the brain. The retina is a complex tissue, made up of several cell types, distributed in a number of different layers (see Figure 2.6). The outermost layer is the retinal pigment epithelium (RPE), which serves to maintain the health and normal functioning of the photoreceptors and absorb stray light. Anterior to the RPE lies the photoreceptor layer, which is composed of rod and cone cells. These photoreceptors are specialised cells which contain photopigments. Rod cells contain the photopigment rhodopsin which is sensitive to light under dim lighting conditions. Three different opsins have been identified in the colour sensitive cone cells of man: these are sensitive to wavelengths corresponding to blue, green and red light. Therefore, cone cells are responsible for the perception of colour, and are mainly found in the retinal area concerned with central vision. The layer adjacent to the photoreceptors is composed of a network of nerve cells, which include horizontal (H), bipolar, Müller and amacrine cells. The ganglion cells make up the innermost layer of the retina. Their axons converge at the optic disc and leave the retina as the optic nerve, synapsing at the lateral geniculate nucleus in the thalamus and then project on to the cortex. In the rat, most of the visual fibres of one eye project on to the contralateral visual cortex in the posterior part of the brain.

Figure 2.6 Schematic diagram of the retina showing relationship between main cell types and the generation of the ERG and VEP



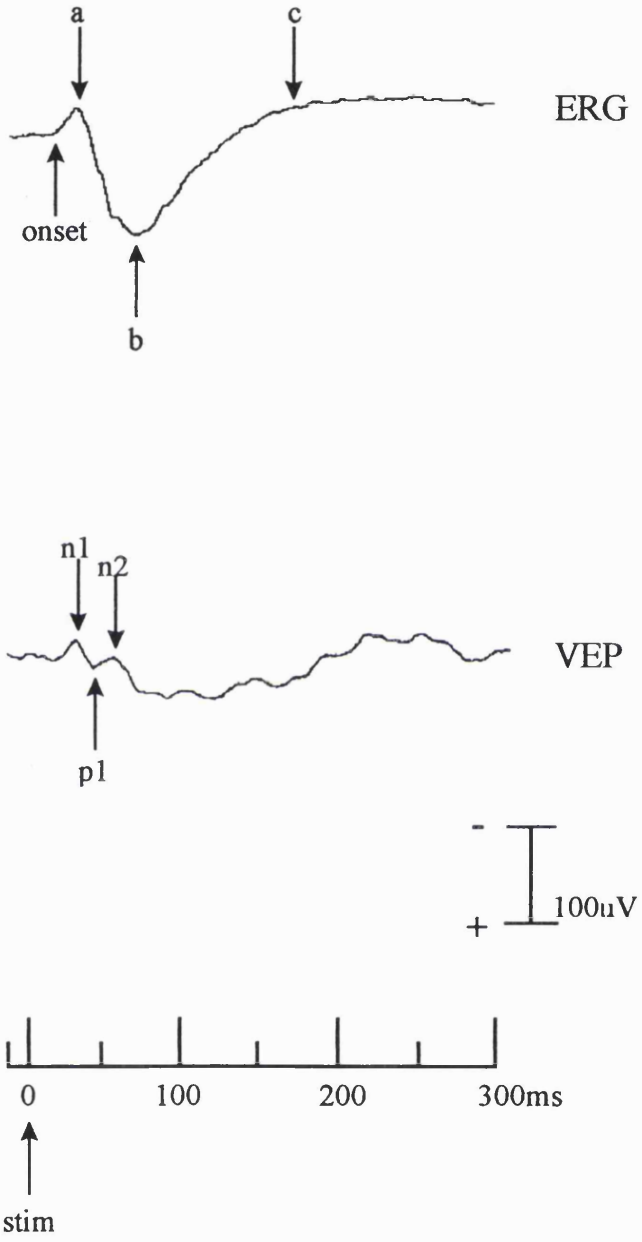
The flash ERG represents electrical activity arising in the outer, middle and innermost retinal layers. It has three major components: the a, b and c waves (see Figures 2.6 and 2.7). The first major negative component is the a wave, which reflects hyperpolarisation of the photoreceptors by incident light (Falk, 1991). A major class of bipolar cells are depolarised by photoreceptor activation, causing the release of potassium ions which are transported into the Müller cells, which are of glial origin. The spread of the ionic current associated with this process produces the positive b wave (Newman & Frishman 1991). This current flow also spreads into the distal parts of the Müller cells, with depletion of the amount of potassium between the receptors and the RPE. The ionic imbalance across the apical membrane of the RPE results in a positive wave which interacts with a small negativity produced by distal parts of the Muller cells, and the net result is the positive c wave (Griff 1991). The c wave follows the b wave, but this is not always reliably recorded by surface electrodes.

The VEP is recorded over the occipital scalp, at the level of the lambda suture between the ears. It has a complex waveform with 2 negative (n1 and n2) and a positive (p1) peak, as shown in Figure 2.7. The waveform reflects events in the visual cortex following the arrival of the afferent volley from the retino-geniculate pathway.

2.3.3.2 Measurement of ERGs and VEPs

Single flash ERGs and VEPs were recorded following induction of anaesthesia. The rats were dark-adapted for a minimum period of 16 hours before undergoing the test procedures, to enable maximal responses from rods. The recordings took place under fully darkened laboratory conditions. A 15W red light was used to enable accurate

Figure 2.7 Typical flash ERG and VEP waveforms



'a' reflects hyperpolarisation of the photoreceptors by incident light
'b' is the large positivity representing the spread of ionic current through Müller cells
'c' is the negative component often found after b wave, but difficult to reliably record with surface electrodes.

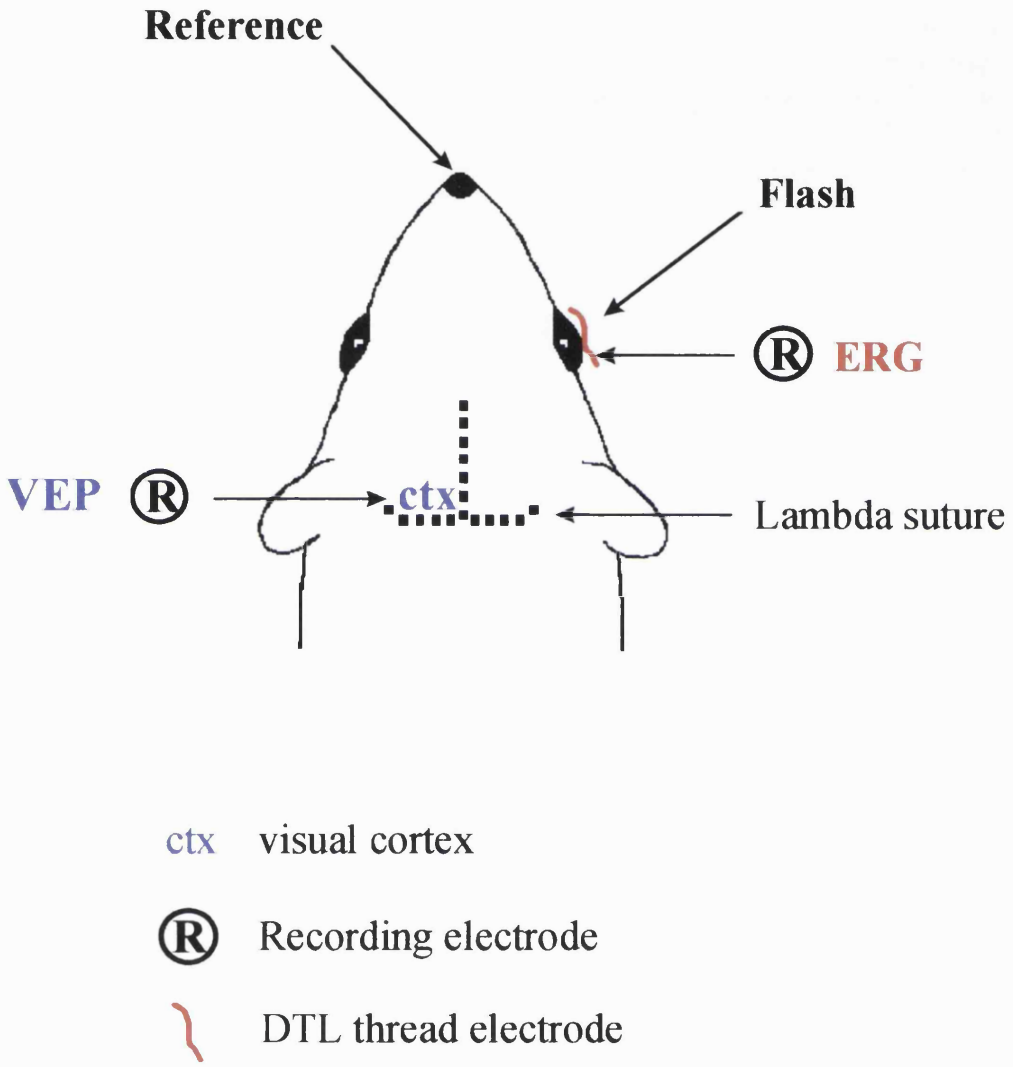
'n1' and 'n2' are the negative components, and 'p1' the major positive component of the VEP. These peaks relate to events within the visual cortex.

electrode placement, but this did not significantly affect dark adaptation and was switched off during the electrophysiological recordings. The rat's right pupil was dilated with 1% Tropicamide (Chauvin Pharmaceuticals Ltd, UK) and 1% methyl cellulose was applied to prevent damage to the cornea due to drying.

Flash ERGs were recorded using a DTL thread electrode (Dawson *et al*, 1979) placed across the upper aspect of the cornea of the right eye (see Figure 2.8). The reference was a needle electrode inserted at the snout. The flash VEP was recorded from an electrode inserted subcutaneously at the level of the lambda suture, and its reference was also the snout electrode. ERGs and VEPs were recorded simultaneously in response to a series of single flash stimuli of 10μs duration. The flash lamp was positioned 15cm above the right eye, in line with the visual axis, and flash stimulation was at a rate of 0.5Hz. The amplifier band width was set at 1-60Hz. Visual recordings were made in response to both dim blue (intensity 1, with blue filter placed over flash-lamp) and bright white (intensity 4, no filter) flashes. Dim flashes gave a response solely from rod cells, whereas the bright flashes gave a mixed rod and cone ERG. 16 responses were averaged for each run using the Medelec Sensor, and runs were repeated 4 times.

The latencies of the ERG b wave and VEP n1 peak were recorded. The difference in μV between the ERG a and b waves was used as a measure of the amplitude of the ERG response. VEP amplitude was calculated as the difference between the n1 and p1 peaks.

Figure 2.8 Electrode placement for visual electrophysiological recordings



2.4 Dissection procedure and tissue removal

The rats were killed by an intraperitoneal injection of 60mg sodium pentobarbital (Sagatal, May & Baker, UK). The thoracic cavity was opened and approximately 5ml blood taken by cardiac puncture and plasma separated. A cannula was then inserted into the aorta, via the left ventricle, and 250ml ice cold physiological saline perfused through the tissues at a rate of 40ml/min. The right atrium was cut to allow the blood and perfusate to flow away. Once removed, all tissues were stored at -70°C prior to analyses.

2.4.1 Brain removal

The brain was separated from the spinal cord by means of a cut between the occipital and the highest cervical vertebra (C1). Using fine bone clippers, the interparietal, parietal, squamosal and surrounding bones were removed, leaving the whole brain exposed. The brain was then separated from the olfactory lobes and gently teased away from the base of the skull. After cutting the optic nerves and trigeminals, the whole brain could be removed intact and kept on ice prior to further dissection.

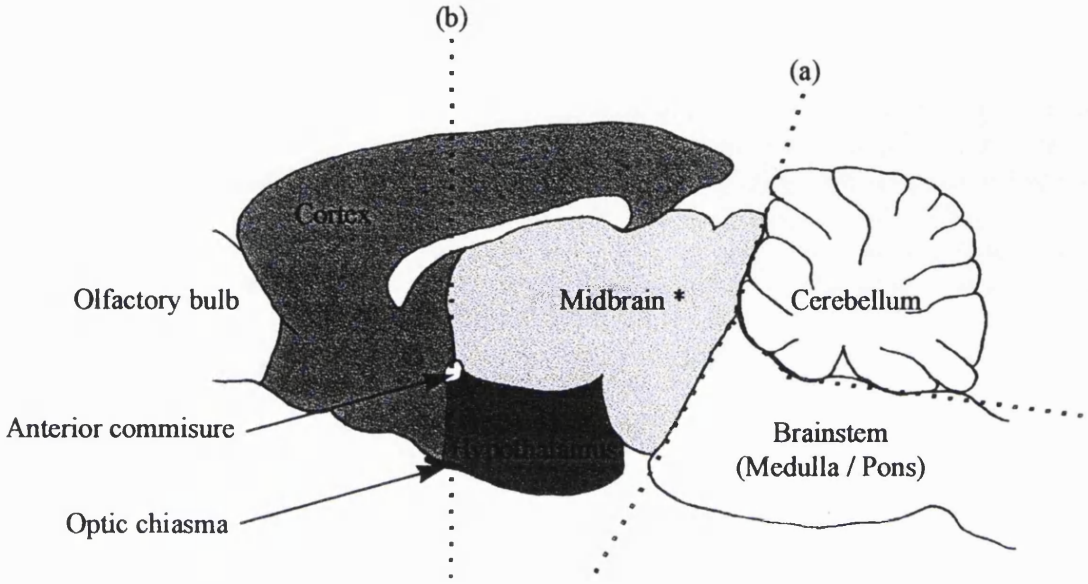
2.4.2 Brain dissection

Each brain was divided into 6 distinct anatomical regions by a similar method to that reported by Glowinski & Iversen (1966). The regions were the cerebellum, medulla/pons, striatum (including the putamen nuclei, caudate nuclei and basal nuclei), hypothalamus, midbrain (including the thalamus and subthalamus) and cortex.

With the dorsal surface facing upward, a tranverse cut ((a) in Figure 2.9) was made between the thalamic regions and the area incorporating the cerebellum and brainstem. The cerebellum was then separated from the brainstem - which incorporates the medulla oblongata and the pons.

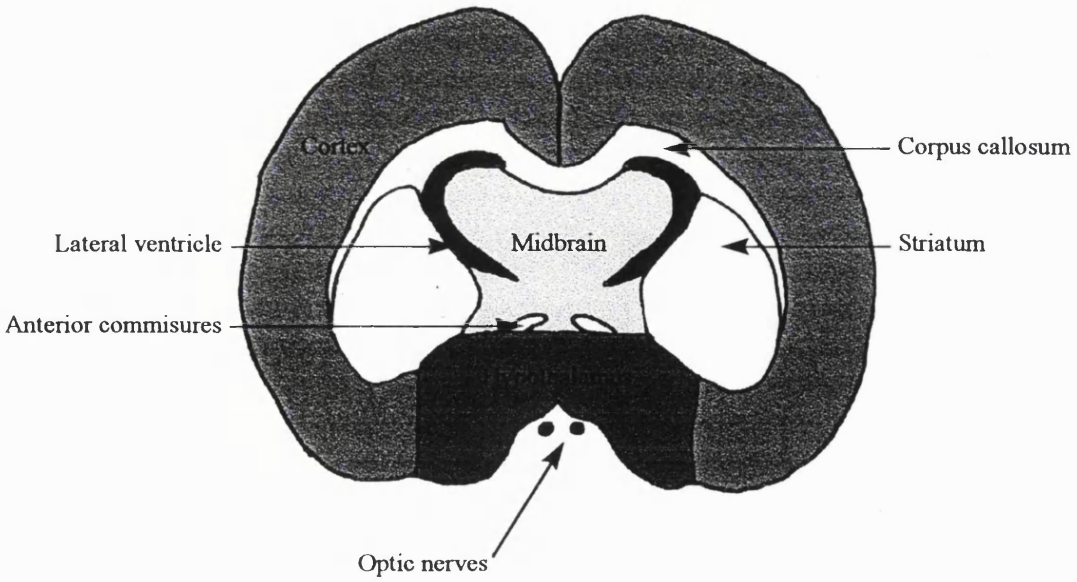
The brain was then turned onto its ventral surface and the forebrain separated from the rest of the cerebral cortex, by means of a transverse cut (b) made at the level of the optic chiasma (Figure 2.9). The head of the caudate nucleus was removed from both sides of the forebrain and pooled with the striatum region, whilst the reminder of the forebrain was pooled with the cortex region. Using the anterior commisures as a horizontal reference, the hypothalamus was dissected out from the midbrain (shown in coronal aspect in Figure 2.10). The striatum regions were removed from both sides using the lateral ventricles as the internal reference and the corpus callosum as the external reference. Finally, the midbrain was carefully separated from the rest of the cortex.

Figure 2.9 Brain dissection: Saggital aspect



* Including Thalamus / Subthalamus

Figure 2.10 Brain Dissection: Coronal aspect (Bregma 9.30mm)



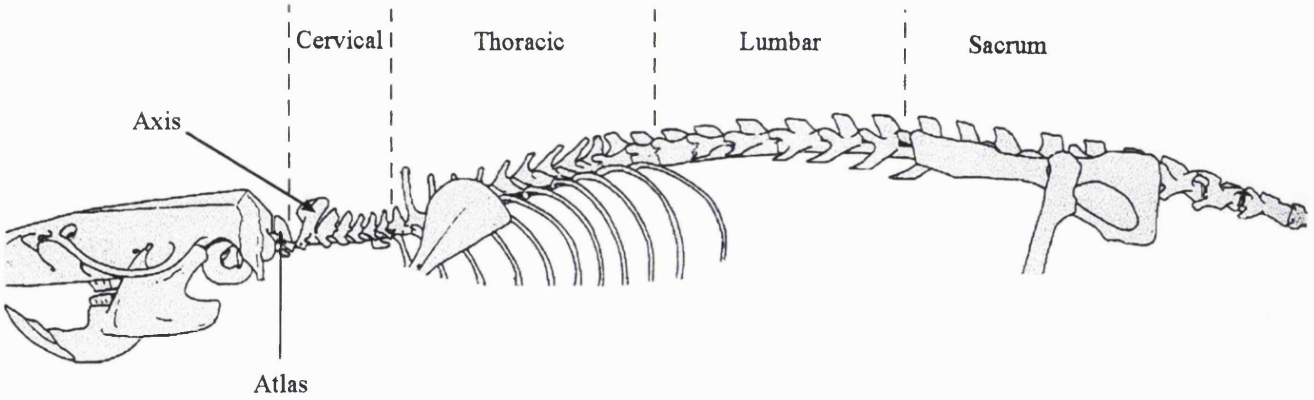
2.4.3 Removal of neural tissues

Skin, muscle and nerves were cut away to reveal the spine. A transverse cut was made between the atlas and axis (first and second cervical vertebrae, respectively) to separate the cervical spinal cord from the brainstem (see Figure 2.11). The fact that thoracic vertebrae possess long dorsal processes, and are attached to the ribs, made separation of the cervical and thoracic regions possible. The rat has six lumbar vertebrae between the thoracic region and the sacrum (region of fused bone). A transverse cut was therefore made after counting six vertebrae up from the sacrum (see Figure 2.11). In this way, the thoracic and lumbar regions of the spinal cord could be reliably divided. The right hindlimb sciatic and tibial nerves were dissected away from surrounding tissues and removed. The eyeballs were also removed for analysis.

2.4.4 Non-neural tissues

The heart, liver, adrenals, testis and gastrocnemius muscle were also removed.

Figure 2.11 Spine of rat, showing landmarks for sectioning the cord



2.5 Biochemical Assays

2.5.1 Tissue α-tocopherol concentrations

2.5.1.1 Measurement of α-tocopherol in tissues and plasma

The concentration of α-tocopherol present in the tissues was measured using high performance liquid chromatography (HPLC) with fluorimetric detection, by a modification (Metcalfe et al. 1984) of the method of Buttriss and Diplock (1983). This method has been validated and used extensively in our Department for the estimation of α-tocopherol concentrations in plasma samples and experimental tissues. Approximately 100mg of tissue was homogenised in 1ml 75% ethanol (Hayman Ltd. UK) using an Omni-TH Homogeniser until a consistent homogenate was formed (30s - 3min, depending on tissue). Hydrophobic species were extracted into 1ml of HPLC grade hexane (Rathburn Chemicals, UK) by vortex mixing for 1min. The samples were then centrifuged at 2500rpm for 10min. Typically 20µl of the hexane phase was injected onto a 15cm, 3µm silica direct phase column with an internal diameter of 4.6mm (Jones Chromatography Ltd). The mobile phase was hexane containing 1% methanol (1ml/min). Any water present in the mobile phase was removed with 4 angstrom molecular sieves. Fluorescence was monitored with a Shimadzu RF-535 fluorimeter using an excitation cut off filter at 290nm and a 325nm emission filter.

To determine the α-tocopherol concentration in plasma, 200µl plasma replaced 100mg tissue in the above method and no homogenisation was required.

2.5.1.2 Standardisation, reproducibility and sensitivity of method

Unknown concentrations of α-tocopherol were determined by direct comparison with 5μM all-rac-α-tocopherol (Sigma UK, in hexane), used as an external standard.

To ensure consistent results, a number of quality control samples (QCs) were injected onto the HPLC at various points during each run. These QCs were outdated plasma samples from normal healthy subjects, obtained from the Great Ormond Street Hospital blood bank, which underwent the same extraction procedure as the test samples. Previously, 10 QC samples were extracted each day for 3 consecutive days and the α-tocopherol concentrations of each determined. From the results, a QC range (mean +/- 2SD) was identified. In subsequent test runs, a QC sample was injected onto the HPLC column every sixth sample. If the α-tocopherol concentration of the QC fell within range, the results for the test samples were deemed acceptable. In this way, reproducibility within day and between days was ensured.

The detection limit for α-tocopherol was apparently 1pmol, equivalent to approximately 0.25μg/g wet weight. When a tocopherol peak could not be detected, 750μl of the hexane phase was aspirated and concentrated to dryness under a constant stream of nitrogen. It was then reconstituted with 100μl hexane, resulting in a 7.5 fold increase in the α-tocopherol concentration of the hexane phase. In this way, samples with very low α-tocopherol concentrations could be assayed.

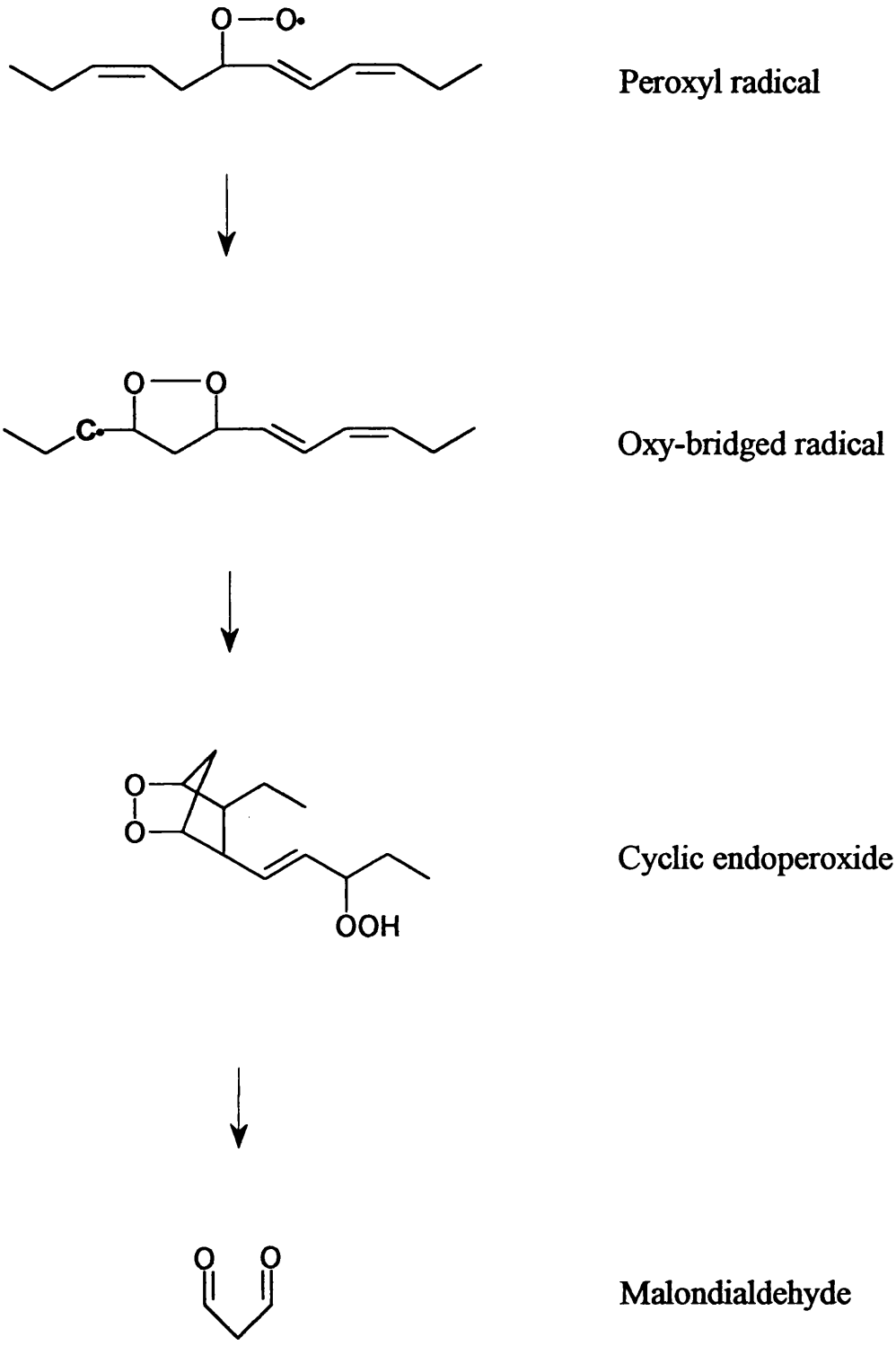
2.5.2 Malondialdehyde

2.5.2.1 Introduction

A variety of methods exist to assess lipid peroxidation in tissues. The most direct approach is the measurement of primary products (hydroperoxides). However, since these species are unstable, lipid peroxidation is more often assessed using indirect methods which determine concentrations of secondary or end-products derived from the metabolism of hydroperoxides. An example is malondialdehyde (MDA), which has been extensively studied and its concentration is the most frequently quoted index of lipid peroxidation. MDA is a volatile, low molecular weight, three carbon compound which is found either in its free form or bound to a variety of molecules such as proteins and DNA. It is formed from lipid peroxides of fatty acids with three or more double bonds via cyclisation of the lipid peroxyl radical to form an oxybridged radical, followed by the formation of a cyclic endoperoxide, which subsequently decomposes to MDA (see Figure 2.12).

There are methods available which either measure MDA directly or measure a derivative of MDA. The most frequently used method uses the latter approach and exploits the reaction of MDA with thiobarbituric acid (Kohn & Liversedge, 1944). Under conditions of low pH and elevated temperature, this reaction produces fluorescent and pigmented adducts (thiobarbituric acid reactive substances - TBARS) which can be easily quantified (Nair & Turner, 1984). However, other low molecular weight aldehydes can also react with thiobarbituric acid making this method non-

Figure 2.12 Formation of malondialdehyde



specific (Kosugi & Kikugawa, 1989). In addition, the severity of the reaction conditions causes increased lipid peroxidation and consequently increased MDA concentrations (Asakawa & Matsushita, 1980).

In this study, a direct and specific assay for MDA using HPLC with UV detection was employed based on a modification (MacEvilly and Muller, 1996) of the method of Esterbauer *et al* (1984), which exploits the fact that in aqueous solution at pH > 7.0, MDA exists as an enolate ion and can therefore be separated on an aminophase HPLC column. This method had been previously used and validated in our Department for various rat tissues (MacEvilly & Muller, 1996).

2.5.2.2 Measurement of tissue MDA concentrations

Free and total malondialdehyde (MDA) concentrations were determined essentially as described by MacEvilly and Muller (1996). Separations were carried out on a 4.6 x 250mm Spherisorb S5NH2 column (Waters, UK) using a mobile phase of 0.03M trizma, pH 7.4 / acetonitrile (21:79 v/v) at a flow rate of 1ml/min (Shimadzu LC-10AS pump). The detector (LDC Analytical SpectroMonitor 3100) was set at a wavelength of 267nm. The injection volume was 20µl. Due to the small size of rat tissues and also time constraints, a single extract (i.e. no replicates) from each sample was assayed. However, out-lying results could be easily identified as tissues from 6 rats in each treatment group were analysed.

Approximately 100mg of tissue were homogenised in 1ml 40mM trizma pH 7.4 until a consistent homogenate was obtained (30 - 90s). To extract free MDA, equal volumes of homogenate and acetonitrile were vortexed in an eppendorf tube before spinning for

5 minutes at 13 000rpm in an eppendorf microfuge. The supernatant was aspirated off and kept at room temperature until injected onto the column (see below for stability studies).

MDA is capable of binding to various biomolecules including proteins and DNA, and this bound fraction was estimated by subtracting the free MDA concentration from the total concentration, estimated by a modification of the alkaline hydrolysis method of Lee and Csallany (1987). In brief, 80µl homogenate was mixed with 10µl promethazine (0.5mg/ml in 40mM trizma pH 7.4) and 12µl NaOH (saturated NaOH diluted 1:4 with dH_2O), and then incubated in the dark at 60°C for 70min. Promethazine was added as an aqueous antioxidant to prevent oxidation taking place during the procedure. After cooling to room temperature, 17µl HCL (concentrated HCL diluted 1:4 with dH_2O) was added. The sample was vortexed for 30s before spinning for 5min at 13 000rpm in an eppendorf microfuge. The supernatant was aspirated off and kept in the dark until injection onto the column (see below for stability studies). The supernatants were analysed on the same HPLC system as used to measure free MDA. All results were expressed as nmol MDA per mg protein (see 2.5.2.6 for protein assay).

2.5.2.3 Standardisation

An MDA standard of known concentration was prepared from 1,1,3,3-tetramethoxypropane (TMP, supplied by Sigma UK) by acid hydrolysis. 1,1,3,3-TMP was diluted 1 in 500 (v/v) with 1% sulphuric acid (total volume 50ml) and left at room temperature for two hours. A stock standard solution was prepared by diluting this

initial solution 1 in 100 (v/v), using 1% sulphuric acid (total volume 50ml). The precise concentration of the stock solution was determined spectrophotometrically (molar extinction coefficient = 13750 at 245nm) using a CE 2021 spectrophotometer (Cecil instruments, Cambridge, UK), and was usually found to be approximately 100µmol/l. Stock standard solutions were prepared weekly and stored at 4°C. Working standards were prepared daily by dilution of the stock with 0.1M trizma pH 7.4/acetonitrile (50:50 v/v). Calibrations were made using a three point standard curve, with a concentration range 1.0 to 5.0µmol/l. A typical standard curve is shown in Figure 2.13.

2.5.2.4 Sample storage

Since the HPLC run time for each sample was 30 min (free MDA) and 50min (total MDA), storage experiments were undertaken to determine the stability of the samples over the length of a typical HPLC run. Liver from rats fed a vitamin E deficient diet was used for storage and reproducibility tests.

a) Homogenate stability

Liver was homogenised (3 replicates for each treatment) as described above. Free MDA was either extracted immediately after the homogenate had been prepared or after 6 hours storage at 4°C, -30°C or -80°C. The results are shown in Figure 2.14. An increase in the MDA peak areas (22-149%) was seen after storing the homogenates, compared to the areas obtained from the extracts prepared immediately after homogenisation. It was therefore decided to perform the extraction procedure immediately after preparing the homogenate.

Figure 2.13 MDA standard curve

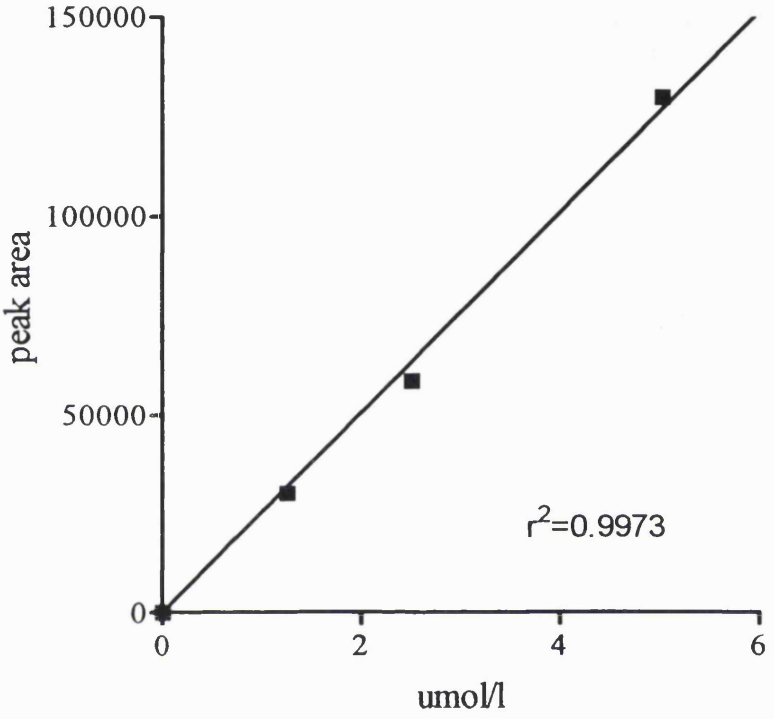


Figure 2.14 Homogenate stability (free MDA)

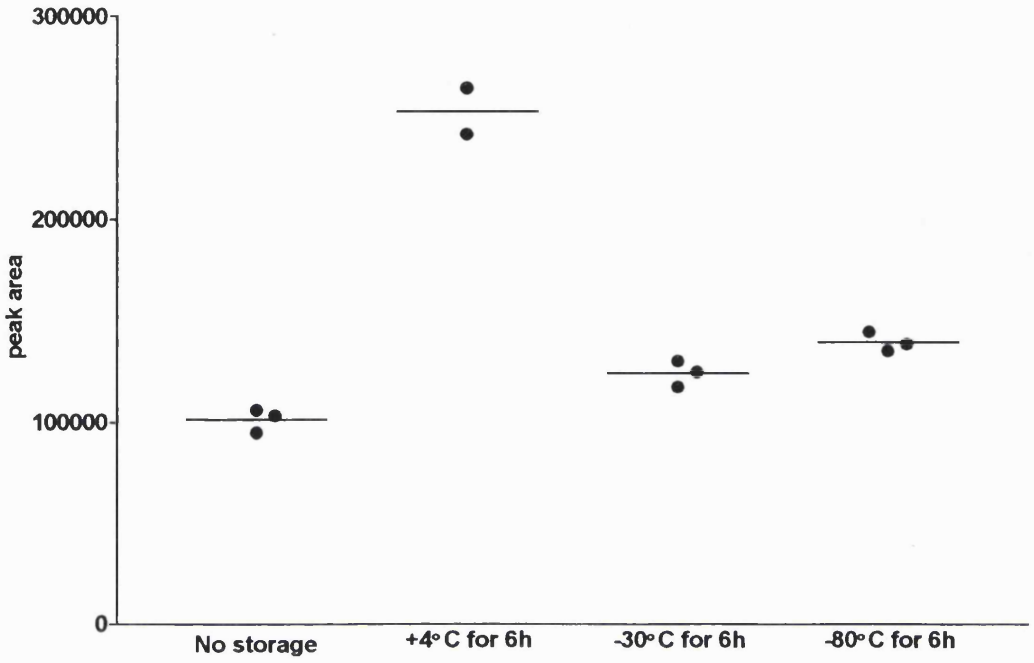
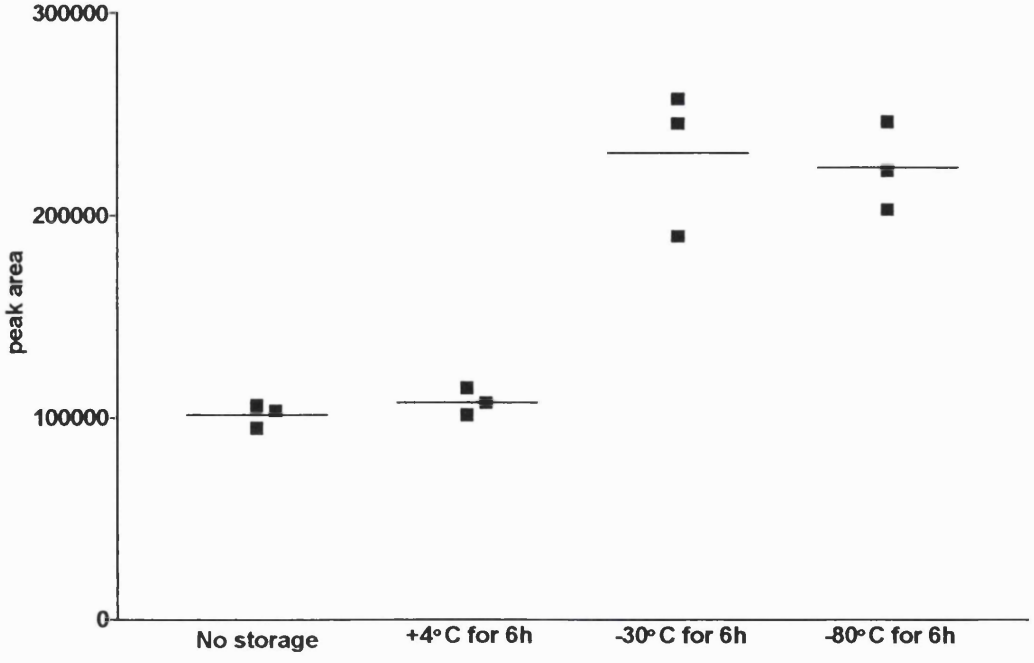


Figure 2.15 Extract stability (free MDA)



b) Extract stability

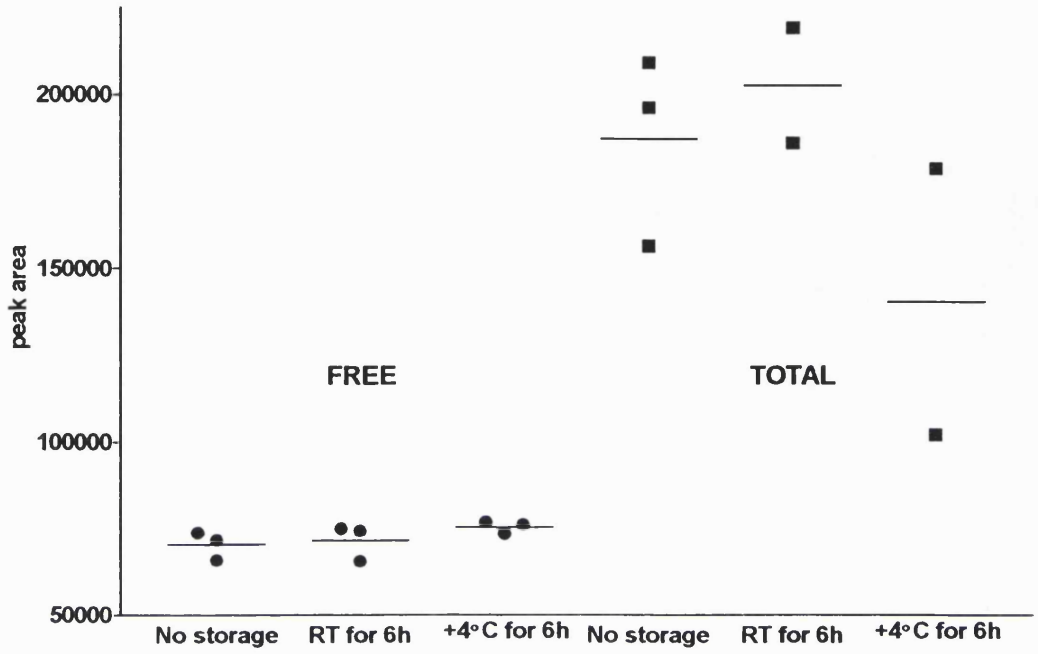
Replicate extractions (n=3 for each treatment) were immediately prepared from a fresh liver homogenate and analysed for free MDA either immediately or after storage for 6 hours at 4°C, -30°C or -80°C. The results, shown in Figure 2.15, indicated that the samples stored at 4°C gave similar results to those analysed immediately. Increased MDA concentrations (120-127%) were detected in the extracts which had been stored at either -30 or -80°C, probably due to increased lipid peroxidation during thawing. The experiment was then repeated with free and total MDA extracts, stored at either room temperature or 4°C for 6 hours. The results are shown in Figure 2.16 and indicated that both free and total MDA extracts were stable kept at either room temperature or 4°C for this length of time.

In subsequent MDA analyses, homogenates were prepared fresh each day and the extraction procedure performed immediately. Samples were then stored at room temperature until injected onto the column, which was typically no longer than 4 hours.

2.5.2.5 Validation and reproducibility of method

The reproducibility of the free MDA extraction method was assessed by calculating the variability of replicate extractions. Ten extractions of free MDA were prepared from a single liver homogenate; the mean MDA concentration was 1.12nmol/mg protein with a coefficient of variation (cv) of 4.64%. Free MDA was also extracted

Figure 2.16 Free and Total MDA extract stability



from six different homogenates, prepared from a single liver sample. This gave a mean MDA concentration of 0.41nmol/mg protein with a cv of 21.26%.

The between-day variation was also assessed. Free MDA concentrations in 4 replicates, extracted from a single liver homogenate, were determined on 9 occasions over 9 successive days. A fresh homogenate was used each day. The mean (n=36) free MDA concentration over the 9 days was 1.17nmol/mg protein with a cv of 31.81%. These results represent the combined variation of the extraction procedure and MDA determination by HPLC.

To ensure consistency between runs, free MDA extractions from α-tocopherol deficient liver homogenates were used as QCs. The homogenate was prepared fresh each day, and liver from the same animal was used for each series of analyses. QCs

were injected onto the column at the beginning, middle and end of each run of free and total MDA analyses.

Extracts of tissues from rats in the different dietary groups were mixed in each run, to ensure that within day and between day variation did not account for observed differences.

2.5.3 Bicinchoninic Acid Protein Assay

The protein assay used was based on the method of Smith *et al* (1985). In brief, 5µl homogenate (100mg tissue in 1ml 40mM trizma pH 7.4 as before) was made up to 50µl with dH_2O and 1ml bicinchoninic acid (Sigma UK) added. After mixing, the solution was incubated at 37°C for 10min. 20µl copper sulphate solution (4% w/v $CuSO_4.5H_2O$, Sigma UK) was then added and the mixture incubated at 37°C for a further 20 min. The absorbance at 562nm was determined using a CE 2021 spectrophotometer (Cecil instruments, Cambridge, UK). The protein concentration was calculated from a standard 5 point calibration curve with a concentration range between 0 and 1mg/ml using bovine serum albumin (BSA, Sigma UK) as a standard. Curves were always linear with r^2 consistently >0.995.

Chapter 3

Effect of anaesthesia on electrophysiological parameters

3.1 Introduction

A wide range of chemical agents with a diverse range of chemical structures can, under appropriate conditions, lead to a state of anaesthesia. At present, however, there is little agreement about the site and mode of action of general anaesthetics.

The brain stem reticular formation (BSRF) is believed to be important in the generation of the anaesthetic state, since its role in co-ordinating sensory and motor mechanisms related to the level of arousal in animals was elucidated by Magoun and his co-workers (1949). The BSRF is a complex of discrete cellular regions in the medial part of the brain stem, which includes the medulla, pons and mesencephalon (Angel, 1993). Magoun *et al* (1946) showed that the BSRF controlled the excitability of spinal motor neurones. High frequency electrical stimulation gave rise to generalised electrocortical arousal, while low frequency stimulation produced generalised electrocortical synchronisation and sleep 'de-arousal' (Moruzzi & Magoun, 1949). During anaesthesia, the reticular influences on the basal ganglia, cerebellar and motor cortical neurones appear to impede the production of volitional movements since the ascending sensory information from kinaesthetic receptors is disrupted at its thalamic site of transfer (Angel, 1993). The planning (basal ganglia), programming (cerebellum) and executive motor neurones are thereby deprived of their appropriate information and its transfer. Reticular influences on vestibulospinal, cerebellar and spinal motor neurones can be used to explain disruption in high level (righting and protective) reflexes, and in stretch reflexes.

Anaesthetic agents appear to act primarily by modifying the transfer of information across the thalamic sensory relay synapse (Angel, 1993). For most anaesthetics, including ketamine and isoflurane, this results in a decrease in the amount of information entering the cerebral cortex which seems to be associated with diminished conscious awareness of sensory messages. For α_2 adrenoceptor agonists (e.g. medetomidine), there is an increase in the overall response rate of thalamic sensory relay neurons, but the timing of signals transferred to the cortex is disrupted, which has been interpreted as 'garbling' of the transmitted information (Mason & Angel, 1983). The effect of reduced or garbled transmission of information along the sensory pathway has been used to explain the occurrence of amnesia commonly found following anaesthetic administration.

Anaesthesia can be divided into a number of components including drug-induced loss of consciousness or hypnosis and analgesia. There is a wide range of anaesthetic agents available commercially and these vary in their hypnotic and analgesic properties. The somatosensory evoked potential (SEP) has been shown to be influenced by the hypnotic properties of an anaesthetic (Thornton *et al*, 1992) and affected to varying extents by different anaesthetic agents. However, vitamin E deficiency also leads to changes in evoked potential latencies and amplitudes due to neuropathological changes in nerves. In order to carry out repeated longitudinal studies of vitamin E deficiency in rats, it was therefore necessary to find an agent which had the least deleterious effects on SEPs, and also on the general welfare of the animal.

The effects of ketamine-xylazine and pentobarbitone on somatosensory, brainstem auditory and peripheral sensory-motor potentials have been compared previously in the rat (Goss-Sampson & Kriss, 1991). Neither of the anaesthetic agents had an effect on peripheral sensory-motor conduction or the early components of the brainstem auditory response, but pentobarbital caused an increase in the latencies of the initial positive and following negative peaks of the cortical SEP. Ketamine-xylazine produced less profound and shorter-lasting effects on cortical evoked potentials. This agent, however, is no longer recommended for use in rodents since it causes an increase in blood pressure and muscle tone, and severe respiratory depression which can sometimes be fatal (Flecknell 1996). In addition, the high dose rates required for surgical anaesthesia lead to a slow recovery. A pilot study was, therefore, undertaken to find an appropriate anaesthetic to replace ketamine-xylazine and the results of this study have subsequently been published (Hayton *et al*, 1999).

The effects of the following four anaesthetic agents on the latencies and amplitudes of SEPs were compared:

1) ketamine-xylazine - an injectable agent;
2) medetomidine - an injectable agent which is reversed by atipamezole;
3) isoflurane - a gaseous anaesthetic;
4) fentanyl/fluanisone-midazolam - an injectable agent which is partially reversed by buprenorphine.

Ketamine-xylazine has been previously shown to cause a dose-dependent increase in the latencies and decrease in the amplitudes of cortical SEPs in rats (Angel & Gratton 1982, Goss-Sampson & Kriss 1991). In humans, however, an increase in the cortical

SEP amplitudes has been reported in response to ketamine administration whereas cortical SEP latencies were unaffected (Schubert *et al*, 1990). The effects of medetomidine on SEPs do not appear to have been previously reported. Isoflurane has consistently been shown to increase the latencies and decrease the amplitudes of cortical SEPs in humans (Sebel *et al* 1986; Thornton *et al* 1992), and in rats (Berg-Johnsen & Langmoen, 1986). These SEP changes are similar to those reported for other volatile anaesthetics (Thornton *et al* 1992). To date, there have been no previous studies which have compared the effects of inhalation and injectable anaesthetic agents on SEPs. Fentanyl, given alone to human adults, had no effect on the latencies or amplitudes of median nerve SEPs (Loughnan *et al* 1987). Midazolam has been reported to cause a small increase in the latencies and decrease in the amplitudes of cortical SEPs in humans (Sloan *et al* 1990). A combination of fentanyl/fluanisone and midazolam produces profound analgesia and good muscle relaxation in rats, with a low intra- and post-anaesthetic mortality rate (Flecknell & Mitchell 1984). There have been, however, no previous studies on the effects of this anaesthetic regimen on SEPs in rats.

3.2 Materials & Methods

3.2.1 Experimental animals

Conventional male Wistar rats (250-320g) were obtained from B&K Universal Ltd, UK and housed, in groups of two or three animals, in standard solid-bottomed rat cages. The rats were kept at a controlled temperature of 21 $\pm$ 1°C, relative humidity of

55 ± 5% and a 12/12h light/dark cycle. Fresh drinking water and pelleted diet (Rat Maintenance Diet RM1, Special Diet Services, UK) were provided *ad libitum*.

The rats were arbitrarily assigned to one of four groups (n=8), with each group receiving a different anaesthetic regimen, as follows:

1) A combination of 90mg/kg ketamine (Vetelar, Parke-Davis Veterinary, UK) and 10mg/kg xylazine (Rompun, Bayer, UK), mixed in the same syringe, and administered intraperitoneally (i.p.).
2) Medetomidine (Domitor, SmithKline Beecham, UK) administered at 0.3mg/kg intramuscularly (i.m.) with atipamezole (Antisedan, SmithKline Beecham, UK) given at 1mg/kg i.p. to reverse the anaesthesia.
3) Isoflurane (Abbott Laboratories, UK) administered in oxygen at a flow rate of 2L/min via an induction chamber (IMS, UK). A concentration of 5% isoflurane was used to induce anaesthesia and 2% for maintenance.
4) A combination of fentanyl/fluanisone (Hypnorm, Janssen, UK) and midazolam (Hypnovel, Roche, UK) was administered. One part Hypnorm (fentanyl 0.315mg/ml; fluanisone 10mg/ml) plus one part Hypnovel (5mg/ml) were mixed in the same syringe with two parts sterile water (Merial Animal Health Ltd, Dublin) and administered at a dose of 2.7ml/kg i.p. To aid recovery, buprenorphine (Vetergesic 0.3mg/ml, Reckitt & Coleman, UK) was injected at 0.1mg/kg i.m.

The drug concentrations chosen were the minimum required to produce surgical anaesthesia in the rat (Flecknell 1996). The depth of anaesthesia was regularly assessed by testing behavioural responses to pedal and palpebral (corneal) stimulation. Rectal temperature was maintained throughout between 35°C and 37°C by placing a

heated blanket under the rat. Each animal was given 3ml physiological saline i.p. to correct for fluid loss during the procedure.

The rats were weighed before being anaesthetised and on each of the following 5 days. The time taken to induce a surgical level of anaesthesia (i.e. loss of corneal and pedal withdrawal reflexes) and to regain the righting reflex were recorded, together with any adverse effects displayed by the animals.

3.2.2 Recording of electrophysiological parameters

Identical inducing and recording procedures were carried out in the four groups of rats, and all recordings were completed within 20min of induction of anaesthesia. Upper and lower limb SEPs were recorded in response to electrical nerve stimulation, as detailed in Section 2.3.2. The latencies and peak-to-peak amplitudes of the selected SEP components from each group of rats were compared using one-way analysis of variance (ANOVA) across the groups and the significance of difference between mean values was calculated by the Student-Newman-Keuls test (SPSS 6.0 statistical software program, SPSS Inc.) with $P<0.05$ the criterion for statistical significance.

3.3 Results

3.3.1 Induction and duration of anaesthesia

Mean induction and recovery times are presented in Table 3.1. There were significant differences between the groups when compared using one-way ANOVA ($P<0.0001$ for both induction and recovery times). Anaesthesia with isoflurane gave the shortest induction and recovery times when compared with the other anaesthetics (mean induction and recovery times 3.33 and 5.33min respectively). Medetomidine had the longest induction time (10.38min), but recovery was rapid after administration of the antidote atipamezole (8.00min). Ketamine-xylazine and fentanyl/fluanisone-midazolam had similar induction times (6.00 and 4.90min respectively), but recovery from ketamine-xylazine was much slower (103.63 compared with 29.78min).

Ketamine-xylazine, isoflurane and fentanyl/fluanisone-midazolam all produced good levels of surgical anaesthesia for the duration of the test procedure, i.e. no responses to pedal or corneal stimulation could be elicited. Medetomidine produced a deep level of anaesthesia in five of the eight rats, but the remaining three animals continued to display pedal and corneal reflexes, and were therefore unable to undergo testing.

Table 3.1 Induction and recovery times for each anaesthetic

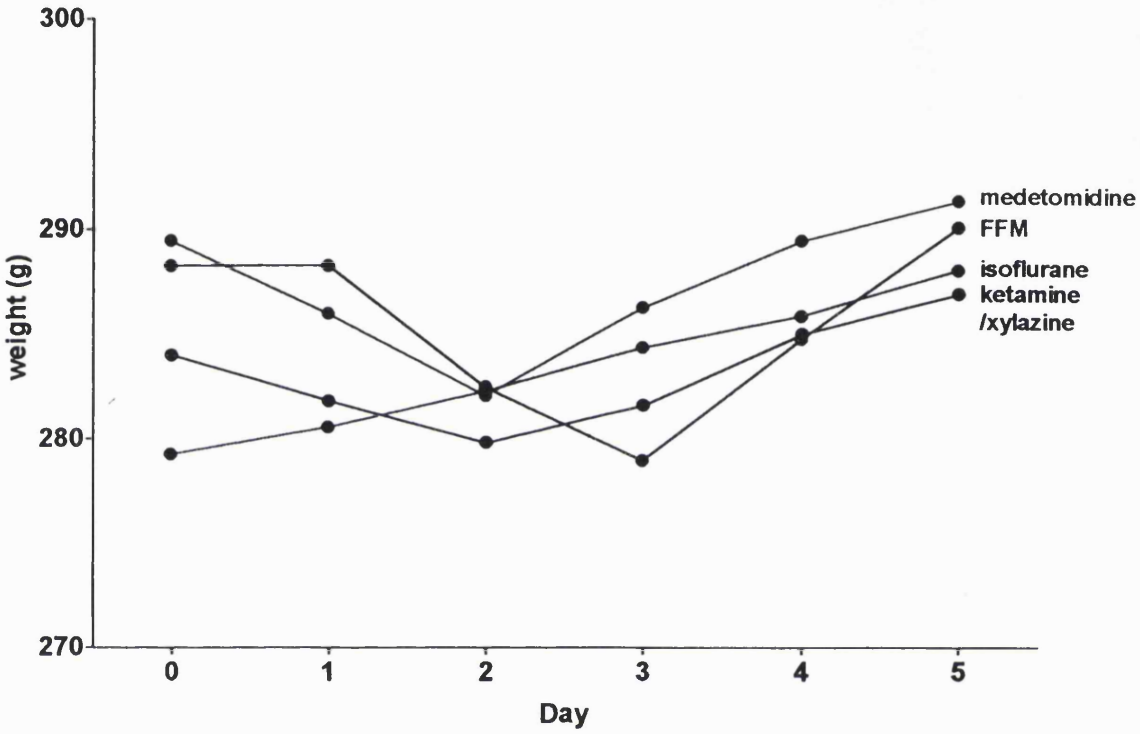
(n=8 in each group)

	Induction time (min)		Recovery time (min)	
Anaesthetic agent	mean	S.D.	mean	S.D.
Ketamine-xylazine	6.00	(1.15)	103.63	(32.08)
Medetomidine	10.38	(3.74)	8.00	(2.20)
Isoflurane	3.33	(0.78)	5.33	(1.15)
Fentanyl/fluanisone-midazolam	4.90	(0.74)	29.78	(10.74)

3.3.2 Weight loss

Rats anaesthetised with either medetomidine or ketamine-xylazine showed a decrease in weight (means of 4.1 and 2.5g respectively) on the first or second day after the procedure and then gained weight steadily (see figure 3.1). Animals which received fentanyl/fluanisone-midazolam showed a decrease in weight on the second or third day after the procedure (means 5.8g and 3.5g, respectively), and then gained weight steadily. Rats anaesthetised with isoflurane did not show consistent loss of weight during the five days following the procedure.

Figure 3.1 Mean weight changes in rats (n=8 per group) after general anaesthesia with different agents



FFM - fentanyl/fluanisone-midazolam

3.3.3 Somatosensory evoked potentials

For each anaesthetic agent, consistent responses were recorded at the cervical and cortical regions after stimulation of the median nerve at the wrist. The mean onset latency of the SEP recorded at C3 was significantly different ($P<0.02$) when the groups were compared using ANOVA (see Table 3.2). The C3 onset latency was significantly shorter with ketamine-xylazine than isoflurane (1.21 +/- 0.12 compared with 1.41 +/- 0.16ms respectively, $P<0.05$). The mean latencies for all components of the cortical response were longest in rats which received the ketamine-xylazine combination. There were significant differences between the groups in mean cortical onset ($P<0.05$) and P1 ($P<0.001$) latencies. Animals anaesthetised with fentanyl/fluanisone-midazolam had significantly ($P<0.05$) shorter cortical onset latencies than ketamine-xylazine (7.69 +/- 0.36 compared with 8.94 +/- 0.91ms respectively) as shown in Table 3.2 and Figure 3.2 (dotted line). The P1 latencies were significantly ($P<0.001$) shorter with fentanyl/fluanisone-midazolam compared with all the other agents and the latency with isoflurane was significantly shorter than with ketamine-xylazine (see Table 3.2 and Figure 3.2). There were no significant differences in the CI and CII components of the cervical response, or in the N1 component of the cortical response when the anaesthetic groups were compared.

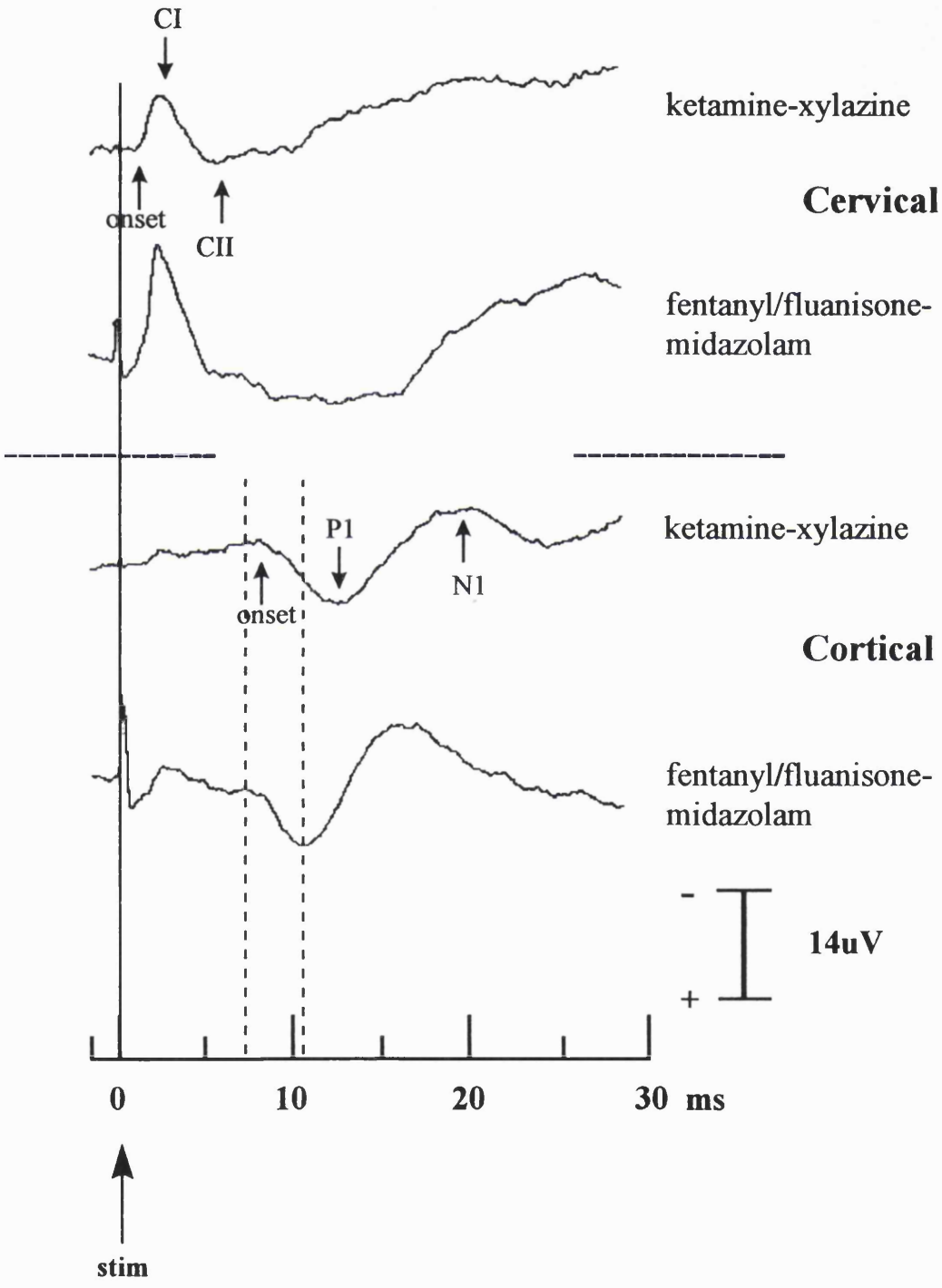
Table 3.2 Latencies (ms) of somatosensory evoked potentials for each anaesthetic (n=8 in each group).

		Ketamine-xylazine		Medetomidine		Isoflurane		Fentanyl/fluanisone-midazolam		ANOVA
		mean	(SD)	mean	(SD)	mean	(SD)	mean	(SD)	*P* value
Forelimb SEPs										
C3	Onset	1.21	(0.12)	1.33	(0.10)	1.41	(0.16)$^{\nabla}$	1.30	(0.10)	<0.02
	CI	2.57	(0.05)	2.66	(0.07)	2.70	(0.15)	2.62	(0.17)	NS
	CII	5.74	(0.46)	5.34	(0.20)	5.29	(0.87)	5.85	(0.40)	NS
Cortex	Onset	8.94	(0.91)*	8.38	(0.85)	8.50	(0.55)	7.69	(0.36)	<0.05
	P1	12.28	(0.91)*	11.89	(0.82)*	11.09	(0.76)*$^{\nabla}$	9.95	(0.53)	<0.001
	N1	17.49	(1.33)	17.15	(1.55)	15.47	(2.39)	15.47	(1.22)	NS
Hindlimb SEPs										
L5	Onset	1.58	(0.25)*	1.34	(0.24)	1.58	(0.42)*	1.10	(0.28)	<0.005
	LI	2.29	(0.08)	2.26	(0.15)	2.45	(0.42)*	2.09	(0.22)	<0.05
	LII	3.37	(0.19)	3.40	(0.18)	3.78	(0.77)	3.24	(0.46)	NS
Cortex	Onset	11.85	(0.88)*	12.82	(1.00)*	12.21	(0.71)*	9.25	(2.34)	<0.001
	P1	15.98	(0.60)*	16.44	(0.77)*	15.24	(0.79)	14.33	(1.67)	<0.005
	N1	20.53	(0.78)	20.17	(1.11)	19.75	(1.17)	19.85	(2.21)	NS

* Significantly different from fentanyl/fluanisone-midazolam ($P<0.05$)

$^{\nabla}$ Significantly different from ketamine-xylazine ($P<0.05$

Figure 3.2 Averaged SEPs from two rats obtained during ketamine-xylazine (K-X) and fentanyl/fluanisone-midazolam (F/F-M) anaesthesia following stimulation of upper limb



Following stimulation of the tibial nerve at the ankle, consistent peripheral (lumbar (L5) region of the spinal cord) and central (cortex) responses were recorded for each group. Significant differences in mean lumbar onset ($P<0.005$) and LI ($P<0.05$) latencies were recorded between the groups (Table 3.2). Fentanyl/fluanisone-midazolam gave significantly ($P<0.05$) shorter mean L5 onset latency (1.10 +/- 0.28ms) than ketamine-xylazine (1.58 +/- 0.25ms) and isoflurane (1.58 +/- 0.42ms) and a significantly shorter LI latency than isoflurane (2.09 +/- 0.22 compared with 2.45 +/- 0.42ms respectively, see also dotted line on Figure 3.3). No significant differences were found when the LII components were compared. The mean cortical onset and P1 latencies were also found to be significantly different ($P<0.001$ and $P<0.005$, respectively) in rats anaesthetised with different agents (Table 3.2 and Figure 3.4). Significantly shorter cortical onset latencies were recorded from animals given fentanyl/fluanisone-midazolam when compared with the other three anaesthetics (see dotted lines on Figure 3.4). It was also found that P1 latencies were significantly ($P<0.05$) shorter with fentanyl/fluanisone-midazolam (14.33 +/- 1.67ms) than with ketamine-xylazine (15.98 +/- 0.60ms) or medetomidine (16.44 +/- 0.77ms). There were no significant differences in the N1 peak latencies.

Figure 3.3 Averaged SEPs from four rats, recorded at L5 during anaesthesia with the four agents following stimulation of the lower limb

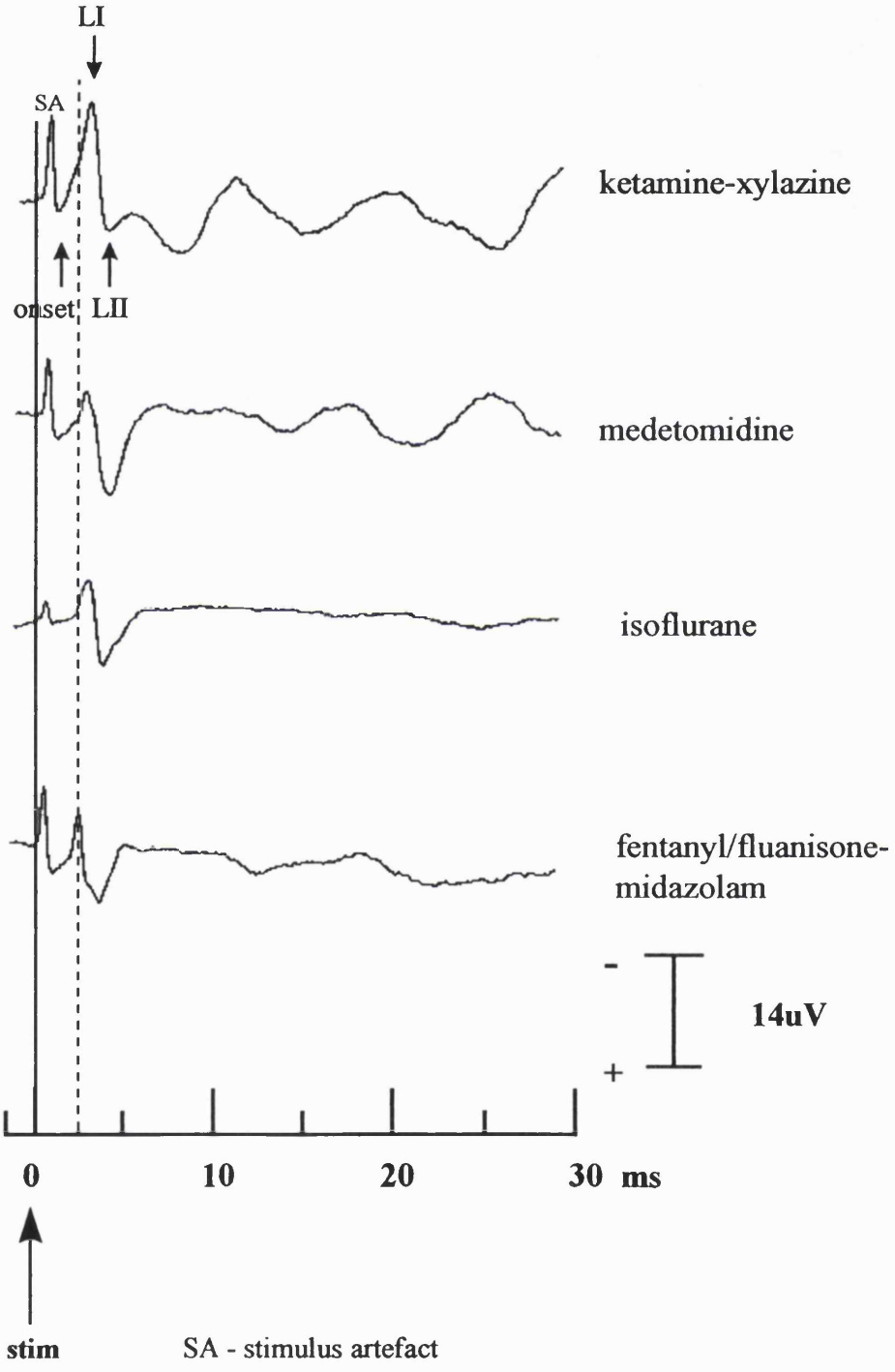
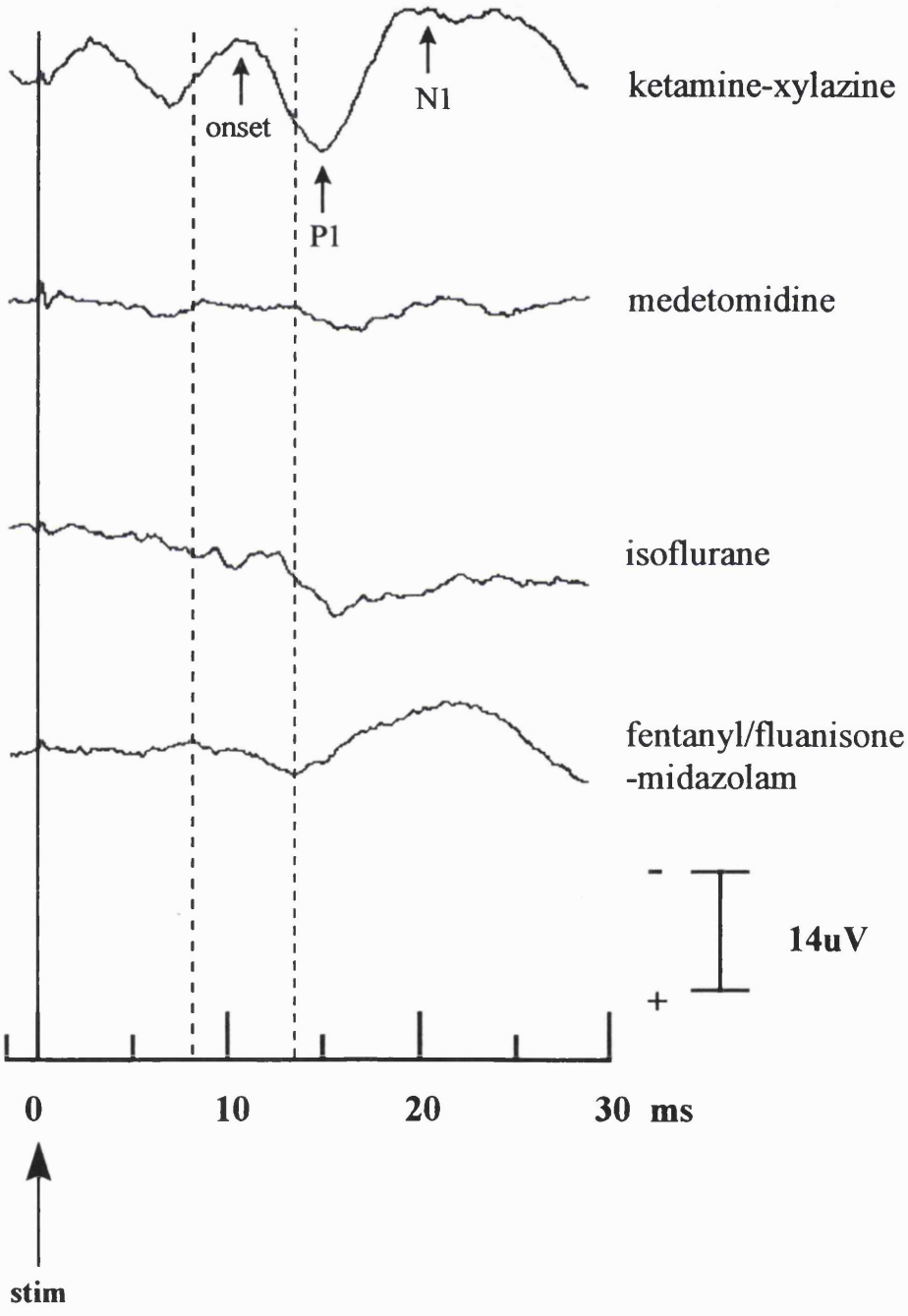


Figure 3.4 Averaged SEPs from four rats, recorded over the somatosensory cortex during anaesthesia with the four agents following stimulation of the lower limb



The effect of different anaesthetic agents on mean SEP amplitudes is shown in Table 3.3. Isoflurane tended to depress the amplitude of both forelimb and hindlimb SEP components to a greater extent than the other agents. The cervical (C3 onset to CI and CI to CII) and cortical (P1 to N1) responses showed significant differences in mean amplitude ($P<0.05$, 0.002 and 0.02, respectively, see also Figure 3.2), whereas there were no significant differences in the amplitude of the cortex onset to P1 responses. Following forelimb stimulation, fentanyl/fluanisone-midazolam gave significantly larger cervical CI to CII amplitudes than all the other agents, and a larger C3 onset to CI amplitude than isoflurane (15.18 +/- 6.43 compared with 10.17 +/- 2.70μV respectively, $P<0.05$). The cortical P1 to N1 wave amplitude was significantly greater in rats given fentanyl/fluanisone-midazolam than those given isoflurane, and was also significantly greater in those given medetomidine than isoflurane.

Following hindlimb stimulation there were no significant differences in amplitudes in the lumbar region, whereas significant differences were found between the groups in cortex onset to P1 ($P<0.001$) and P1 to N1 ($P<0.005$) responses. These cortical amplitudes were significantly higher with ketamine-xylazine than with the other anaesthetics.

Table 3.3 Amplitudes (μV) of somatosensory evoked potentials for each anaesthetic (n=8 in each group)

		Ketamine-xylazine		Medetomidine		Isoflurane		Fentanyl/fluanisone -midazolam		ANOVA
		mean	(SD)	mean	(SD)	mean	(SD)	mean	(SD)	*P* value
Forelimb SEPs										
C3	Onset to CI	11.42	(3.15)	9.75	(3.39)	10.17	(2.70)*	15.18	(6.43)	<0.05
	CI to CII	14.10	(2.93)*	12.66	(2.78)*	10.83	(3.39)*	18.39	(6.16)	<0.002
Cortex	Onset to P1	8.21	(4.99)	7.52	(3.54)	5.41	(2.64)	6.29	(2.64)	NS
	P1 to N1	10.76	(7.59)	14.84	(7.67)	5.00	(2.16)*°	16.00	(5.61)	<0.02
Hindlimb SEPs										
L5	Onset to LI	6.28	(2.37)	5.05	(1.77)	4.92	(2.15)	7.20	(3.28)	NS
	LI to LII	15.88	(3.56)	10.68	(5.34)	9.61	(4.10)	9.82	(6.07)	NS
Cortex	Onset to P1	7.25	(1.17)	3.22	(1.42)$^{\nabla}$	3.69	(1.38)$^{\nabla}$	4.01	(1.94)$^{\nabla}$	<0.001
	P1 to N1	8.55	(4.24)	4.05	(1.94)$^{\nabla}$	3.30	(1.09)$^{\nabla}$	5.10	(1.92)$^{\nabla}$	<0.005

* Significantly different from fentanyl/fluanisone-midazolam ($P<0.05$)

$^{\nabla}$ Significantly different from ketamine-xylazine ($P<0.05$)

° Significantly different from medetomidine ($P<0.05$)

3.4 Discussion

This pilot study investigated the effects of four different anaesthetic agents on the latencies and amplitudes of SEPs in the rat. The early components of the SEP waves appeared to be more affected by the different agents than the later components, with isoflurane and medetomidine having the greatest effect on evoked potentials. In agreement with previous studies isoflurane increased the latencies and decreased the amplitudes of the responses (Sebel *et al* 1986; Thornton *et al* 1992). Medetomidine was found to have similar effects to isoflurane in this study. Basic pharmaco-physiological studies concerned with synaptic transmission have demonstrated that both drugs exert their effects by reducing the efficacy of pre- and post-synaptic transmission. Isoflurane predominantly affects glutamate-releasing neurones (Richter *et al* 1977; Hablitz & Langmoen 1982), which results in a decrease of cortical activation by the primary afferent input. Medetomidine is a selective and specific full agonist of central and peripheral α_2-adrenoceptors, but the drug's anaesthetic action involves the central receptors only (Doze *et al* 1989). It inhibits noradrenergic neurones in the locus coerulus (Virtanen 1989) causing a complete disruption of the timing of signals passed from the ventrobasal thalamus to the cerebral cortex (Angel 1993). Since a greater number of synapses have to be crossed to reach the higher regions of the tract, brainstem and cortical components are more affected than those generated more peripherally.

L-glutamate is the major excitatory neurotransmitter in the vertebrate CNS and N-methyl-D-aspartate (NMDA) is an important class of ionotropic glutamate receptor channel. Ketamine is a non-competitive NMDA receptor antagonist (Thomson *et al*

1985; Yamamura *et al* 1990) which binds to a site within the lumen of the NMDA-activated channel of the neuronal membrane (MacDonald *et al* 1987). Xylazine, like medetomidine, produces sedation by activation of central α_2-adrenoceptors in the locus coerulus (Goodchild *et al* 1996). The results of this study indicate that ketamine-xylazine had no significant effect on peripheral conduction. This observation is in agreement with earlier work carried by Goss-Sampson and Kriss (1991), who suggested that ketamine-xylazine affects the sensory pathway beyond the level of the medial lemniscus. The cervical SEP response is associated with pre-synaptic activity at the level of the cuneate nucleus (CI) followed by post-synaptic activity at the level of the medial lemniscus (Claus *et al* 1985). Ketamine-xylazine anaesthesia led to an increase in cortical SEP latencies, although the amplitudes of the cortical SEPs were less affected. Changes in latency reflect the hypnotic component of anaesthesia (Thornton *et al* 1992) and it appears that ketamine-xylazine may have a strong hypnotic effect, producing a profound loss of consciousness. Schubert and his colleagues (1990) found that earlier waves of the SEP were more resistant to the effects of anaesthetics including ketamine. The results presented here, however, do not support this view. Significant differences were found between the anaesthetics, when early wave latencies and amplitudes were compared.

In general, fentanyl/fluanisone-midazolam was found to have the least effect on SEPs, compared with the other anaesthetics tested. Midazolam is a water soluble imidazo-benzodiazepine which has both sedative and amnesiac properties, mediated by interaction with the GABA system. By binding to GABA receptor recognition sites, benzodiazepine makes more free GABA available (Bahar *et al* 1997). GABA possesses analgesic properties and is found in the dorsal root in high concentrations.

Benzodiazepines do not block the transmission of sensory impulses through the nerve fibres, whereas fentanyl does block sensory impulses. This block occurs before impulses are transmitted to more rostral structures responsible for the identification of pain (Thornton *et al* 1992).

Midazolam has been previously shown to produce only a small increase in the latency and decrease in the amplitude of cortical SEPs (Sloan *et al* 1990), and fentanyl given alone has been shown to have no effect (Loughnan *et al* 1987). These results agree with the findings that indicate that the opioid fentanyl/fluanisone has a strong analgesic action (Thornton *et al* 1992) and midazolam has mild hypnotic properties only (Sloan *et al* 1990). It has been suggested (Thornton *et al* 1992) that analgesia can affect structures situated between the medial lemniscus and the primary somatosensory cortex. Both the thalamus and primary cortical area appear to contribute to the generation of the N1 component (Chiappa, 1985), and this may account for fentanyl/fluanisone-midazolam having less effect on the latency of the P1 peak than the other agents, but not the N1 peak. Both of these previous studies were performed on humans, whereas the results of this study show, for the first time, that this anaesthetic combination has little effect on SEPs in the rat.

When considering which anaesthetic would be the most appropriate for use in experimental longitudinal studies, it was necessary to find a balance between the general welfare of the animal, with respect to long-term deleterious effects, and the effects of the agent on SEPs which may confound the experimental variables studied. There were marked differences in the induction and recovery times between the groups. The route of administration affects the induction, as this will dictate the rate of

absorption. The differences in recovery time can be explained by the different rates of metabolism and tissue clearance of each drug.

Isoflurane, unlike the other agents used, is a gaseous anaesthetic and appeared to cause less stress to the rat when administered, as no physical restraint was necessary. In addition, the drug is almost totally expelled with each exhalation, making the depth of anaesthesia easy to control and leaving no residue in the tissues which could prolong recovery. However, isoflurane had a profound degrading effect on SEPs and was, therefore, rejected as a suitable anaesthetic for longitudinal electrophysiological studies.

Medetomidine is an injectable anaesthetic agent, which is fast-acting and rapidly reversed with atipamezole. A small amount of drug is required, thus lowering the risk of complications arising from tissue residues. The reversible nature of this anaesthetic reduces the problems associated with a slow recovery, such as hypothermia. The results, however, showed that medetomidine severely affected SEPs (especially amplitude) and was, therefore, not considered suitable for future work.

Ketamine-xylazine has been used previously in rats for this type of study (Goss-Sampson & Kriss 1991), since it was shown to have less effect on SEPs than pentobarbital. Indeed, the data presented here indicate that ketamine-xylazine has less deleterious effect than isoflurane or medetomidine. However, respiration was generally severely depressed which could lead to hypoxia and ultimately death, as was the case in one rat. In addition, recovery was slow (mean 103.63min) and external heat had to be applied to prevent hypothermia. The recovery time was somewhat longer than that

reported by Goss-Sampson and Kriss (1991), who found that all the animals had recovered after 80 minutes. There was not an obvious explanation for the difference between the two studies. Ketamine-xylazine was not chosen for use in further electrophysiological studies because of the deleterious effect on the welfare of the rats. In addition, the duration of anaesthesia provided by this agent was far longer than the 30 minutes needed to complete the experimental procedure.

Rats which received ketamine-xylazine, medetomidine or fentanyl/fluanisone-midazolam lost weight in the days following the experimental procedure. This may have been caused by a decrease in food intake or by water loss as a result of evaporation from the respiratory tract. Since saline was administered to each animal to prevent a fluid deficit, the former is a more likely explanation. The rats were observed to sleep for much of the 12 to 24 hours after the injection of the anaesthetic agent, resulting in a lower food intake than would normally be expected.

The results of the pilot study showed that fentanyl/fluanisone-midazolam generally had less effect on SEPs in the rat than the other anaesthetics tested. This combination produced surgical anaesthesia for up to 45 minutes and the effects of fentanyl could be reversed by administering buprenorphine. This reduced the recovery time and the risk of hypothermia. Furthermore, Flecknell (1996) stated that this anaesthetic combination was the agent of choice for rats. For these reasons, fentanyl/fluanisone-midazolam was chosen as the most appropriate anaesthetic for longitudinal studies of evoked potentials in the rat. This agent was used in all the following longitudinal electrophysiological studies.

Chapter 4

Study I: all-rac-α-tocopheryl acetate

4.1 Introduction

In a number of previous animal studies of α-tocopherol deficiency, two experimental groups were compared: the first fed a diet totally deficient in vitamin E, the second fed a normal diet containing adequate amounts of vitamin E (e.g. Machlin *et al*, 1977; Towfighi, 1981; Nelson *et al*, 1981; Goss-Sampson *et al*, 1988, 1990). This study, however, compared groups of rats with different dietary intakes of α-tocopherol in order to determine the critical concentration necessary to prevent the neural abnormalities associated with vitamin E deficiency. A concentration of 36mg all-rac-α-tocopheryl acetate/kg diet was chosen as the control diet, since this level had been established as being adequate in previous vitamin E studies within the department.

4.2 Experimental diet

75 weanling male rats were randomly allocated to one of five groups (n=15 in each) and housed as described in section 2.1. The animals in each group were numbered from 1 to 15 by means of a code of holes punched in the pinna. This procedure was carried out under gaseous anaesthesia (isoflurane, Abbott Laboratories, UK) to minimise discomfort and stress. Each group received a different amount of deuterated all-rac-α-tocopheryl acetate via the diet (i.e. deficient*, 1.25, 2.5, 5 or 36mg/kg diet). The different diets were distinguished by the addition of inert coloured dyes.

(* The deficient diet here and elsewhere refers to 0mg/kg diet)

4.3 Protocol for longitudinal electrophysiological recordings

Electrophysiological parameters were measured in the same rats (n=12 from each group) at monthly intervals for 14 months. The use of twelve animals from each group provided statistically meaningful results and allowed for wastage due to anaesthetic complications, congenital defects and other unforeseen eventualities. SEPs were recorded at the level of the lumbar spine and cerebral cortex in response to lower limb (tibial nerve) stimulation, and at the level of the cervical spine and cerebral cortex following upper limb (median nerve) stimulation (as detailed in section 2.3.2). VEPs and the ERG were recorded in response to dim (blue-filtered) and then bright white flash stimuli (see section 2.3.3). The responses recorded were characterised in terms of their peak latencies (i.e. time in milliseconds between delivery of stimulus and peak) and peak-to-peak amplitudes in μV. Conduction velocities were derived from the peak latencies and distances in mm between the stimulating and recording electrodes (see section 2.3.2.3).

After 14 months, the rats were killed and various tissues collected, as detailed in section 2.4. Tissue α-tocopherol and malondialdehyde concentrations were then determined (see section 2.5).

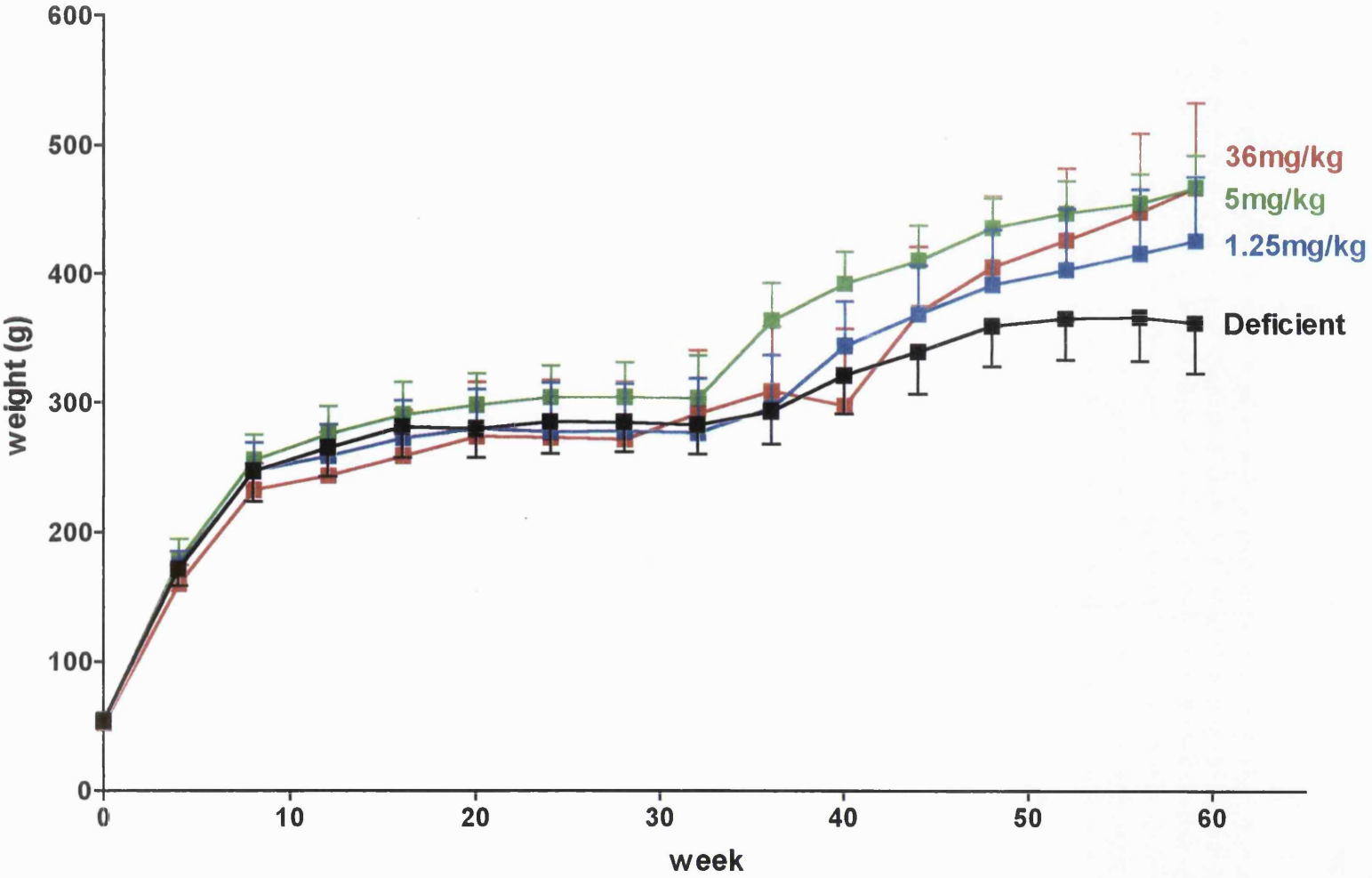
4.4. Growth and condition of rats

The group of animals which received 2.5mg α-tocopheryl acetate/kg diet behaved very differently from the other groups. The animals ate more voraciously, gaining weight at

a faster rate and both somatosensory and visual responses became abnormal after only 4 months. A red dye was used to distinguish this diet, which was thought to be harmless. However, this dye appeared to be metabolised in the gut, whereas the blue, yellow and green (made by mixing blue and yellow) dyes used to colour the other diets passed through the animals' digestive tracts unchanged, i.e. coloured faecal pellets were observed. It appears, therefore, that the red dye had an undocumented toxic effect and this group was excluded from the study. Time precluded systematic investigation of this observation and this aspect warrants further study.

The rats were weighed weekly for the duration of the experiment, and the mean monthly weights of the 4 remaining groups plotted (see Figure 4.1). The animals gained weight rapidly for the first 8 weeks and then the growth rate slowed. The weights tended to remain relatively constant from 16-32 weeks before increasing again. The 1.25, 5 and 36mg/kg groups continued to gain weight for the duration of the study, whereas no further increase in mean body weight was seen in the deficient group after 48 weeks. The weights of the 36mg/kg group remained relatively constant until 40 weeks. After this point, there was a rapid increase in weight and by the end of the study, the mean weight of the 36mg/kg group was similar to that of the 5mg/kg group. This 'growth spurt' was more marked than in any of the other groups and may be explained by problems of diet storage and 'shelf-life'. The commercially prepared diets were stored below 5°C and assumed to be stable for many months. On further investigation, however, it was ascertained from Dyets Inc. (Pennsylvania, USA) that the diets had a 'shelf-life' of only 3 months. Greater amounts of the 36mg/kg diet were ordered and therefore only the rats in this group received a diet that was out-of-date. This resulted in changes in the general

Figure 4.1 Mean monthly weights (+/- 1SD) for deficient, 1.25mg/kg, 5mg/kg and 36mg/kg groups



condition of the rats, so it was decided to use the 5mg/kg group as the positive control for the study.

By 33 weeks the rats in the 5mg/kg group were significantly heavier than those receiving the deficient diet (317.5 +/- 29.5g compared with 269.5 +/- 20.8g, $P<0.05$). Also at this time, a significant difference ($P<0.05$) was observed between the 5mg/kg and 1.25mg/kg groups (mean 270.3 +/- 40.63g). After 47 weeks, significant differences ($P<0.05$) were observed in the mean weights of the deficient group (355.1 +/- 29.26g) compared with the 1.25mg/kg group (389.0 +/- 42.36g) and with the 36mg/kg group (396.3 +/- 53.33g). No significant differences were found, at any time point, between the 1.25mg/kg and 36mg/kg groups.

The general condition of the rats fed a vitamin E deficient diet began to deteriorate after 20 weeks. Their coats became greasy and towards the end of the study, the fur looked matted and unkempt. The first signs of neurological dysfunction were seen after 45 weeks, when two of the deficient rats were observed with an irregular gait and impaired balance. Over the next fourteen weeks, the condition of all the deficient animals became progressively worse with visible muscle wastage especially around the spine and hind limbs. At the end of the study, eight of the eleven rats were dragging their hind limbs while the remaining three had a severely abnormal gait.

Rats fed the diet containing 1.25mg/kg all-rac-α-tocopheryl acetate showed no general deterioration until week 33. After this time, changes in coat condition were observed. After 52 weeks, impaired balance was seen in ten animals, three of which were dragging their hind limbs. Two rats from this group displayed no ill effects throughout

the duration of the study. The 5mg/kg and 36mg/kg group rats remained healthy through the entire study, with no visible changes in coat condition or neural function.

4.5 Results - Electrophysiology

The onset and major peak latencies of the SEP waveforms were analysed statistically and similar results obtained for each SEP component. For this reason, only onset latency data are presented here.

4.5.1 Lower limb SEPs

Figure 4.2 shows representative SEP waveforms recorded at the fifth lumbar vertebra at month 14 of the study. The potentials recorded in the deficient and 1.25mg/kg rats showed reduced peak-to-peak amplitudes, compared with rats which received higher dietary intakes of vitamin E, but these did not reach significance when the group data were analysed (see Figure 4.8).

Peripheral conduction velocities

Figure 4.3 shows the mean peripheral conduction velocities (pCVs) for the deficient, 1.25mg/kg, 5mg/kg and 36mg/kg groups. The mean pCVs of all four groups increased for the first 5 months, after which those of the 5mg/kg and 36mg/kg groups plateaued, whereas those of the deficient and 1.25mg/kg groups tended to decrease. The mean pCV of the deficient group became consistently significantly slower than the

Figure 4.2 Lumbar (L5) SEP waveforms recorded at month 14 in representative rats from the deficient, 1.25mg/kg, 5mg/kg and 36mg/kg groups

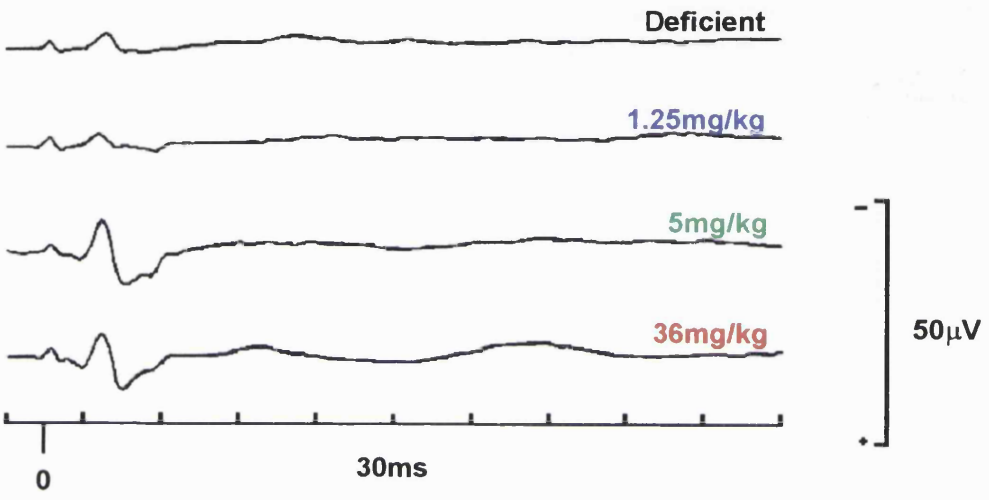


Figure 4.3 Group mean peripheral conduction velocities (pCVs) - lower limb

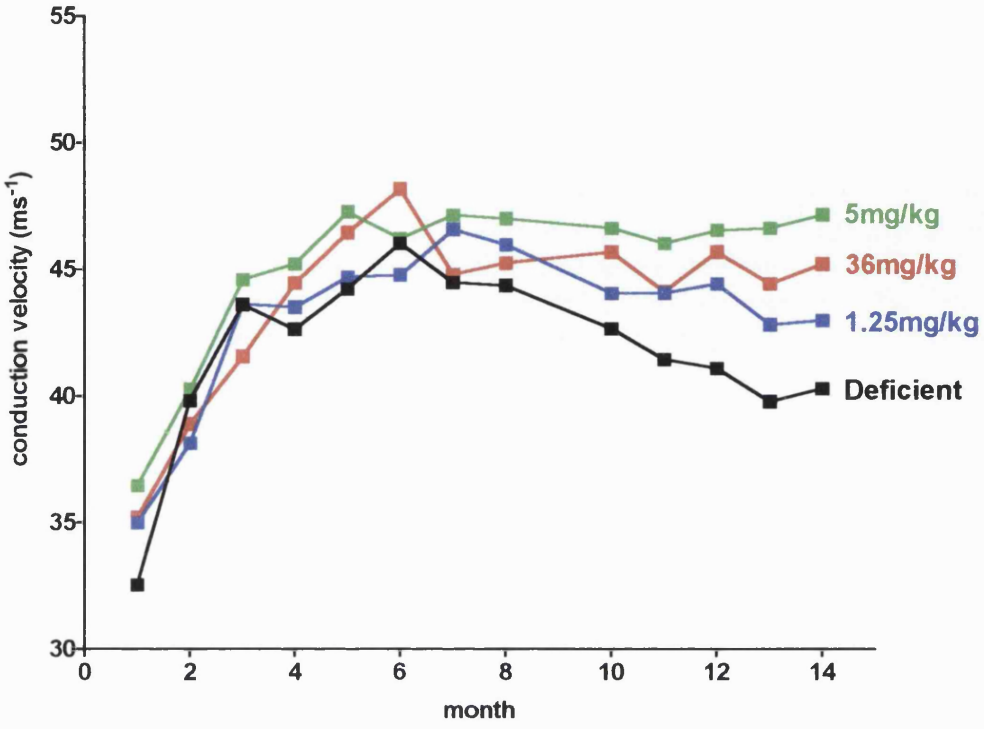


Figure 4.4 Group mean pCVs +/- 1SD - deficient v 1.25mg/kg (lower limb)

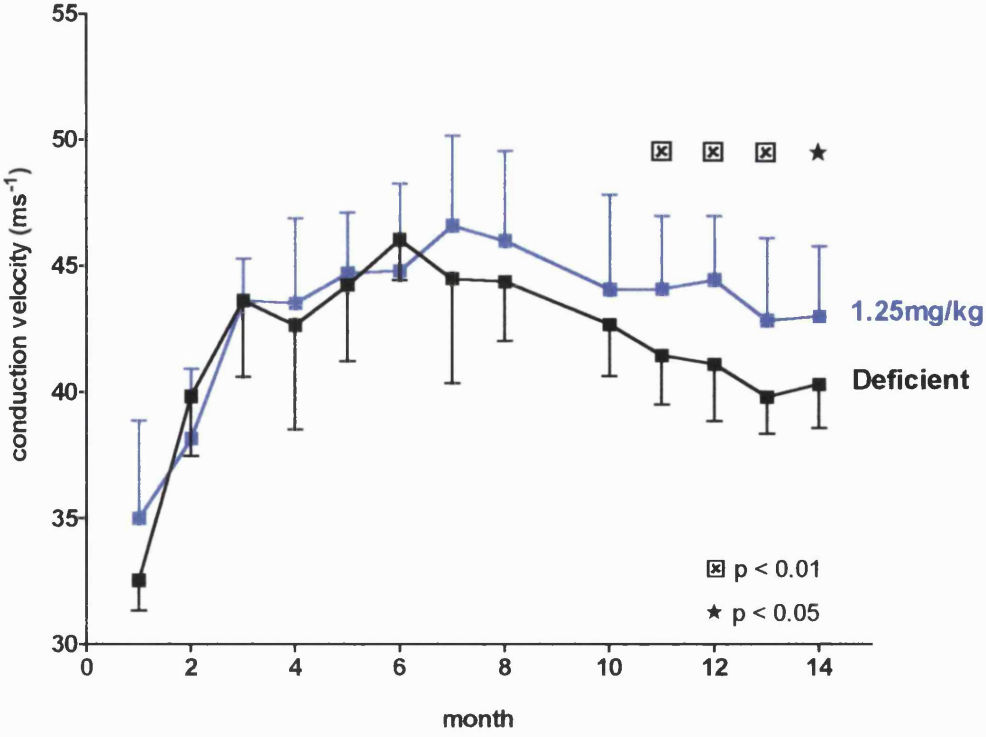


Figure 4.5 Group mean pCVs +/- 1SD - deficient v 5mg/kg (lower limb)

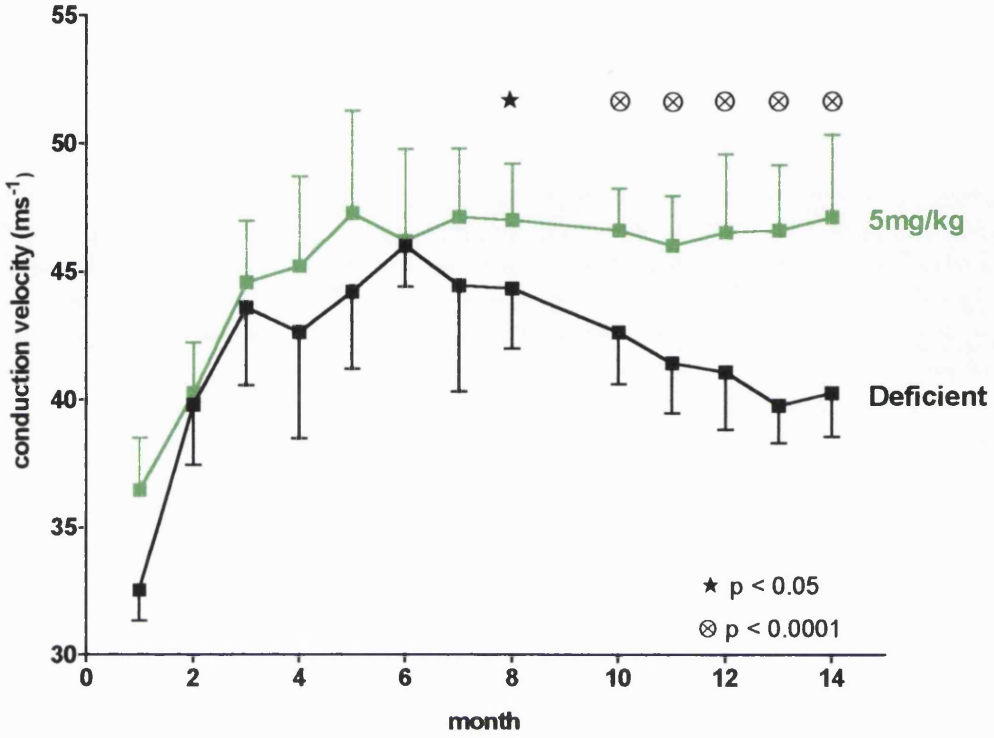
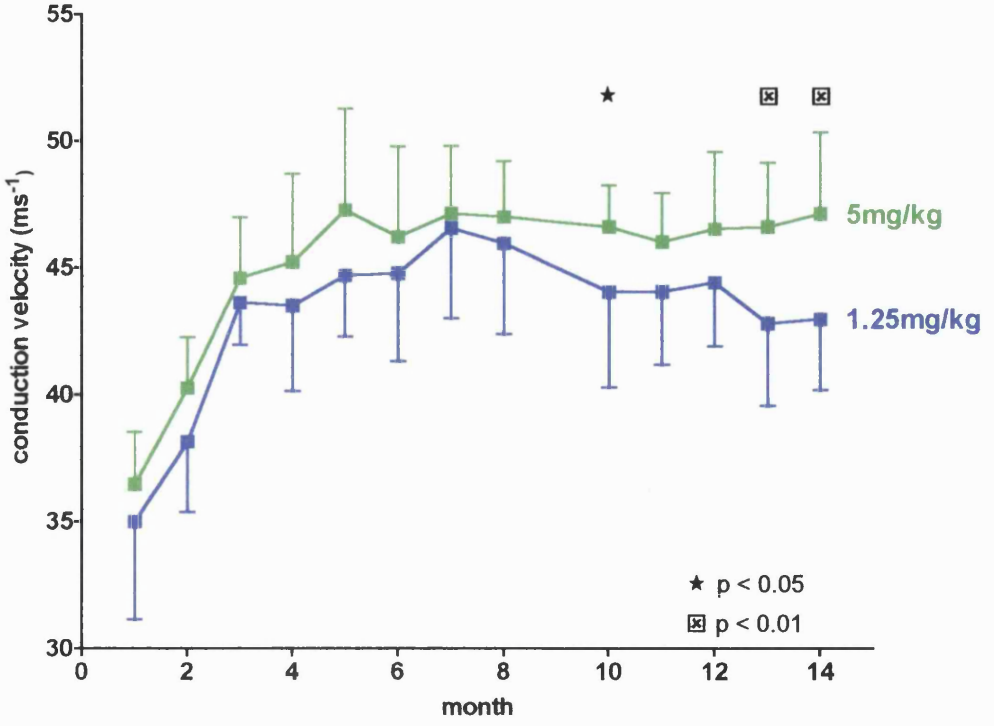


Figure 4.6 Group mean pCVs +/- 1SD - 1.25mg/kg v 5mg/kg (lower limb)



1.25mg/kg group after 11months of testing (Figure 4.4) and slower than the 5mg/kg group after 8 months (Figure 4.5). There were also significant differences after 13 months in the pCV of rats receiving 1.25mg/kg compared with those receiving 5mg/kg all-rac-α-tocopheryl acetate (Figure 4.6). There were no consistent significant differences between the 5mg/kg and the 36mg/kg groups at any time point during the course of the study.

Central conduction velocities

The mean central conduction velocities for each group tended to increase for the first 5 months of the study before reaching a plateau, as shown in Figure 4.7. There were no significant differences between the groups at any time point during the study.

SEP amplitudes

Figures 4.8 and 4.9 show group mean peak-to-peak amplitudes of SEPs recorded at the level of the spine (L5) and cortex, respectively. The amplitudes recorded at L5 tended to decrease during the course of the study whereas those recorded at the cortex were more variable. There were no significant differences between the groups at any time point.

Figure 4.7 Group mean central conduction velocities (lower limb)

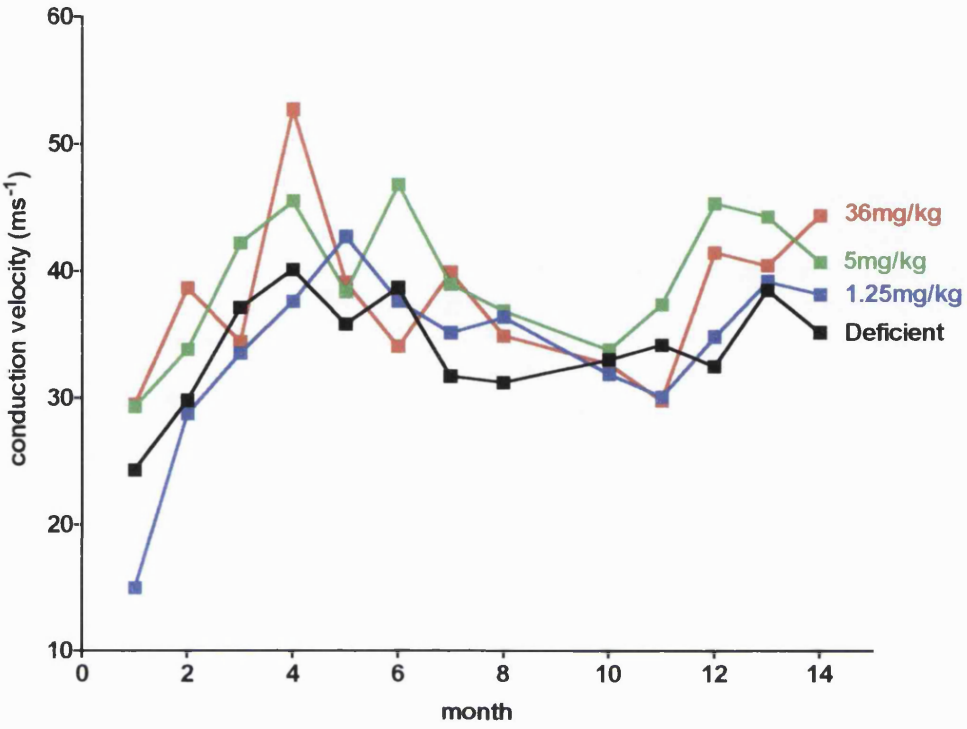


Figure 4.8 Group mean lumbar (L5) peak-to-peak amplitudes

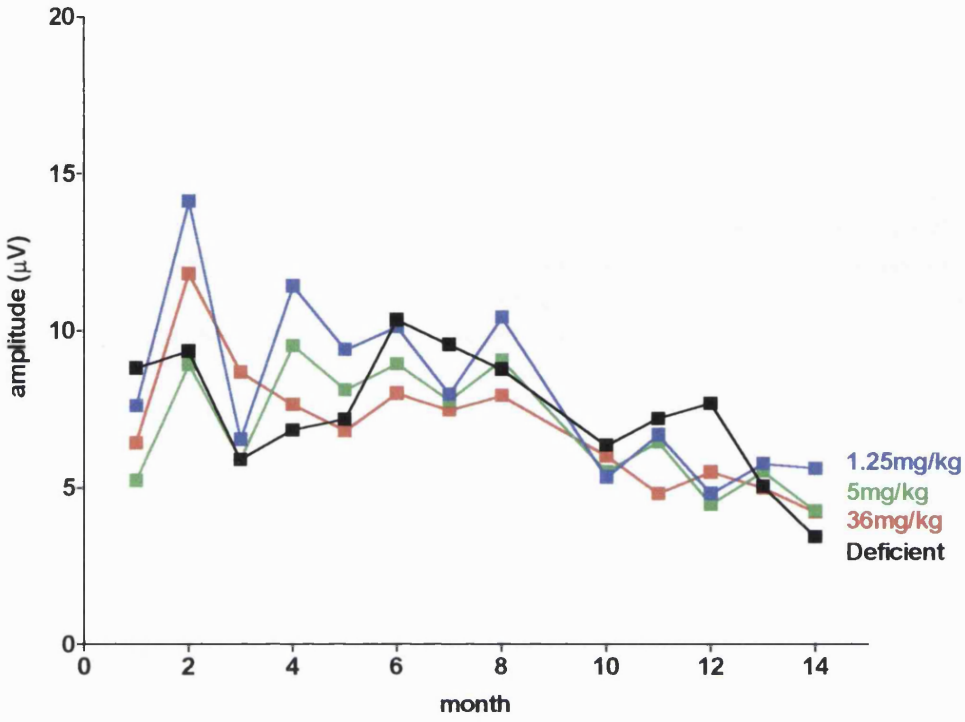
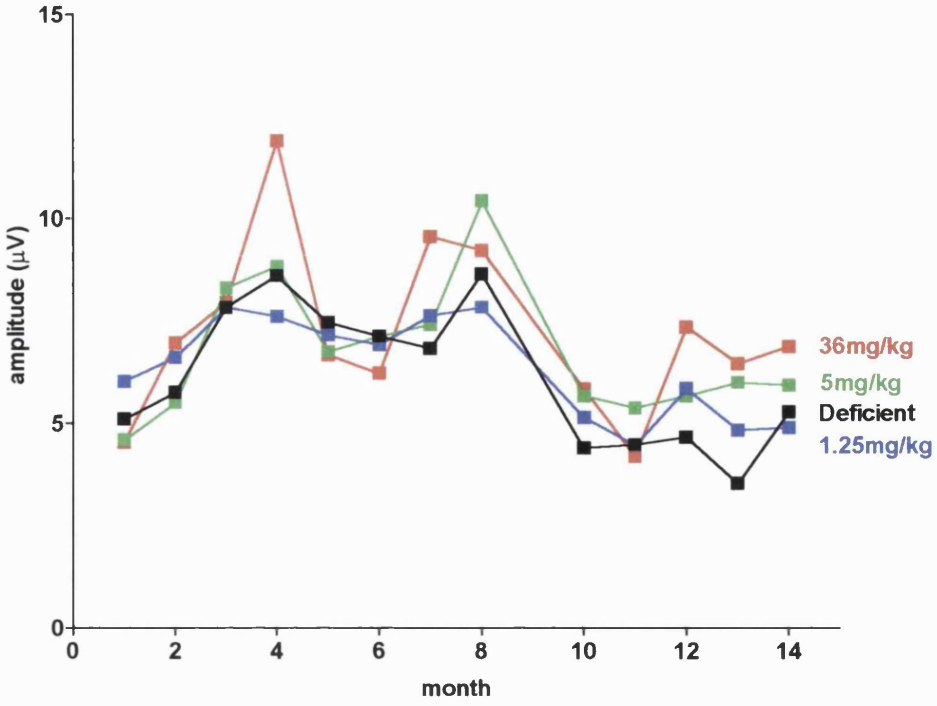


Figure 4.9 Group mean cortical peak-to-peak amplitudes (lower limb)



4.5.2 Upper limb SEPs

Peripheral conduction velocities

Figure 4.10 shows the mean peripheral conduction velocities for all four groups, which increased rapidly until month 4 and then tended to plateau. After month 7, the mean pCVs of the deficient and 1.25mg/kg groups decreased while those of the 5mg/kg and 36mg/kg groups remained relatively constant. The mean pCVs of the deficient group became consistently significantly slower ($P<0.001$) than the 5mg/kg group after 11months (50.56 +/- 3.70 compared with 57.28 +/- 3.30ms^{-1}, Figure 4.11) and the 1.25mg/kg group became significantly slower ($P<0.05$) than the 5mg/kg group after 10 months (51.19 +/- 3.44 compared with 55.08 +/- 2.95 ms^{-1}, Figure 4.12). There were no significant differences when either the deficient and 1.25mg/kg groups, or the 5mg/kg and 36mg/kg groups were compared.

Central conduction velocities

The mean central conduction velocities for all the groups remained relatively constant until month 8, as shown in Figure 4.13, when the groups began to diverge. The mean cCV of the 5mg/kg group became consistently significantly faster than the deficient group after 10 months (Figure 4.14). Deficient rats had a significantly slower mean group cCV than the 1.25mg/kg group when compared at 11, 12 and 13 months (Figure 4.15). At month 14, however, there were no significant differences between the two groups. There were no significant differences in mean cCV when the 5 and 36mg/kg groups were compared.

Figure 4.10 Group mean peripheral conduction velocities (upper limb)

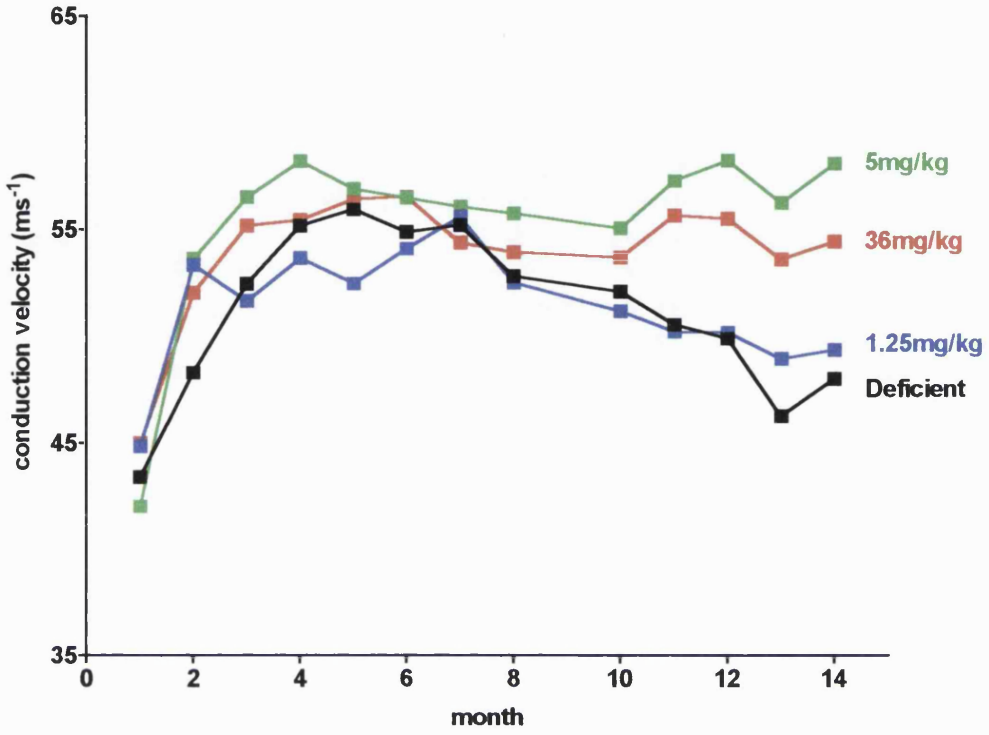


Figure 4.11 Group mean pCVs +/- 1SD - deficient v 5mg/kg (upper limb)

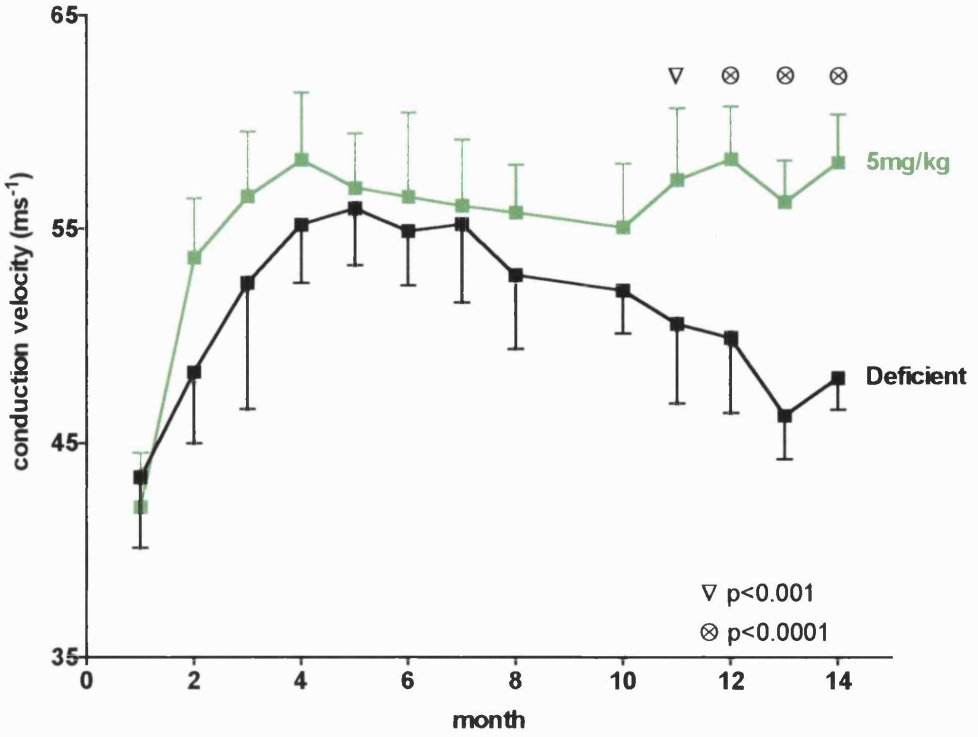


Figure 4.12 Group mean pCVs +/- 1SD - 1.25 v 5mg/kg (upper limb)

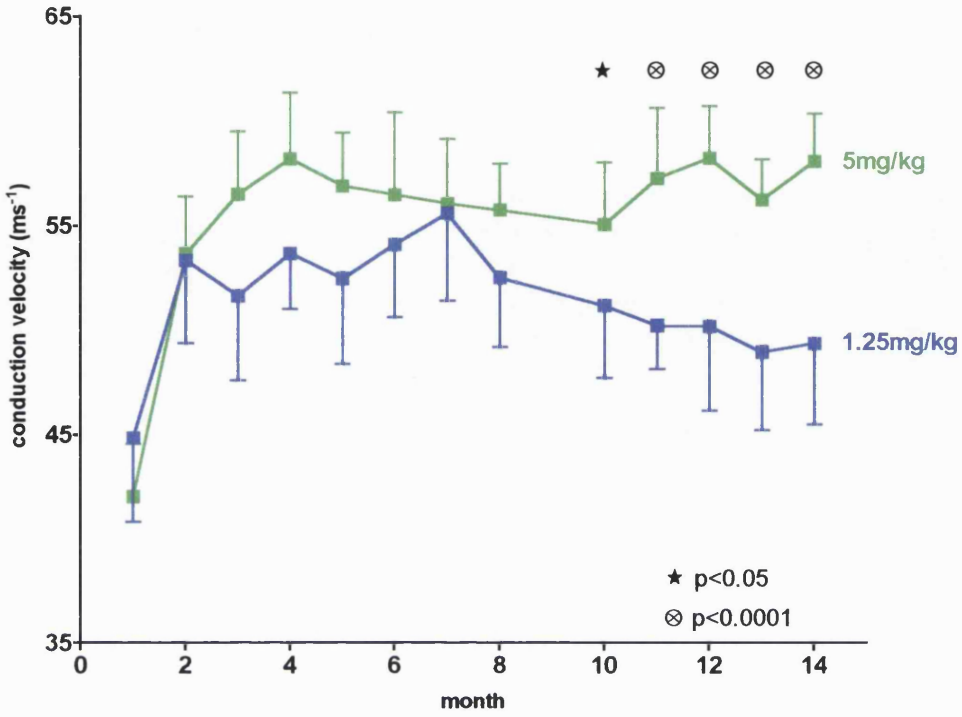


Figure 4.13 Group mean central conduction velocities (cCVs) - upper limb

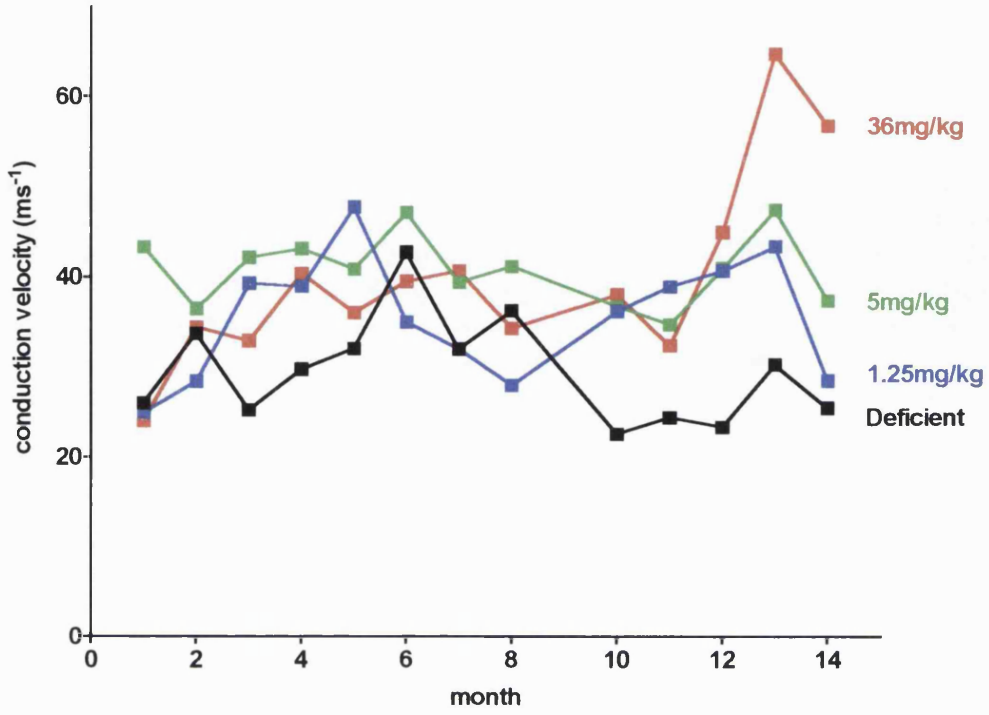


Figure 4.14 Group mean cCVs +/- 1SD - deficient v 5mg/kg (upper limb)

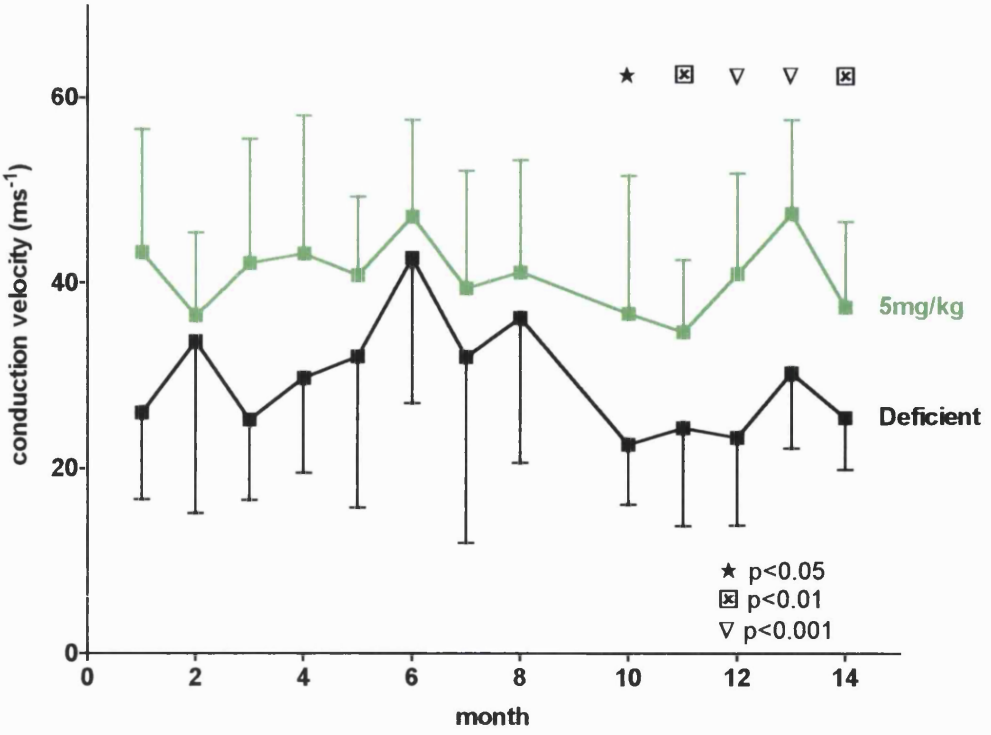
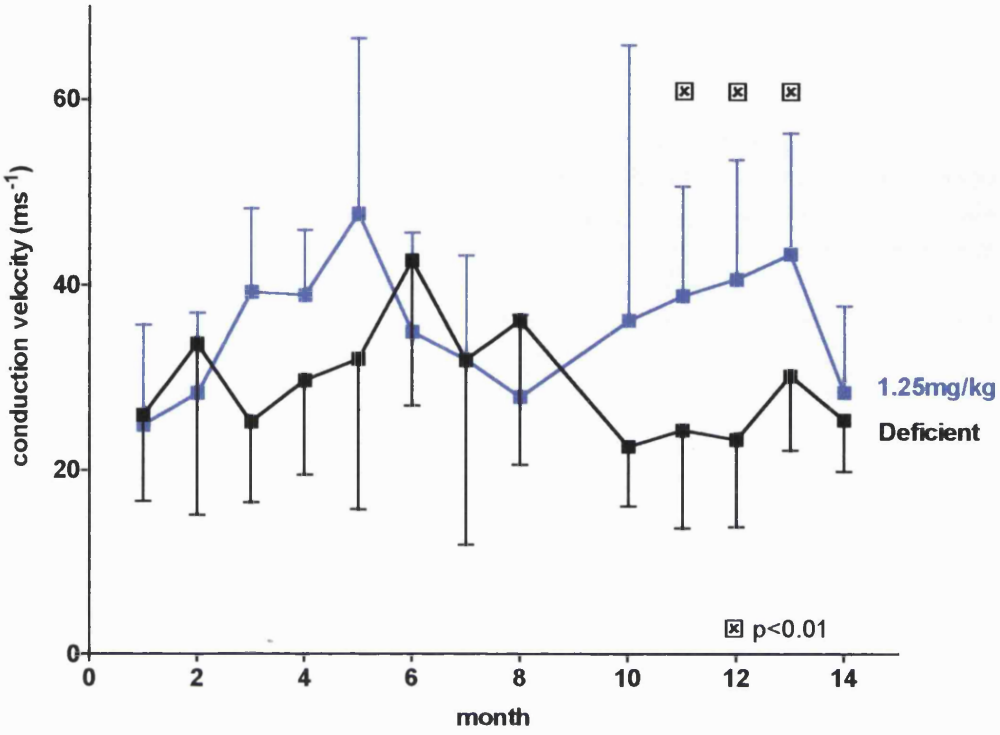


Figure 4.15 Group mean cCVs +/- 1SD - deficient v 1.25mg/kg (upper limb)



SEP amplitudes

Figure 4.16 shows the mean amplitudes for all the groups, of SEPs recorded at C3 in response to median nerve (upper limb) stimulation. The amplitudes remained relatively constant until month 8 and then the groups began to diverge. Consistently significant reductions in amplitude were found when the deficient group was compared with the 5mg/kg group after 11 months (Figure 4.17). A significant reduction in amplitude was seen at 14 months in the 1.25mg/kg group compared to the 5mg/kg group (12.70 +/- 1.46 compared with 15.12 +/- 3.03μV, $P<0.05$). Rats fed a vitamin E deficient diet had a significantly smaller group mean C3 amplitude after 13 months than those fed 1.25mg/kg α-tocopheryl acetate (Figure 4.18). There were no significant differences in C3 amplitudes between the 5mg/kg and 36mg/kg groups at any time point.

The group mean amplitudes of the cortical SEP for all the groups are presented in Figure 4.19. When cortical response amplitudes were compared, the 5mg/kg group were found to have consistently significantly larger amplitudes than the deficient group after 13 months (Figure 4.20). A significant reduction in amplitude was seen at 14 months in the 1.25mg/kg compared to the 5mg/kg group (6.70 +/- 2.48 compared with 8.88 +/- 2.41μV, $P<0.05$). There were no significant differences between the 5mg/kg and 36mg/kg, or the deficient and 1.25mg/kg, groups.

Figure 4.16 Group mean cervical (C3) amplitudes (upper limb)

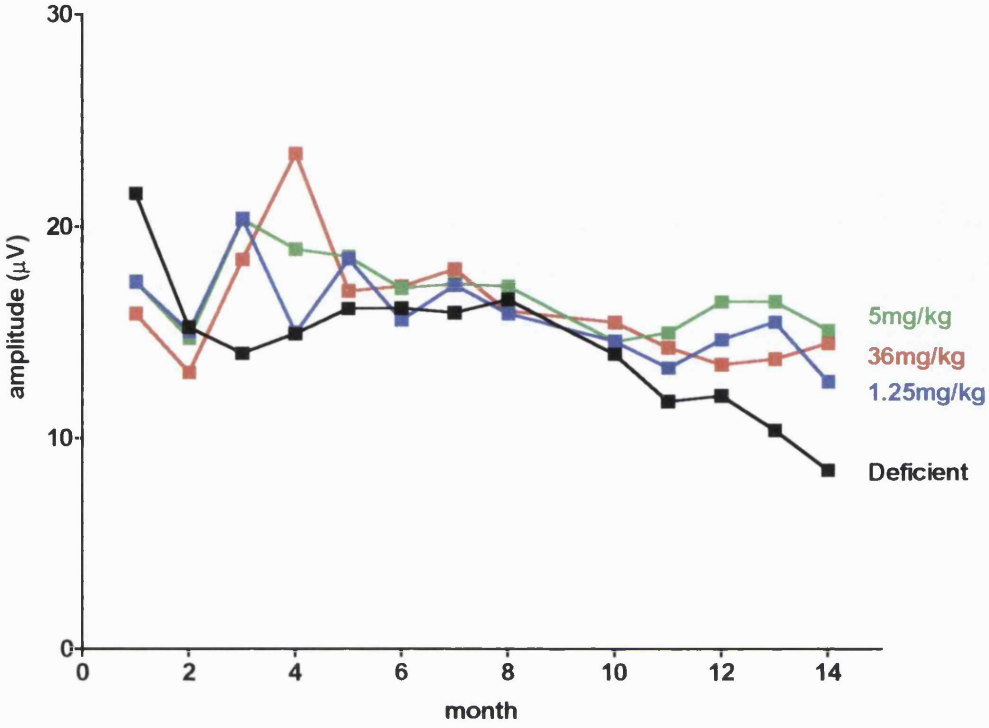


Figure 4.17 Group mean C3 amplitudes +/- 1SD - deficient v 5mg/kg (upper limb)

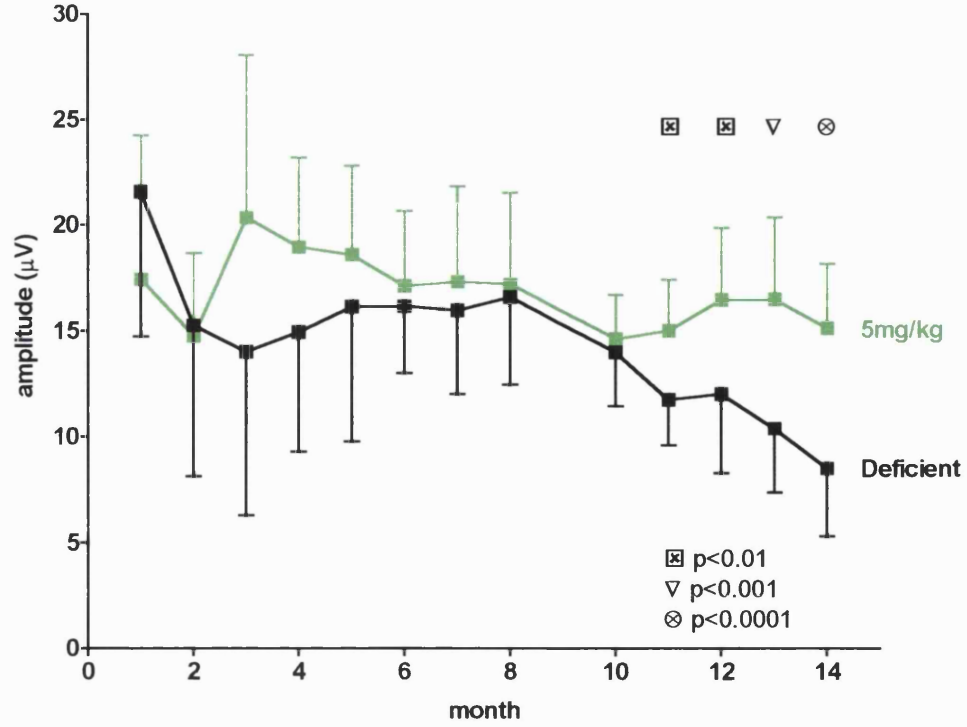


Figure 4.18 Group mean C3 amplitudes +/- 1SD - deficient v 1.25mg/kg (upper limb)

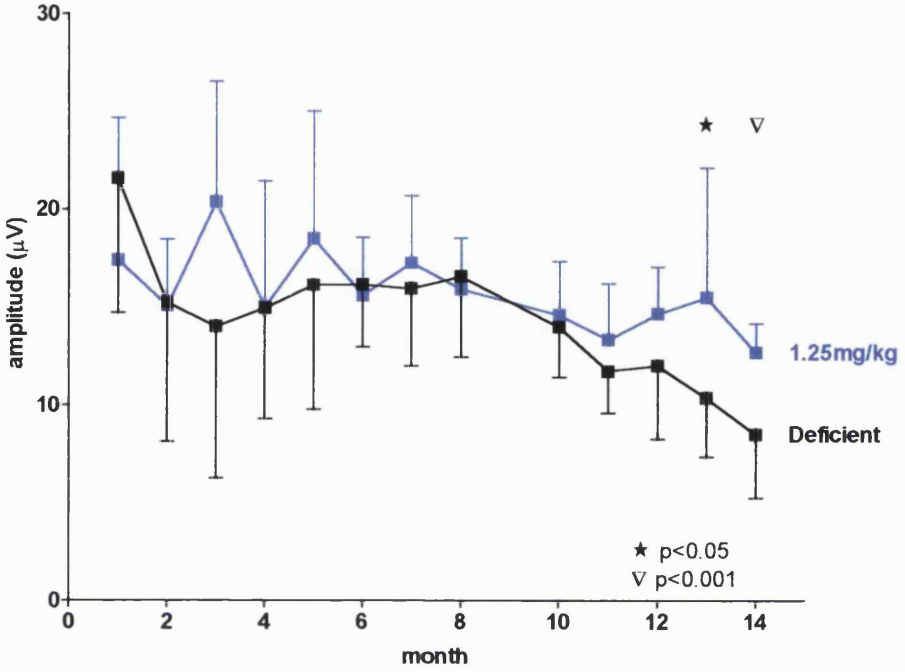


Figure 4.19 Group mean cortical amplitudes (upper limb)

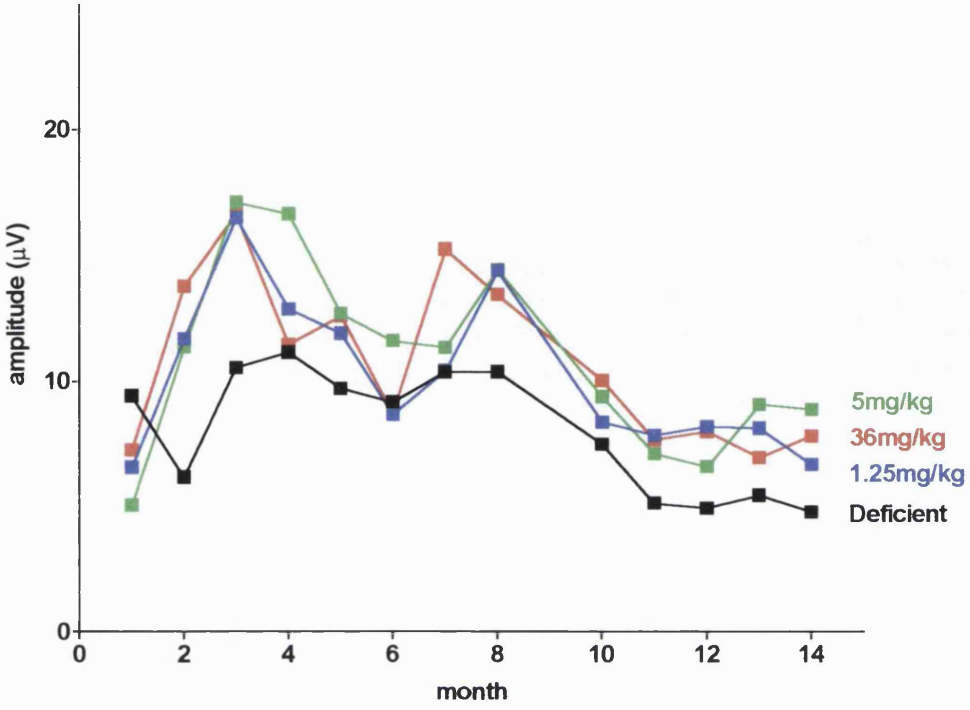
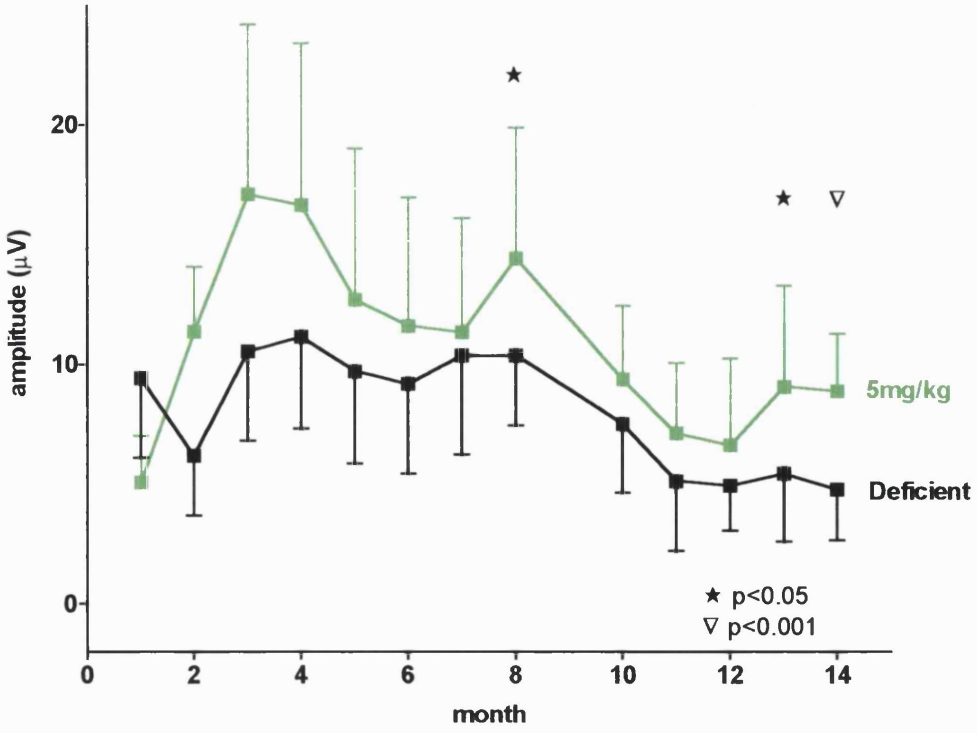


Figure 4.20 Group mean cortical amplitudes +/- 1SD - deficient v 5mg/kg (upper limb)



4.5.3 Visual function results

Representative rod mediated flash ERGs recorded in response to dim (blue-filtered) flash stimuli at month 14 are shown in Figure 4.21. Rats in the deficient and 1.25mg/kg groups showed markedly longer b wave latencies (indicated by arrows on Figure 4.21) and smaller peak-to-peak amplitudes, compared with rats in the 5mg/kg and 36mg/kg groups, which had latencies which were within normal limits.

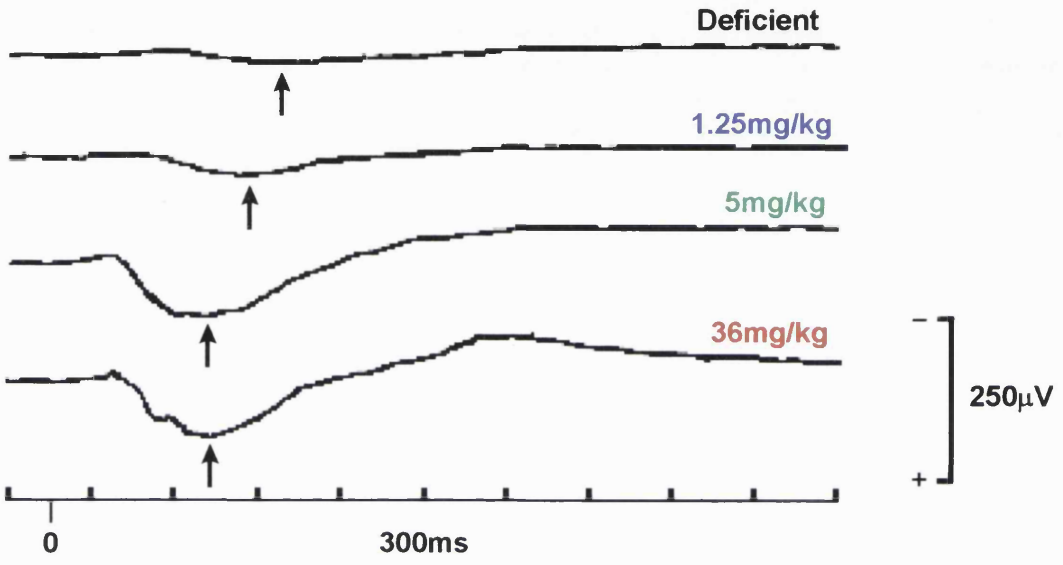
Dim (blue-filtered) ERG latencies

Figure 4.22 shows the group mean ERG b wave latencies for all four groups, recorded following dim blue flash stimulation. The mean latencies of the deficient group were consistently significantly longer than those of the 5mg/kg group after 8 months and the 1.25mg/kg group after 12 months (Figures 4.23 and 4.24, respectively). When the mean b wave latency of the 1.25mg/kg group was compared to that of the 5mg/kg group, significant differences were seen after 11 months, with the exception of the 13 months time point (Figure 4.25). No significant differences were found when the 5mg/kg group was compared with the 36mg/kg group.

Dim (blue-filtered) ERG amplitudes

The mean ERG amplitudes for all the groups are presented in Figure 4.26. The deficient group was found to have significantly reduced amplitudes compared with those of the 1.25mg/kg group after 13 months (Figure 4.27) and compared with the 5mg/kg group after 14 months. When the 5mg/kg group was compared to the 1.25 and 36mg/kg groups, no significant differences in ERG amplitude were found.

Figure 4.21 Representative flash ERGs recorded in response to dim flash stimuli at month 14



Black arrows indicate the latency of the ERG b wave

Figure 4.22 Group mean ERG b wave latencies (dim blue flash)

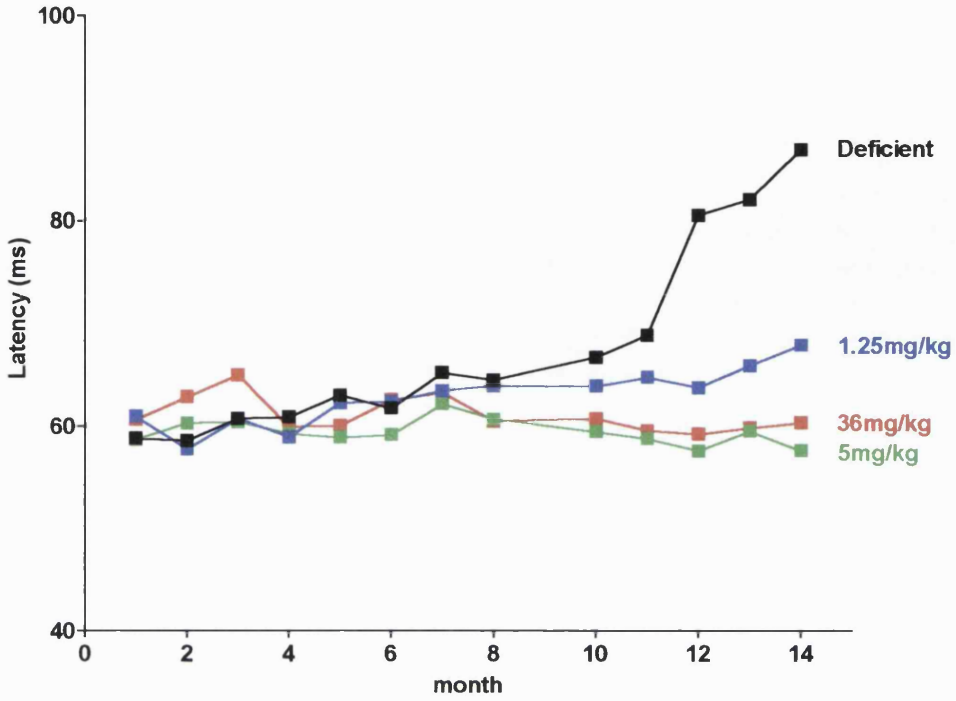


Figure 4.23 Group mean ERG b wave latencies +/- 1SD
- deficient v 5mg/kg (dim blue flash)

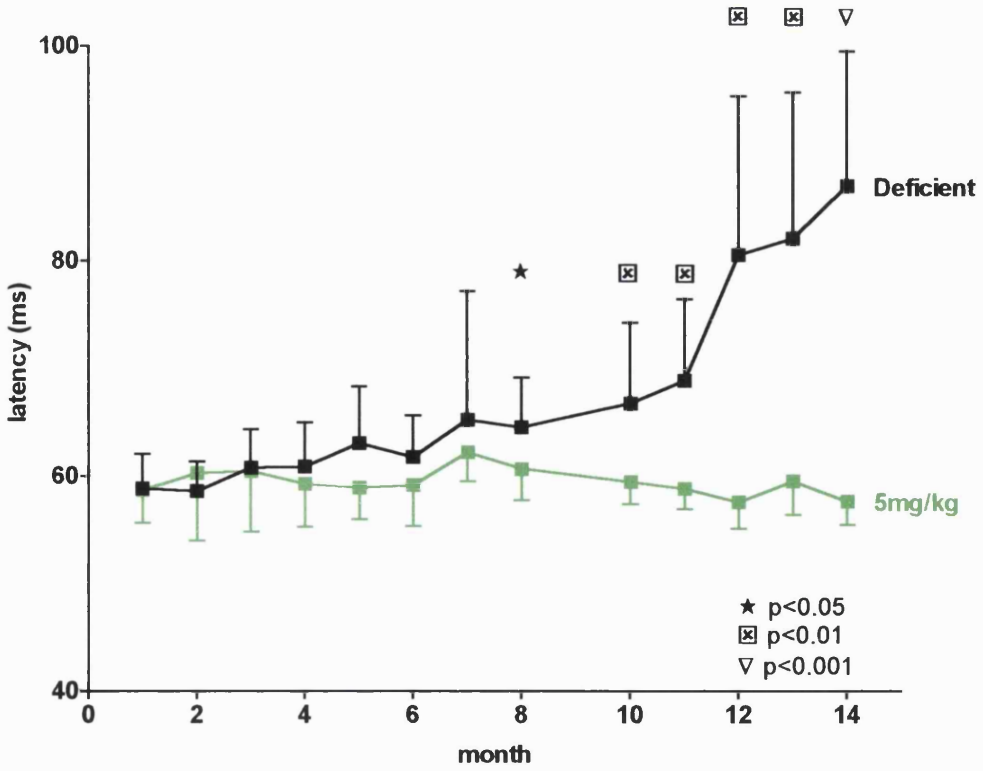


Figure 4.24 Group mean ERG b wave latencies +/- 1SD
- deficient v 1.25mg/kg (dim blue flash)

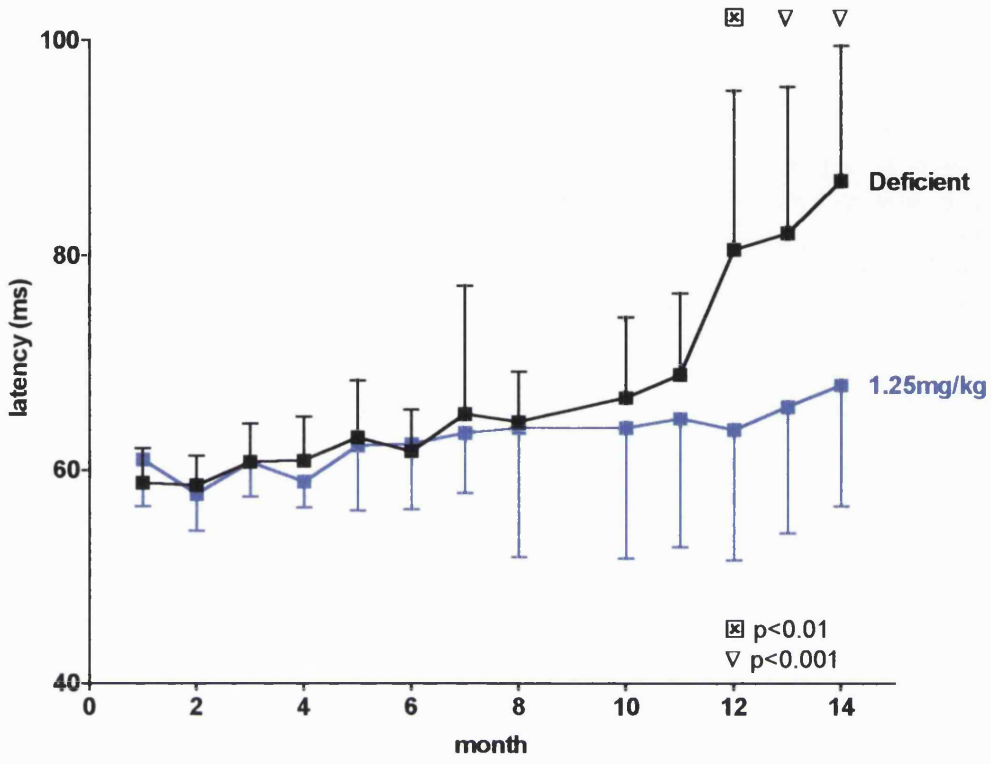


Figure 4.25 Group mean ERG b wave latencies +/- 1SD
- 1.25mg/kg v 5mg/kg (dim blue flash)

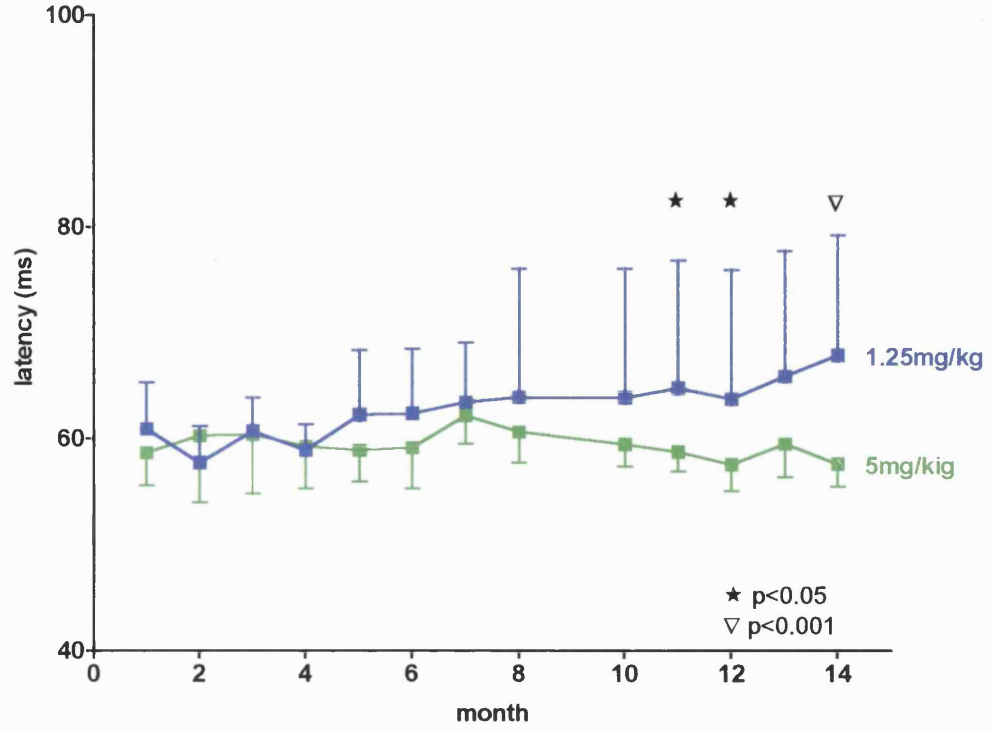


Figure 4.26 Group mean ERG amplitudes (dim blue flash)

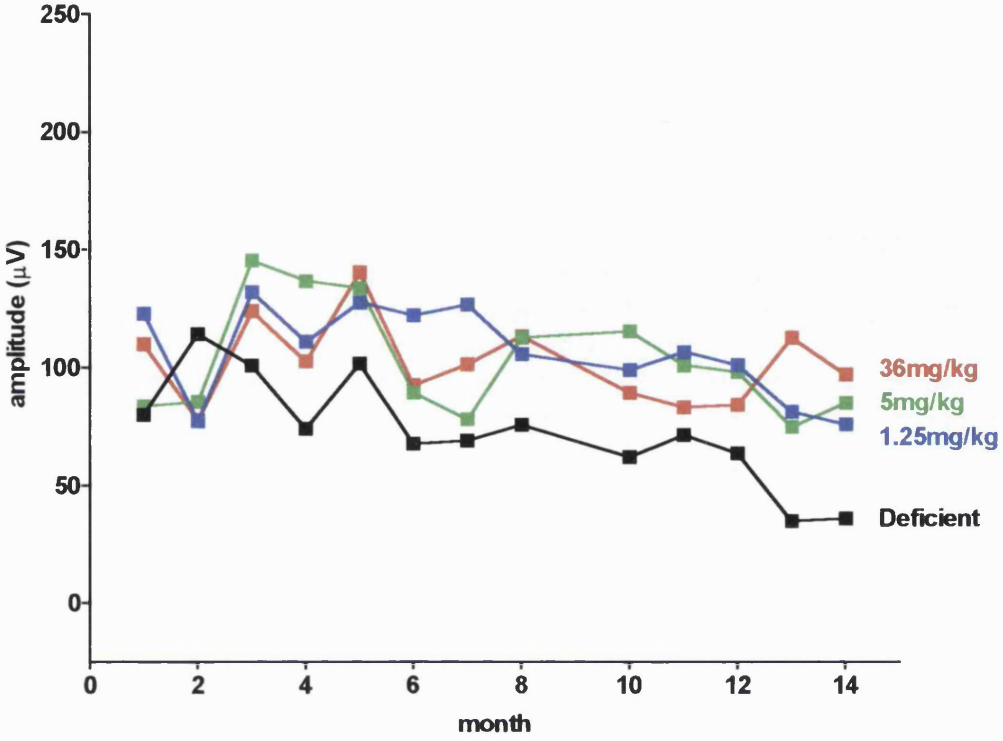
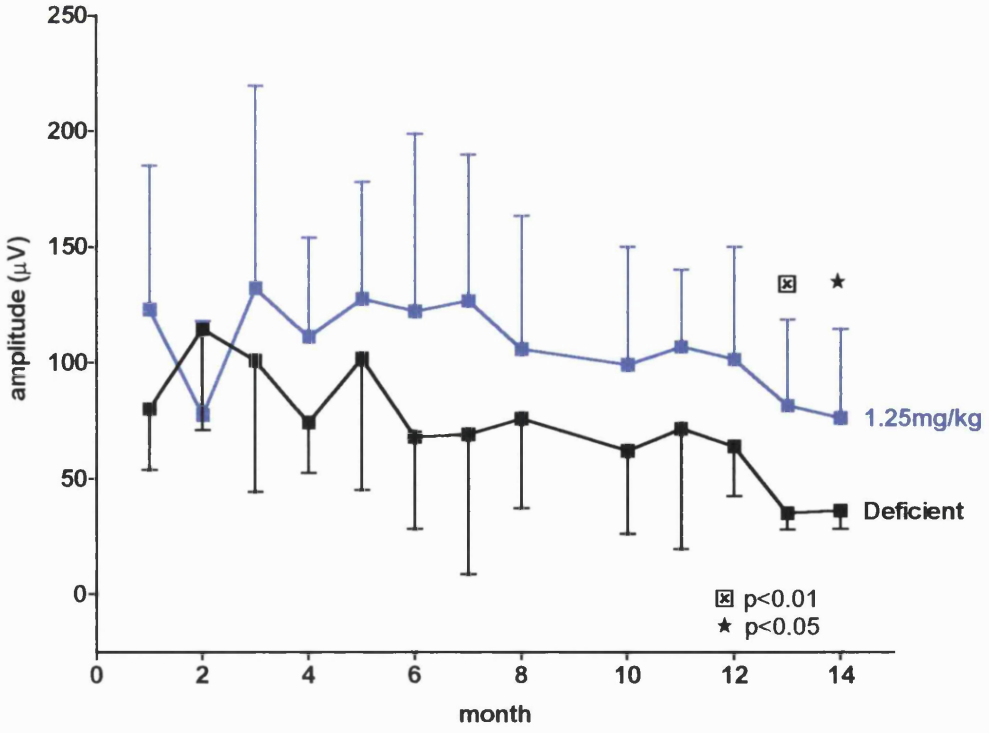


Figure 4.27 Group mean ERG amplitudes +/- 1SD
- deficient v 1.25mg/kg (dim blue flash)



Dim (blue filtered) VEP latencies

Figure 4.28 shows the mean VEP latencies, recorded in response to dim flash stimuli, for all four groups. The group mean VEP latencies of the deficient group were consistently significantly longer than those of the 1.25mg/kg group after 10 months (Figure 4.29) and the 5mg/kg group after 11 months (Figure 4.30). No significant differences in latency were observed when the 5mg/kg group was compared to either the 1.25 or 36mg/kg groups.

Dim (blue filtered) VEP amplitudes

No significant differences were found between any of the groups when mean VEP amplitudes were compared (Figure 4.31).

Figure 4.28 Group mean VEP latencies (dim blue flash)

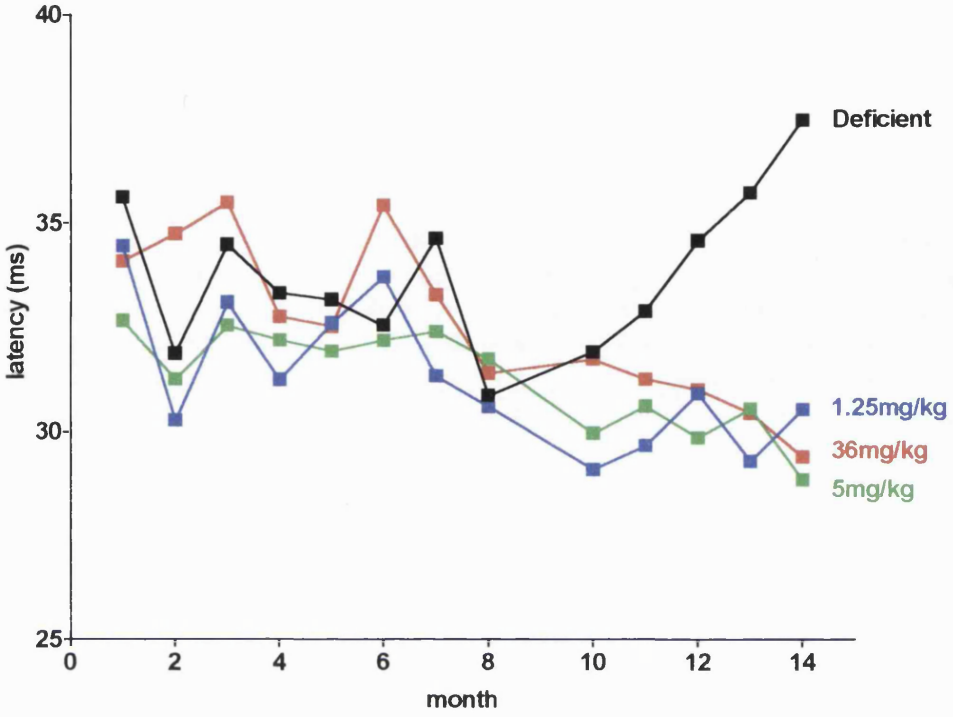


Figure 4.29 Group mean VEP latencies +/- 1SD
- deficient v 1.25mg/kg (dim blue flash)

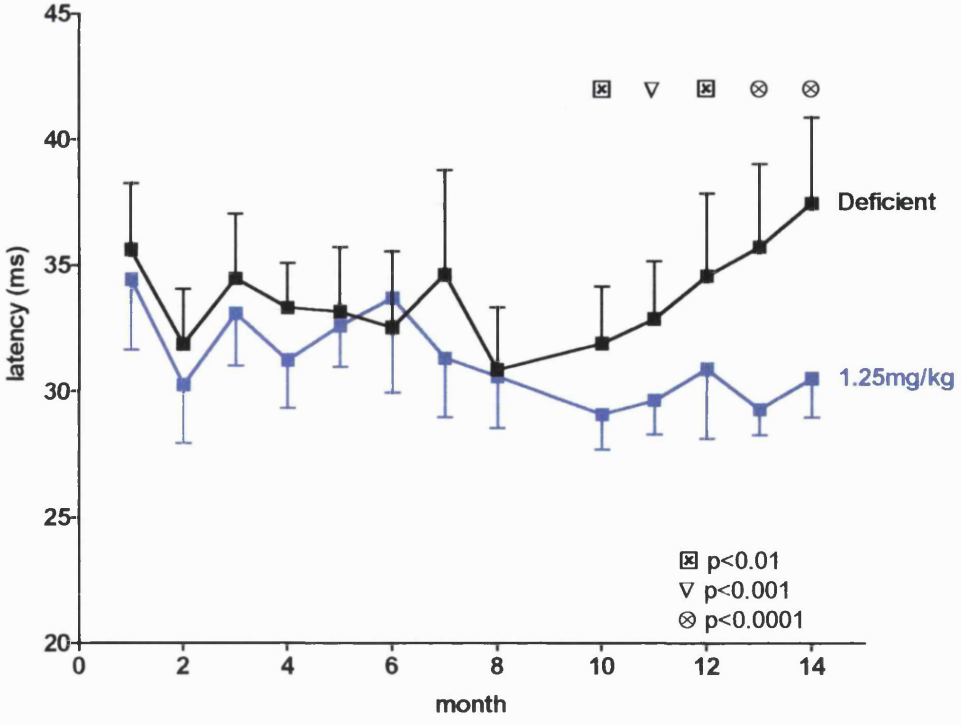


Figure 4.30 Group mean VEP latencies +/- 1SD
- deficient v 5mg/kg (dim blue flash)

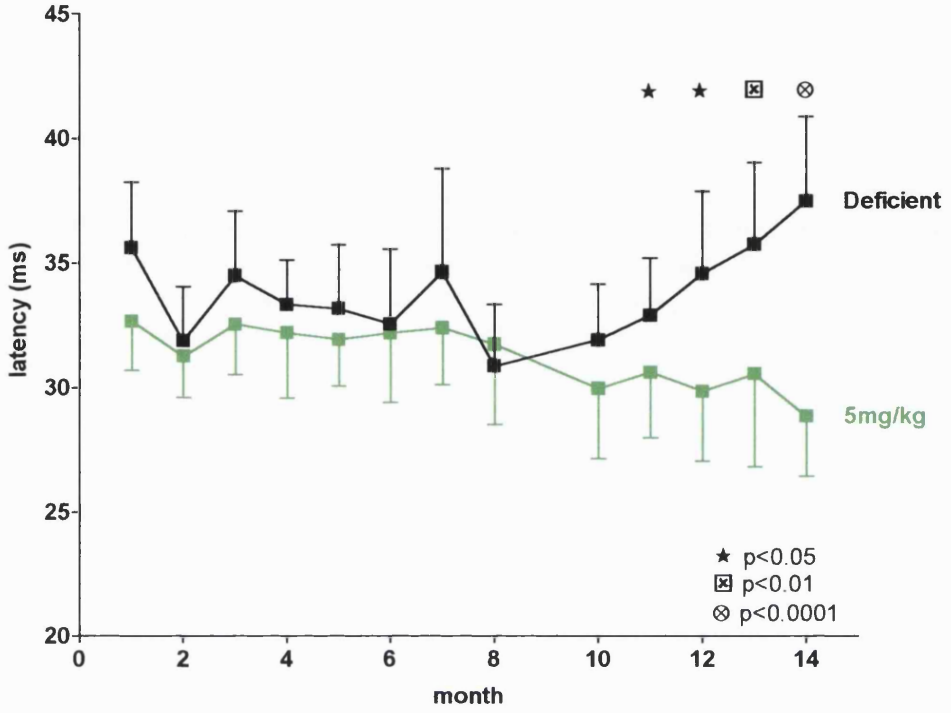
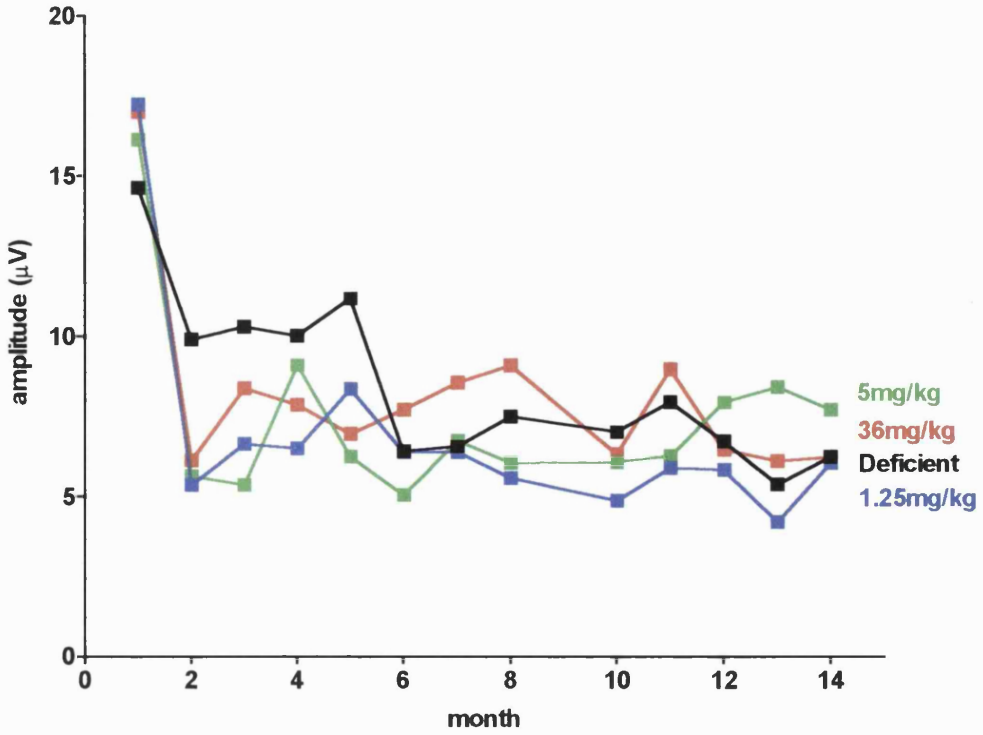


Figure 4.31 Group mean VEP amplitudes (dim blue flash)



Bright white ERG latencies

The mean ERG b wave latencies, recorded in response to bright white flash stimuli, for all the groups are shown in Figure 4.32. The latencies for all groups remained relatively constant for the first 7 months of the study, then the latencies of the deficient and 1.25mg/kg increased. The deficient group had significantly longer ($P<0.05$) ERG latencies than the 5mg/kg group after 10 months (72.02 +/- 13.67 compared with 60.75 +/- 4.08ms, Figure 4.33) and significantly longer latencies ($P<0.05$) than the 1.25mg/kg group after 13 months (81.24 +/- 16.45 compared with 66.29 +/- 15.94ms, Figure 4.34). There were no significant differences between the 5mg/kg and 36mg/kg groups at any point during the study.

Bright white ERG amplitudes

The mean ERG peak-to-peak amplitudes remained relatively similar in all the groups until month 8, after which they began to diverge (Figure 4.35). The amplitudes of the deficient group became consistently significantly smaller than the 5mg/kg group after 12 months (Figure 4.36) and smaller than the 1.25mg/kg group after 13 months (Figure 4.37). The ERG amplitudes of the 1.25mg/kg group tended to fall after 12 months, but were not found to be consistently significantly different from those of the 5mg/kg or 36mg/kg groups. No significant differences were found between the 5mg/kg and 36mg/kg groups throughout the duration of the study.

Figure 4.32 Group mean ERG b wave latencies (bright white flash)

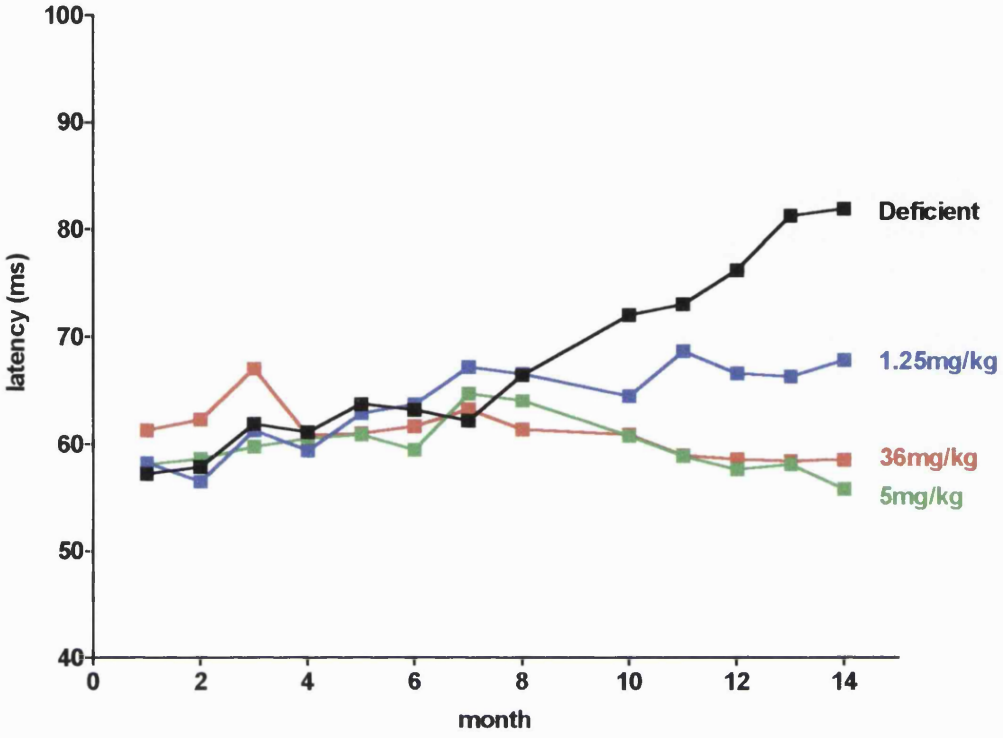


Figure 4.33 Group mean ERG b wave latencies +/- 1SD
- deficient v 5mg/kg (bright white flash)

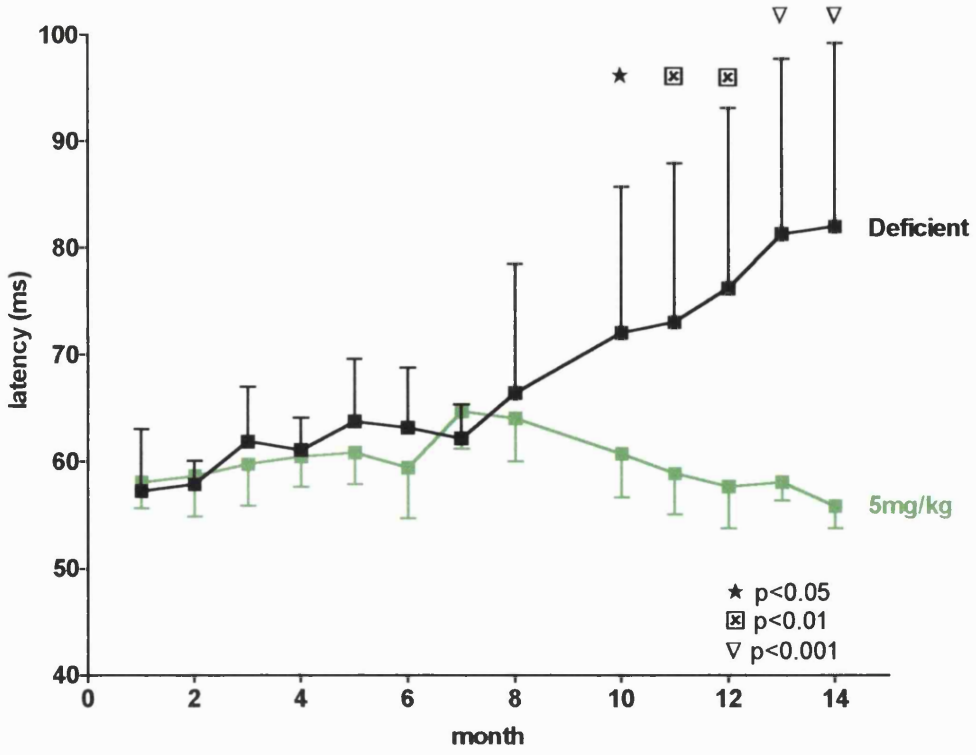


Figure 4.34 Group mean ERG b wave latencies +/- 1SD
- deficient v 1.25mg/kg (bright white flash)

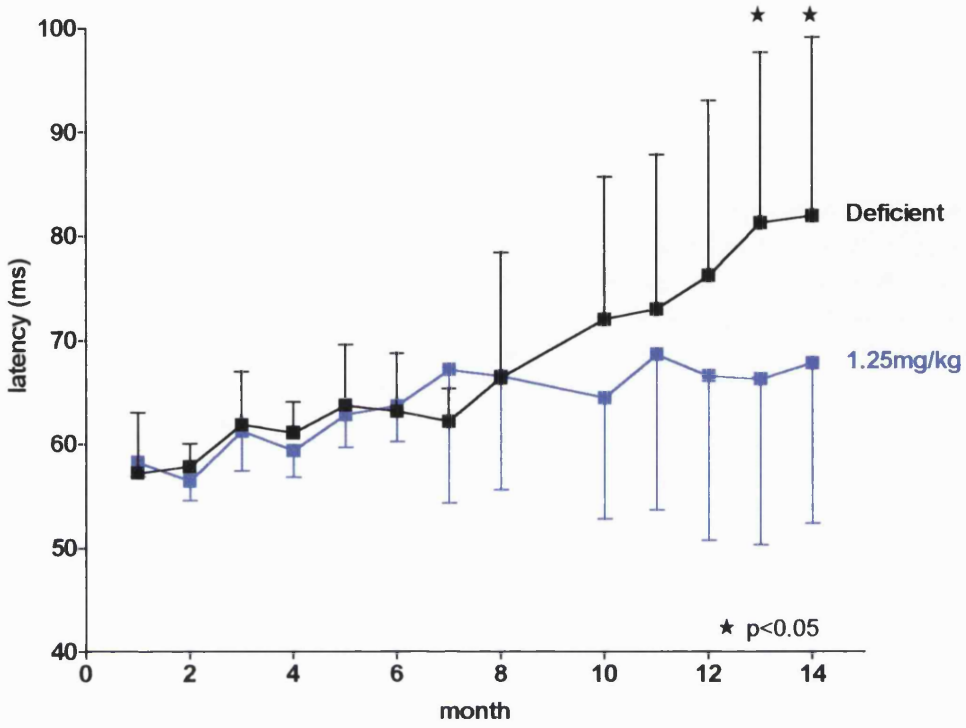


Figure 4.35 Group mean ERG amplitudes (bright white flash)

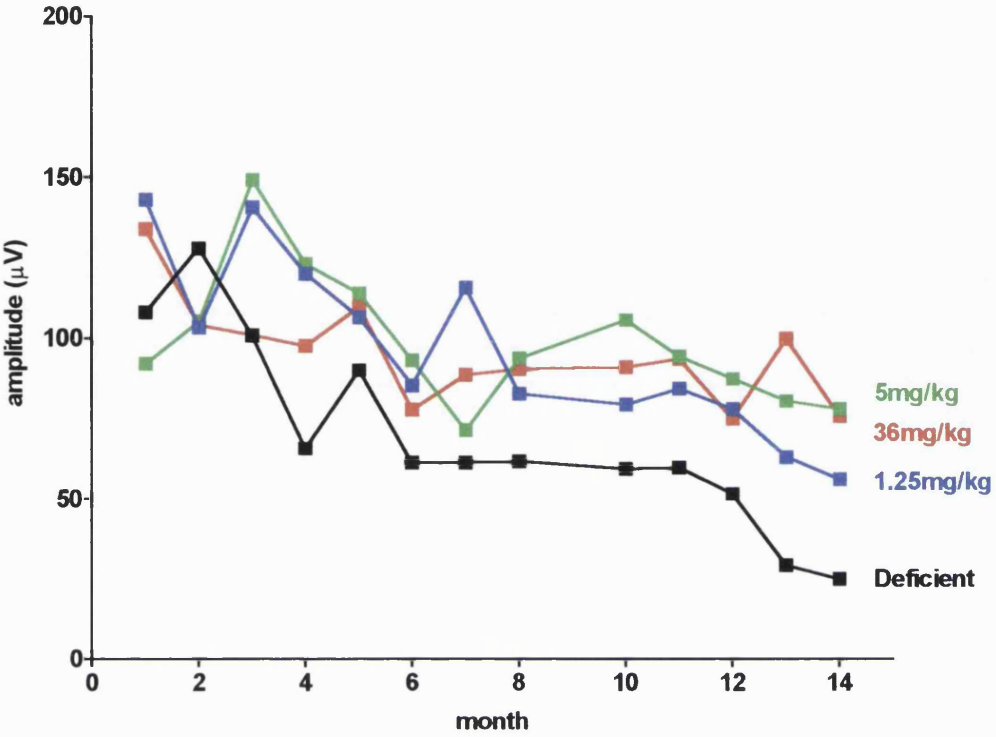


Figure 4.36 Group mean ERG amplitudes +/- 1SD
- deficient v 5mg/kg (bright white flash)

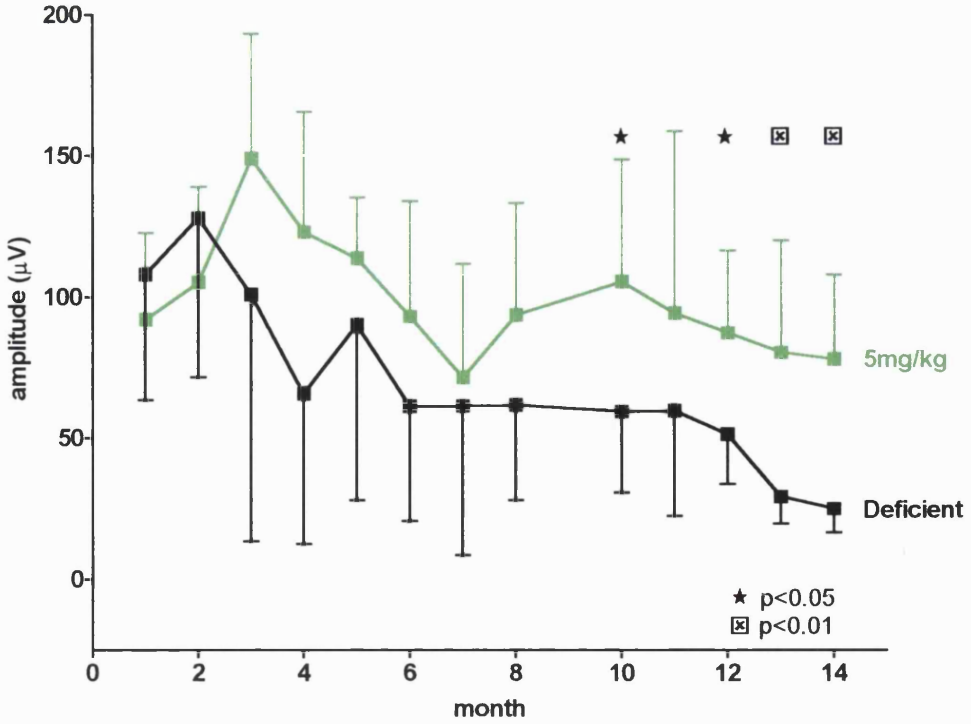
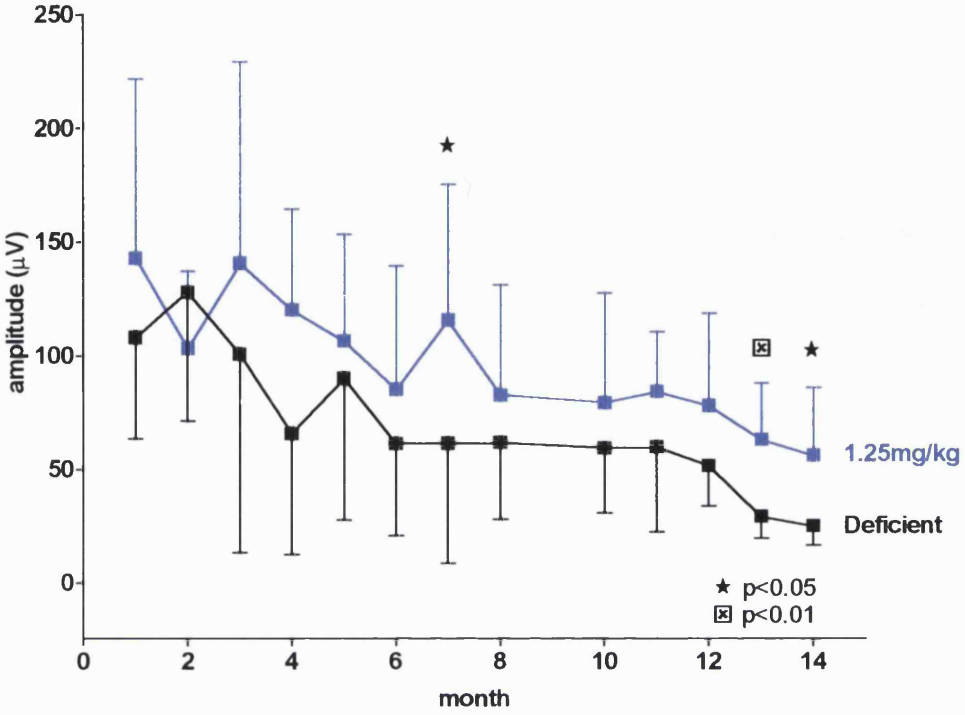


Figure 4.37 Group mean ERG amplitudes +/- 1SD
- deficient v 1.25mg/kg (bright white flash)



Bright white VEP latencies

The group mean VEP latencies of the deficient group increased after 8 months, while those of the other groups remained relatively constant throughout the study (Figure 4.38). The latencies of the deficient group were consistently significantly longer than those of the 5mg/kg (Figure 4.39) and 1.25mg/kg (Figure 4.40) groups after 12 months. No consistently significant differences were found when the 1.25mg/kg, 5mg/kg and 36mg/kg groups were compared.

Bright white VEP amplitudes

There were no significant differences between the groups when VEP amplitudes were compared (Figure 4.41).

Figure 4.38 Group mean VEP latencies (bright white flash)

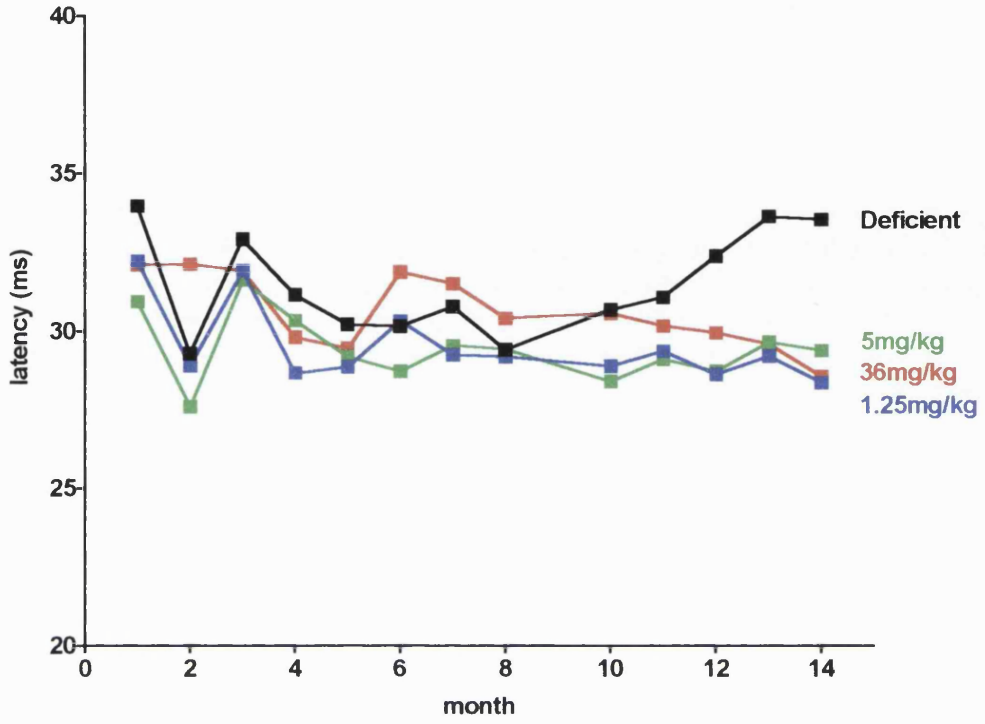


Figure 4.39 Group mean VEP latencies +/- 1SD
- deficient v 5mg/kg (bright white flash)

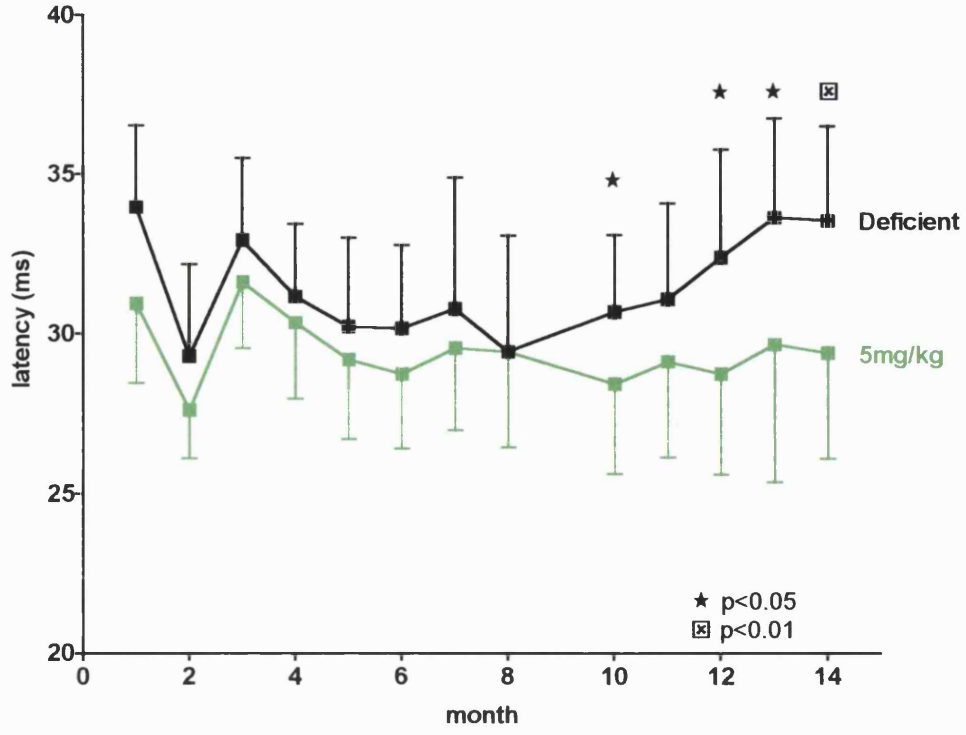


Figure 4.40 Group mean VEP latencies +/- 1SD
- deficient v 1.25mg/kg (bright white flash)

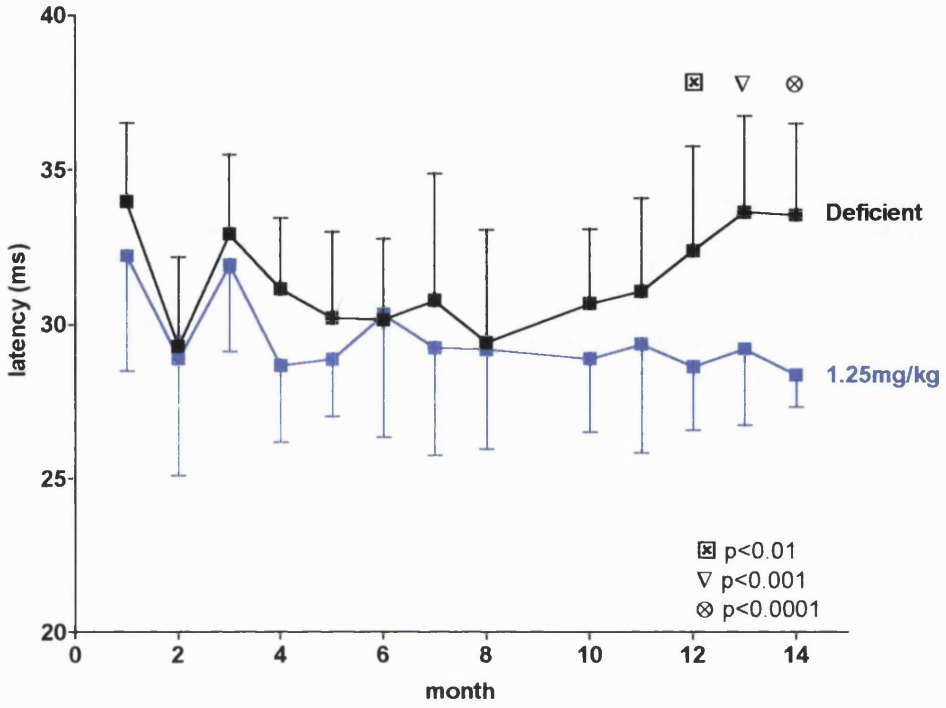
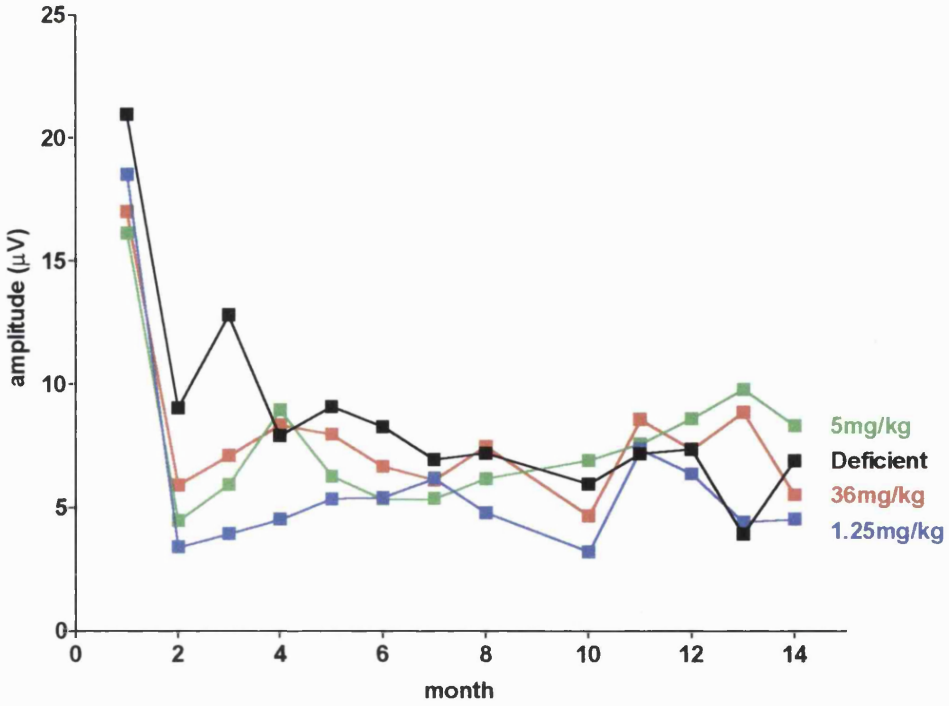


Figure 4.41 Group mean VEP amplitudes (bright white flash)



4.6 Results - Biochemistry

4.6.1 Tissue α-tocopherol concentrations

The concentrations of α-tocopherol were measured in tissues from six rats from each group after 14 months on the respective diets. Figure 4.42 shows the concentrations found in non-neural tissues and Figure 4.43, those in neural tissues. The expected increase in concentration with increasing dietary intakes of α-tocopherol was observed.

α-tocopherol was undetectable in plasma, liver, gastrocnemius muscle, heart and testis from the deficient group. α-tocopherol was however detectable in the sciatic nerve, thoracic cord and cerebral cortex of deficient animals (means 0.06, 0.45 and 0.27μg/g wet weight, respectively). The rats which received 1.25mg/kg α-tocopheryl acetate were found to have low concentrations of tocopherol in the plasma, liver, heart, testis and gastrocnemius (means 0.64μmol/l and 0.44, 1.06, 0.35 and 0.29μg/g wet weight, respectively). However, this group had more tocopherol in the nervous tissues - sciatic nerve 0.53μg/g, thoracic cord 1.32μg/g and cerebral cortex 1.39μg/g wet weight - than the deficient group.

Figure 4.43 also shows the concentrations found in eyes from rats in the deficient, 1.25mg/kg and 36mg/kg groups (n=6 per group). Those taken from the deficient group were found to have undetectable levels of α-tocopherol, compared to a small amount in eyes from the 1.25mg/kg group (mean 0.10μg/g) and a mean of 4.63μg/g in the 36mg/kg group.

The mean concentration of α-tocopherol present in the tissues was plotted against the dietary intake of tocopherol, as shown in Figures 4.44 and 4.45 for non-neural and neural tissues respectively. For the sciatic nerve and eye, the relationship was linear up to a dietary intake of 36mg/kg. For the other tissues, however, the relationship was linear up to 5mg/kg but then tended to plateau. There was a linear relationship in the majority of tissues between the concentrations of α-tocopherol found in plasma and the various tissues (see Figures 4.46 and 4.47).

Figure 4.42 Concentrations of α-tocopherol in non-neural tissues

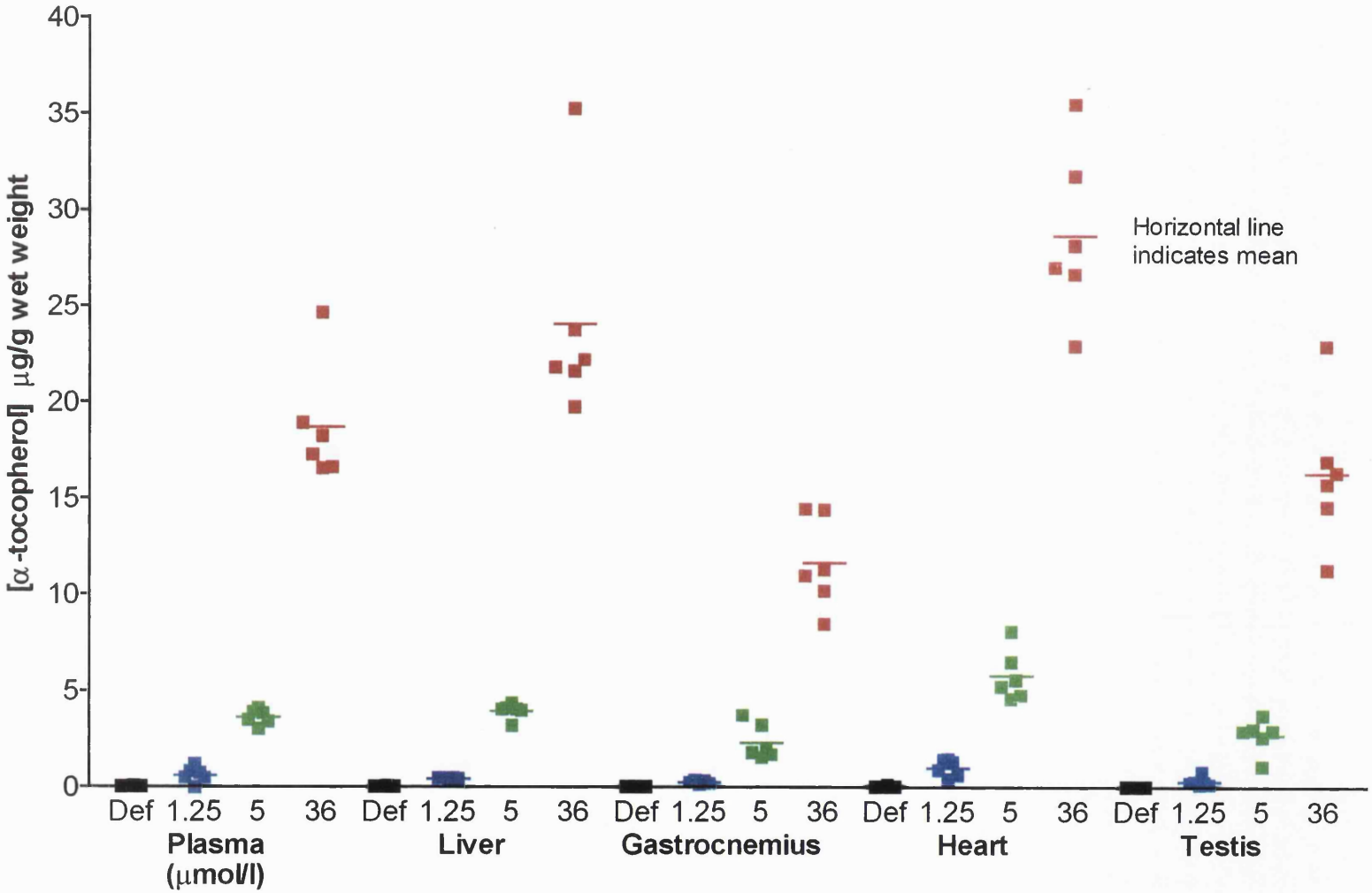


Figure 4.43 Concentrations of α-tocopherol in neural tissues

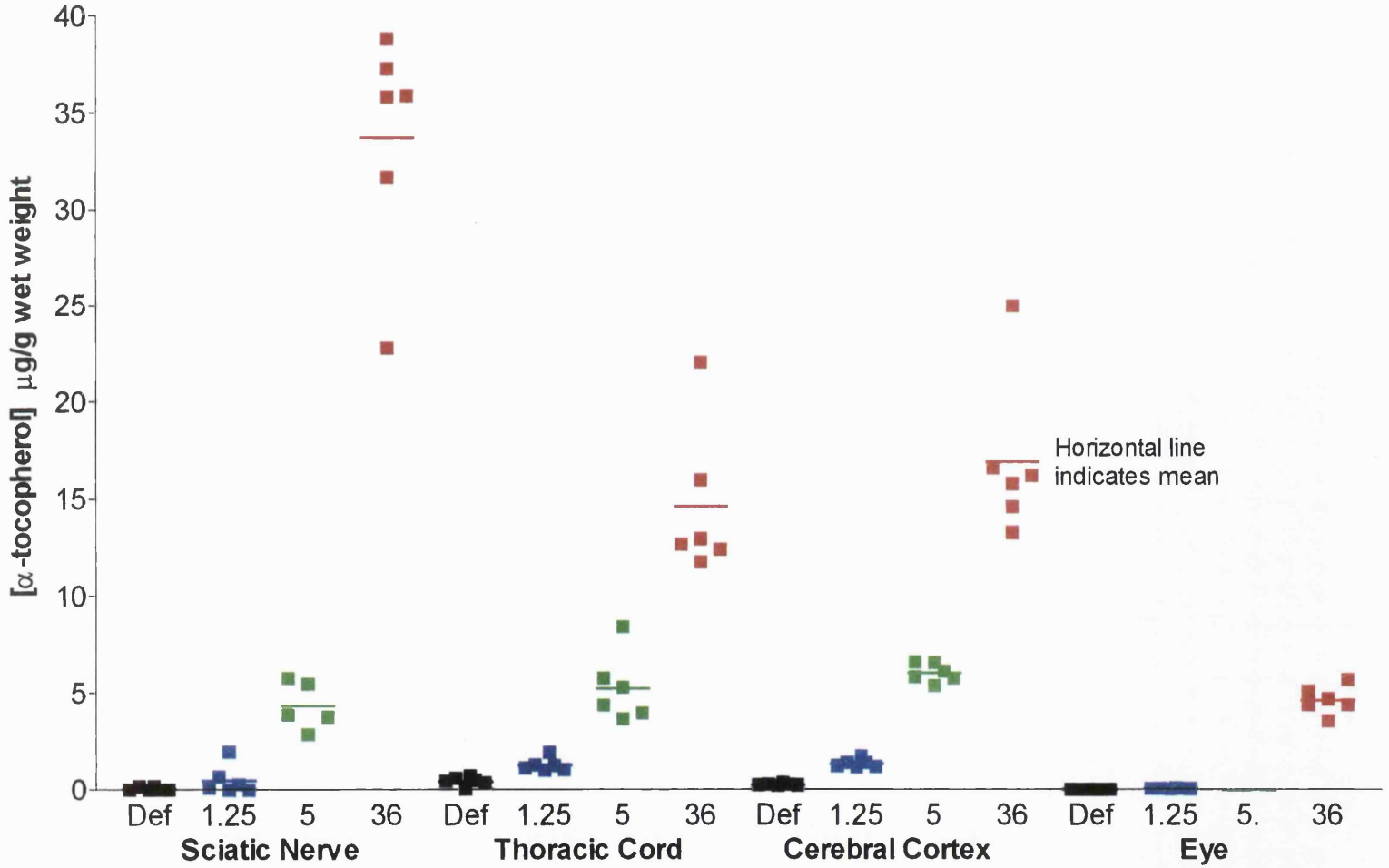
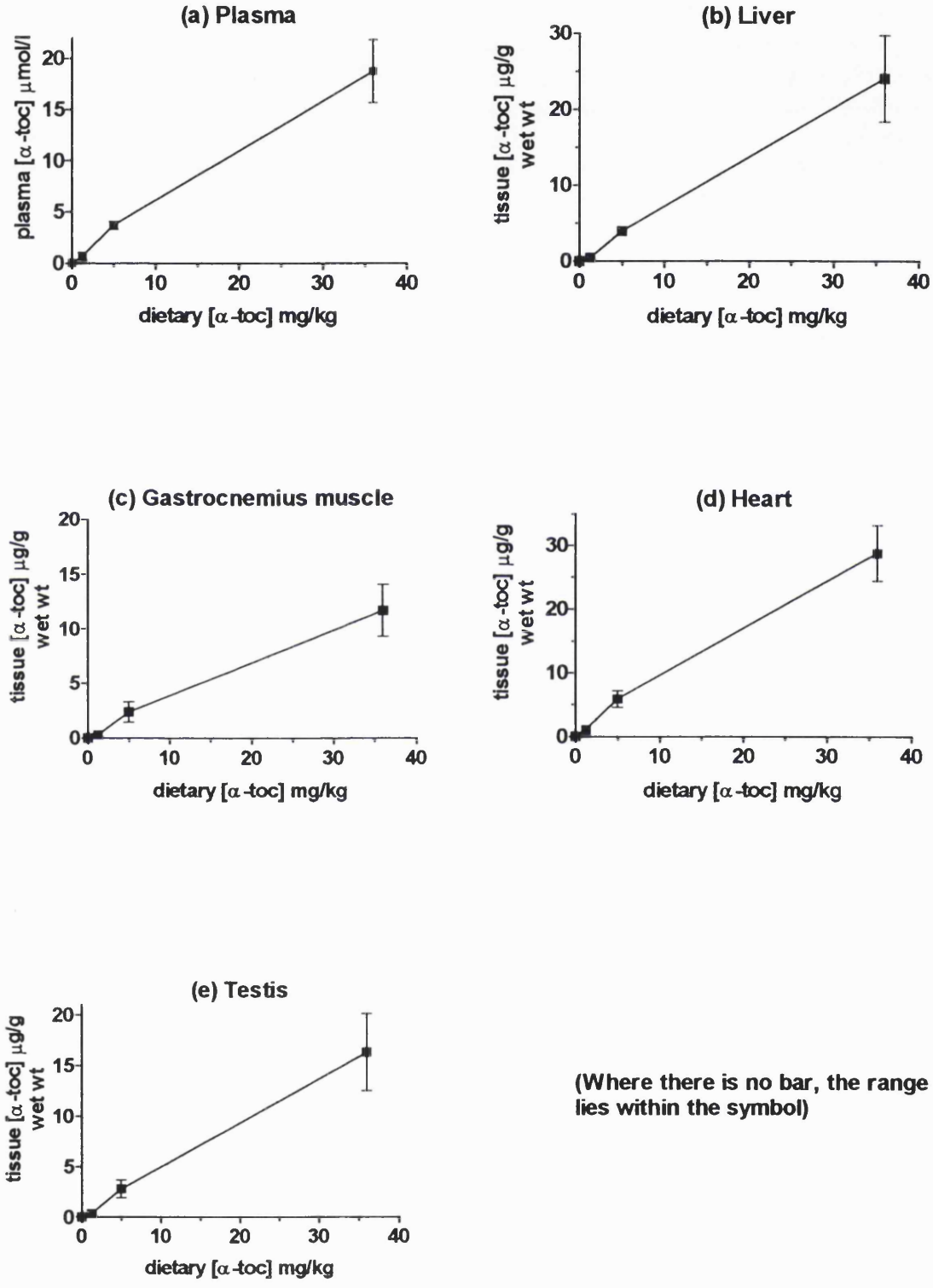
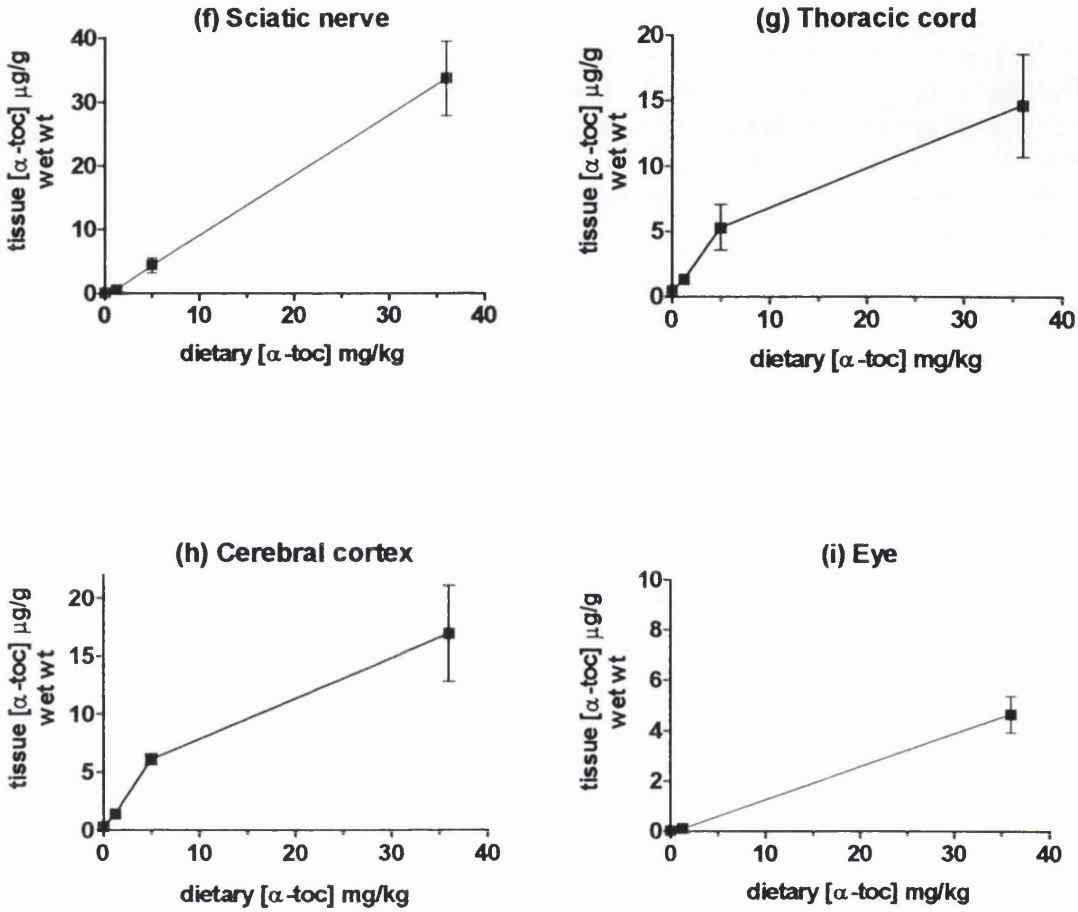


Figure 4.44 Relationship between dietary intake of α-tocopherol and its concentration in non-neural tissues (mean +/- 1SD)



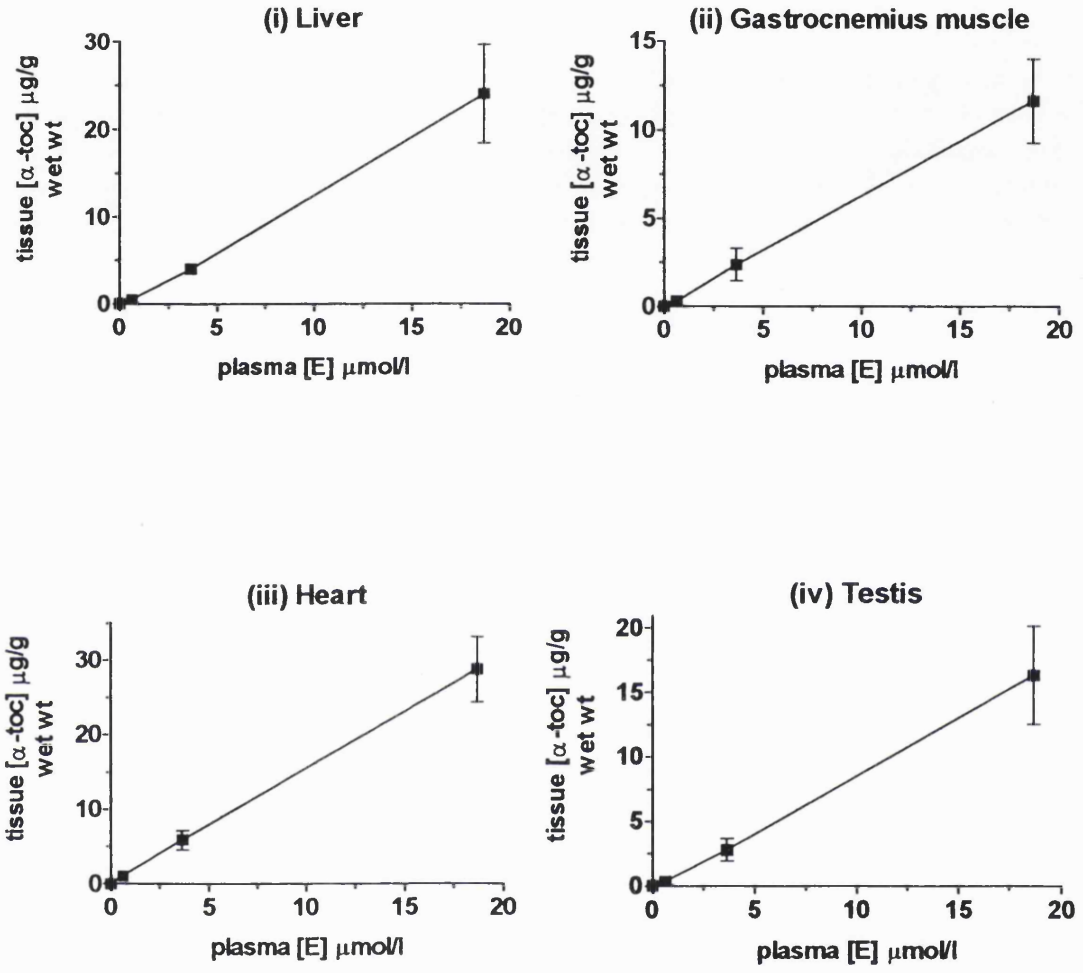
(Where there is no bar, the range lies within the symbol)

Figure 4.45 Relationship between dietary intake of α-tocopherol and its concentration in neural tissues (mean +/- 1SD)



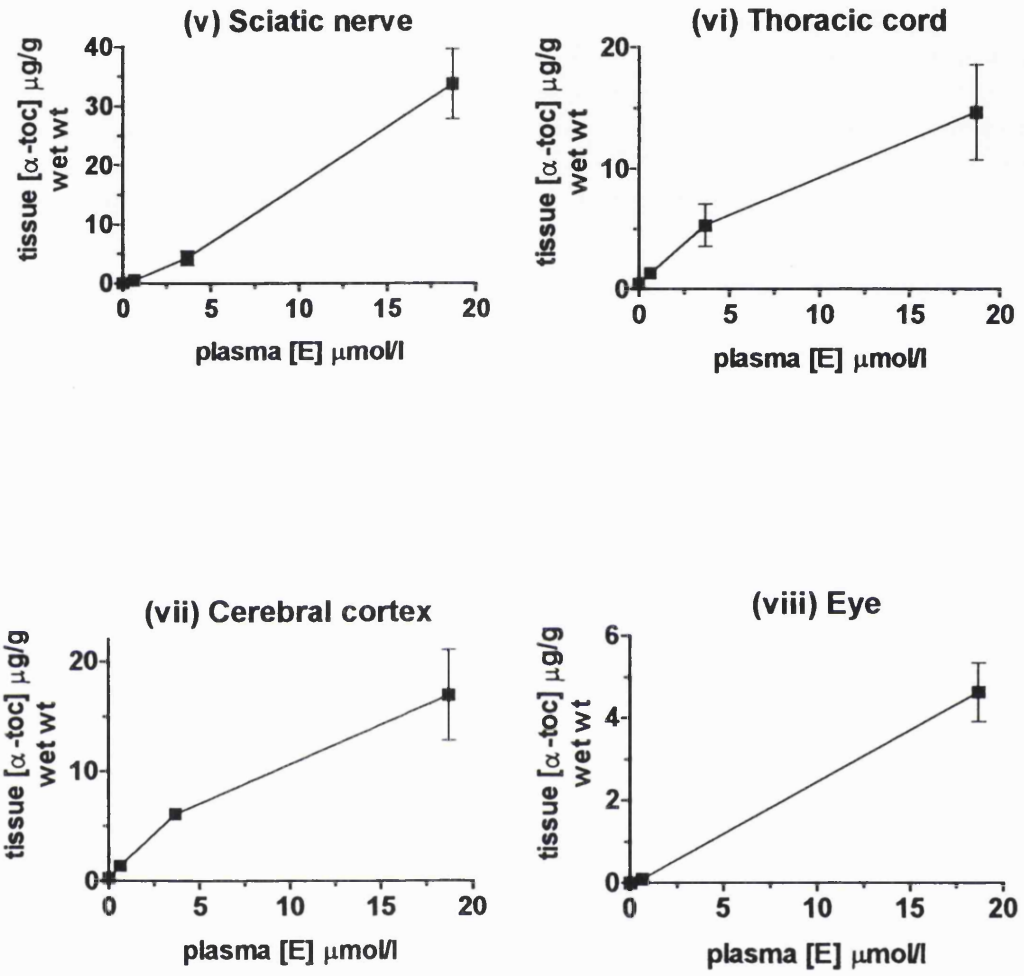
(Where there is no bar, the range lies within the symbol)

Figure 4.46 Relationship between α-tocopherol concentrations in plasma and non-neural tissues (mean +/- 1SD)



(Where there is no bar, the range lies within the symbol)

Figure 4.47 Relationship between α-tocopherol concentrations in plasma and neural tissues (mean +/- 1SD)



(Where there is no bar, the range lies within the symbol)

4.6.2 Tissue malondialdehyde (MDA) concentrations

Free, total and bound MDA concentrations were determined in liver, gastrocnemius muscle, heart, cerebral cortex and eye from 9 rats from both the deficient and 36mg/kg groups. The spread of results obtained from tissues from the deficient group was much greater than that of the 36mg/kg group. Significant increases were seen in the concentrations of free MDA in all the tissues from the deficient compared with the 36mg/kg group (Figure 4.48). For example, liver from the deficient group contained a mean concentration of free MDA of 0.85 +/- 0.30 compared with 0.19 +/- 0.11nmol/mg protein in the 36mg/kg group (P=0.0001). The differences were greater in the non-neural tissues than the cerebral cortex and eye.

When total MDA concentrations were compared, significant differences were found between the deficient and 36mg/kg groups in the gastrocnemius muscle (mean 0.86 +/- 0.46nmol/mg protein in the deficient group compared with 0.27 +/- 0.19nmol/mg protein in the 36mg/kg group) and heart (0.85 +/- 0.39nmol/mg protein compared with 0.21 +/- 0.09nmol/mg protein), but not in the liver (Figure 4.49). When the neural tissues were investigated, the cortex of the deficient rats had a significantly higher concentration of total MDA compared with the 36mg/kg group (0.68 +/- 0.67nmol/mg protein compared with 0.33 +/-nmol/mg protein, respectively), but there were no significant differences in total MDA in the eyes from the two groups. No significant differences in concentrations of bound MDA were seen in gastrocnemius muscle, heart, cerebral cortex or eye (Figure 4.50), but the liver of the deficient animals had a significantly reduced (P<0.05) concentration.

Figure 4.48 Free MDA concentrations

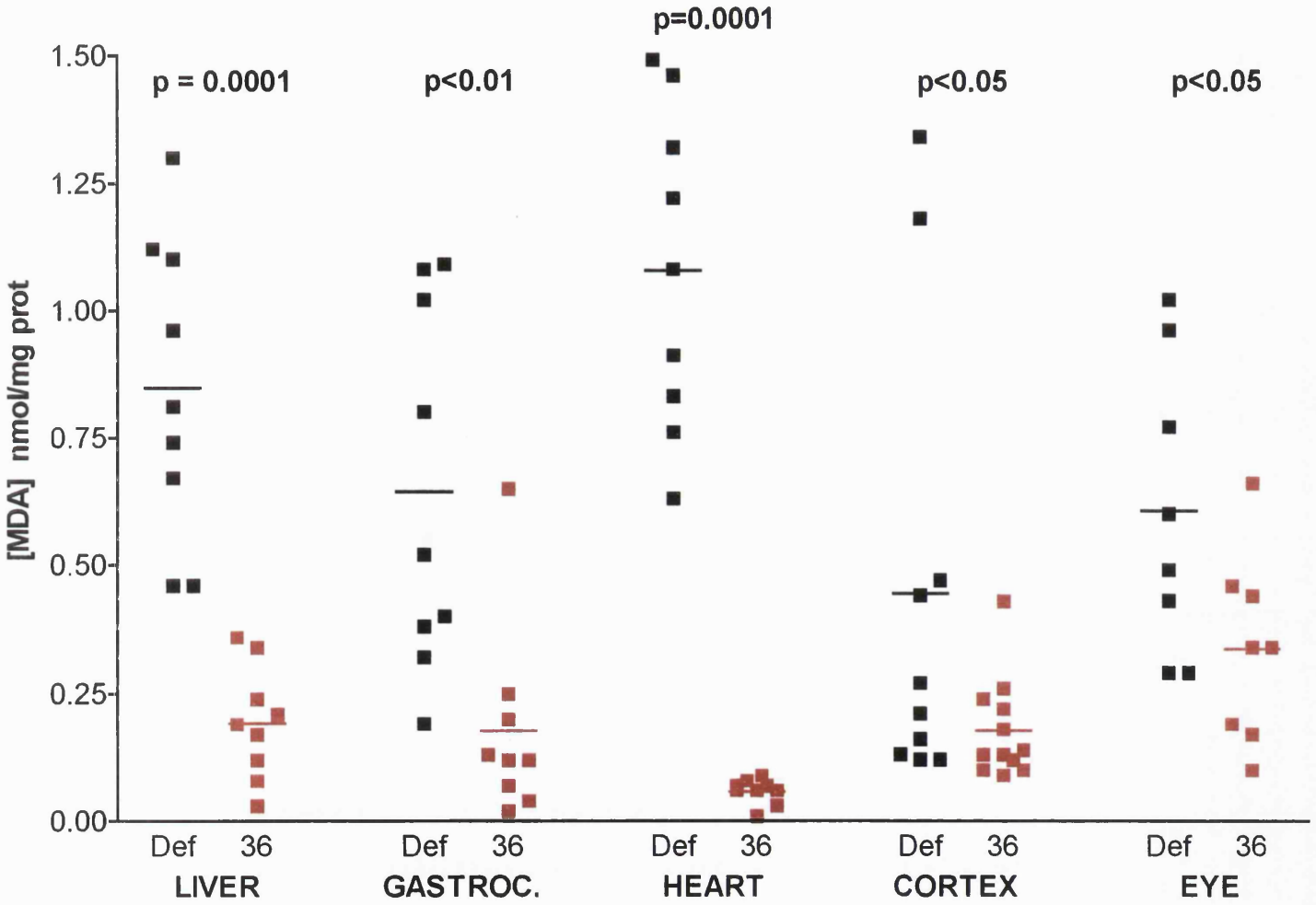


Figure 4.49 Total MDA concentrations

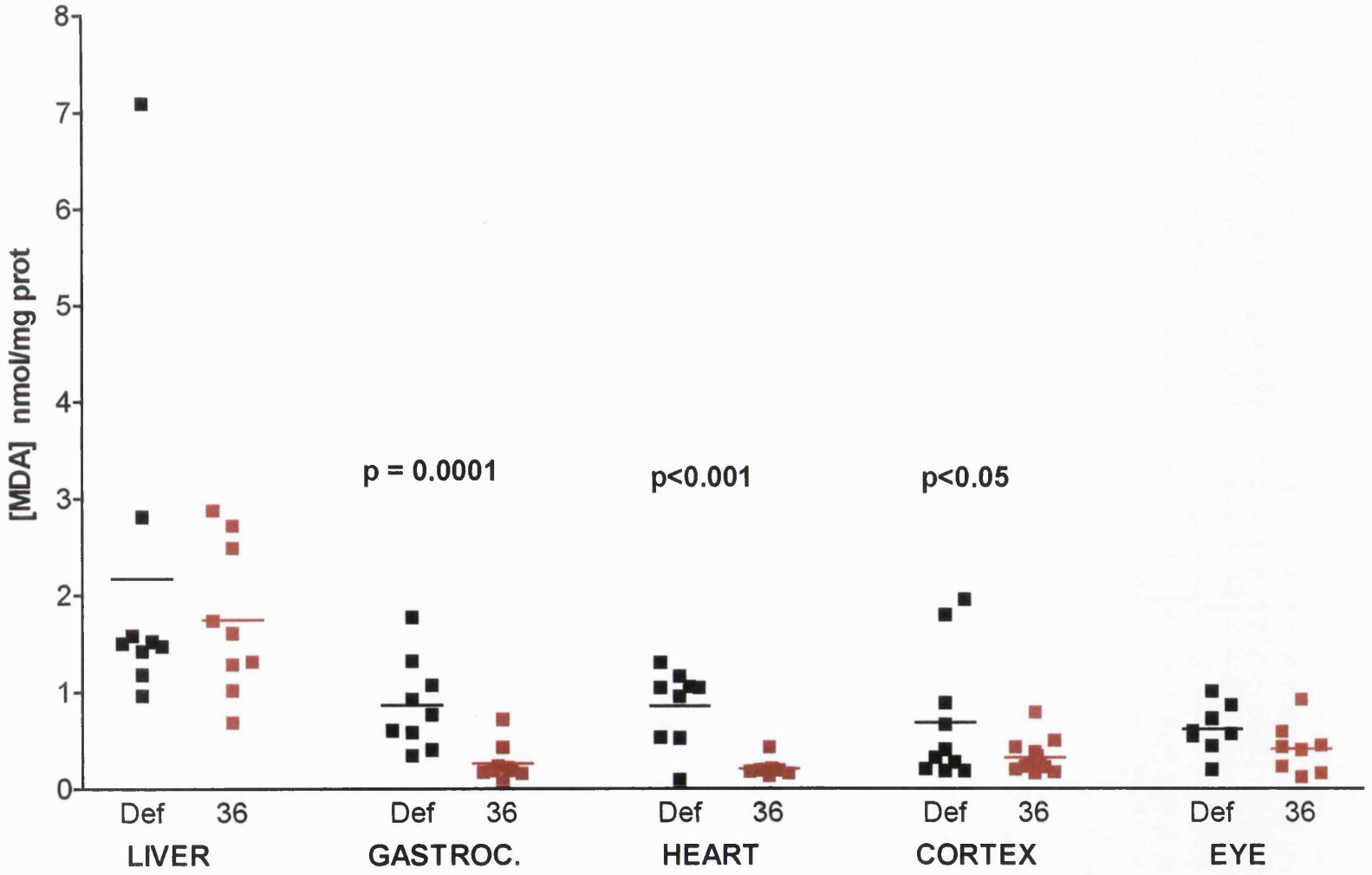
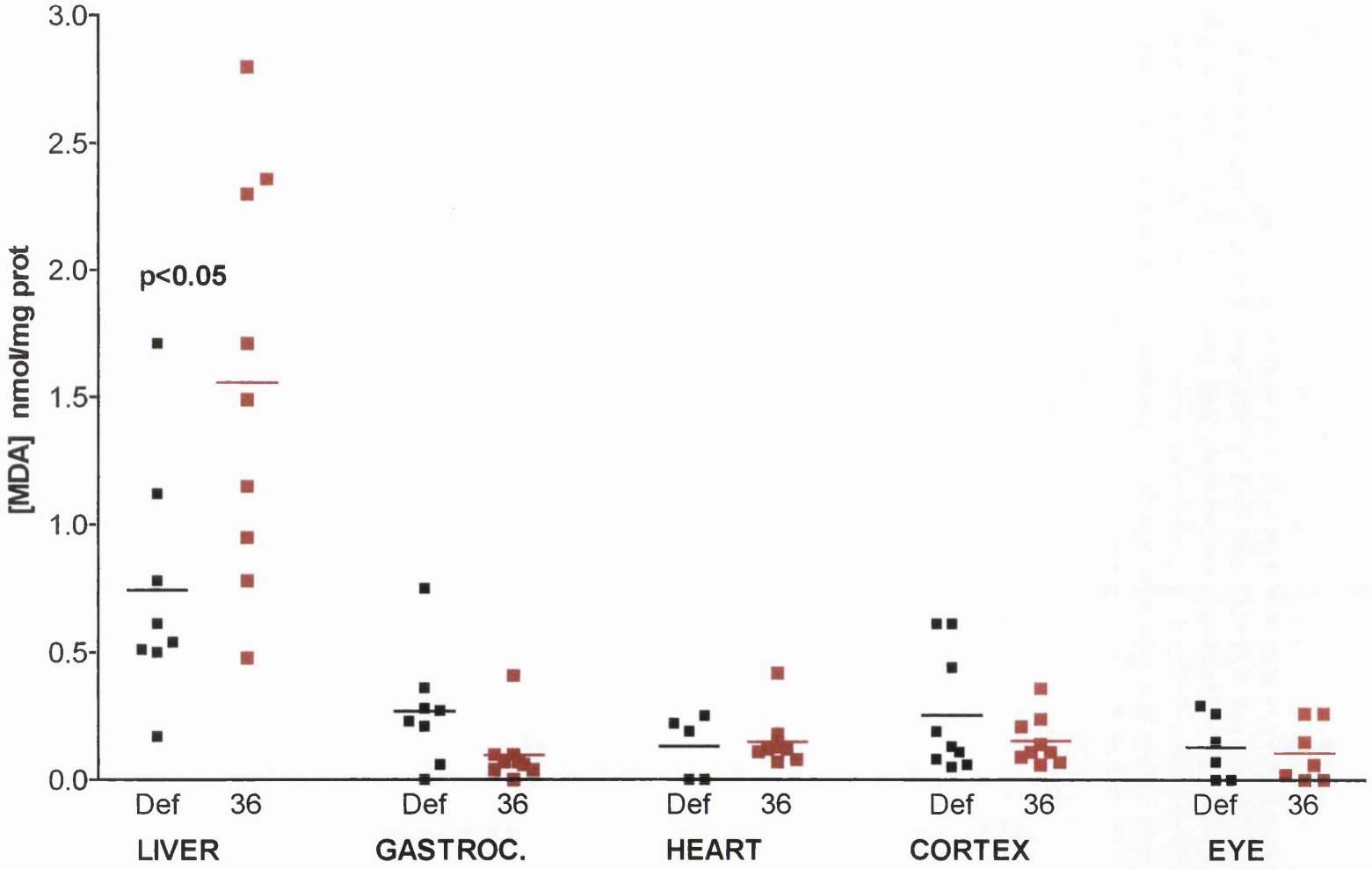


Figure 4.50 Bound MDA concentrations



The electrophysiological and biochemical results from this study using all-rac-α-tocopheryl acetate will be discussed in Chapter 6 together with those obtained in Study II using predominantly RRR-α-tocopheryl acetate.

Chapter 5

Study II: RRR-α-tocopheryl acetate

5.1 Introduction

The major aim of Study I was to determine the minimum dietary intake of α-tocopherol necessary to prevent abnormalities in neural function. The results of the study, as detailed in Chapter 4, indicated that the minimum requirement was approximately 1.25mg/kg all-rac-α-tocopheryl acetate. For Study II, dietary α-tocopherol concentrations were chosen to confirm the marginal protection of the 1.25mg/kg diet in neural tissues. Another aim of the study was to compare the biological activity of synthetic all-rac-α-tocopherol with that of the natural form (RRR-α-tocopherol). For this reason, rats in study II were fed diets containing various levels of RRR-α-tocopheryl acetate, apart from one group which received the all-racemic form to provide a direct comparison between the two studies.

5.2 Experimental diets

The same vitamin E deficient diet (HLR 814, Dyets Inc USA) was used as for study I to which various levels of deuterated α-tocopheryl acetate were added (see Table 5.1). The different diets were distinguished, as before, by the addition of inert coloured dyes. On this occasion the red dye was not used.

Table 5.1 Concentrations of deuterated α-tocopherol added to the deficient diet

Concentration of deuterated α-tocopheryl acetate (mg/kg diet)	Form of α-tocopheryl acetate	all-rac-α-tocopheryl acetate equivalents
0.00	RRR	0
0.40	RRR	0.54
1.00	**all-rac**	**1.00***
0.75	RRR	1.02*
5.00	RRR	6.80

(* 1.00mg/kg all-rac- is approximately equivalent in activity to 0.75mg/kg RRR-α-tocopheryl acetate)

5.3 Protocol for longitudinal recordings

Since no consistent significant differences were seen between the groups in study I until month 8, electrophysiological recordings in study II were not undertaken until the animals had been fed the experimental diets for 5 months. In study I, results were obtained from lower and upper limb stimulation but time constraints allowed only lumbar and cortical SEPs (in response to tibial nerve stimulation) to be measured during study II, as detailed in section 2.3.2.3. Similar results were also obtained in response to dim (blue-filtered) and bright white flash stimuli in study I. VEPs and the ERG were, therefore, only recorded following bright white flash stimuli during study II as described in section 2.3.3.2. The potentials recorded were then described in terms of their peak latencies and peak-to-peak amplitudes. Recordings were made in the

12 animals from each group at monthly intervals from months 5 to 14. After 14 months, the rats were killed and various tissues collected, as detailed in section 2.4. Tissue α-tocopherol and malondialdehyde concentrations were then determined (see section 2.5).

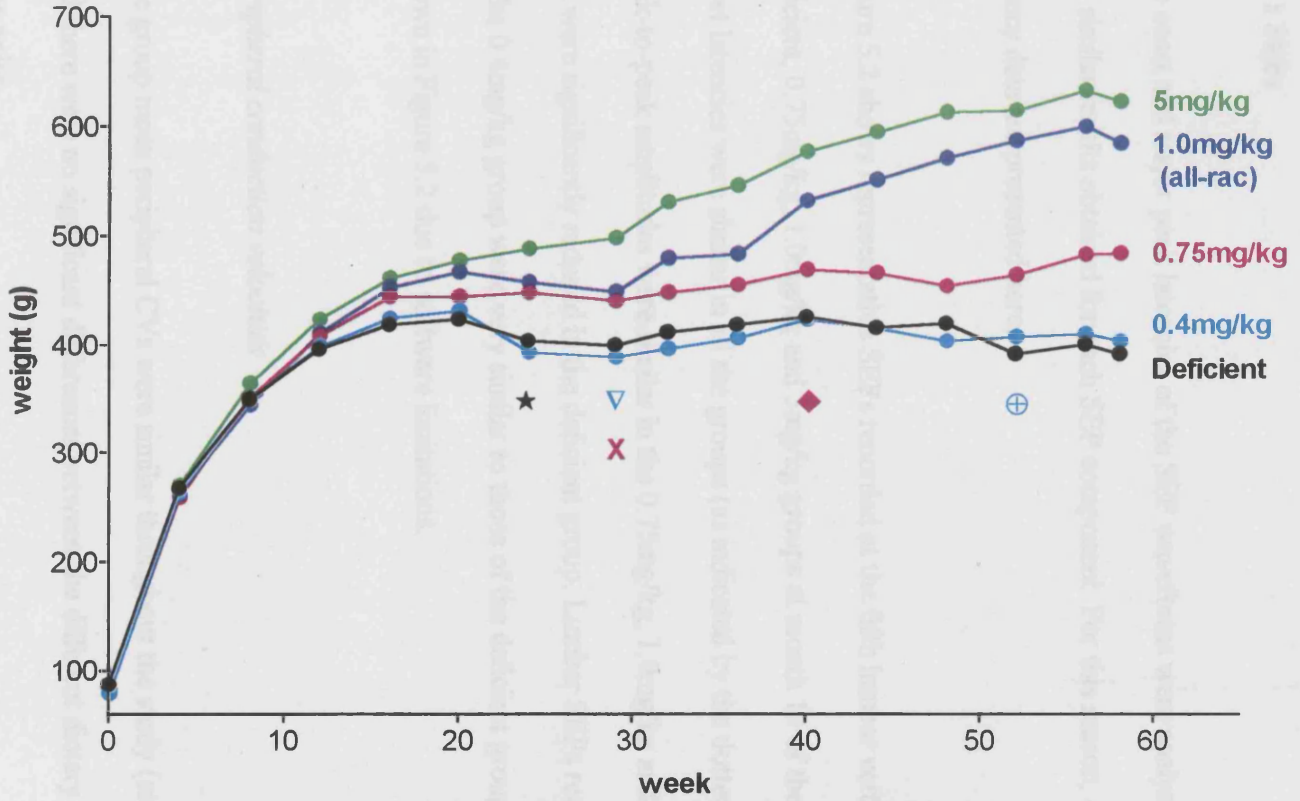
5.4 Growth and condition of the rats

The rats were weighed every week throughout the study period and growth curves plotted from the monthly group means (+/- 1sd) as shown in Figure 5.1. The animals in all the groups rapidly increased in weight for the first 16 weeks of the study, after which the rate of increase slowed. The mean weights of the deficient, 0.4mg/kg and 0.75mg/kg groups did not show an appreciable increase from week 16, whereas the other two groups continued to gain weight for the duration of the study. The deficient rats were found to have a significantly lower mean weight than the 0.75mg/kg ($P<0.05$), 1.0mg/kg ($P<0.01$) and 5mg/kg ($P<0.01$) groups after 24 weeks of the study. No significance differences, however, were found at any time point, when the mean weights of the deficient and 0.4mg/kg groups were compared. At 29 weeks, the 0.4mg/kg group had a significantly lower mean weight than the 1.0mg/kg ($P<0.001$) and 5mg/kg groups ($P<0.0001$). However, the mean weight of the 0.4mg/kg group did not differ significantly from that of the 0.75mg/kg group until week 52 ($P<0.05$). The group mean weight of the 0.75mg/kg rats became significantly different from that of the 5mg/kg animals at 29 weeks ($P<0.01$) and from that of the 1.0mg/kg animals after 40 weeks of study ($P<0.01$). The rats fed a diet containing 5mg/kg RRR-α-tocopheryl acetate had a consistently higher mean weight than those receiving 1.0mg/kg

all-rac-α-tocopheryl acetate, but this difference never reached statistical significance.

The vitamin E deficient rats, and those fed 0.4mg/kg RRR-α-tocopheryl acetate, showed a progressive deterioration in their general condition after 20 weeks. As in study I, the animals' coats became increasingly greasy and matted. At 43 weeks, the first signs of neural problems were seen in 4 deficient rats and 3 animals from the 0.4mg/kg group. These rats had impaired balance and irregular gait, which became progressively worse leading to splaying and finally dragging of the hind limbs with muscle wastage being evident. Animals which received the 0.75mg/kg diet did not display any symptoms of neural dysfunction until week 48, when 4 animals were observed with irregular gait, after which time they rapidly deteriorated. At the end of the study (week 58), all the rats in the deficient, 0.4mg/kg and 0.75mg/kg groups showed some degree of muscle wastage, and ataxia. Three rats fed a diet containing 1.0mg/kg all-rac-α-tocopheryl acetate started to display abnormal balance and gait after 50 weeks, with 7 animals affected at the end of the study. The 5mg/kg (control) group rats did not show any signs of neural deficit or deterioration in appearance for the duration of the study.

Figure 5.1 Growth curves



★ Deficient group significantly different from 0.75mg/kg, 1.0mg/kg (all-rac) and 5mg/kg groups
▽ 0.4mg/kg group significantly different from 1.0mg/kg (all-rac) and 5mg/kg groups
X 0.75mg/kg group significantly different from 5mg/kg group
◆ 0.75mg/kg group significantly different from 1.0mg/kg (all-rac) group
⊕ 0.4mg/kg group significantly different from 0.75mg/kg group

5.5 Results of electrophysiological recordings

5.5.1 SEPs

The onset and major peak latencies of the SEP waveforms were analysed statistically and similar results obtained for each SEP component. For this reason, only onset latency data are presented here.

Figure 5.2 shows representative SEPs recorded at the fifth lumbar vertebra in the deficient, 0.75mg/kg, 1.0mg/kg and 5mg/kg groups at month 14 of the study. The onset latencies were similar in all the groups (as indicated by the dotted line). The peak-to-peak amplitudes were similar in the 0.75mg/kg, 1.0mg/kg and 5mg/kg groups, but were significantly reduced in the deficient group. Lumbar SEPs recorded from rats in the 0.4mg/kg group were very similar to those of the deficient group, but are not shown in Figure 5.2 due to software limitations.

Peripheral conduction velocities

The group mean peripheral CVs were similar throughout the study (see Figure 5.3), and there were no significant differences between the different dietary groups, at any time point.

Figure 5.2 SEPs recorded at month 14 in representative rats from the deficient, 0.75mg/kg, 1.0mg/kg and 5mg/kg groups

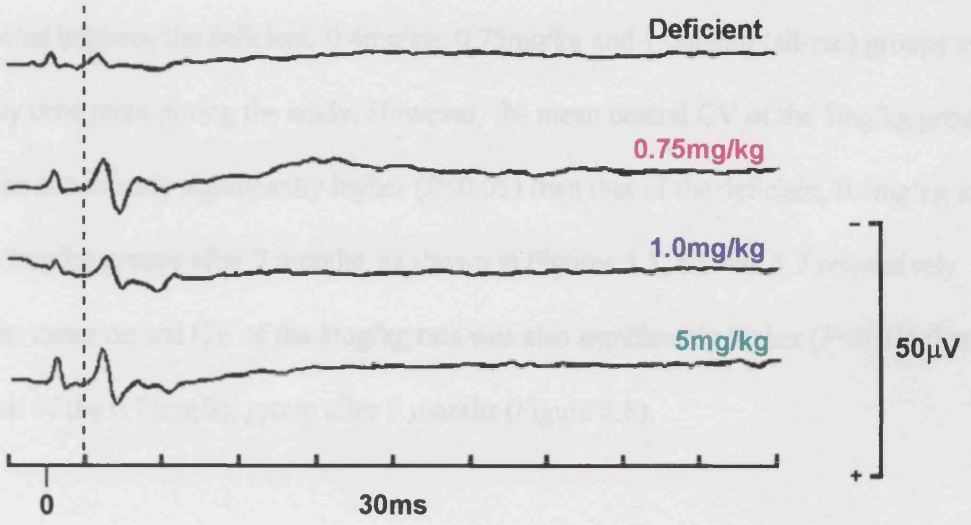
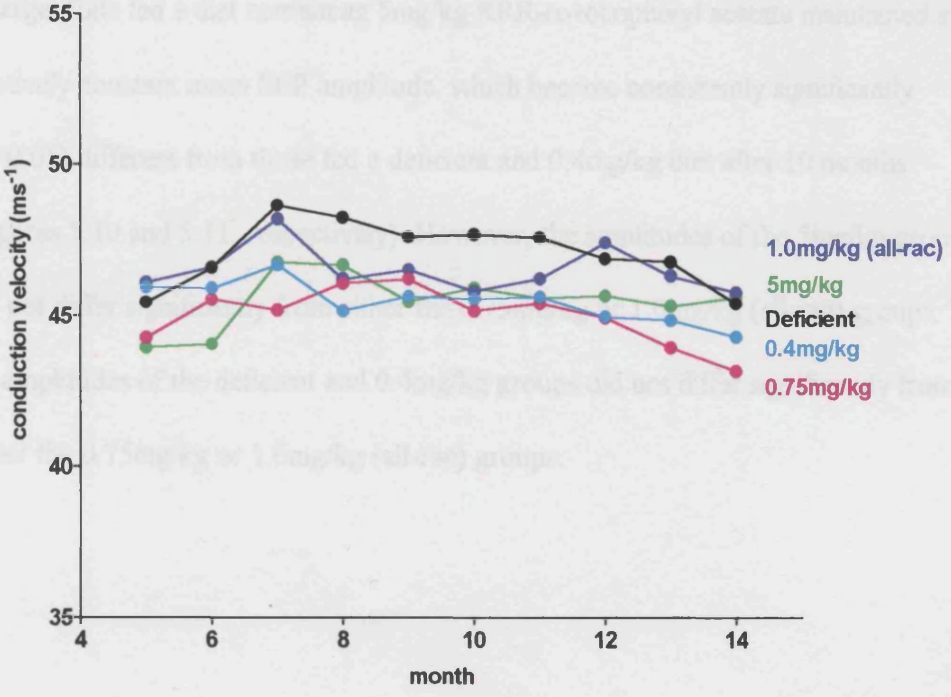


Figure 5.3 Group mean peripheral conduction velocities (lower limb)



Central conduction velocities

With the exception of the 5mg/kg group, the mean central conduction velocities of all the other groups decreased after month 9 (Figure 5.4). No significant differences were found between the deficient, 0.4mg/kg, 0.75mg/kg and 1.0mg/kg (all-rac) groups at any time point during the study. However, the mean central CV of the 5mg/kg group was consistently significantly higher ($P<0.05$) than that of the deficient, 0.4mg/kg and 1.0mg/kg groups after 7 months, as shown in Figures 5.5, 5.6 and 5.7 respectively. The mean central CV of the 5mg/kg rats was also significantly higher ($P<0.01$) than that of the 0.75mg/kg group after 9 months (Figure 5.8).

L5 SEP peak-to-peak amplitudes

The group mean amplitudes of the responses recorded at L5 are shown in Figure 5.9. All the groups gave similar results from months 5 to 8, after which time they began to diverge. Rats fed a diet containing 5mg/kg RRR-α-tocopheryl acetate maintained a relatively constant mean SEP amplitude, which became consistently significantly ($P<0.05$) different from those fed a deficient and 0.4mg/kg diet after 10 months (Figures 5.10 and 5.11, respectively). However, the amplitudes of the 5mg/kg group did not differ significantly from either the 0.75mg/kg or 1.0mg/kg (all-rac) groups. The L5 amplitudes of the deficient and 0.4mg/kg groups did not differ significantly from either the 0.75mg/kg or 1.0mg/kg (all-rac) groups.

Figure 5.4 Group mean central conduction velocities (lower limb)

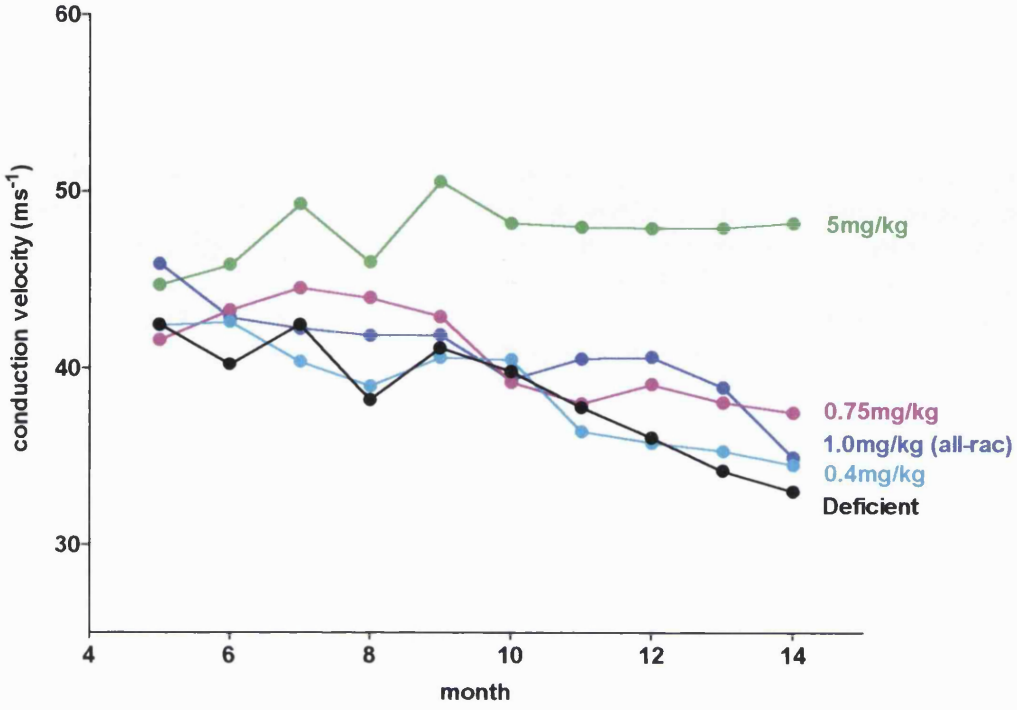


Figure 5.5 Group mean central CVs +/- 1SD - deficient v 5mg/kg (lower limb)

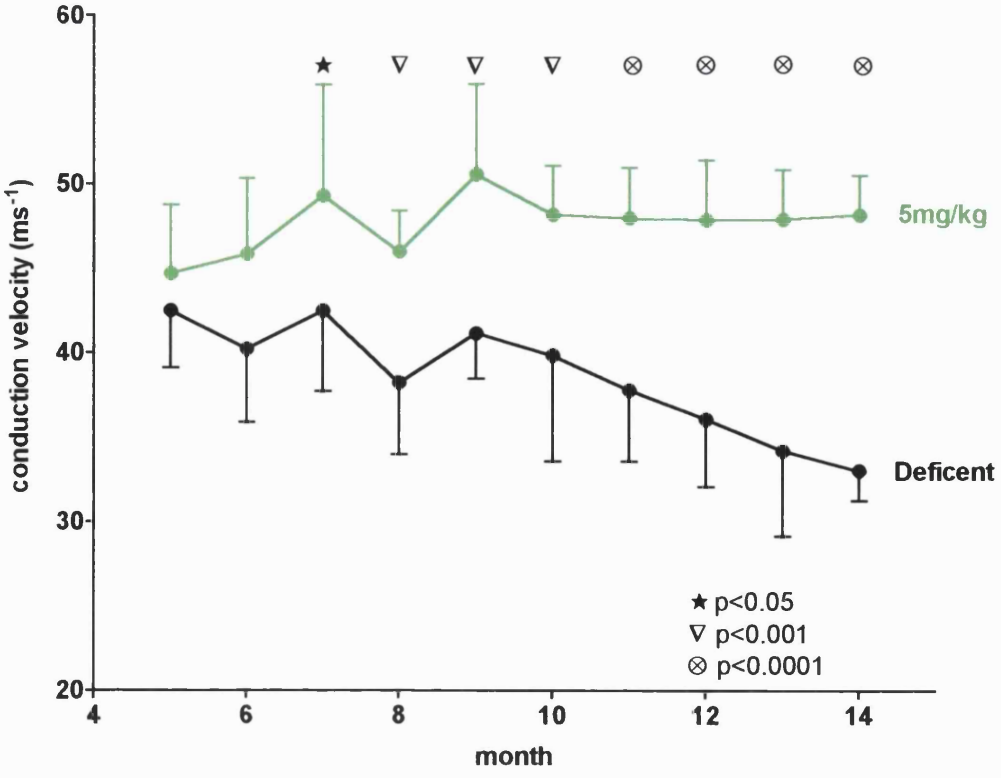


Figure 5.6 Group mean central CVs +/- 1SD - 0.4mg/kg v 5mg/kg (lower limb)

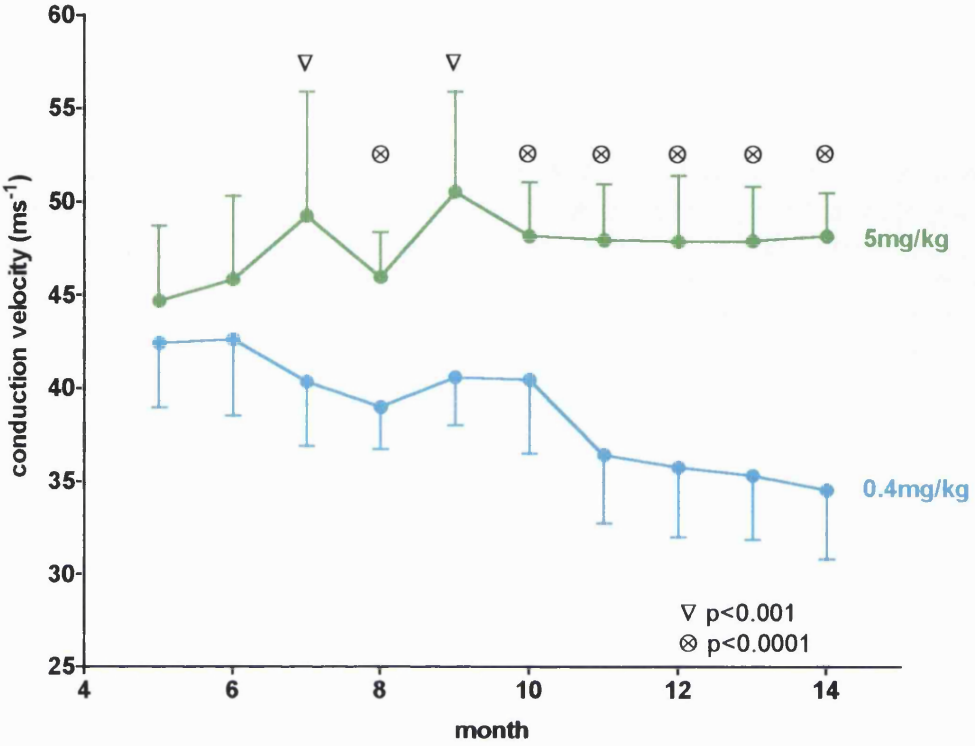


Figure 5.7 Group mean central CVs +/- 1SD - 1.0mg/kg (all-rac) v 5mg/kg (lower limb)

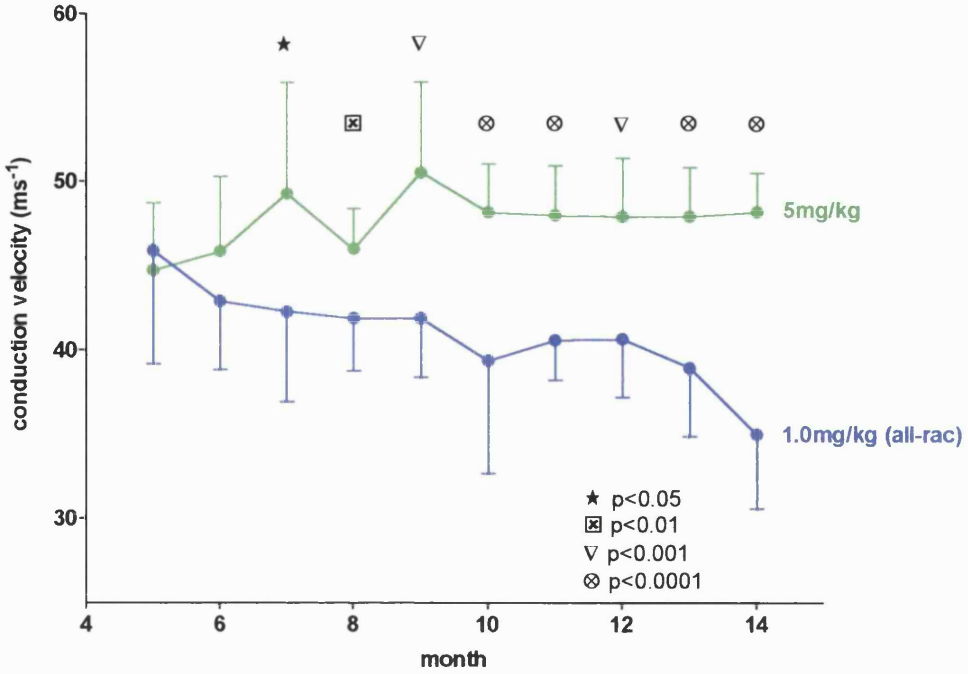


Figure 5.8 Group mean central CVs +/- 1SD - 0.75mg/kg v 5mg/kg (lower limb)

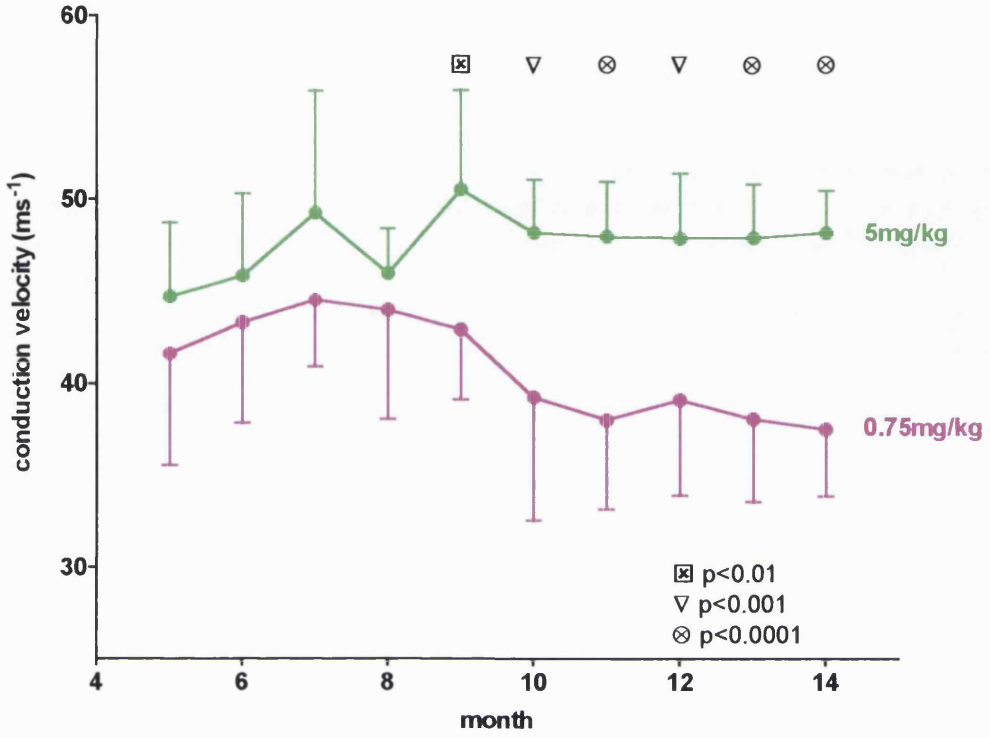


Figure 5.9 Group mean L5 SEP amplitudes (lower limb)

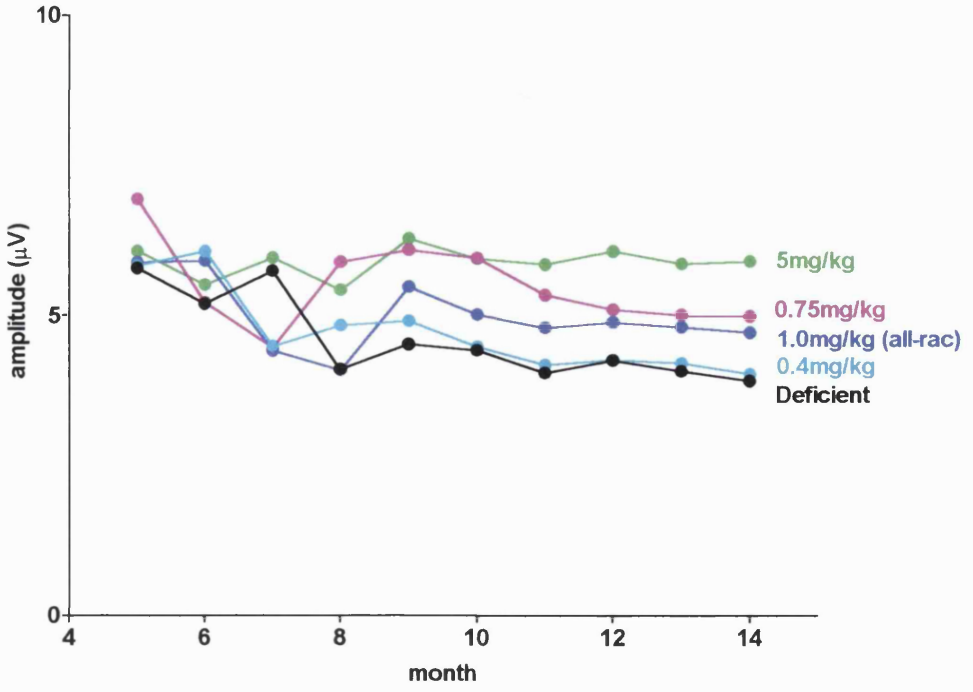


Figure 5.10 Group mean L5 SEP amplitudes +/- 1SD - deficient v 5mg/kg (lower limb)

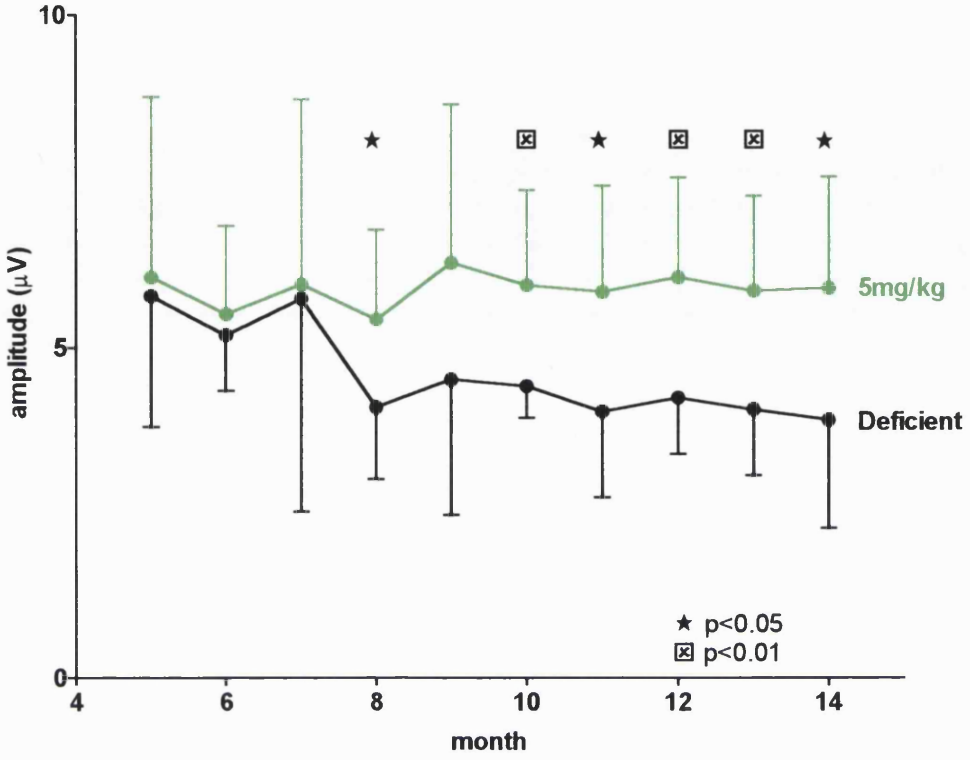
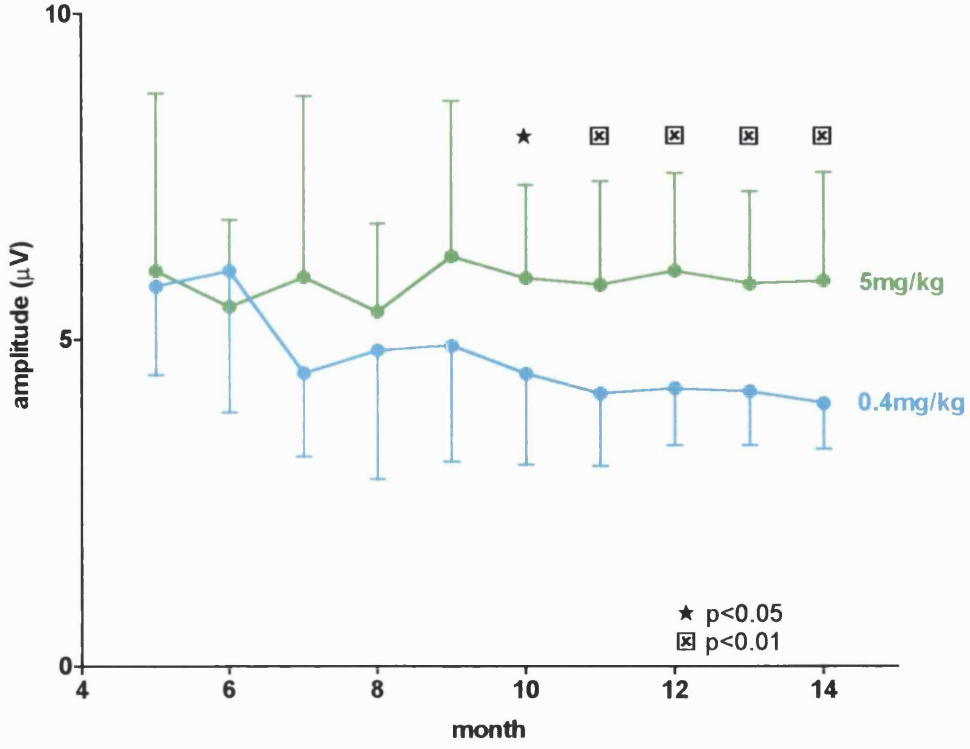


Figure 5.11 Group mean L5 SEP amplitudes +/- 1SD - 0.4mg/kg v 5mg/kg (lower limb)



Cortical SEP peak-to-peak amplitudes

Figure 5.12 shows the group mean cortical SEP amplitudes, recorded in response to tibial nerve stimulation. After month 6, the mean amplitudes of the deficient, 0.4mg/kg, 0.75mg/kg and 1.0mg/kg (all-rac) groups decreased, whereas those of the 5mg/kg remained constant. The mean amplitudes of the 5mg/kg group were consistently significantly greater than those of the deficient group after 12 months (3.27 +/- 0.91 compared with 2.04 +/- 0.26μV respectively, Figure 5.13) and than the 0.75mg/kg or 1.0mg/kg (all-rac) groups after 14 months. At months 10, 11 13 and 14, the rats fed a diet containing 5mg/kg also had significantly higher (P<0.05) cortical amplitudes than the 0.4mg/kg group (Figure 5.14). No significant differences were observed between the deficient, 0.4mg/kg, 0.75mg/kg and 1.0mg/kg (all-rac) groups at any time point.

Figure 5.12 Group mean cortical amplitudes (lower limb)

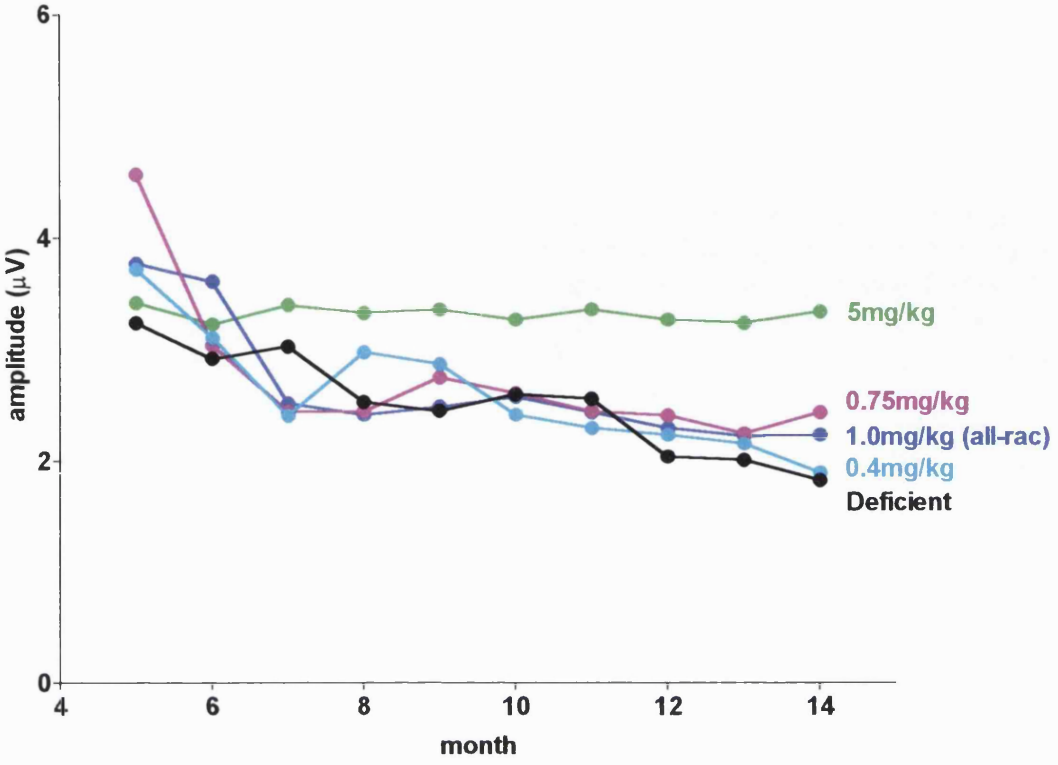


Figure 5.13 Group mean cortical amplitudes +/- 1SD - deficient v 5mg/kg (lower limb)

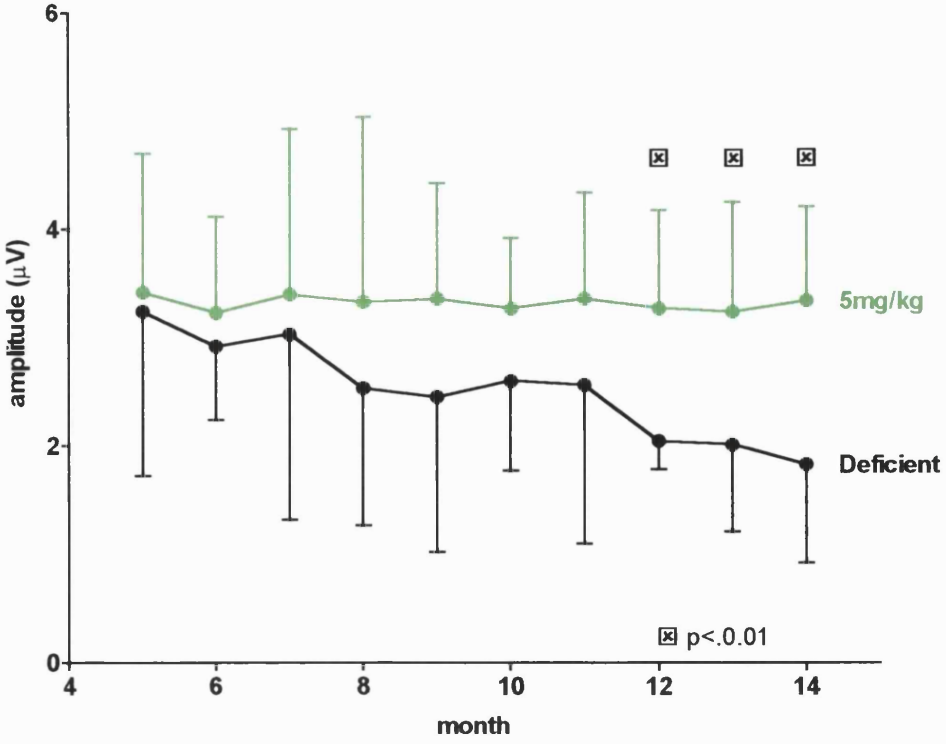
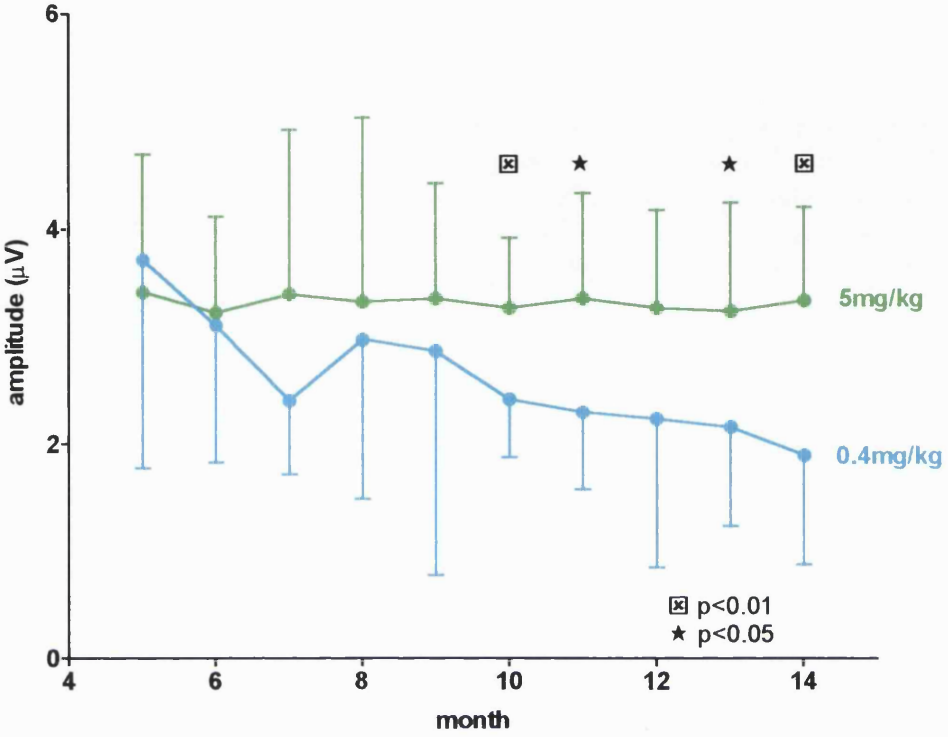


Figure 5.14 Group mean cortical amplitudes +/- 1SD - 0.4mg/kg v 5mg/kg (lower limb)



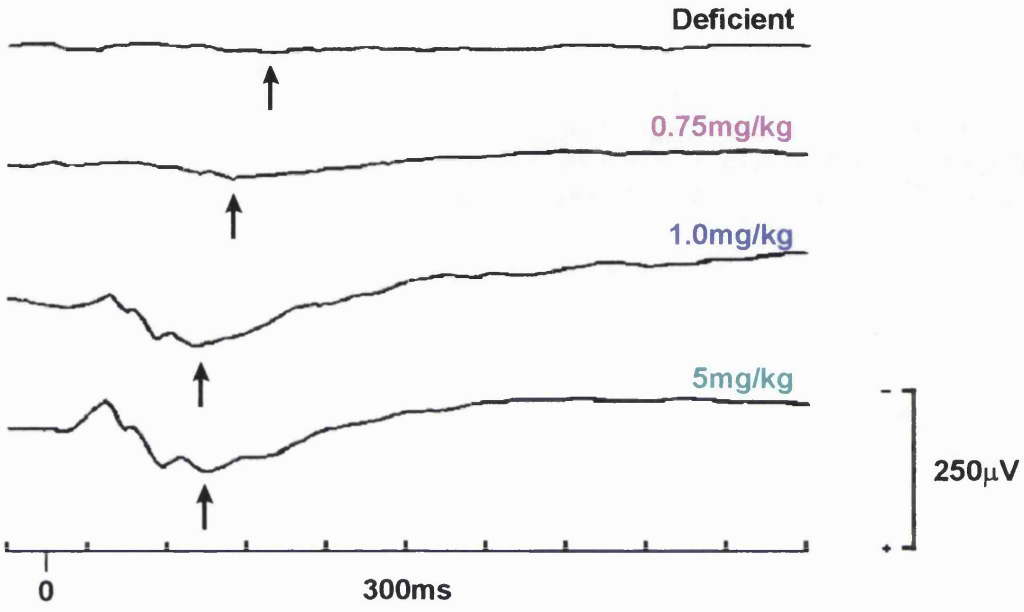
5.5.2 Visual function

Representative flash ERGs, recorded in response to bright white flash stimuli at month 14, are shown in Figure 5.15. There were marked increases in the b wave latencies (as indicated by the arrows) and reductions in the amplitudes of ERGs recorded in rats in the deficient and 0.75mg/kg groups. The 0.4mg/kg gave responses which were indistinguishable from those obtained in the deficient group.

ERG b wave latencies (bright white flash)

Figure 5.16 shows the group mean ERG b wave latencies for all the groups. The animals fed 5mg/kg RRR-α-tocopheryl acetate displayed constant ERG latencies throughout the duration of the study, whereas the latencies increased with time in the other groups. The 5mg/kg group had significantly shorter mean latencies than the deficient and 0.4mg/kg groups after 8 months, and than the 0.75mg/kg group after 9 months (Figures 5.17, 5.18 and 5.19, respectively). When the 5mg/kg group was compared to the 1.0mg/kg (all-rac) group, consistently significant differences were observed after 11 months (Figure 5.20). After 14 months, the mean ERG b wave latencies of the deficient group of rats were found to be significantly longer ($P<0.05$) than the 0.4mg/kg, 0.75mg/kg and 1.0mg/kg (all-rac) groups.

Figure 5.15 Representative flash ERGs recorded at month 14 in response to bright white flash stimuli



Black arrows indicate b wave

Figure 5.16 Group mean ERG b wave latencies (bright white flash)

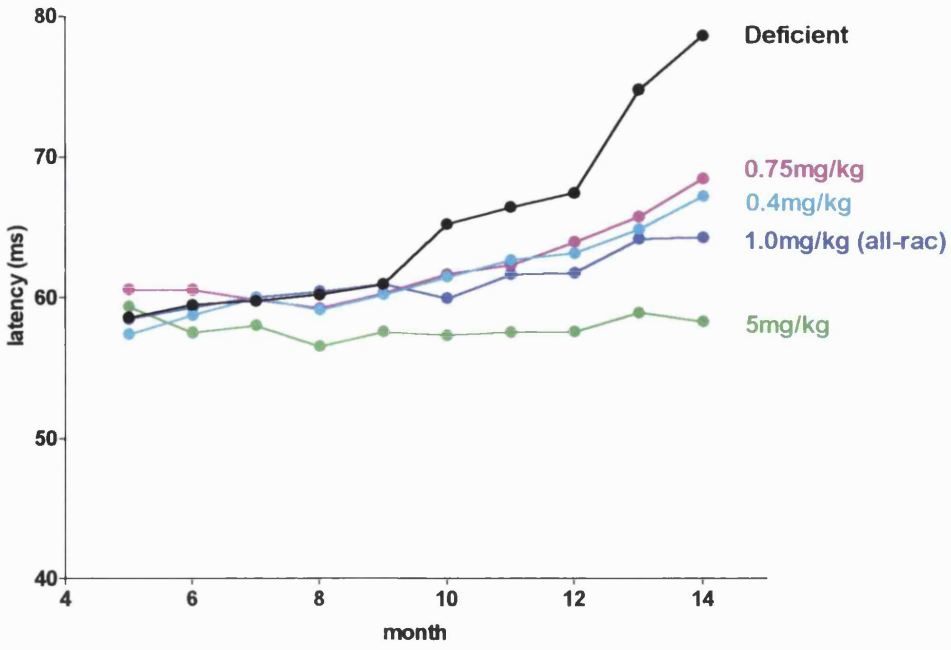


Figure 5.17 Group mean ERG b wave latencies +/- 1SD
- deficient v 5mg/kg (bright white flash)

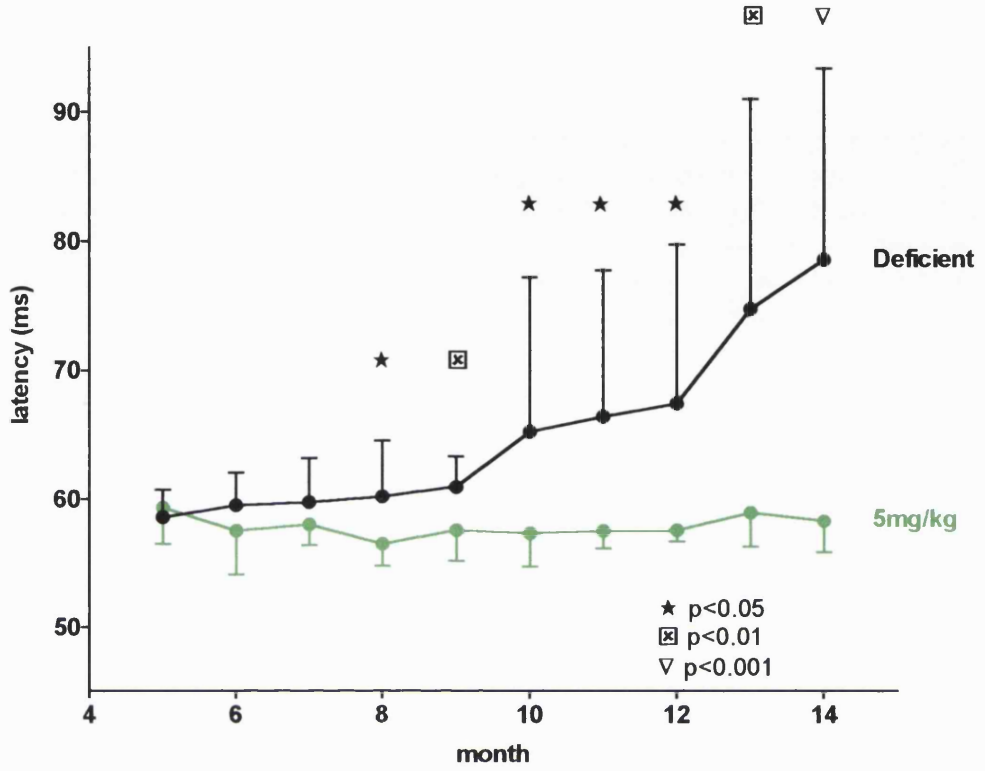


Figure 5.18 Group mean ERG b wave latencies - 0.4mg/kg v 5mg/kg

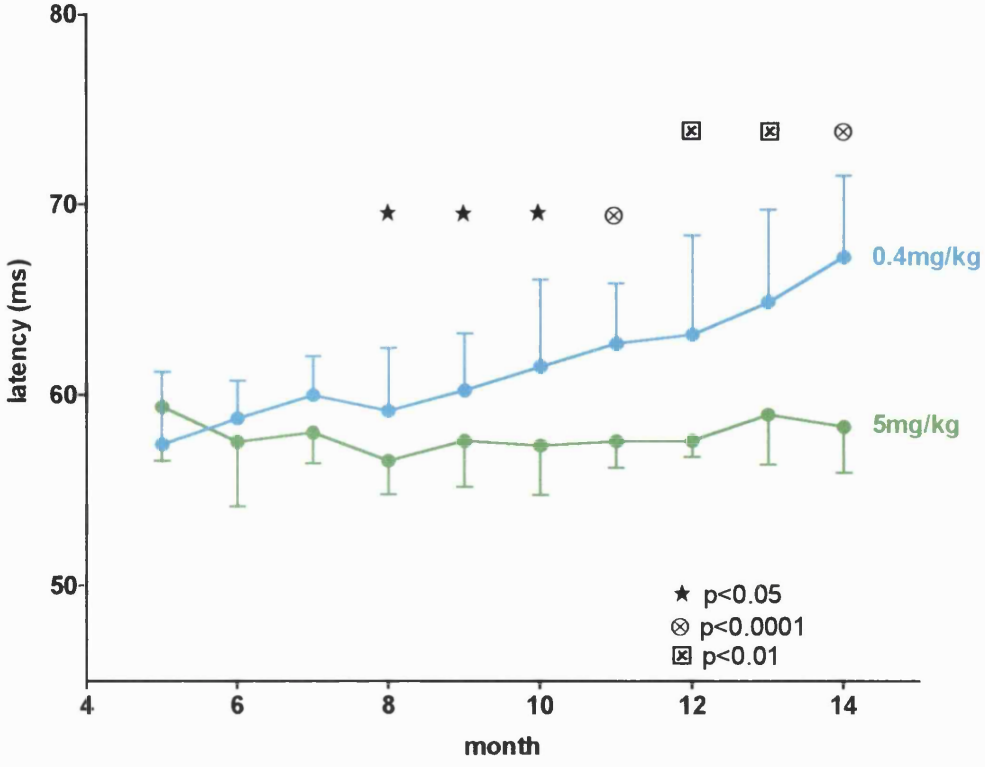


Figure 5.19 Group mean ERG b wave latencies +/- 1SD
- 0.75mg/kg v 5mg/kg (bright white flash)

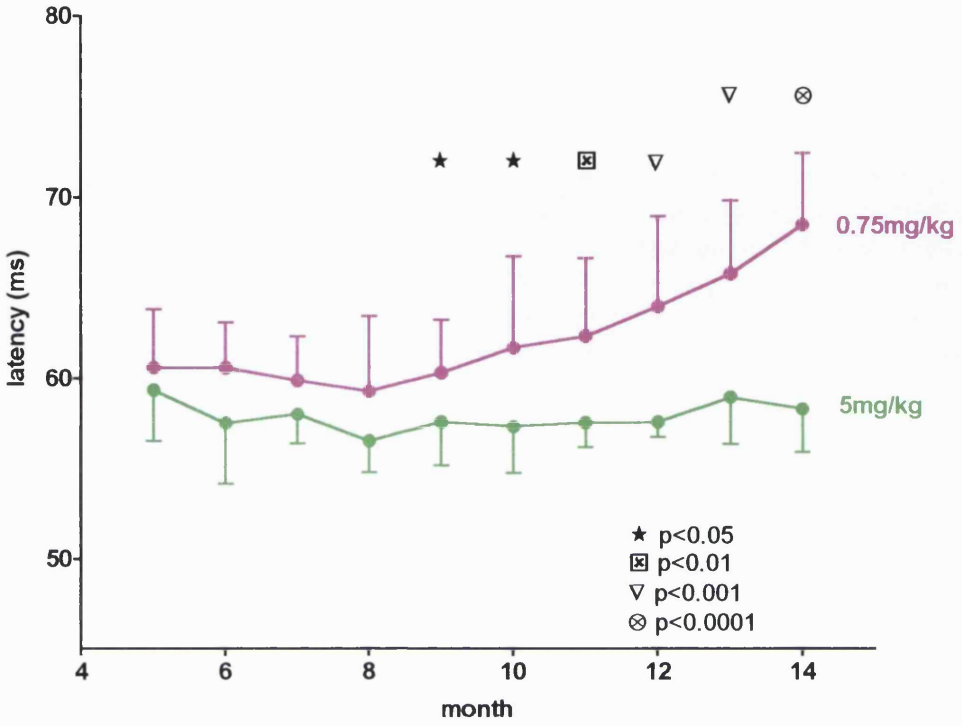
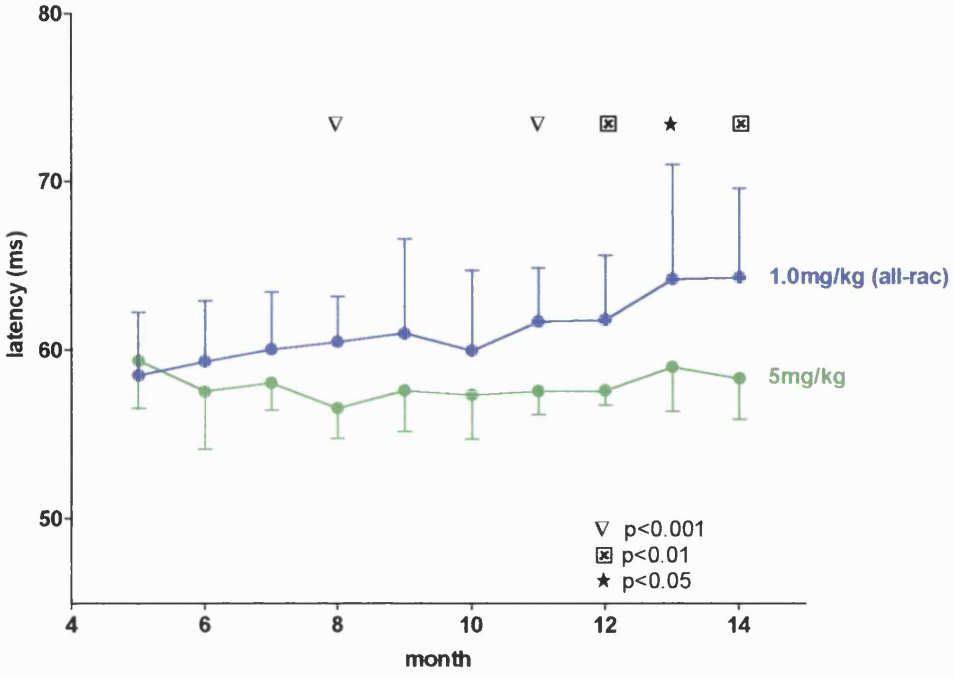


Figure 5.20 Group mean ERG latencies +/- 1SD
- 1.0mg/kg (all-rac) v 5mg/kg (bright white flash)



ERG peak-to-peak amplitudes (bright white flash)

Over the course of the study, all the groups showed a decrease in mean ERG amplitudes, with the exception of the 5mg/kg (control) group, as shown in Figure 5.21. The control group of rats had significantly larger mean ERG amplitudes when compared with the deficient group after 7 months (Figure 5.22) and the 0.4mg/kg group after 8 months (Figure 5.23). The 5mg/kg rats also had larger mean ERG amplitudes than those of the 0.75mg/kg group after 6 months and the 1.0mg/kg (all-rac) group after 8 months, as shown in Figures 5.24 and 5.25 respectively.

VEP latencies (bright white flash)

The group mean VEP onset latencies increased during the study in all the groups except the 5mg/kg group, as shown in Figure 5.26. The 5mg/kg group was found to have consistently significantly shorter onset latencies than the deficient group after 8 months (Figure 5.27), than the 0.4mg/kg group after 6 months, although significance was not found at 10 and 14 months probably as a result of increased variability, (Figure 5.28) and the 1.0mg/kg (all-rac) group after 12 months (Figure 5.29). The mean VEP onset latencies of the 0.75mg/kg group were consistently significantly longer than those of the 5mg/kg group throughout the study, with the exception of month 8 where no significant differences were found (Figure 5.30). No statistically significant differences were found when the deficient, 0.4mg/kg, 0.75mg/kg and 1.0mg/kg (all-rac) groups were compared.

Figure 5.21 Group mean ERG peak-to-peak amplitudes (bright white flash)

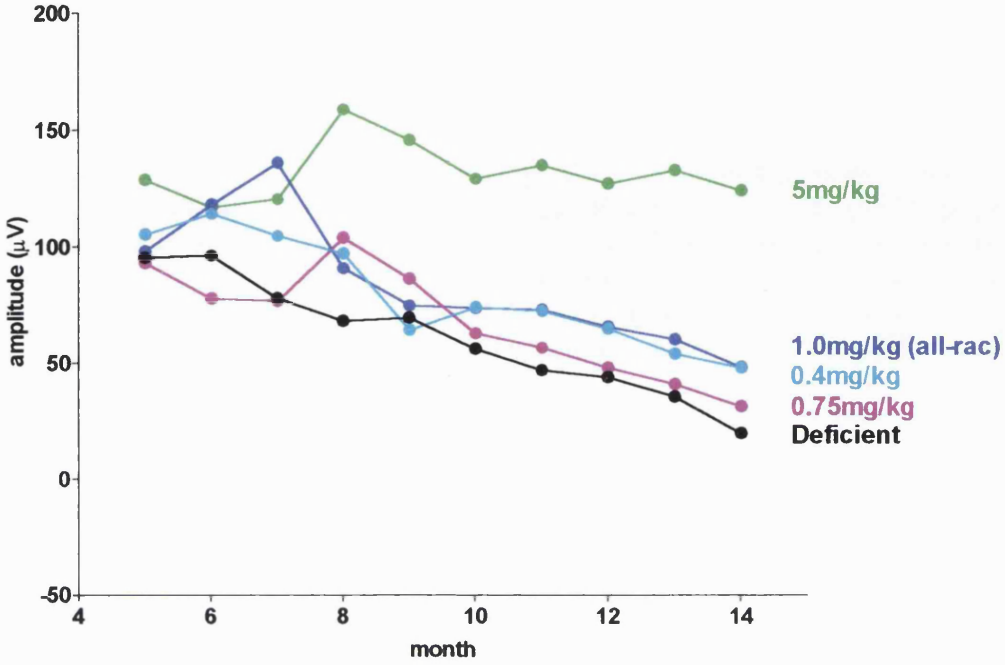


Figure 5.22 Group mean ERG amplitudes +/- 1SD - deficient v 5mg/kg (bright white flash)

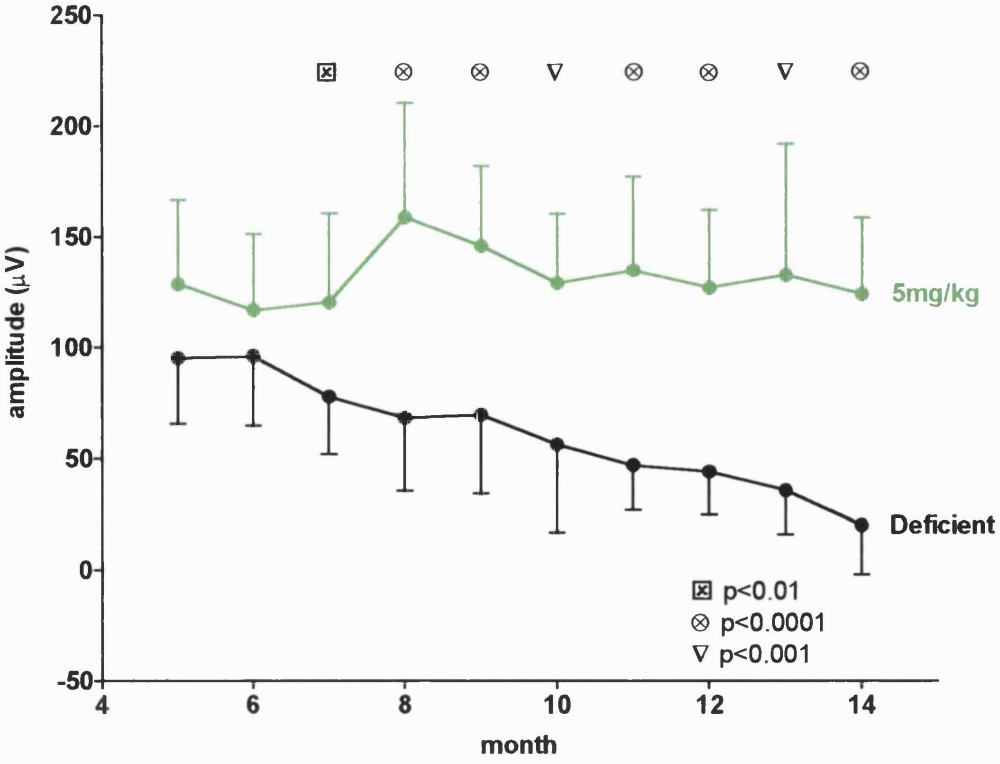


Figure 5.23 Group mean ERG amplitudes +/- 1SD - 0.4mg/kg v 5mg/kg (bright white flash)

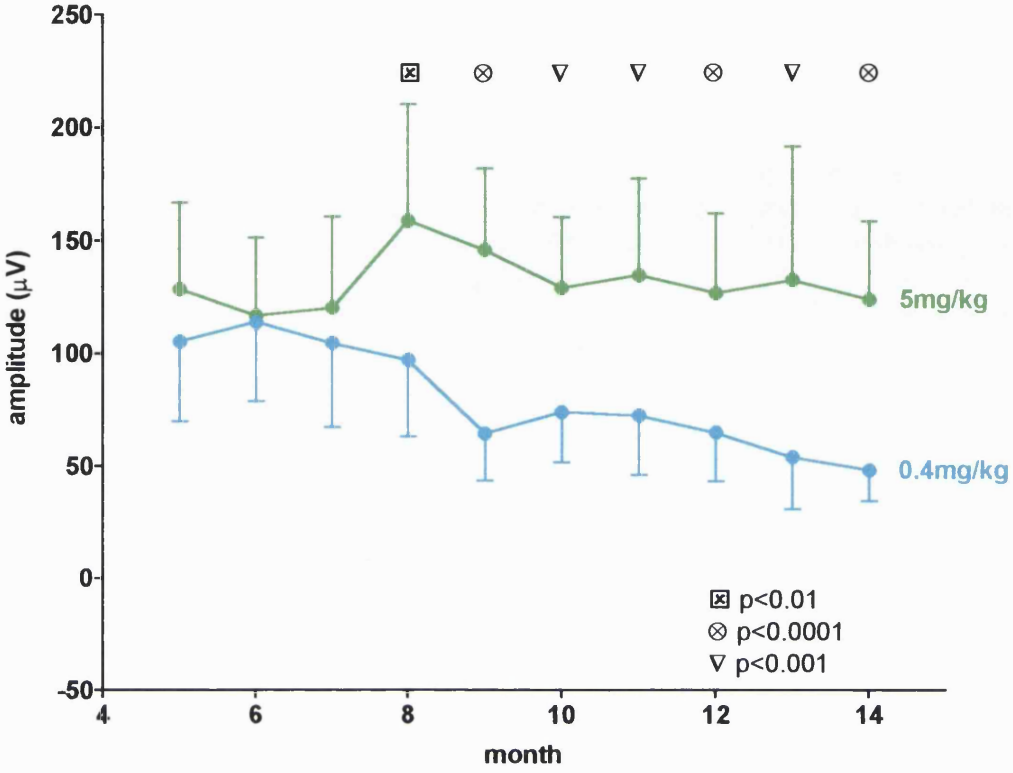


Figure 5.24 Group mean ERG amplitudes +/- 1SD - 0.75mg/kg v 5mg/kg (bright white flash)

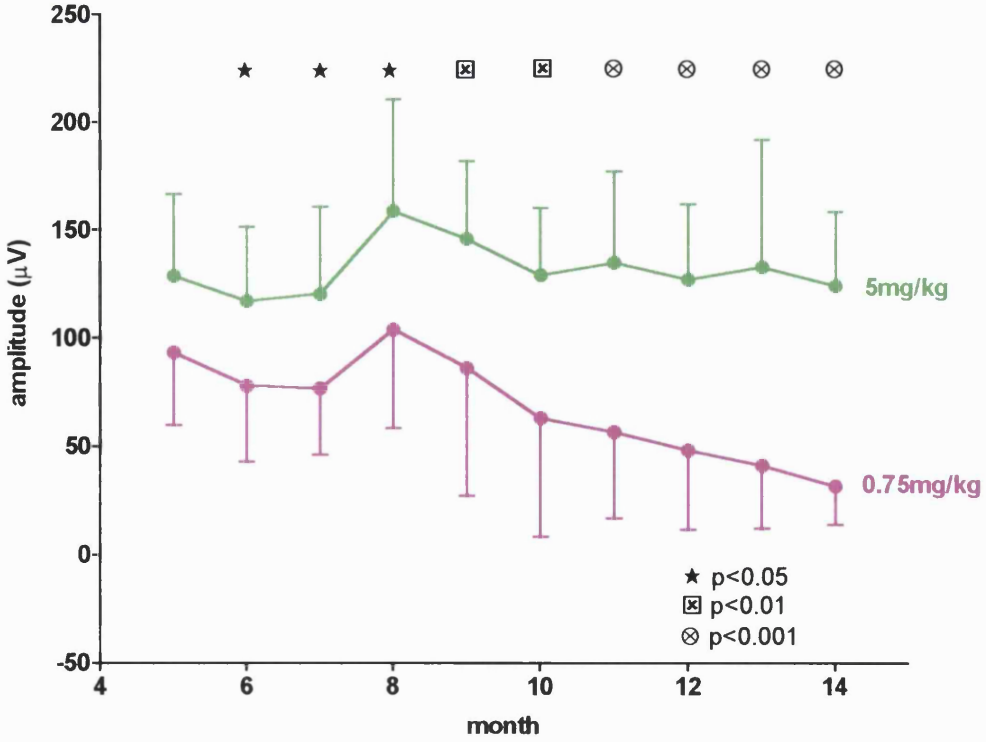


Figure 5.25 Group mean ERG amplitudes +/- 1SD
- 1.0mg/kg (all-rac) v 5mg/kg (bright white flash)

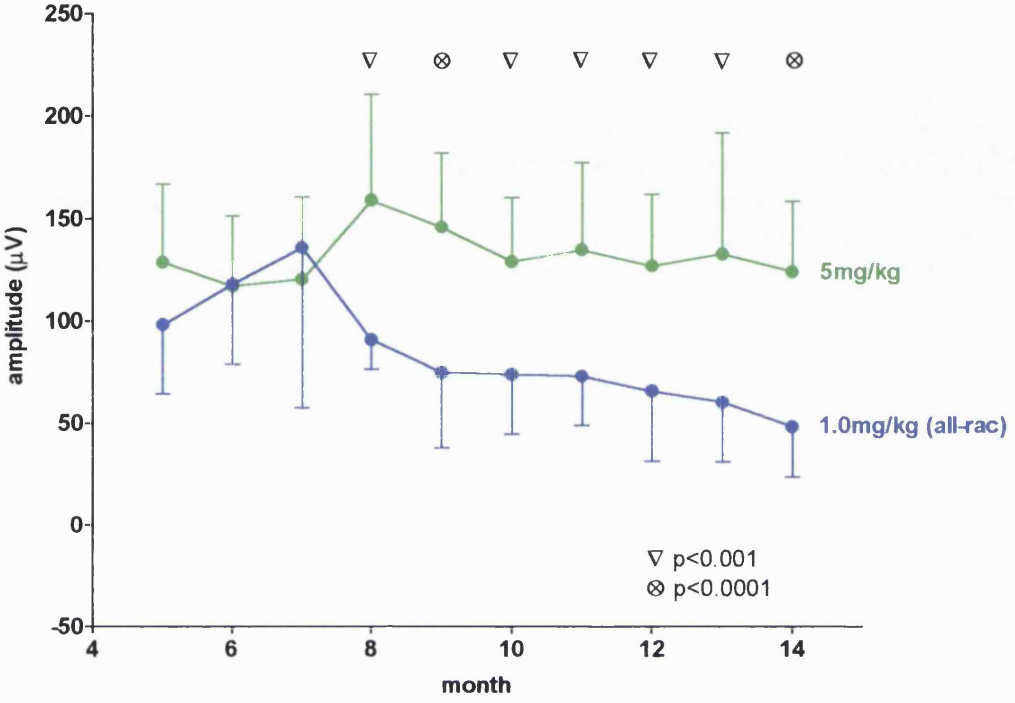


Figure 5.26 Group mean VEP onset latencies (bright white flash)

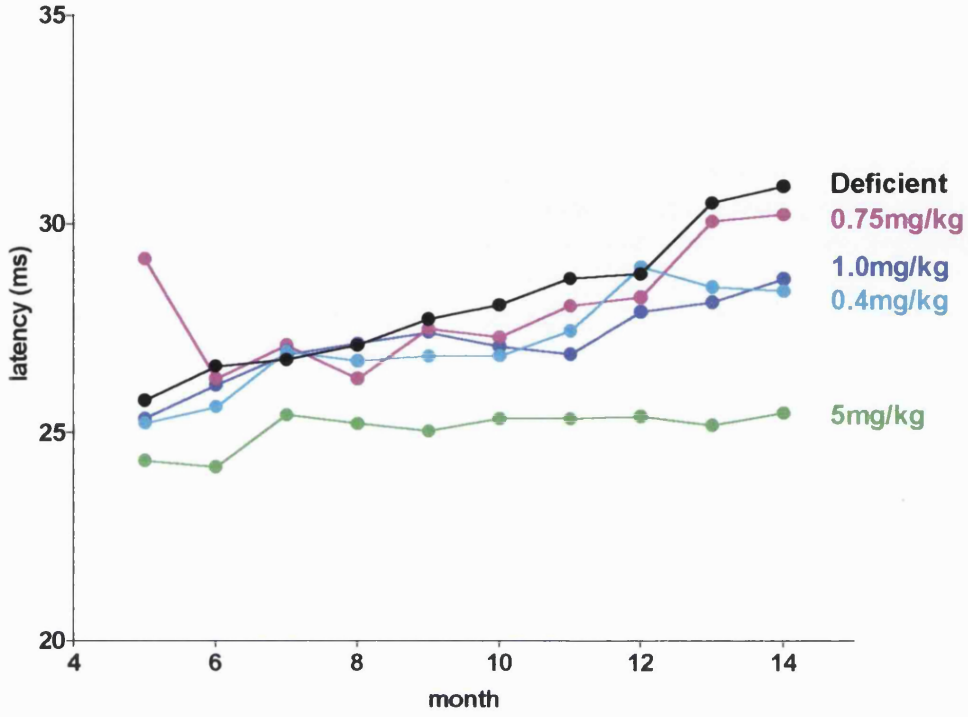


Figure 5.27 Group mean VEP onset latencies +/- 1SD - deficient v 5mg/kg (bright white flash)

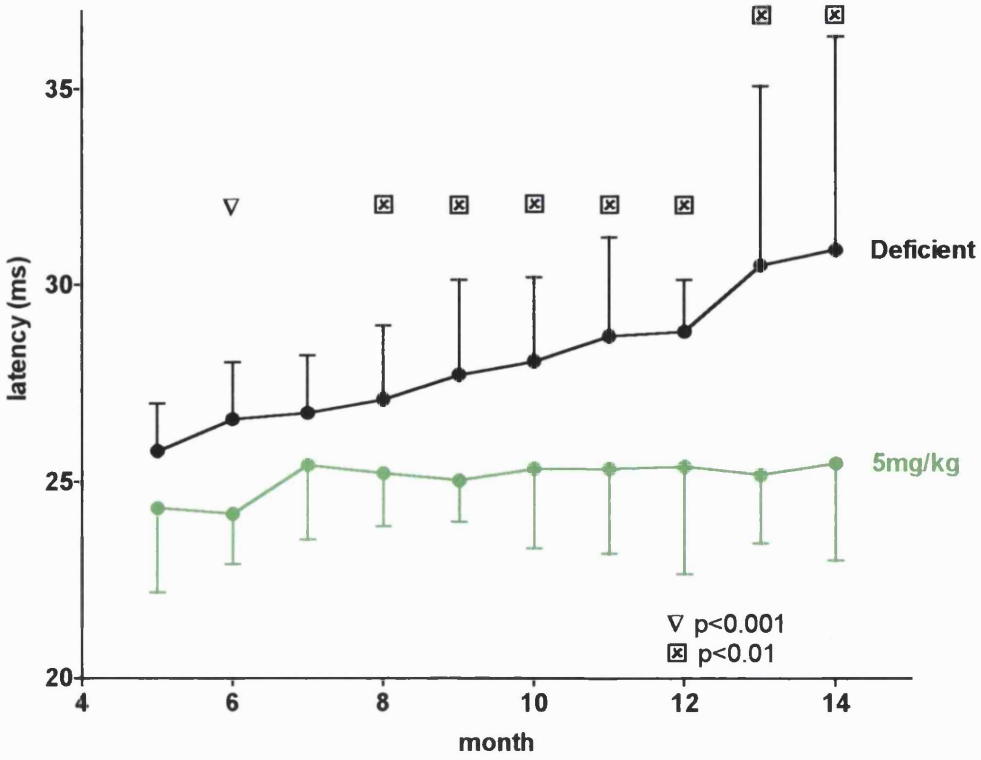


Figure 5.28 Group mean VEP onset latencies +/- 1SD
- 0.4mg/kg v 5mg/kg (bright white flash)

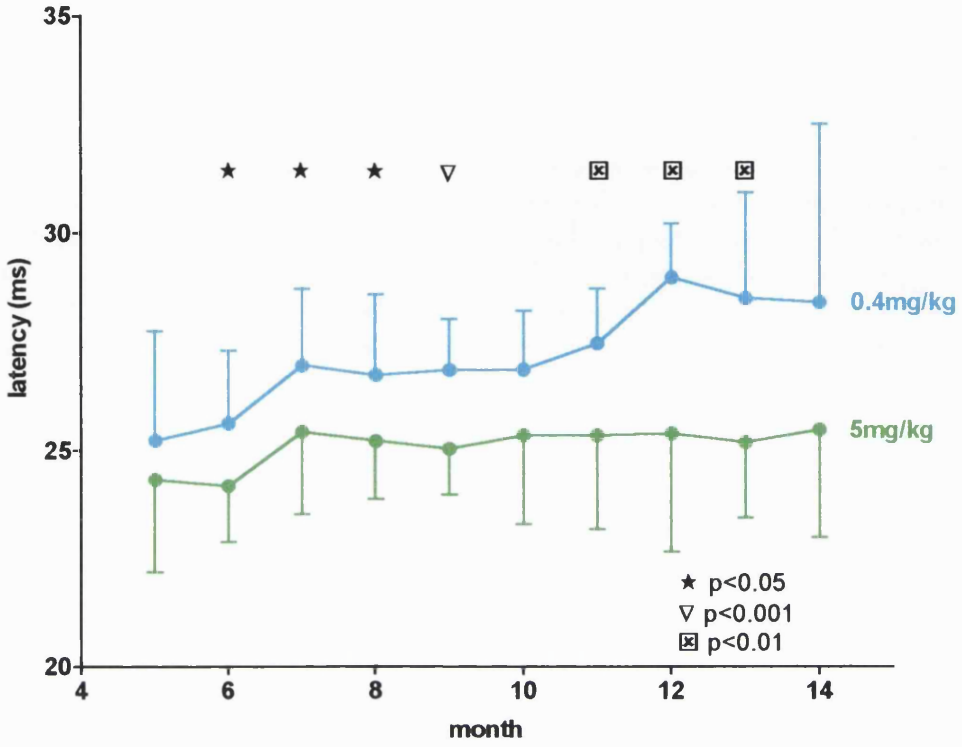


Figure 5.29 Group mean VEP latencies +/- 1SD
- 1.0mg/kg (all-rac) v 5mg/kg (bright white flash)

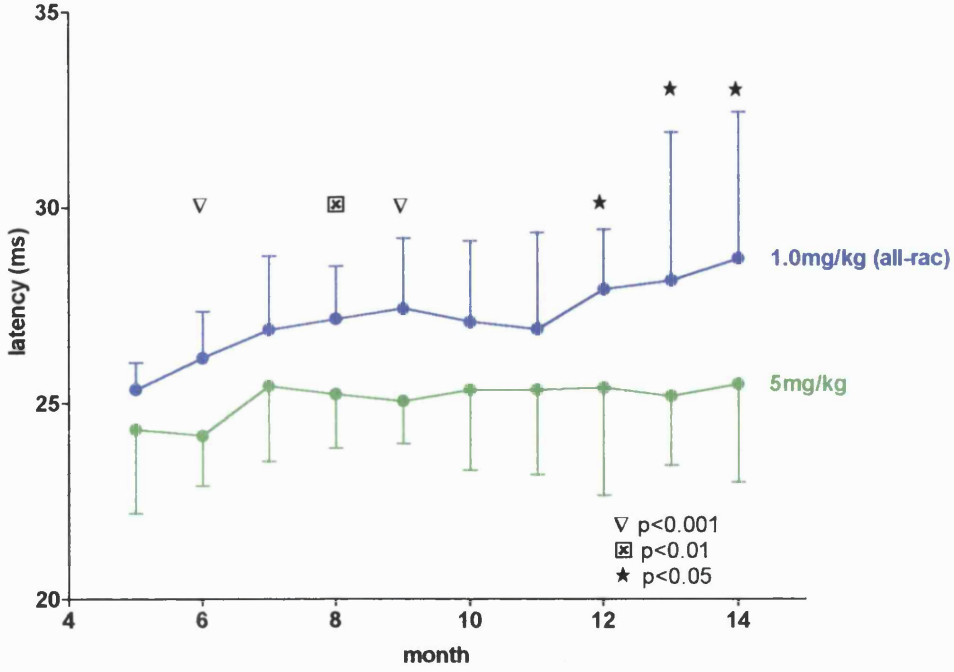
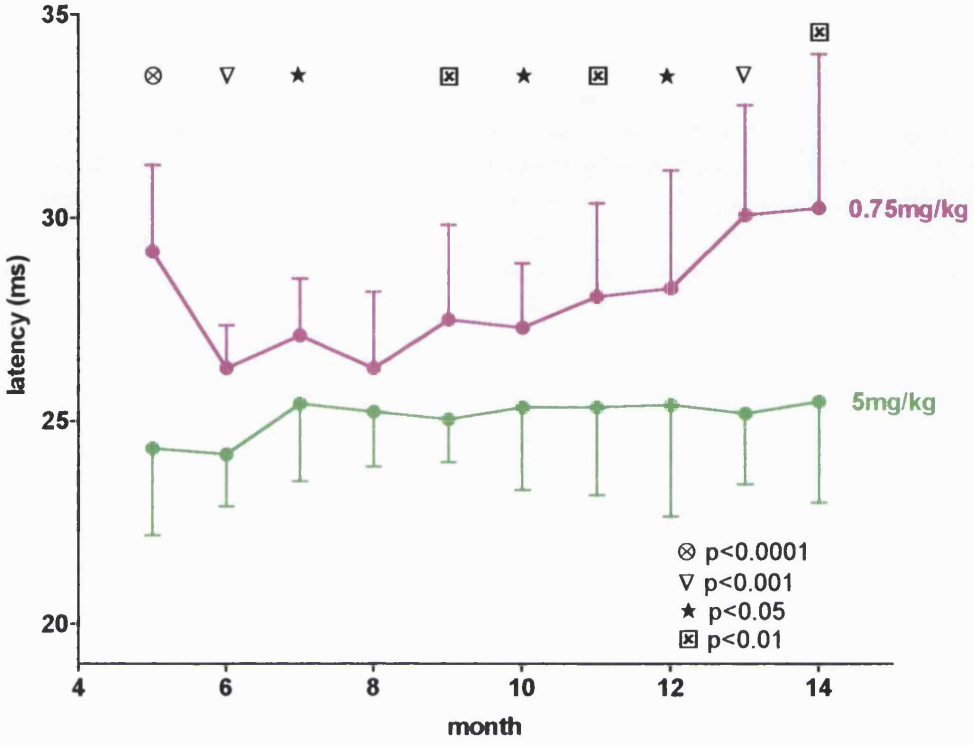


Figure 5.30 Group mean VEP onset latencies +/- 1SD
- 0.75mg/kg v 5mg/kg (bright white flash)



VEP peak-to-peak amplitudes

The animals fed 5mg/kg RRR-α-tocopheryl acetate displayed constant VEP amplitudes throughout the duration of the study, whereas the amplitudes decreased over time in all the other groups (see Figure 5.31). The difference in group mean amplitudes became significant after 13 months, when the 5mg/kg group was compared to the deficient (Figure 5.32), 0.4mg/kg (Figure 5.33) and 0.75mg/kg (Figure 5.34) groups. A significant difference was observed ($P<0.01$) between the 5mg/kg and 1.0mg/kg (all-rac) groups after 14 months.

Figure 5.31 Group mean VEP amplitudes (bright white flash)

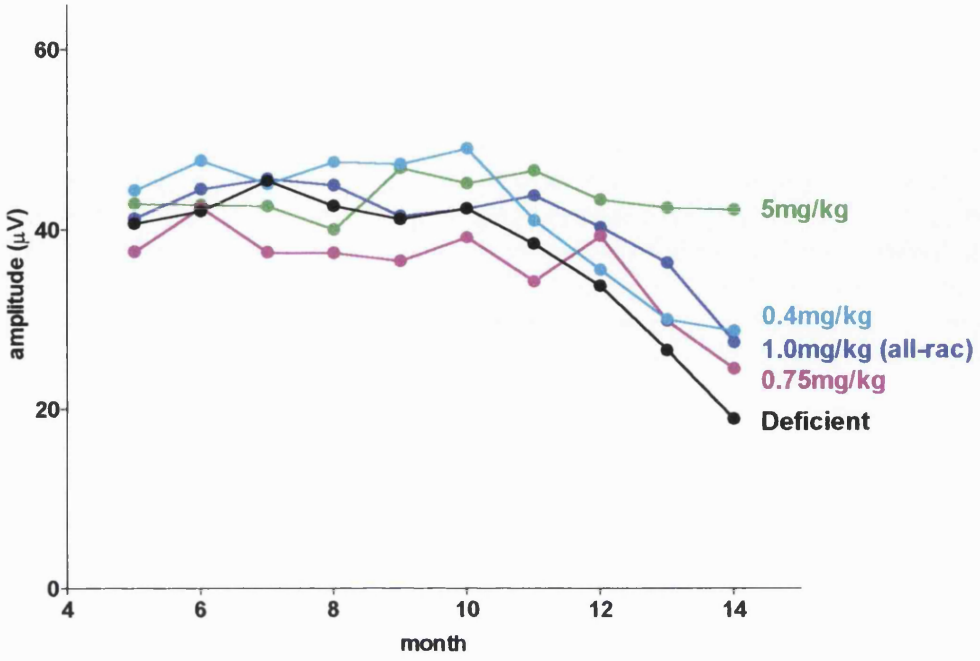


Figure 5.32 Group mean VEP amplitudes +/- 1SD - deficient v 5mg/kg (bright white flash)

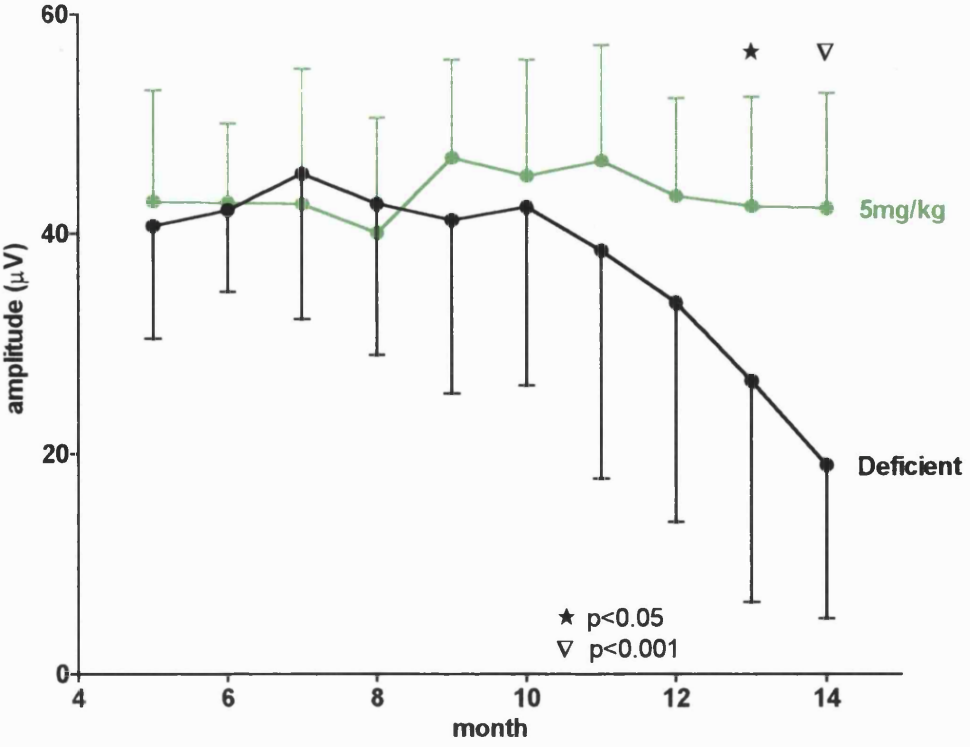


Figure 5.33 Group mean VEP amplitudes +/- 1SD - 0.4mg/kg v 5mg/kg (bright white flash)

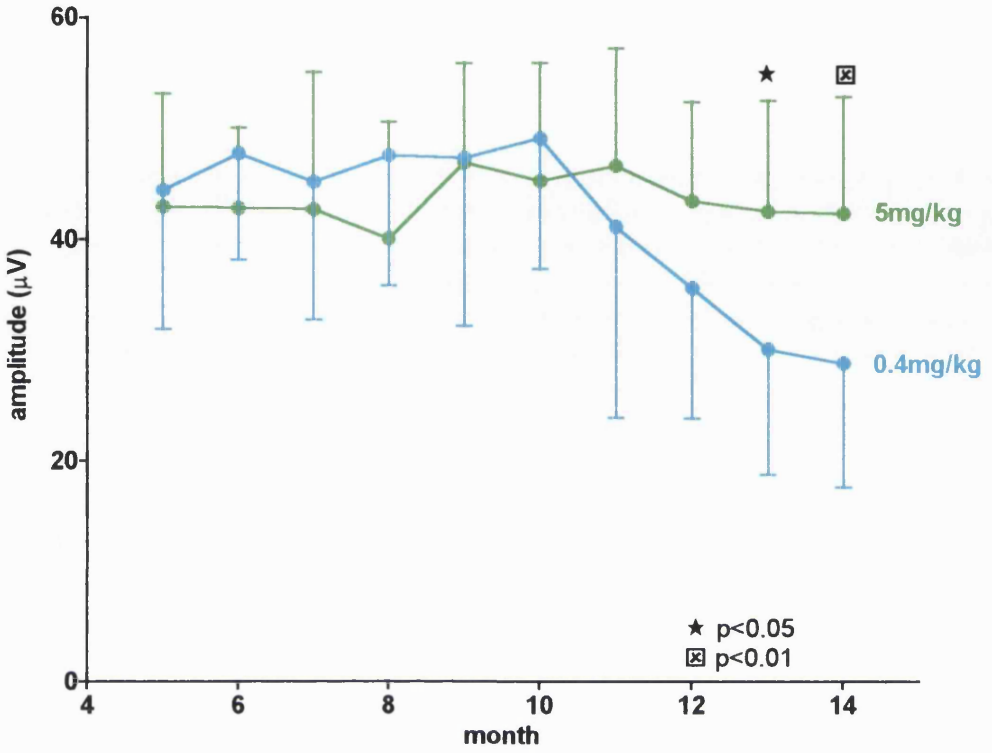
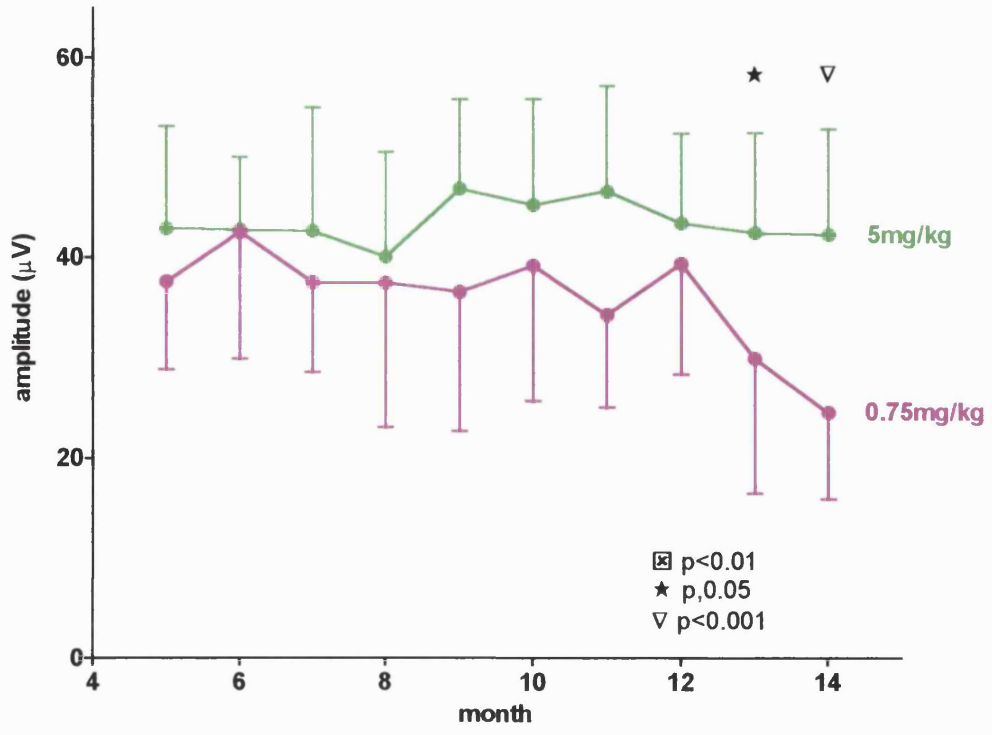


Figure 5.34 Group mean VEP amplitudes +/- 1SD - 0.75mg/kg v 5mg/kg (bright white flash)



5.6 Results of biochemical analyses

5.6.1 Tissue α-tocopherol concentrations

The rats were killed after 14 months and various tissues collected and stored as described in section 2.4. The concentration of α-tocopherol was determined in various non-neural and neural tissues from six rats from each group, as shown in Figures 5.35 and 5.36, respectively. The expected increase in concentration with increasing dietary intake of α-tocopherol was observed.

α-tocopherol was undetectable in plasma, the non-neural tissues (liver, gastrocnemius muscle, heart, testis) and in the sciatic nerve and eye of deficient rats. α-Tocopherol was, however, detectable in the thoracic cord and cerebral cortex of these animals (means 0.69 and 0.47μg/g wet weight, respectively). All tissues from the 0.4mg/kg group rats, except the sciatic nerve, were found to contain small amounts of α-tocopherol (0.08 - 0.63μg/g wet weight). Similar concentrations of α-tocopherol were found in the tissues from the 0.75mg/kg and 1.0mg/kg (all-rac) groups. The sciatic nerve samples from rats in these two groups contained very low concentrations of α-tocopherol (means 0.21 and 0.13μg/g wet weight, respectively). Concentrations of α-tocopherol were much higher in tissues from the 5mg/kg group animals than the other groups.

In general, a linear relationship was found between the dietary intake of α-tocopheryl acetate and the mean concentration of the vitamin in both non-neural and neural tissues (Figures 5.37 and 5.38, respectively). There was also a linear relationship between the concentration of α-tocopherol in plasma and in the tissues, as shown in Figures 5.39 and 5.40.

Figure 5.35 α-tocopherol concentrations in non-neural tissues

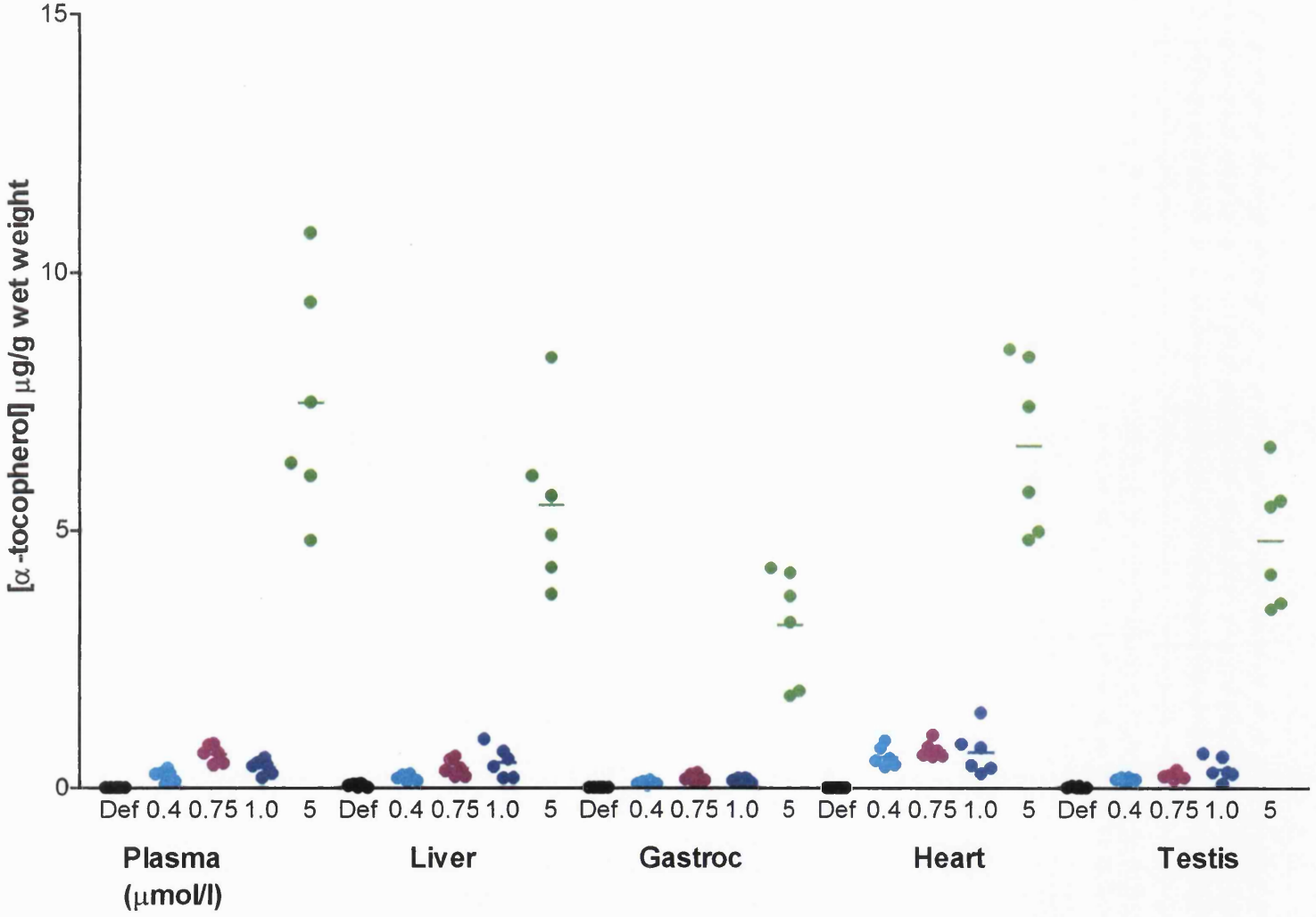


Figure 5.36 α-tocopherol concentrations in neural tissues

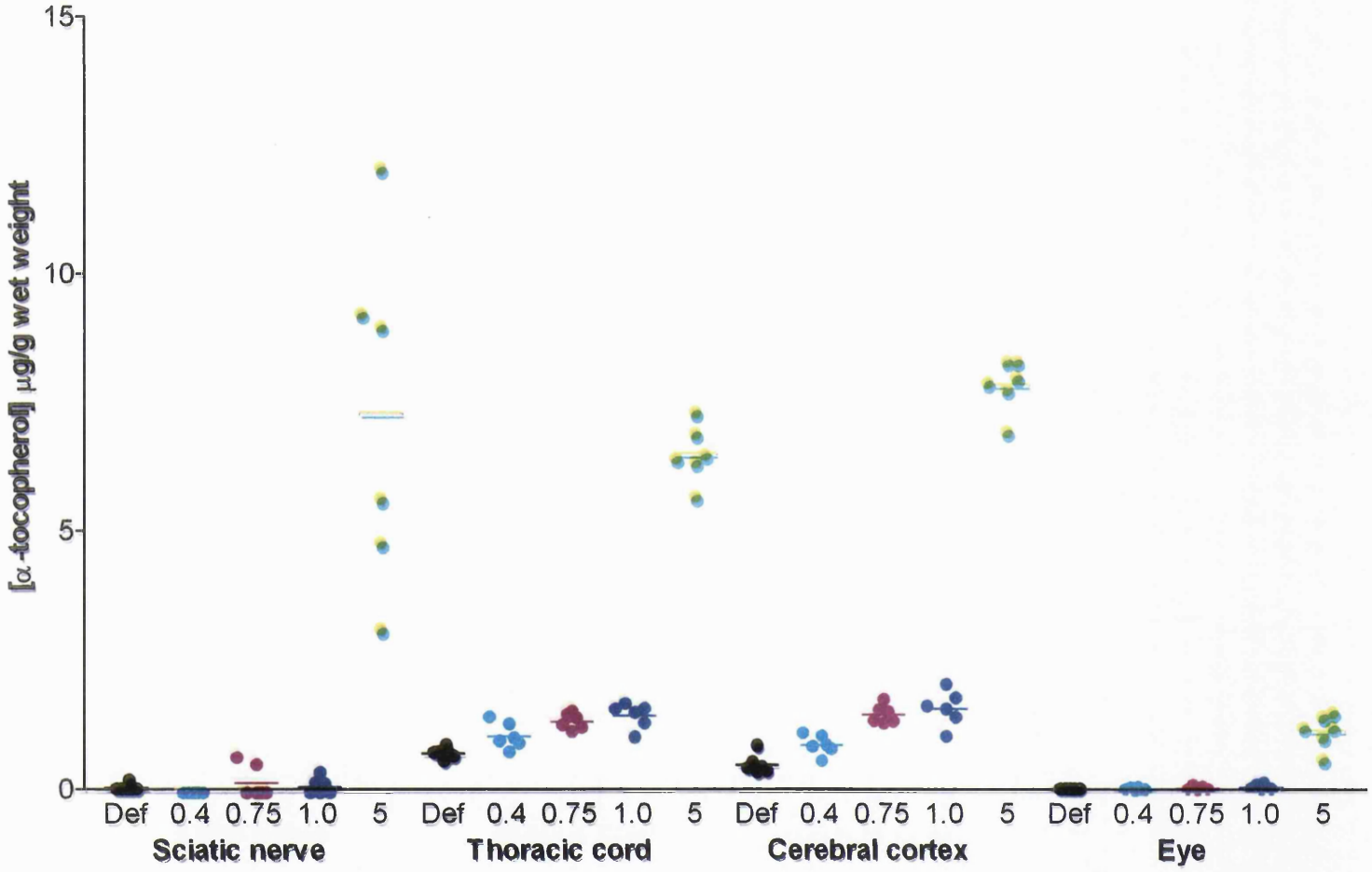
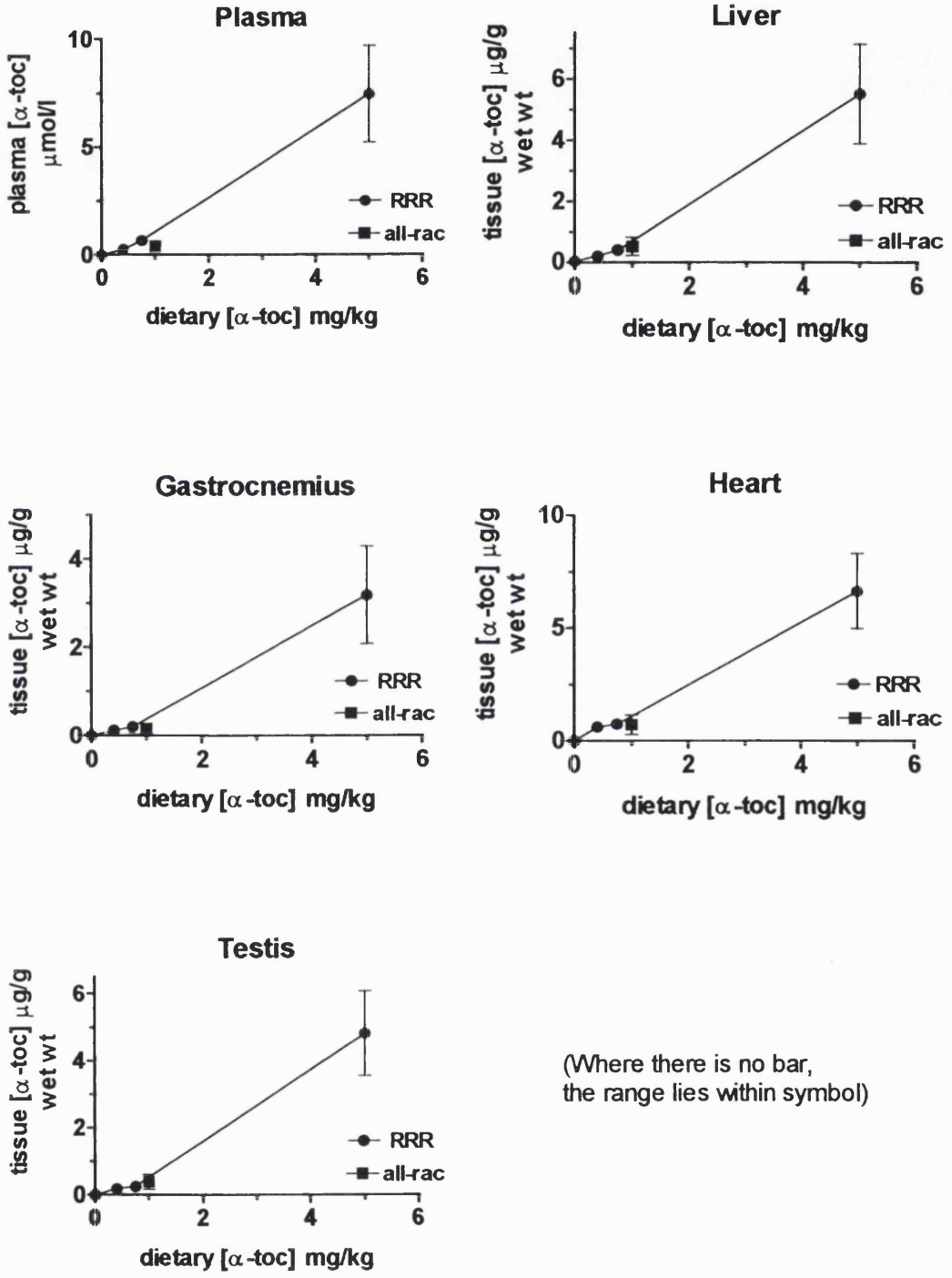
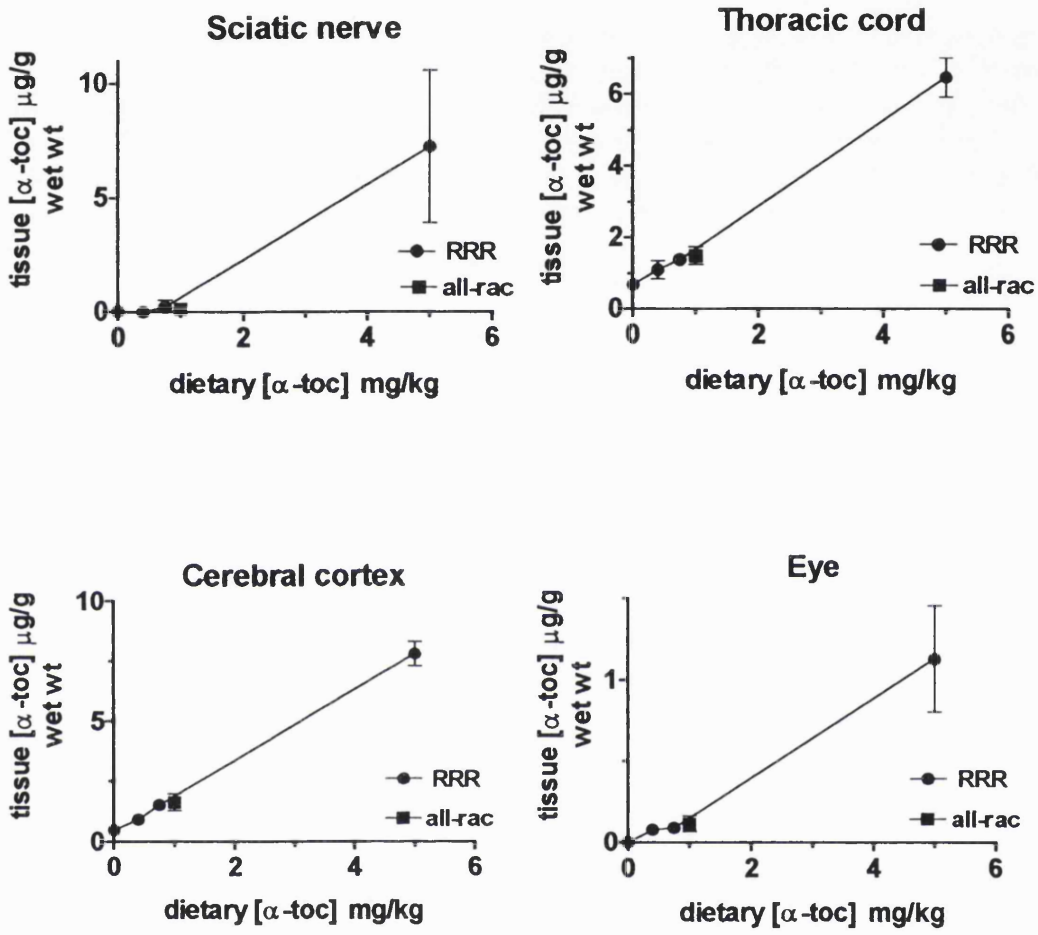


Figure 5.37 Relationship between dietary intake and concentrations of α-tocopherol in non-neural tissues



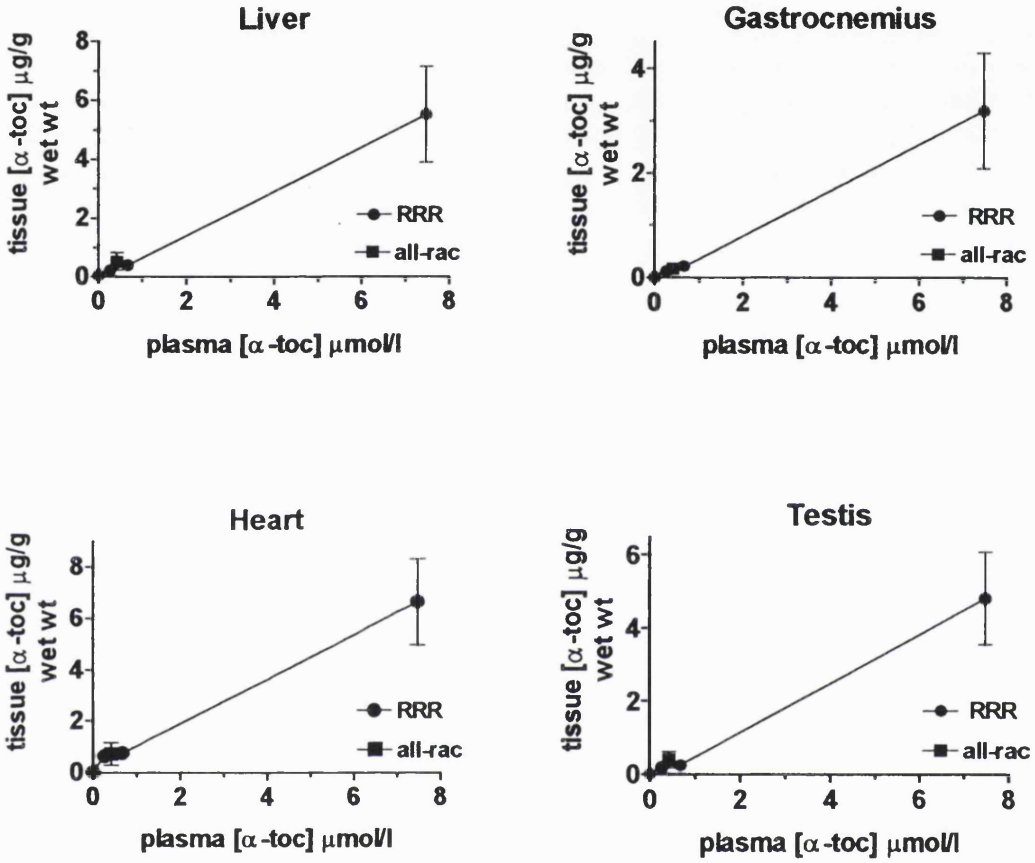
(Where there is no bar, the range lies within symbol)

Figure 5.38 Relationship between dietary intake and concentrations of α-tocopherol in neural tissues



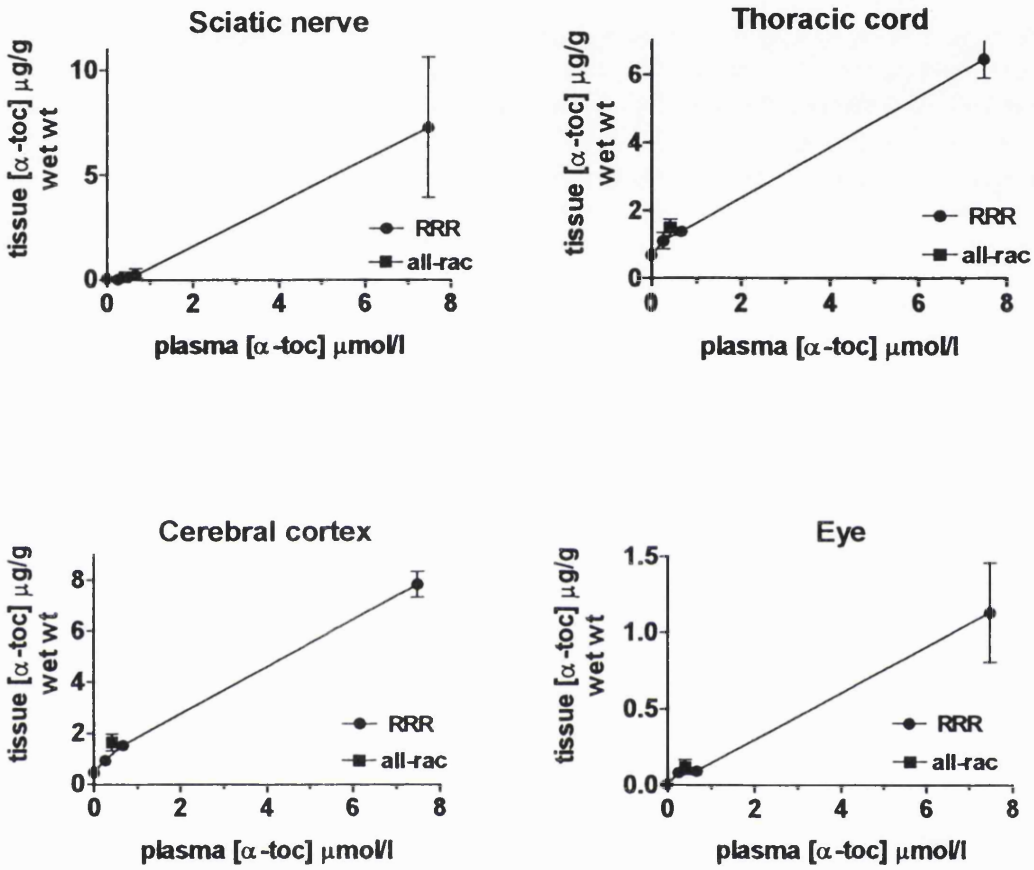
(Where there is no bar, the range lies within symbol)

Figure 5.39 Relationship between α-tocopherol concentrations in plasma and non-neural tissues



(Where there is no bar, the range lies within symbol)

Figure 5.40 Relationship between α-tocopherol concentrations in plasma and neural tissues



(Where there is no bar, the range lies within symbol)

5.6.2 Tissue malondialdehyde concentrations

Concentrations of free, total and bound MDA were determined in the heart, liver, cerebral cortex and eye of 6 rats from the deficient and 5mg/kg groups. The concentration of free MDA tended to be greater in the deficient tissues compared to those from 5mg/kg group rats (see Figure 5.41). However, these differences were only significant ($P<0.05$) for heart (means 0.65 compared to 0.24nmol/mg protein) and liver (0.75 compared to 0.55nmol/mg protein). When total MDA concentrations were compared, all the deficient tissues had higher mean concentrations than the corresponding tissue from the 5mg/kg group of animals, although these differences were not statistically significant (see Figure 5.42). No significant differences were found between the two groups when bound MDA concentrations were compared.

The electrophysiological and biochemical results from this study will be discussed in Chapter 6 together with those obtained in Study I using all-rac-α-tocopheryl acetate.

Figure 5.41 Free MDA concentrations

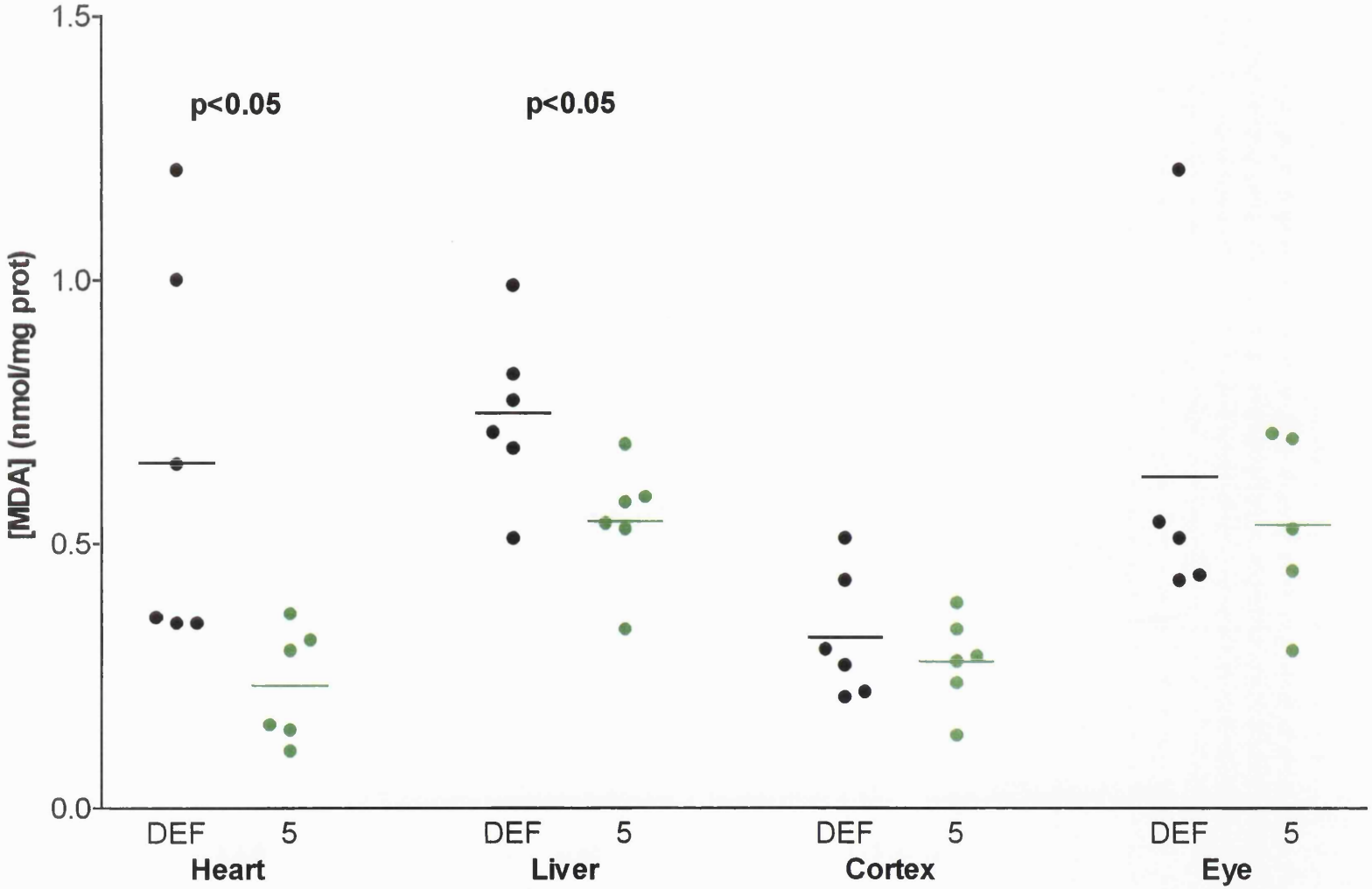
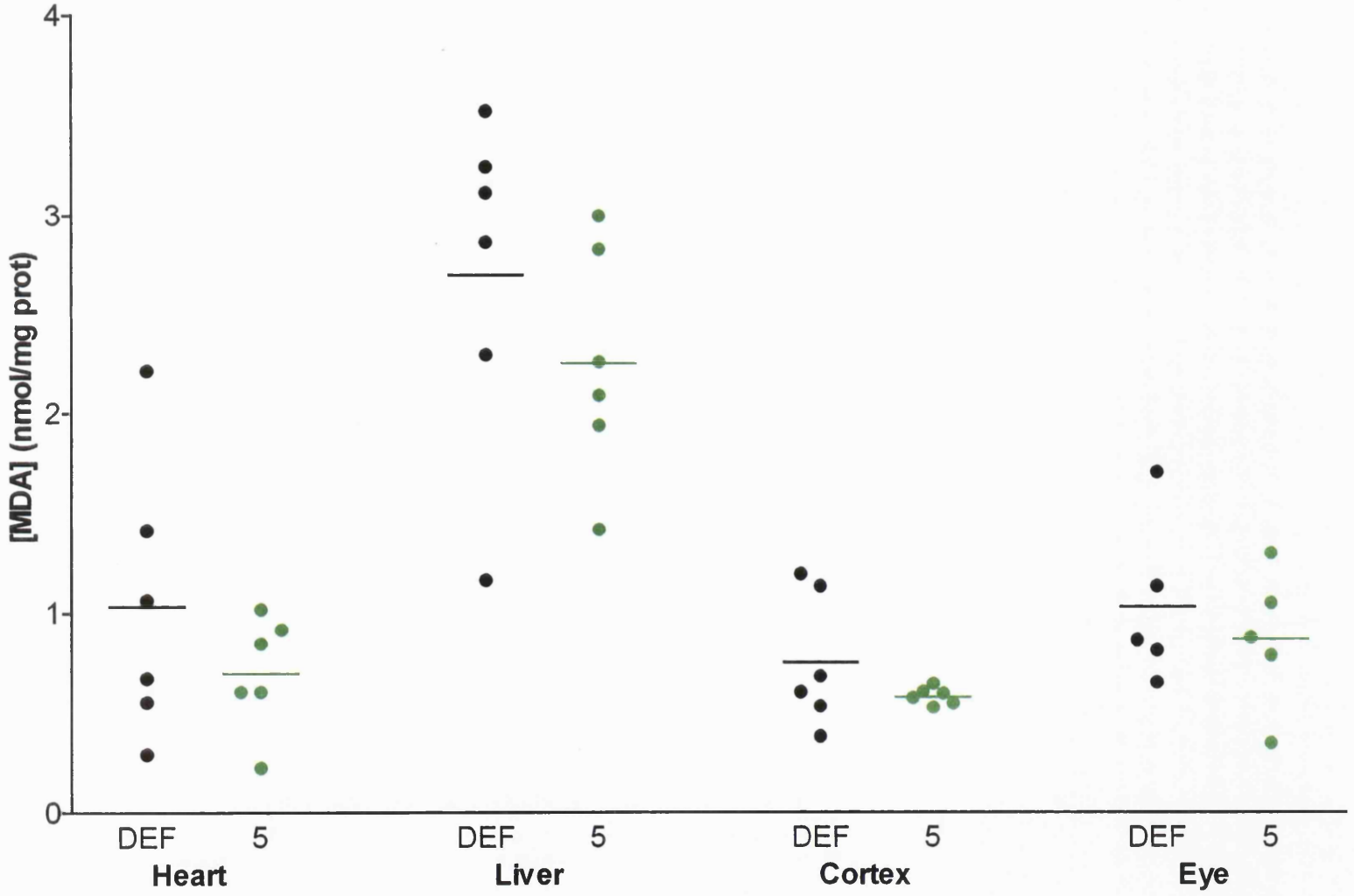


Figure 5.42 Total MDA concentrations



Chapter 6

Comparison and discussion of the effects of various dietary intakes of all-rac- (study I) and RRR- α-tocopheryl acetate (study II) on neural function

The principal aims of the study were to define the minimum dietary requirements of α-tocopheryl acetate necessary to prevent neurological sequelae, and to compare the biological activities of the synthetic (all-rac) and natural (RRR) forms of the vitamin. This chapter briefly summarises the results obtained in study I (described in Chapter 4) and study II (Chapter 5), and then discusses the findings in relation to a) the principal aims of the study and b) other studies in experimental animals and man. Finally, a possible mechanism of action of vitamin E in nerves is also discussed.

6.1 Summary of results

Table 6.1 lists the different experimental diets used in the two studies, together with their biological activities as determined by classical foetal resorption assays (Bunyan *et al*, 1961), where all-rac-α-tocopheryl acetate is assigned an activity of 1.0. Therefore, for example, 1mg of all-rac-α-tocopheryl acetate is approximately equivalent to 0.75mg RRR-α-tocopheryl acetate, allowing a direct comparison to be made between the two diets. The results from study I indicated that a dietary intake of 1.25mg/kg all-rac-α-tocopheryl acetate gave marginal protection from the neurodegenerative effects of vitamin E deficiency. Concentrations close to and below 1.25mg/kg were chosen for study II.

Table 6.1 Amounts, forms and biological activities of α-tocopheryl acetate in the various experimental diets

all-rac-tocopheryl acetate (mg/kg diet)		**RRR-α-tocopheryl acetate** (mg/kg diet)	**all-rac-α-tocopheryl acetate equivalents*** (mg/kg diet)
Study I	Study II	Study II	
0		0	0
		0.40	0.54
	1.00		1.00
		0.75	1.02
1.25			1.25
5.00			5.00
		5.00	6.80
36.00			36.00

* Assuming that the activity of RRR-α-tocopheryl acetate is greater than that of all-rac-α-tocopheryl acetate by a factor of 1.36 (Bunyan *et al*, 1961)

6.1.1 Growth

The groups of rats in both studies showed broadly similar patterns of growth, i.e. an initial phase of rapid growth followed by a second, longer phase of slower weight gain. The groups which were fed very small amounts or no α-tocopheryl acetate did not show an appreciable increase in weight after week 16, whereas the 1.25mg/kg (study I), 1.0mg/kg (study II) and control groups (both studies) continued to gain weight for the duration of the study. The 0.75mg/kg group also did not show an appreciable increase in weight after 16 weeks. However, this group had gained weight more rapidly than the deficient and 0.4mg/kg groups during the initial weeks of the study, hence the 0.75mg/kg group was consistently heavier than the other two groups.

6.1.2 General condition

The general condition of the deficient animals in both studies, and the 0.4mg/kg group in study II, deteriorated after 20 weeks. Their coats became greasy and matted, and there was an increase in porphyrin secretion. At 43 weeks, the first signs of neural problems (impaired balance and irregular gait) were seen in rats from the deficient and 0.4mg/kg groups. Neural signs developed in the other tocopherol-restricted groups (0.75, 1.0 and 1.25mg/kg) from weeks 48 to 52. No signs of neural deficit or deterioration in appearance was observed in rats in the control (5 and 36mg/kg) groups.

6.1.3 Electrophysiology

Table 6.2 compares the time when consistently significant differences in the various electrophysiological parameters were seen between the deficient and 5mg/kg groups in studies I and II. During the course of both study I and study II, there were significant increases in the latencies and decreases in the amplitudes of flash ERGs, in the rats which received the deficient diet. These differences were seen earlier in study II than study I. Similar changes were observed in the flash ERGs of the 0.4, 0.75, 1.0 and 1.25mg/kg groups, but these occurred later than in the deficient group. When the VEP results of the two groups were compared, significant differences were seen in the latencies recorded in both studies and the amplitudes in study II only. The 1.25mg/kg group (study I) gave similar VEP results to the control group, whereas the 0.4, 0.75 and 1.0mg/kg groups in study II behaved like the deficient group.

Significant decreases were observed in the peripheral conduction velocities of the SEPs in the deficient group compared with the controls in study I after 8 months, but significant differences were not seen in study II. The mean monthly peripheral CVs of the 1.25mg/kg (study I) group also decreased, but to a lesser extent than those of the deficient and control groups. The reverse was the case for central conduction velocities, where significant differences were seen after 7 months in study II but no significant differences were seen in study I. No significant differences in the amplitudes of either the peripheral or central SEPs were seen in study I, whereas significant differences were observed in study II. The peripheral SEP amplitudes of the 0.4mg/kg group, and the central amplitudes of the 0.4, 0.75 and 1.0mg/kg (all-rac) groups were similar to those obtained in the deficient group.

Table 6.2 Comparison of electrophysiological results between studies I and II.

	Time (months) from which consistently significant differences were seen	
	Deficient v 5mg/kg[a]	
	Study I	Study II
ERG[b] (white flash)		
- latency	10	8
- amplitude	12	7
VEP[b] (white flash)		
- latency	12	8
- amplitude	ns	13
Peripheral SEP[c] (lower limb)		
- conduction velocity	8	ns
- amplitude	ns	10
Central SEP[d] (lower limb)		
- conduction velocity	ns	7
- amplitude	ns	12

[a] all-rac-α-tocopheryl acetate in study I; RRR-α-tocopheryl acetate in study II

[b] Bright white flash produces mixed rod/cone response

[c] SEP recorded at the 5th lumbar vertebra (L5)

[d] SEP recorded over contralateral somatosensory cortex

The mean peripheral SEP amplitudes of the 0.75mg/kg and 1.0mg/kg (all-rac) groups fell between those of the deficient and control groups.

6.1.4 α-tocopherol concentrations

Tissue α-tocopherol concentrations gave the expected increase with increasing dietary intake. In both studies, α-tocopherol was present at very low or undetectable concentrations in the non-neural tissues and eyes from the deficient rats, though detectable concentrations were present in the thoracic spinal cord and cerebral cortex. Only small concentrations of tocopherol were found in the plasma and tissues of the 0.4mg/kg group, with the exception of the sciatic nerve which had no detectable tocopherol. Concentrations of α-tocopherol in tissues from rats maintained on the 0.75mg/kg (RRR) and 1.0mg/kg (all-rac) diets were similar.

When the mean concentrations of α-tocopherol were plotted against the dietary intake of tocopherol, a linear relationship was found for the sciatic nerve and eye, up to a dietary intake of 36mg/kg. For the other tissues the relationship was linear up to 5mg/kg. There was, in general, a linear relationship between the concentration of α-tocopherol in plasma and the tissues.

6.1.5 Lipid peroxidation

The concentration of malondialdehyde (MDA) was measured in the tissues of deficient and control animals, as an index of lipid peroxidation. Increased concentrations of free

and total MDA were found in all deficient tissues compared with controls, and this increase was greater and more significant in non-neural tissues.

6.2 Discussion of results

6.2.1 Growth

The results of the present study are similar to those found in other studies of vitamin E deficiency in rats. Machlin *et al* (1977) noted that growth was reduced, compared to controls, in deficient male rats at 12 weeks and in females at 17 weeks of age. Other studies reported a significant difference in weight between deficient and control animals after 20 (Goss-Sampson *et al*, 1988, 1990) and 20-24 weeks (Southam *et al*, 1991).

6.2.2 General condition

The physical signs seen in the deficient groups (poor coat condition, muscle wasting, kyphoscoliosis and ataxia) were also similar to those reported previously (Machlin, 1977; Towfighi, 1981; Goss-Sampson *et al*, 1988, 1990). Some studies, however, have reported additional physical changes, such as skin lesions (Machlin *et al*, 1977) or liver necrosis (Century & Horwitt, 1960). Jager (1972) fed rats a vitamin E deficient diet for 22 months and observed only testicular degeneration and mild muscular dystrophy. Differences between studies may be attributed to a variety of reasons.

Firstly, the age of the rat when dietary manipulation commenced, since older rats have higher tissue stores of α-tocopherol than weanlings. It has also been shown that weanling rats (as used in the present studies) fed a vitamin E deficient diet lose tocopherol at a faster rate than older animals (Bieri, 1972). Therefore older rats will take longer to become deficient due to mobilisation of tocopherol stores from the liver and adipose tissue. The strain of rat used may also influence the results. For example, Sprague-Dawley rats (as used in the study of Southam *et al*, 1991) grow at a much slower rate than the Wistar strain used in the present study and by Goss-Sampson *et al* (1988, 1990) and this may explain differences in the rate of development of signs of vitamin E deficiency.

The composition of the diets used in a vitamin E deficiency study is very important. A deficient diet must be shown to be truly deficient since only low circulating levels of α-tocopherol are required to prevent, or at the very least delay, the onset of physical changes. The dietary fat source is also important. High levels of polyunsaturated fatty acids are likely to add another oxidative stress, and therefore affect the outcome of a study, as they are liable to lipid peroxidation which itself could lead to cell membrane damage. Furthermore, experimental diets must contain adequate concentrations of other essential nutrients, such as vitamin A. The total dietary intake may also exert an effect. Goss-Sampson *et al* (1988) reported hind limb muscle wasting in rats after 28 weeks of tocopherol deficiency, whereas animals in the present study showed no wasting before week 43. A possible explanation for this difference is that rats in the present study had a restricted food intake, while the animals in the study of Goss-Sampson *et al* were fed *ad libitum*. The latter group, therefore, gained weight more rapidly and this may have exacerbated the deficiency symptoms.

In agreement with other studies (Goss-Sampson *et al*, 1988; Machlin *et al*, 1977), the muscle wasting and ataxia noted in the present studies were confined predominantly to the hind limbs. The first indications of neural abnormality were impaired balance and an irregular gait. This progressed to a low gait, where the hind quarters and tail were carried close to the ground, and culminated in hind limb paralysis in a number of animals in the deficient and 0.4mg/kg groups. These observations were probably related to weakness of the paraspinal muscles (Southam *et al*, 1991). Electromyographic recordings from the wasted muscles of chronically vitamin E deficient rats, has shown increased fibrillary insertion activity and positive sharp waves, together with spontaneous fibrillation, indicative of denervation (Southam *et al*, 1991).

6.2.3 Electrophysiology

6.2.3.1 SEP results

The earliest electrophysiological abnormality of the SEPs which was observed was a decrease in the central CVs of the deficient, 0.4 (RRR) and 1.0mg/kg (all-rac) groups after 7 months in study II. No significant differences in peripheral CV were observed in study II. In contrast a significant reduction in peripheral but not central CVs was seen after 8 months in the deficient animals of study I. The abnormal central CVs seen in study II are in agreement with other studies in the rat (Goss-Sampson *et al*, 1988, 1990). Goss-Sampson *et al* (1988) reported a significant decrease in central but not peripheral CVs after 8 months of vitamin E deficiency. In another longitudinal study,

changes in central CV again became significant after 8 months of deficiency, whereas significant changes in peripheral CV at L5 were not seen until 12 months of deficiency (Goss-Sampson *et al*, 1990). Bradley *et al* (1986) were unable to find any significant changes in either central or peripheral CVs up to 12 months of deficiency in the rat. A study in dogs (Pillai *et al*, 1993) did, however, find that peripheral nerves were more responsive to both vitamin E supplementation and deficiency than central nerves.

The CV results obtained in the deficient rats in study II are also consistent with those reported in vitamin E deficient man. Brin *et al* (1986) recorded SEPs in 9 patients with abetalipoproteinemia, 8 of whom were found to have decreased CVs. This study also found that central conduction was more severely affected than peripheral (Brin *et al*, 1986). Similar results have been reported in other studies of abetalipoproteinemia (Muller *et al*, 1977; Lowry *et al*, 1984; Wichman *et al*, 1985). Delays in central nerve conduction have also been recorded in other conditions involving chronic vitamin E deficiency, such as AVED (Harding *et al*, 1985; Sokol *et al*, 1988) and ileal resection (Harding *et al*, 1982). Reductions in nerve conduction velocities in vitamin E deficiency may be explained by oxidative damage to nerve cell membranes leading to the demyelination of the nerve fibres.

There are several possible explanations for the differences in the CV results observed between studies I and II. Firstly, the rats in study II had faster growth rates than those in study I, and were heavier throughout the study. Secondly, problems with the storage of the experimental diets, as discussed in section 4.4, may have affected the electrophysiological results obtained. Furthermore, the variability in both the central and peripheral data obtained in both studies may have affected statistical significance.

For example, the peripheral CV data in study II were more variable than those obtained in study I, which could have masked significant differences between the groups. The reverse was the case for central CV data, which was less variable in study II, especially for the 5mg/kg (control) group.

There were no significant differences in SEP amplitudes between the groups in study I. In study II, however, consistently significant reductions in the amplitudes of the responses recorded at L5 were noted in deficient rats after 10 months and significant reductions in the amplitude of the cortical response were seen after 12 months. Furthermore, in the animals with a dietary intake of 0.4mg/kg RRR-α-tocopheryl acetate, both L5 and cortical response amplitudes became significantly reduced after 10 months. The difference in cortical responses between the deficient and 0.4mg/kg groups is probably due to increased variation between the rats in the deficient group. As mentioned above, the rats used in study II were heavier than those in the first study, and this may explain why significant differences were observed in study II and not in study I.

The significant decrease in SEP amplitudes observed in the deficient rats of study II agrees with findings in human studies. Lowry *et al* (1984) recorded cortical SEPs in response to median nerve stimulation in five patients with abetalipoproteinemia and observed reduced amplitudes or an absent response. Wichman *et al* (1985) and Brin *et al* (1986) also reported reductions in SEP amplitudes in patients with abetalipoproteinemia. Diminished amplitudes have also been reported in patients with chronic cholestasis (Landrieu *et al*, 1985) and AVED (Sokol *et al*, 1988). A reduction in SEP amplitude reflects a decrease in the total number of functioning axons, which

will result in a reduction in the number of nerve impulses travelling along the axon bundle.

Electrophysiological abnormalities were recorded approximately 3 months before 'clinical' signs of neural deficit were observed, indicating that these techniques provide a sensitive early index of neural dysfunction. However, Goss-Sampson *et al* (1990) reported significantly reduced CVs at the same time as onset of hindlimb weakness (8 months). As mentioned above, the rats in the study of Goss-Sampson *et al* were fed unrestricted amounts of food, leading to heavier animals, and this may have contributed towards the differences between these studies.

Despite the differences in the SEP results between the two studies as described on pages 223-4, electrophysiological abnormalities were recorded earlier than the clinical signs and therefore provided an early indication of neural dysfunction.

6.2.3.2 Visual function

Increases in latencies and decreases in amplitudes were seen in flash ERGs recorded in the deficient groups from both studies. Significant increases in the ERG b wave latencies, following bright white flash stimulation, were first observed after 10 months in study I, but after only 8 months in study II. The ERG peak-to-peak amplitudes of the deficient group were significantly reduced after 12 months in study I, and after only 7 months in study I. These findings were similar to those reported in a previous study comparing visual function in vitamin E deficient and control rats (Goss-Sampson *et al*, 1998). In the study of Goss-Sampson *et al* (1998), the latencies of both the ERG

a- and b-waves were significantly delayed in deficient animals from 6 months, and significantly smaller wave amplitudes were recorded after 5 months. These changes, therefore, occurred earlier than those observed in the present studies, which may have resulted either from biological variation as discussed above, or that the deficient rats in the study of Goss-Sampson *et al* were heavier.

The flash ERG represents electrical activity arising in the outer and middle layers of the retina. The a-wave corresponds to events at the level of the photoreceptors, while the b-wave reflects the spread of ionic current through the bipolar and Muller cells. The fact that the onset of the a-wave was particularly delayed in the deficient rats is consistent with a primary defect at the level of the photoreceptors. Goss-Sampson *et al* (1998), however, failed to find any morphological changes in the photoreceptors on electronmicroscopic examination, and suggested that the abnormalities may be at a biochemical and/or membrane level. Several studies have, however, reported morphological changes in the retina associated with vitamin E deficiency (Hayes *et al*, 1970; Hayes, 1974; Robison *et al*, 1979). These latter studies used chemically treated natural diets, which may have been deficient in other essential nutrients (e.g. vitamin A) which are known to affect the photoreceptors and pigment epithelium.

VEP onset latencies were more variable than the other visual parameters. In study II, consistently significant increases in VEP latencies occurred between 5 and 8 months in the low tocopherol intake groups, whereas significant increases were not seen in study I until 12 months. A possible contribution to the increased variability of the VEP response is the difficulty of placing the recording electrode in the same position in each animal. Since the visual cortex is small, even minor differences in electrode position

can affect the recorded response. The VEP waveforms were often small and it was, therefore, sometimes difficult to define the onset. These results were, however, similar to those observed by Goss-Sampson *et al* (1998), who reported significantly longer VEP latencies after 6 months in deficient rats compared to controls.

Changes in VEP amplitudes were not observed until month 13 in study II, and not at all in study I, whereas a reduced response had been noted in deficient rats at 6 months by Goss-Sampson *et al* (1998). As before, difficulty in defining the onset and the variability of VEP size and shape could have contributed to the observed differences between the studies. Another factor is that the rats used in the present study were smaller, giving responses which were of a smaller size and, therefore, more difficult to record accurately.

The changes in visual electrophysiology observed in the present studies in rats are similar to those observed in vitamin E deficient man. In a study of 13 children with chronic cholestasis and low serum vitamin E and/or vitamin A concentrations, all were found to have abnormal flash ERGs with marked decreases in the amplitudes of the b waves, and VEPs were abnormal in 6 of the 11 patients tested (Alvarez *et al*, 1983). Cynamon *et al* (1988) reported abnormal delays in the major occipital positive peak (P100) of the VEP in 8 cholestatic children tested, but could not find a consistent relationship with vitamin E status. Abnormal visual electrophysiology has also been noted in patients with abetalipoproteinemia (Brin *et al*, 1986; Wichman *et al*, 1985) and fat malabsorptive disorders (Satya-Murti *et al*, 1986).

The VEP represents electrical activity in the visual cortex in response to flash stimulation. The arrival of nerve impulses from the retinogeniculate pathway, via the optic nerve, causes depolarisation in a specific area (lamina 4c) of the striate cortex (Schroeder *et al*, 1991; Givre *et al*, 1994). Depolarisation spreads to other visually specialised cortical areas, more than 30 of which have been identified in the monkey (Felleman & Van Essen, 1991). In vitamin E deficiency, abnormalities of the VEP tend to appear after changes of the ERG (Alvarez *et al*, 1983). It has been suggested, therefore, that VEP abnormalities are secondary to retinal dysfunction (Goss-Sampson *et al*, 1998). The results of the current studies tend to support this view as significant changes in ERGs generally occurred earlier than in VEPs. However, further studies need to be conducted on the optic nerve as it is possible that VEP abnormalities may in part also reflect optic nerve dysfunction.

6.2.3.3 Minimum dietary requirements for vitamin E in nerves

No statistically significant differences were found between the 5mg/kg and 36mg/kg all-rac α-tocopheryl acetate groups in any of the electrophysiological parameters measured, indicating that a dietary intake of 5mg/kg was sufficient to maintain normal neural and retinal function.

The group which received the 0.4mg/kg RRR-α-tocopheryl acetate diet gave similar electrophysiological results to the deficient rats. In both groups, central CVs became significantly reduced after 7 months when compared with controls. ERG latencies and VEP amplitudes also became significantly different from controls in both groups at the same times (8 and 13 months, respectively).

The results of study I indicated that the minimum dietary requirement for α-tocopherol, necessary to prevent neural and retinal degeneration, was approximately 1.25mg/kg all-rac-α-tocopheryl acetate. In general, it took longer for significant differences between the deficient and 1.25mg/kg groups to become apparent than between the deficient and control groups. Thus, the mean ERG b wave latencies did not become significantly different from the deficient group until month 13, and the peripheral SEP CVs only became significantly slower than the deficient group after 11 months. There were no significant differences in mean ERG b wave latencies between the 1.25 and 5mg/kg, and significant differences in peripheral CVs were not seen until 13 months.

In order to examine in more detail the marginal protection afforded by 1.25mg/kg all-rac-α-tocopheryl acetate, diets containing less than that amount were compared to a control group. When the 0.75 (RRR) and 1.0mg/kg (all-rac) groups were compared with the 5mg/kg (RRR) control group, significant differences were observed in central CV, ERG and VEP latencies and amplitudes. These differences occurred later than in the deficient and 0.4mg/kg groups, indicating that a dietary intake of 0.75mg/kg (RRR) or 1.0mg/kg (all-rac) delays, but does not prevent, the onset of the neural signs of deficiency. It would have also been of interest to examine diets between 1.25 and 5mg/kg to determine what level would prevent the development of neural abnormalities, but time constraints prevented further study.

The results of the present studies indicate that a dietary intake of 1.25mg/kg all-rac-α-tocopheryl acetate provides marginal protection from neural and retinal degeneration in rats.

6.2.3.4 Comparison of the biological activities of all-rac- and RRR-α-tocopheryl acetate

Using the common conversion factor of 1.36, the dietary intakes of 0.75mg/kg RRR-α-tocopheryl acetate and 1.0mg/kg all-rac-α-tocopheryl acetate would have been expected to have similar biological activities and, therefore, to give similar electrophysiological results. The results of the SEP recordings (central and peripheral latencies and amplitudes) were essentially the same in the two groups of animals. However significant changes in visual function tended to be seen earlier in the 0.75mg/kg (RRR) compared to the 1.0mg/kg (all-rac) group. Biological variation may have contributed to the differences between these two groups. For example, the mean ERG latency fell slightly to 59.97 +/- 4.76ms in the 1.0mg/kg (all-rac) group at month 10, bringing it closer to the mean of the 5mg/kg group (57.35 +/- 2.59ms) and therefore not statistically different. At the same time, the mean of the 0.75mg/kg group increased slightly to 61.70 +/- 5.04ms, thereby increasing the difference from the 5mg/kg group mean and giving statistical significance. The VEP traces tended to be more variable than the other electrophysiological parameters recorded, therefore giving more scatter and less significance.

There was, therefore, no evidence that the relative biological activities of the two different forms of α-tocopherol were different in neural tissues from the commonly accepted ratio of 1.36 to 1.0, as determined using the classical foetal resorption assay.

6.2.4 Tissue vitamin E concentrations

The results obtained confirmed previous observations that neural tissues retain α-tocopherol more effectively than non-neural tissues (Goss-Sampson *et al*, 1988; Ingold *et al*, 1987). Goss-Sampson and colleagues (1988) found that, after 16 weeks of deficiency, α-tocopherol was undetectable in rat serum, and concentrations in liver and adipose tissue were <2% of control values. The concentration in nerve, however, was 9%, and in brain and spinal cord 18% of control values. By 52 weeks, neural tissues still retained 3-5% of control concentrations. The results of the present studies showed that the brain and cord of deficient animals retained approximately 3% of the control concentration of α-tocopherol after 60 weeks of deficiency. Ingold *et al* (1987) investigated the half-life of natural (RRR) α-tocopherol in various tissues by sequentially feeding rats unlabelled and deuterated tocopherol. The ratio of deuterated to unlabelled tocopherol was determined by gas chromatography/mass spectrometry and used as a measure of the rate of transfer of RRR-α-tocopherol under steady-state conditions. The results showed that the half-life of α-tocopherol was greatest in neurological tissues (76 days in spinal cord and 29 days in brain, compared to 10-11 days in liver and serum).

The preferential conservation of vitamin E by neural tissues may reflect a reduced rate of turnover compared with other tissues. All tissues appear to show two phases of depletion, an initial rapid loss during the first 4-8 weeks of deficiency, followed by a second phase of slow prolonged depletion. Bieri (1973) suggested that the first phase corresponded to a rapidly mobilised labile pool of vitamin E, and the second phase represented vitamin E which was bound to subcellular or membranous structures. It is

possible that this latter phase relates to the loss of the functional and more critical component of tissue vitamin E. It may, therefore, be significant that neural tissues appear to maintain a greater proportion of α-tocopherol in the second labile pool.

α-tocopherol could not be detected in eyes taken from the deficient group of rats. However, the importance of α-tocopherol for normal retinal function might have suggested that the eye would retain α-tocopherol in a similar way to nervous tissues. An explanation for this result is the rapid and continual turnover of the outer segment membranes of the photoreceptors. Cells in the outermost layer of the retina (retinal pigment epithelium, RPE) phagocytose and degrade the tips of the photoreceptor outer segments that are intermittently shed into the inter-photoreceptor space. In this way, vitamin E is lost from the eye and cannot be replaced by rats on a deficient diet. Vitamin E is likely to act as an antioxidant in the eye since it has been shown that lipofuscin accumulates in the RPE of rats fed a tocopherol deficient diet (Katz *et al*, 1978). Lipofuscin is produced as a result of the peroxidation of polyunsaturated fatty acids (PUFA) and its accumulation in RPE probably reflects lipid peroxidation resulting from the breakdown of the receptor membranes.

Small amounts of α-tocopherol were found in the cerebral cortex (0.27 - 0.92μg/g wet weight) and thoracic spinal cord (0.45 - 1.10μg/g wet weight) of rats from the deficient and 0.4mg/kg groups. However, these concentrations were not sufficient to prevent central electrophysiological abnormalities. Rats in the 0.75, 1.0 and 1.25mg/kg groups had α-tocopherol concentrations in the cortex of 1.39 - 1.63μg/g wet weight, and in thoracic cord of 1.32 - 1.49μg/g wet weight. Although these concentrations

were higher than those found in the deficient and 0.4mg/kg groups, some electrophysiological abnormalities were still observed, albeit later, in these groups. The concentrations of α-tocopherol in the cerebral cortex and thoracic spinal cord in the 0.75, 1.0 and 1.25mg/kg groups correspond to 12.2 - 25.0% of that found in the 5mg/kg control groups.

Increasing the dietary intake of α-tocopheryl acetate resulted in increases in tissue tocopherol concentrations, which were linear up to 5mg/kg dietary intake. Above this level, the relationship between dietary intake and tissue concentrations of α-tocopherol were non-linear, suggesting that the tissues had a finite capacity for taking up tocopherol. There was, in general, a linear relationship between the concentrations of α-tocopherol in the tissues and in plasma. This indicated that plasma tocopherol concentrations gave a good indication of tissue vitamin E status.

6.2.5 Vitamin E deficiency and lipid peroxidation

Free and total MDA concentrations were increased in all tissues from the rats receiving the deficient compared with the control diet, indicating that there was more lipid peroxidation occurring in the deficient tissues. This is consistent with a reduced (or absent) tissue α-tocopherol concentration affording less protection from lipid peroxidation in these tissues and agrees with results obtained by MacEvilly and Muller (1996). The differences in free MDA concentrations between deficient and control tissues were greater in the non-neural tissues than the cerebral cortex and eye. This is consistent with the fact that neural tissues retain α-tocopherol more effectively than

non-neural tissues. MDA was detected in all tissues tested, indicating that some lipid peroxidation is occurring as part of the normal metabolic process of the cell.

Apart from the liver in study I, there were no significant differences in the concentrations of bound MDA between the deficient and control groups. The mean bound MDA concentration present in liver from rats fed 36mg/kg α-tocopheryl acetate was just significantly higher ($P<0.05$) than that found in the deficient group (1.75 compared to 1.56nmol/mg protein). This would not be expected since less lipid peroxidation is likely to occur in tissues from rats receiving 36mg/kg. Similar results have, however, been reported previously (MacEvilly & Muller, 1996). A suggestion put forward, which could explain this observation is that MDA may be produced at a faster rate in deficiency states than can react with various cellular components and that vitamin E deficient liver might be more efficient in clearing toxic free MDA. This could be achieved by an increased activity of aldehyde dehydrogenase or by excreting MDA in urine (Lee & Csallany, 1987).

6.3 Proposed mechanism of action of vitamin E in nerves and the retina

From the data in this and other reported studies, the following mechanism of action of vitamin E in nerves and the retina can be proposed.

Vitamin E is the major lipid soluble chain-breaking antioxidant *in vivo* (Ingold *et al*, 1987). It is able to terminate free radical-generated chain reactions by scavenging peroxyl radicals formed by the action of oxygen-derived free radicals on unsaturated

fatty acids. Vitamin E is, therefore, important for the prevention of lipid peroxidation of the polyunsaturated fatty acids (PUFA) of membrane phospholipids (Tappel, 1962; McCay *et al*, 1972). Southam *et al* (1991) suggested that the neurological syndrome associated with severe and chronic vitamin E deficiency could result from damage to mitochondrial and other intra-axonal membranous structures. The membranes of mitochondria and smooth endoplasmic reticulum contain a high proportion of polyunsaturated fatty acyl chains and may well, therefore, be more susceptible to damage as a result of vitamin E deficiency. In addition, there is increased production of oxygen-derived free radicals in mitochondria as a result of leakage during oxidative phosphorylation. The mitochondrial respiratory chain is composed of 5 protein complexes (I to V), which are located in the inner mitochondrial membrane. Complexes I and III are the main sites for free radical production (Turrens & Boveris, 1980), and complexes I and IV are particularly susceptible to inhibition by oxidative damage (Narabayashi *et al*, 1982; Benzi *et al*, 1991).

The above findings agree with the observations of MacEvilly and Muller (1996), who found mitochondria were more susceptible to oxidative stress compared with other neural fractions (myelin and axolemma). A disturbance of the axonal mitochondria could then lead to the reported abnormalities in fast retrograde transport (Muller & Goss-Sampson, 1990; Southam *et al*, 1991). These processes are energy dependent and result in the accumulation of organelles in the preterminal regions of the axons (Brimijoin, 1984). The terminal axons would then become isolated from the cell body, and ultimately degenerate. This process would then spread centripetally in a 'dying-back' manner, which is the characteristic of neural degeneration in vitamin E deficiency (Southam *et al*, 1991).

A study of muscle mitochondria from vitamin E deficient rats has also provided support for this hypothesis (Thomas *et al*, 1993). Thomas and his colleagues reported significant decreases in the activities of complexes I and IV of the mitochondrial respiratory chain, a reduced respiratory control ratio (indicative of membrane damage) and increased membrane fluidity. Inhibition of complexes I and IV could result from changes in the membrane lipid environment or oxidative damage to the proteins themselves or to mitochondrial DNA (Thomas *et al*, 1993).

The results obtained in this study agree with this proposed mechanism of action of vitamin E in nerves. Rats fed a diet containing no or very little (0.4mg/kg) α-tocopheryl acetate had undetectable or very low tissue concentrations of α-tocopherol. This led to increased lipid peroxidation, indicated by increased MDA concentrations, and presumably damage to cell and organelle membranes. The damage to nerve cell (i.e. axonal) membranes would lead to secondary demyelination of the nerve fibres, which was observed as increases in the latencies (and decreases in the conduction velocities) of both the somatosensory and visual evoked potentials. Also, the resulting loss of axons would lead to a reduced number of nerve impulses which was seen as a reduction in the amplitudes of the evoked potentials.

The results from the ERG recordings in the present study agree with the hypothesis proposed by Goss-Sampson et al (1998). These authors proposed that vitamin E deficiency leads to increased oxidative stress in the retina which causes increased lipid peroxidation with the loss of long-chain PUFA from the photoreceptors. Consequently, there is a change in the photoreceptor membrane microenvironment which could affect membrane bound proteins involved in ion transport. This in turn

would alter phototransduction by impairing the ability of photoreceptors to hyperpolarise and depolarise. Changes in the membrane microenvironment could also affect phototransduction by altering the light induced movement of rhodopsin within the membrane. All these alterations will result in changes in electrical activity as represented by the ERG.

Chapter 7

Effects of repletion with all-rac-α-tocopheryl acetate

7.1 Introduction

There have been very few studies in animals which have investigated the effects of repletion with vitamin E on the neurological signs of deficiency. Nelson *et al* (1981) repleted 2 rhesus monkeys with oral supplements of vitamin E following 30 months of deficiency. This resulted in normal serum vitamin E concentrations and the resolution of the muscle weakness and anaemia seen during the period of deficiency. Furthermore, the repleted monkeys did not exhibit the ultrastructural changes indicative of early axonal injury that were noted in the peripheral nerves of deficient animals.

Neurological function in repleted rats was investigated by Goss-Sampson *et al* (1998). One group of animals were fed a vitamin E deficient diet, while a second (control) group received the same diet to which 40mg all-rac-α-tocopheryl acetate/kg diet had been added. A third group were fed the deficient diet for 30 weeks, before repletion with the control diet for 20 weeks. Single flash ERGs, VEPs and oscillatory potentials were recorded at various time points during the year and the rats were weighed at regular intervals. The deficient animals started to lose weight after 20 weeks, and by 1 year all exhibited the neuromuscular syndrome characteristic of chronic vitamin E deficiency. The repletion group animals also lost weight until they were changed to the control diet, then they showed a rapid catch-up. None of the repleted rats developed neuromuscular signs. After 20-30 weeks of deficiency, there were significant differences in the ERGs, VEPs and oscillatory potentials compared with control animals. Repletion with vitamin E did not correct any of these electrophysiological

abnormalities, although retinal vitamin E concentrations increased from undetectable to 50% of the control level (0.34 +/- 0.04μmol/mol lipid) after 20 weeks of repletion (Goss-Sampson *et al*, 1998).

To date, I am not aware of any studies in vitamin E deficient rats which have investigated the effects of repletion on central and peripheral nerves. This chapter details 2 longitudinal experiments in which electrophysiological parameters (SEP, VEP, ERG) were recorded in groups of rats that were fed a vitamin E deficient diet until neurological abnormalities were evident, and were then changed to a diet containing 36mg/kg all-rac-α-tocopheryl acetate.

7.2 Methods

Repletion with 36mg/kg all-rac-α-tocopheryl acetate was investigated during both study I and study II , described in chapters 4 and 5 respectively. The results obtained were compared with the deficient and control (5mg/kg) groups of the respective study.

7.2.1 Animals and experimental diet

For each study, 15 weanling male rats were maintained on a vitamin E deficient diet (see section 2.2.2 for composition) until electrophysiological changes became apparent. The rats were then changed to a diet containing 36mg/kg all-rac-α-tocopheryl acetate for the remainder of the study. These groups will be termed the repleted groups. For study I, the animals were repleted after 10 months (week 42) and for study II after 9 months (week 38). The reason for this difference is that

electrophysiological testing was not carried out at month 9 in study I, and therefore significant degeneration of the responses was not noted until month 10.

7.2.2 Protocol for longitudinal electrophysiological recordings

Study I

Electrophysiological parameters were recorded in the same 12 rats at monthly intervals for 14 months. SEPs were recorded at the level of the cervical spine (C3) and cerebral cortex in response to upper limb (median nerve) stimulation, and at the level of the lumbar spine (L5) and cortex in response to lower limb (tibial nerve) stimulation, as detailed in section 2.3.2. VEPs and ERGs were recorded in response to dim blue and bright white flash stimuli (see section 2.3.3). The responses recorded were characterised in terms of their peak latencies and peak-to-peak amplitudes, and compared with the results obtained for the deficient and 5mg/kg (all-rac-α-tocopheryl acetate) groups in study I (chapter 4), which were followed concurrently.

Study II

Since electrophysiological changes in study I were not observed until month 8, recordings in study II were not begun until month 5. Thereafter, lower limb SEPs were recorded at the level of the lumbar spine (L5) and cortex in 12 rats at monthly intervals until month 14. Visual function was investigated over the same time period by recording ERGs and VEPs in response to bright white flash stimuli only. As above,

peak latencies and peak-to-peak amplitudes were compared with those obtained from the deficient and 5mg/kg (RRR-α-tocopheryl acetate) groups in study II (chapter 5).

7.2.3 Biochemistry

The same procedure was used in both study I and II. After 14 months, the animals were killed and various non-neural and neural tissues, together with plasma were collected as described in section 2.3. Plasma and tissue α-tocopherol concentrations were then determined, as detailed in section 2.4.

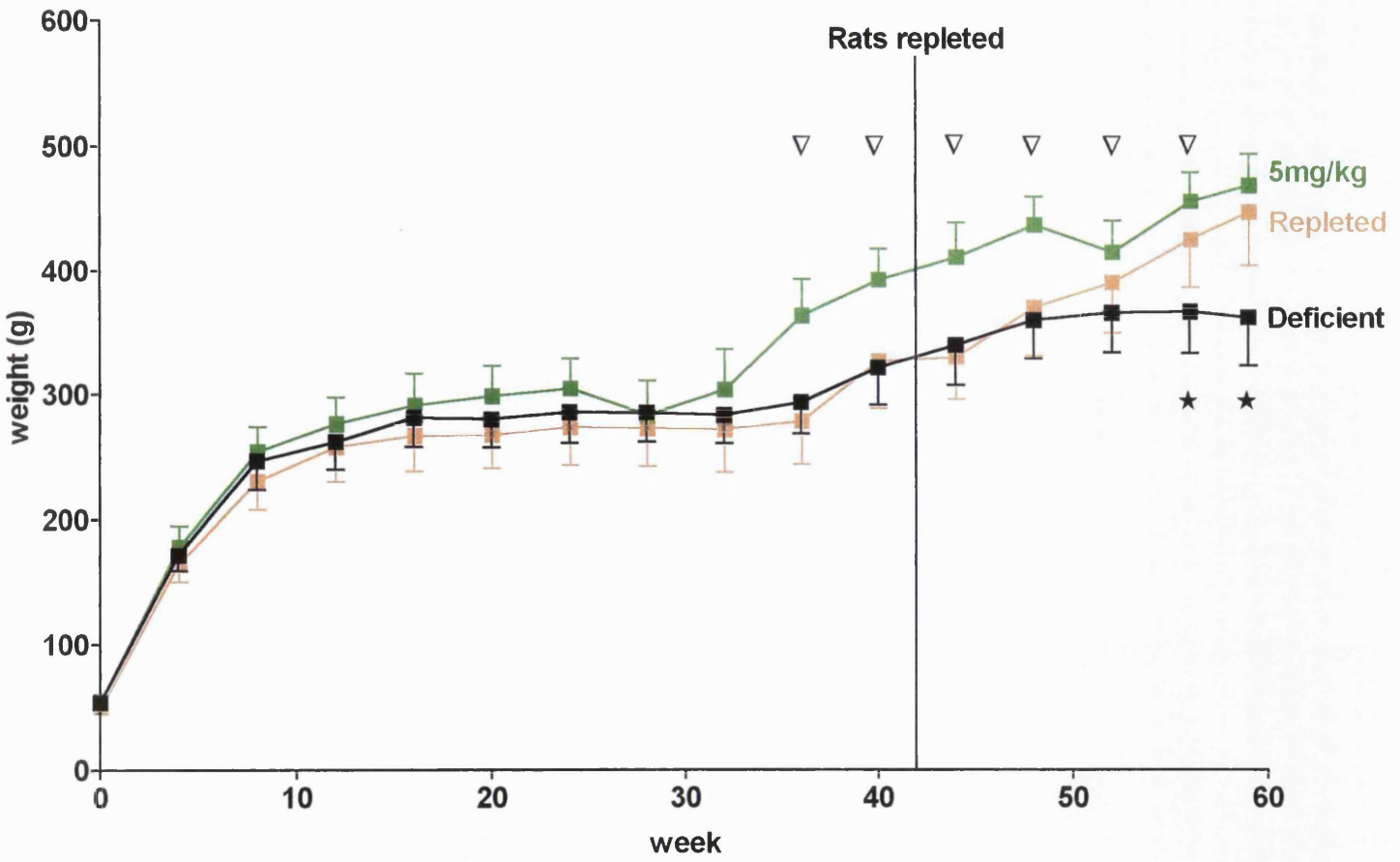
STUDY I

7.3 Growth and condition of rats

The rats in the repleted group were weighed weekly throughout the course of the study, and the mean weights (n=15) at monthly intervals, together with those of the deficient and 5mg/kg groups are shown in Figure 7.1. The repleted group were changed from the deficient diet to one containing 36mg/kg all-rac-α-tocopheryl acetate at week 42. The repleted and deficient groups had similar mean weekly weights until week 52. The repleted group then began to gain weight and became significantly heavier than the deficient group at week 56 (425.0 +/- 38.3 compared with 366.5 +/- 33.8g). By the end of the study (week 59), the repleted group had attained 95.3% of the weight and was not significantly different from the 5mg/kg group (447 +/- 43.0 compared with 468.7 +/- 25.0g, respectively).

The general condition of the repleted group began to deteriorate at the same time as the deficient group i.e. at approximately 20 weeks. Their coats became greasy and there was an increase in porphyrin secretion, giving the fur a pink tinge. After 45 weeks, the first signs of neurological dysfunction, an irregular gait, became apparent in 3 rats of the repleted group. The condition of 2 of these animals remained the same for the remainder of the study, but that of the third worsened. By week 59, this rat had impaired balance, a very abnormal gait and was dragging its hindlimbs.

Figure 7.1 Growth curves for deficient, repleted and 5mg/kg (all-rac-α-tocopheryl acetate) groups



7.4 Electrophysiological results

The results obtained from rats fed a vitamin E deficient diet, or one containing 1.25mg/kg or 5mg/kg all-rac-α-tocopheryl acetate, have been described previously (section 4.5). This chapter will therefore concentrate on the differences between the repleted group and the other groups.

7.4.1 Lower limb SEPs

Peripheral conduction velocities

The group mean peripheral CV for the deficient, repleted, 1.25mg/kg and 5mg/kg groups are shown in Figure 7.2. For the first 8 months, the repleted group gave results which were essentially the same as the deficient group, and therefore data is only shown for the repleted group from this time point. From month 8-14, the peripheral CV of the repleted group remained relatively constant (Figure 7.3). The peripheral CV of the repleted group was significantly different from the deficient at 10 months (i.e. before repletion was commenced) but it continued to diverge and was highly significantly faster than that of the deficient group by the end of the study (46.39 +/- 3.04 compared with 40.29 +/- 1.73ms^{-1} respectively, $P<0.0001$). When the repleted rats were compared with those which received 1.25mg/kg all-rac-α-tocopheryl acetate (Figure 7.4), a significant difference was not recorded until month 13 (46.10 +/- 3.38 compared with 42.82 +/- 3.24ms^{-1}respectively, $P<0.05$). When the repleted group was

compared with the 5mg/kg (all-rac) group, no significant differences were found at any time point.

Central conduction velocities

Figure 7.5 shows the group mean central CVs for the deficient, repleted, 1.25mg/kg and 5mg/kg (all-rac) groups. The central CV of the deficient and 1.25mg/kg groups remained relatively constant from 8 months, while that of the repleted group increased. It became consistently significantly faster than deficient rats (see Figure 7.6) at month 12 (42.39 +/- 7.68 compared with 32.51 +/- 7.00ms^{-1} respectively, $P<0.01$) and the 1.25mg/kg group (Figure 7.7) at month 11 (38.41 +/- 7.66 compared with 30.12 +/- 6.91ms^{-1} respectively, $P<0.05$).

SEP amplitudes

Figures 7.8 and 7.9 show the group mean peak to peak amplitudes of spinal (L5) and cortical SEPs, respectively, following lower limb stimulation. No consistently significant differences were found between the groups.

Figure 7.2 Group mean peripheral conduction velocities (lower limb)

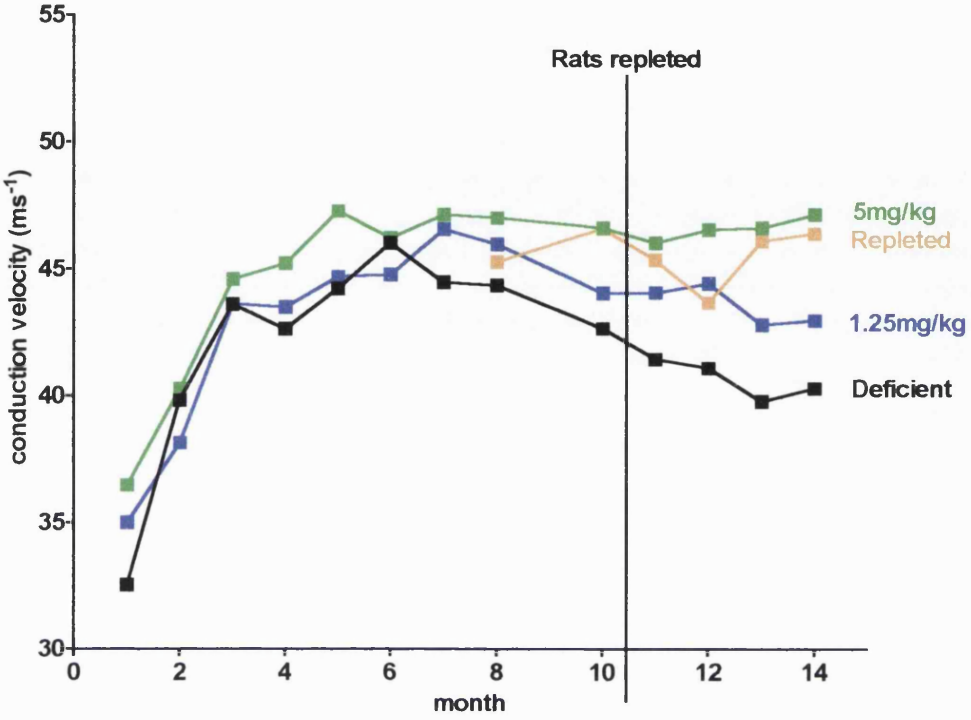


Figure 7.3 Group mean peripheral CV +/- 1SD - deficient v repleted (lower limb)

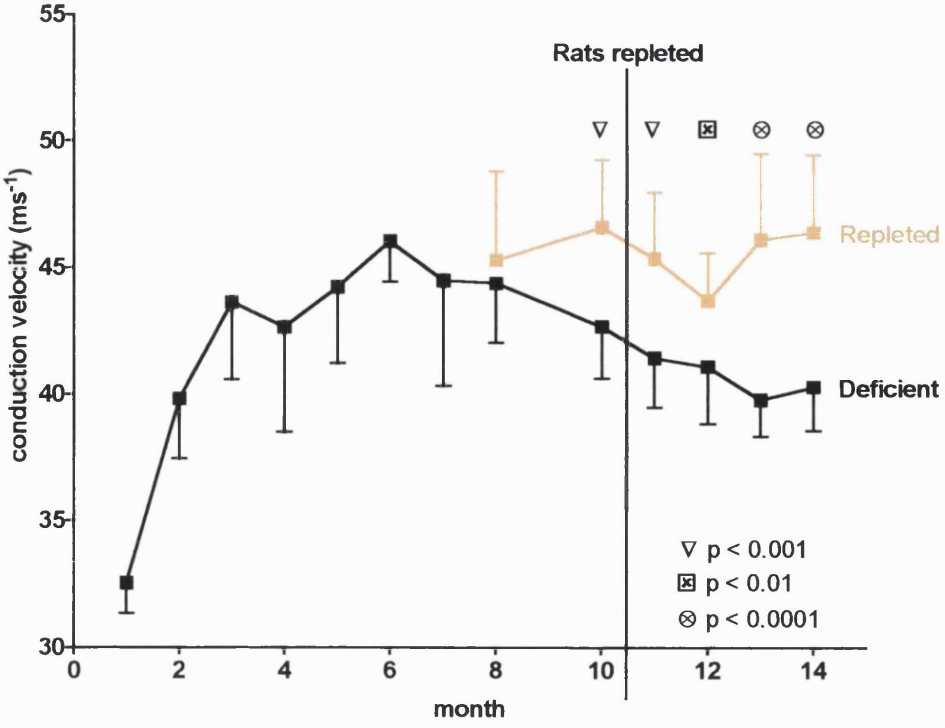


Figure 7.4 Group mean peripheral CV +/- 1SD - 1.25mg/kg v repleted (lower limb)

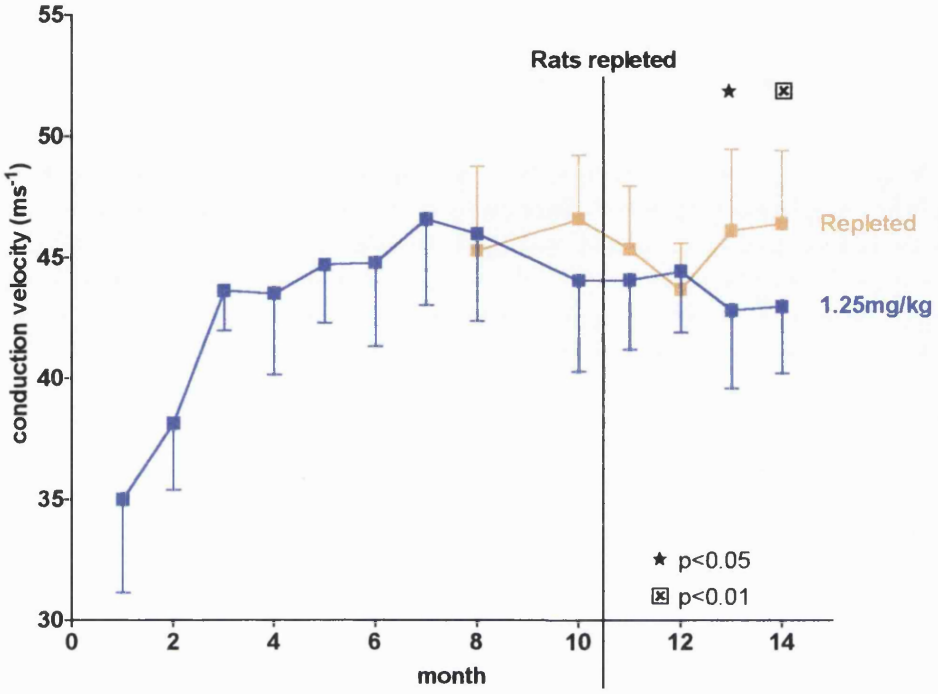


Figure 7.5 Group mean central conduction velocities (lower limb)

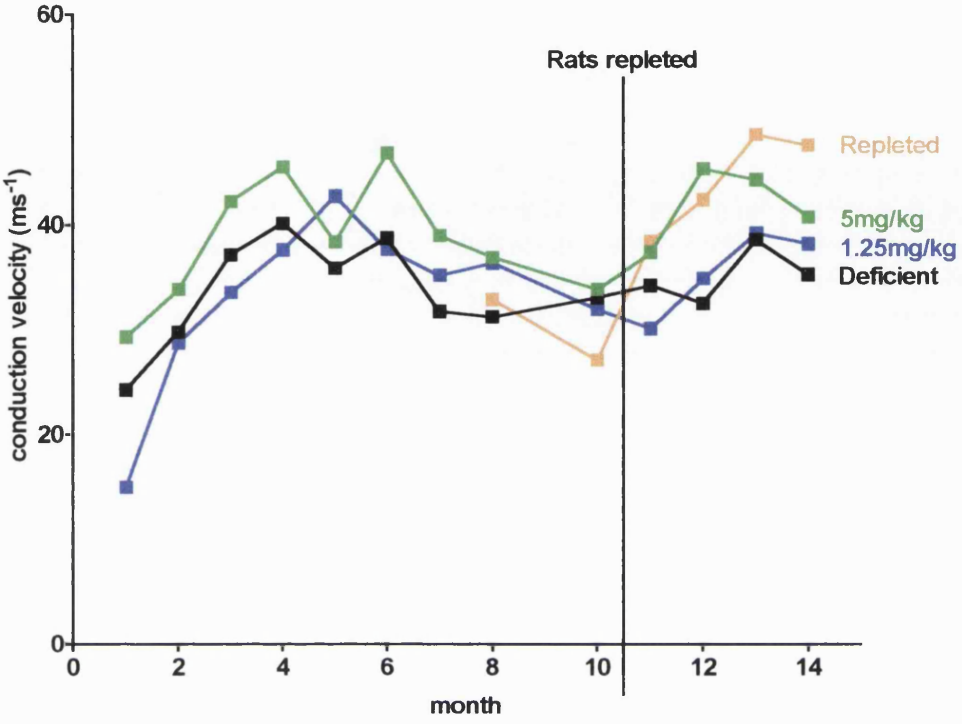


Figure 7.6 Group mean central CV +/- 1SD - deficient v repleted (lower limb)

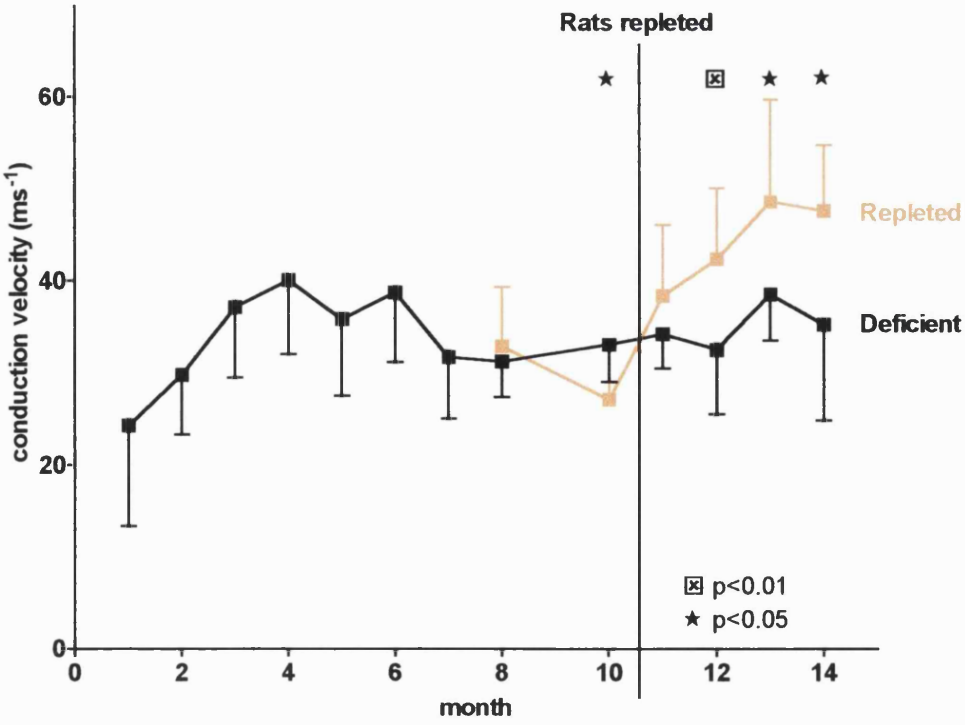


Figure 7.7 Group mean central CV +/- 1SD - 1.25mg/kg v repleted (lower limb)

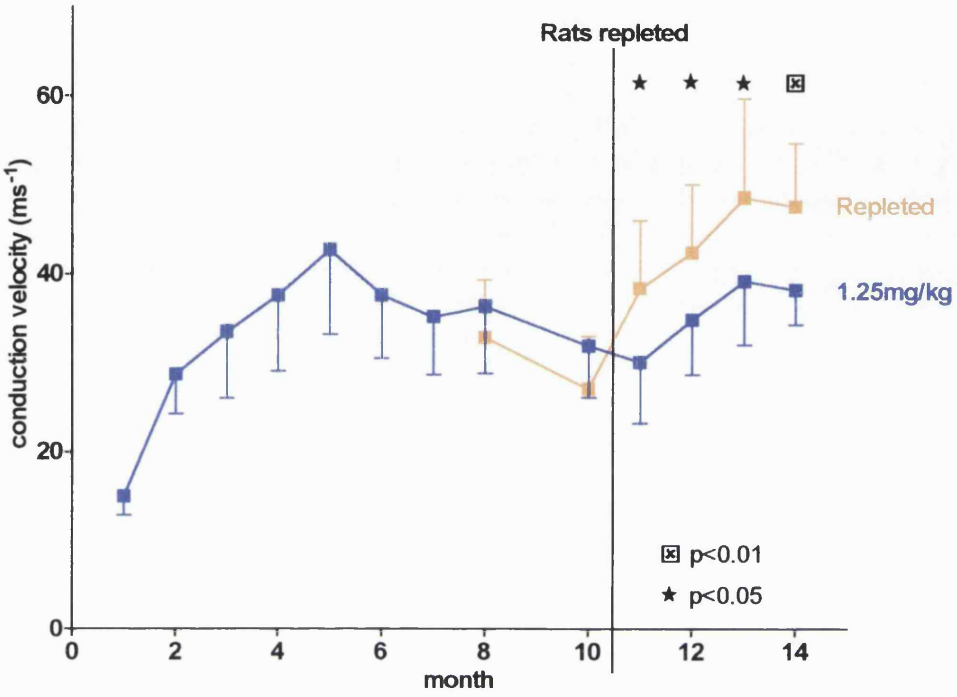


Figure 7.8 Group mean L5 SEP peak-to-peak amplitudes (lower limb)

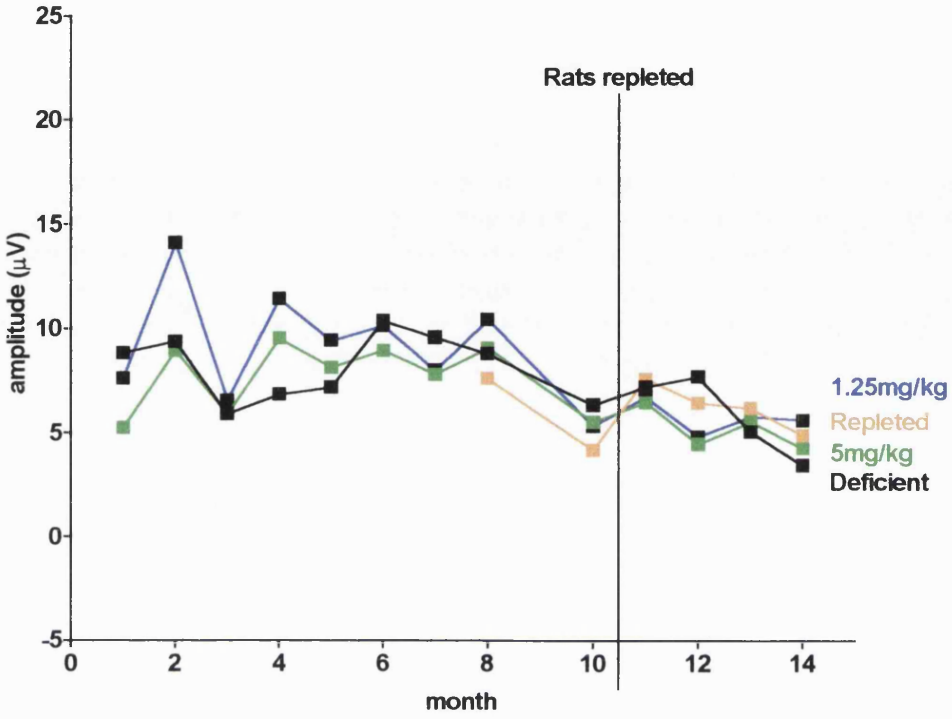
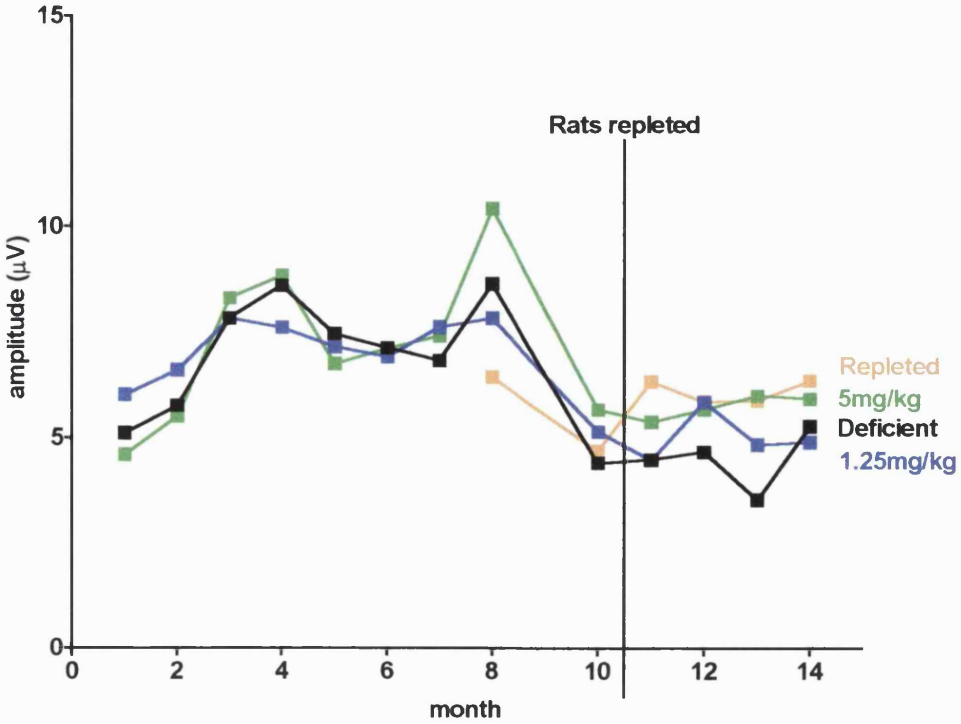


Figure 7.9 Group mean cortical SEP peak-to-peak amplitudes



7.4.2 Upper limb SEPs

Peripheral conduction velocities

The group mean peripheral CV following upper limb stimulation for the deficient, 1.25mg/kg, 5mg/kg and repleted groups are shown in Figure 7.10. The peripheral CV of the repleted group decreased like the deficient and 1.25mg/kg groups from month 7, but then began to increase after repletion. It became consistently significantly faster than the deficient ($P<0.01$, Figure 7.11) and 1.25mg/kg ($P<0.05$, Figure 7.12) groups at month 12. The repleted group had received a diet containing 36mg/kg all-rac-α-tocopheryl acetate for 6 weeks at this time point. When compared with the 5mg/kg group, the repleted group had a significantly slower peripheral CV from month 10 i.e. from before repletion commenced (Figure 7.13).

Central conduction velocities

Figure 7.14 shows the group mean central CV for the deficient, repleted, 1.25mg/kg and 5mg/kg groups. Central CV remained relatively constant in the repleted group until repletion and then it increased, becoming consistently significantly faster than the deficient group at month 12 (38.36 +/- 12.19 compared with 23.33 +/- 9.47ms^{-1} respectively, $P<0.01$) as shown in Figure 7.15 and than the 1.25mg/kg group at month 14 only ($P<0.01$). The central CV of the repleted group was not consistently significantly slower than the 5mg/kg group.

Figure 7.10 Group mean peripheral conduction velocities (upper limb)

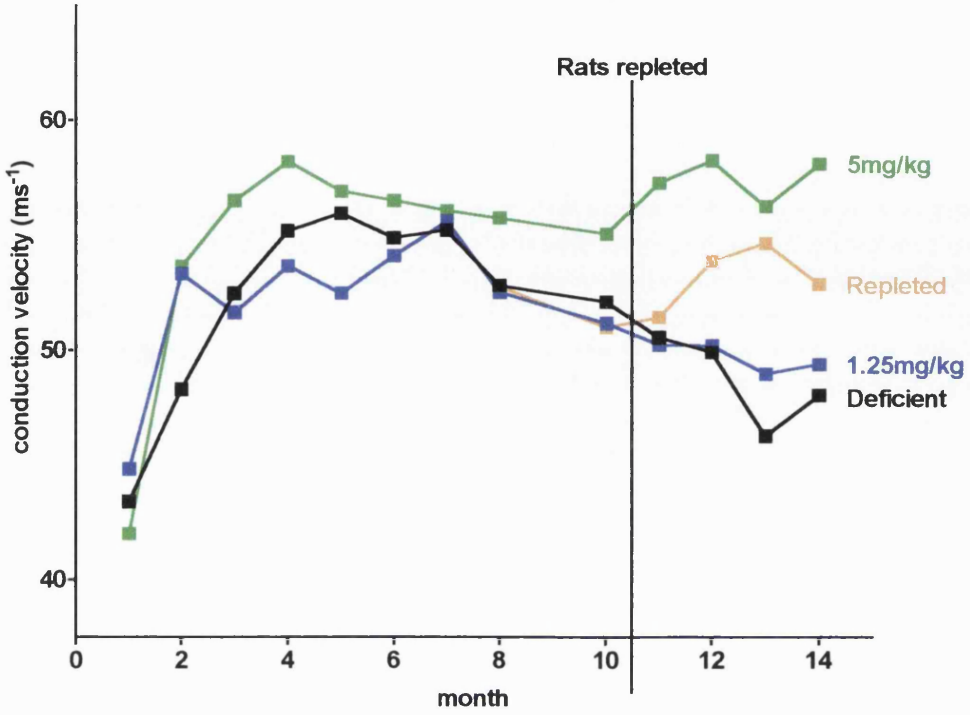


Figure 7.11 Group mean peripheral CV +/- 1SD - deficient v repleted (upper limb)

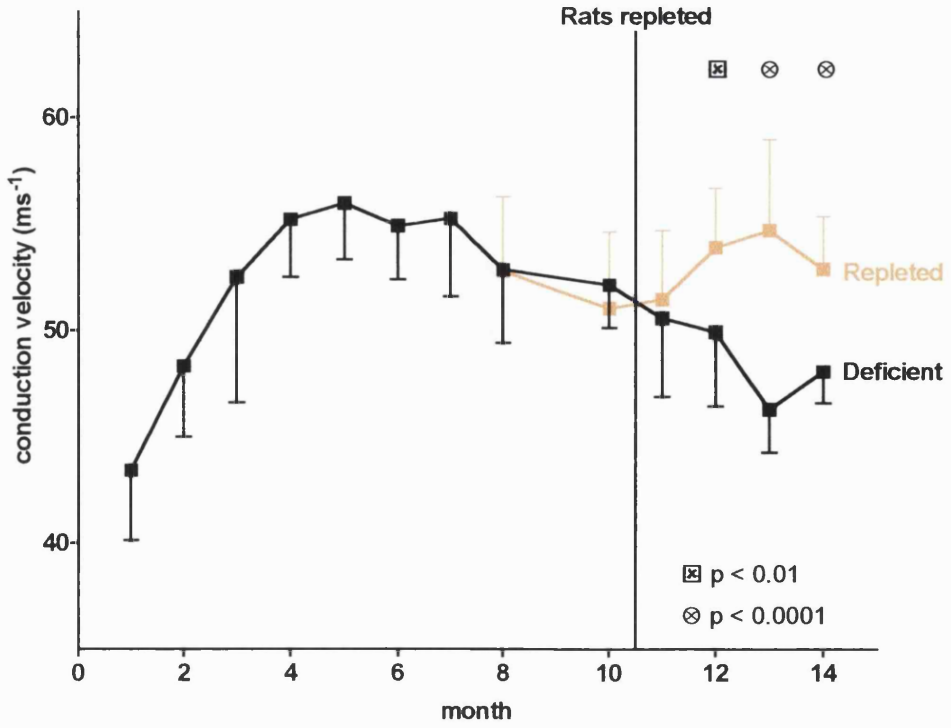


Figure 7.12 Group mean peripheral CV +/- 1SD - 1.25mg/kg v repleted (upper limb)

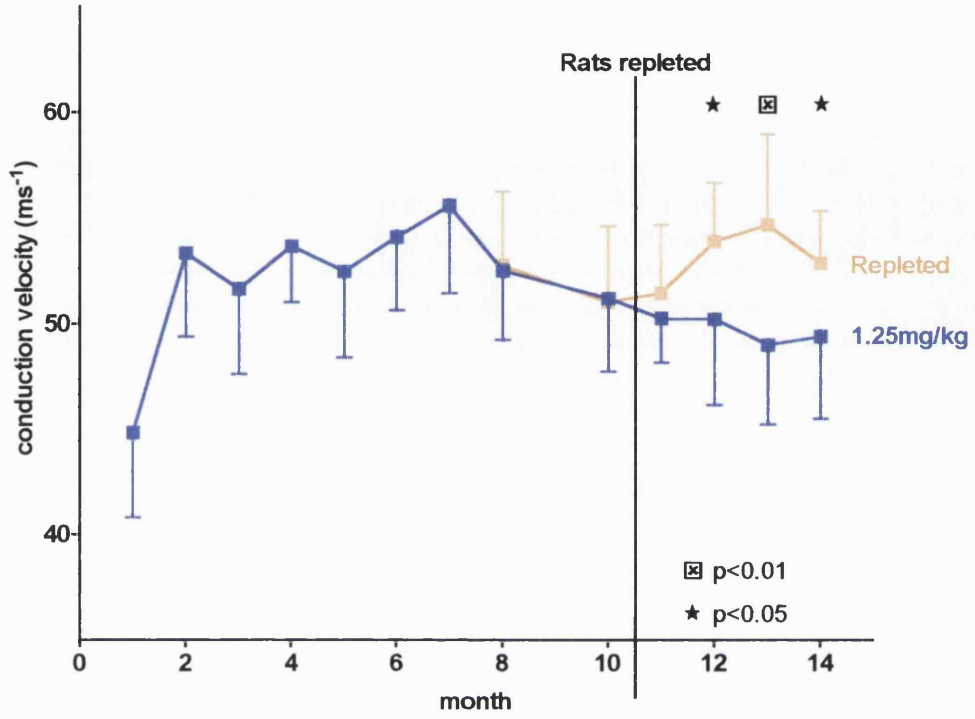


Figure 7.13 Group mean Peripheral CV +/- 1SD - 5mg/kg v repleted (upper limb)

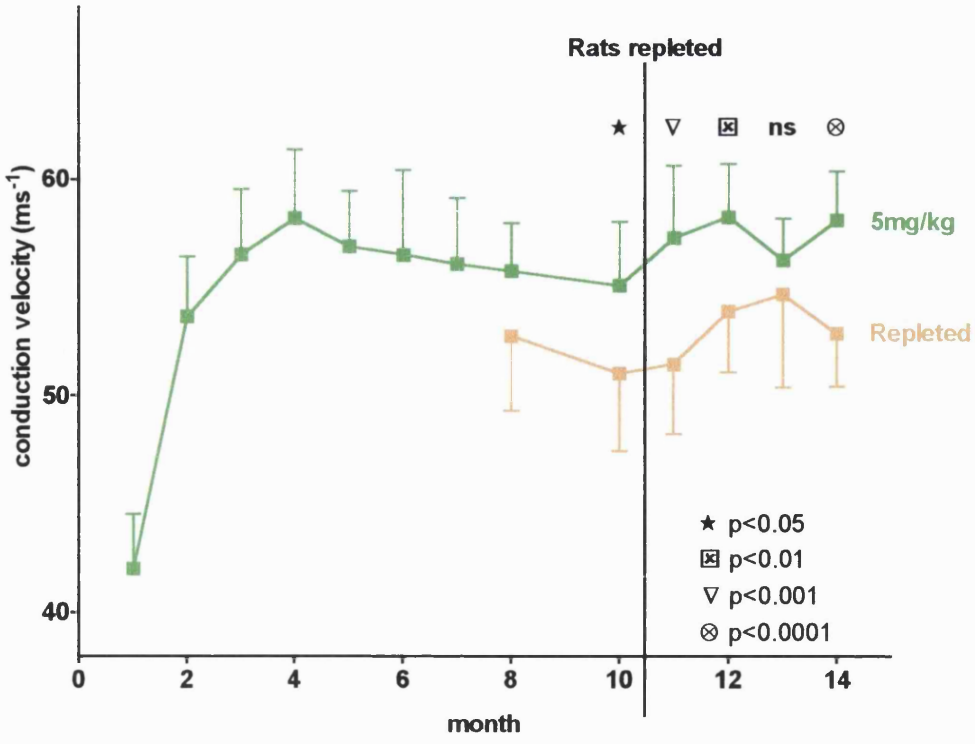


Figure 7.14 Group mean central conduction velocities (upper limb)

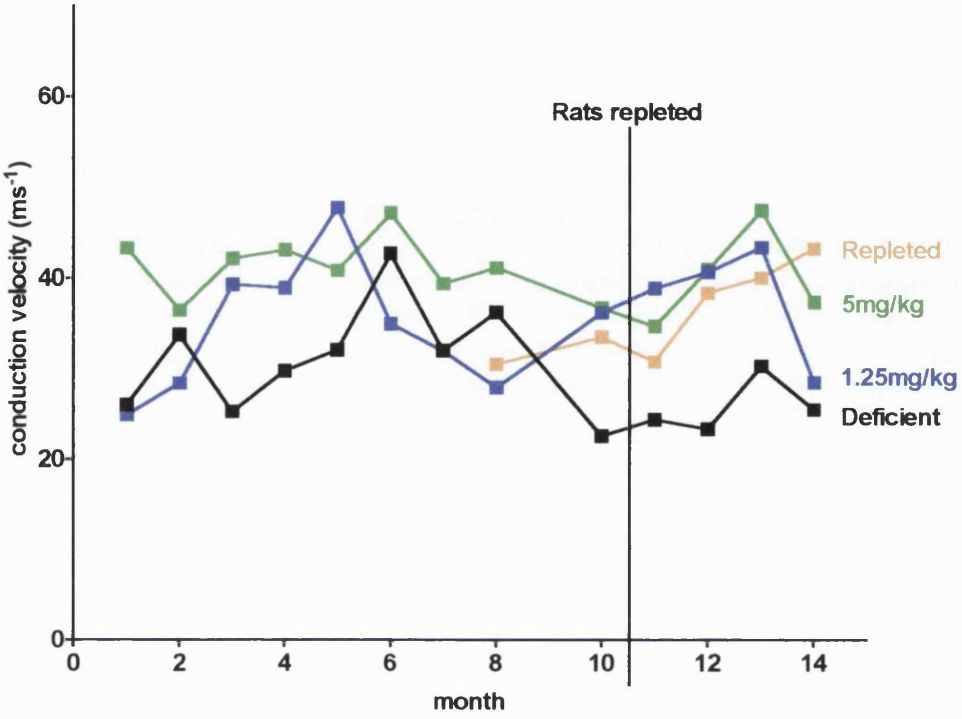
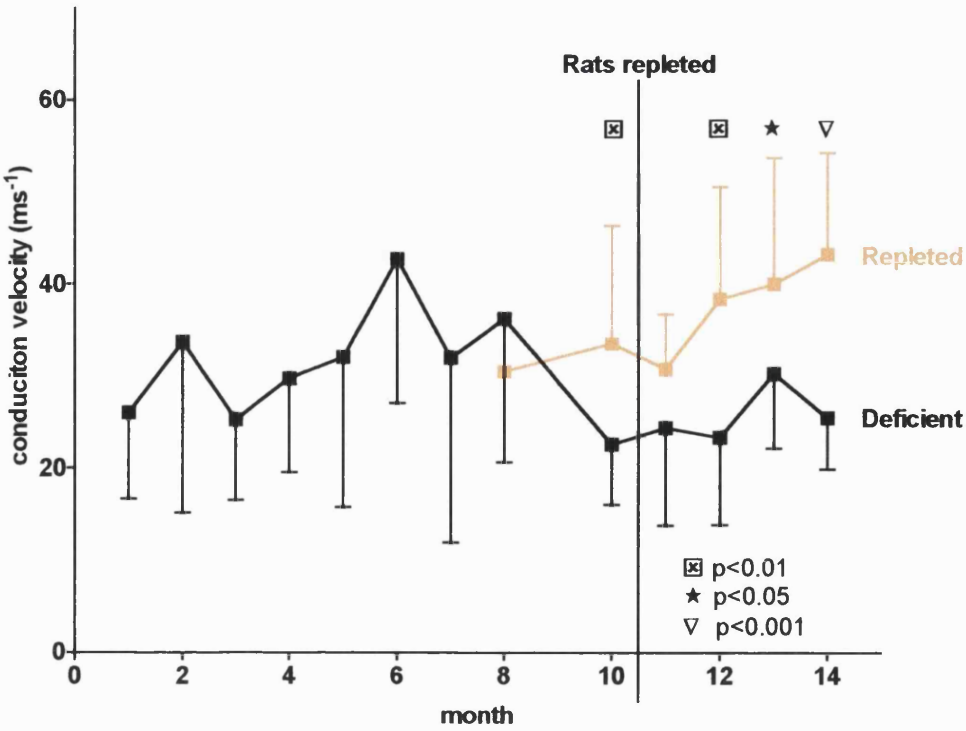


Figure 7.15 Group mean central CV +/- 1SD - deficient v repleted (upper limb)



SEP amplitudes

The group mean amplitudes of the SEP recorded at the level of the cervical spine (C3) following stimulation of the upper limb are presented in Figure 7.16. All the groups gave very similar results until month 10, after which the amplitudes of the deficient group decreased whereas the 1.25mg/kg, 5mg/kg and repleted groups maintained a reletively constant amplitude. The repleted group SEP amplitudes recorded at C3 were not consistently significantly different from the 1.25mg/kg and 5mg/kg groups, but were significantly larger than those of deficient animals after 13 months (15.75 +/- 3.15 compared with 10.38 +/- 3.03μV respectively, $P<0.001$) as shown in Figure 7.17.

Figure 7.18 shows the group mean amplitudes of the cortical SEP, recorded in response to median nerve (upper limb) stimulation, for the deficient, repleted, 1.25mg/kg and 5mg/kg groups. All four groups gave broadly similar results for the first 10 months of the study, then the amplitudes of the deficient group tended to decrease, while the other groups remained relatively constant. After 12 months the repleted group had a consistently significantly larger cortical SEP amplitude than the deficient group (Figure 7.19). No significant differences were found when the repleted group was compared with the 1.25mg/kg or 5mg/kg groups.

Figure 7.16 Group mean C3 peak-to-peak amplitudes (upper limb)

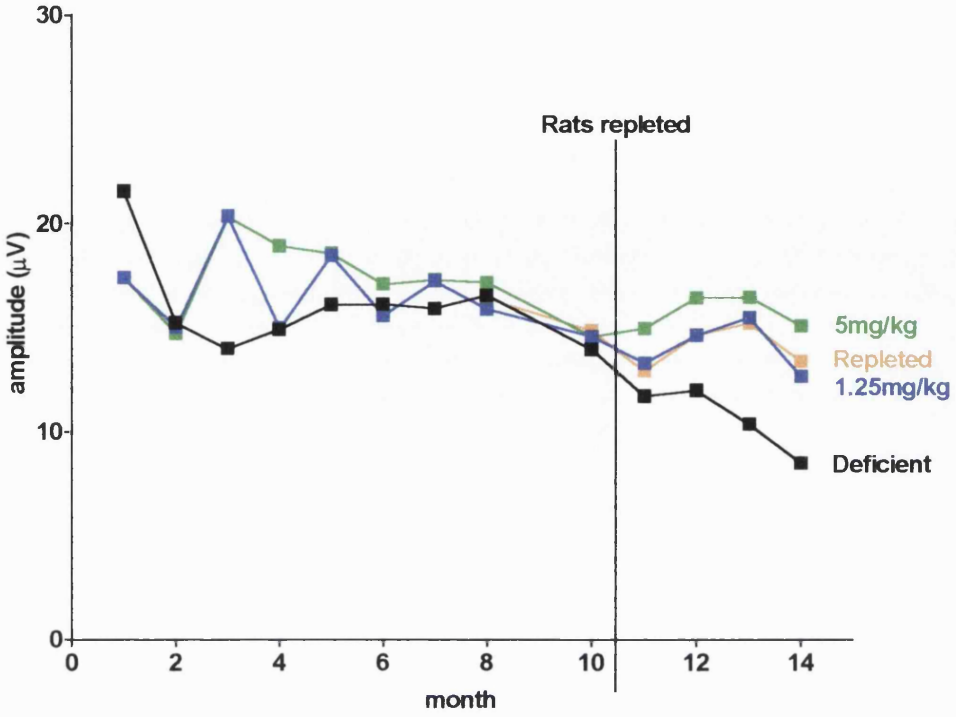


Figure 7.17 Group mean C3 SEP amplitudes +/- 1SD - deficient v repleted (upper limb)

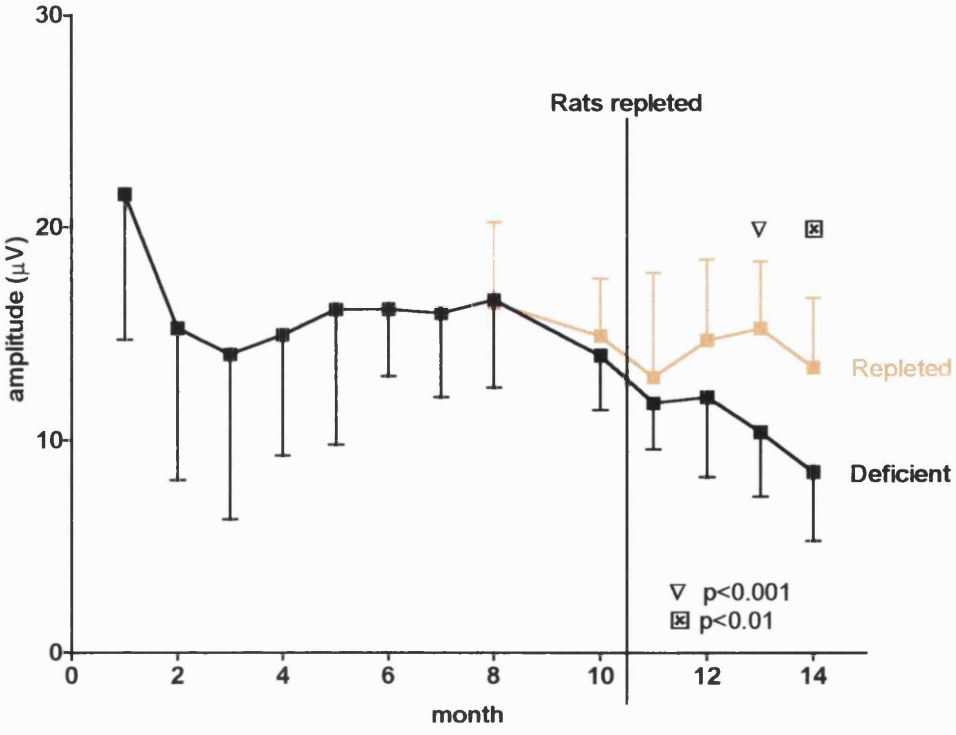


Figure 7.18 Group mean cortical SEP peak-to-peak amplitudes (upper limb)

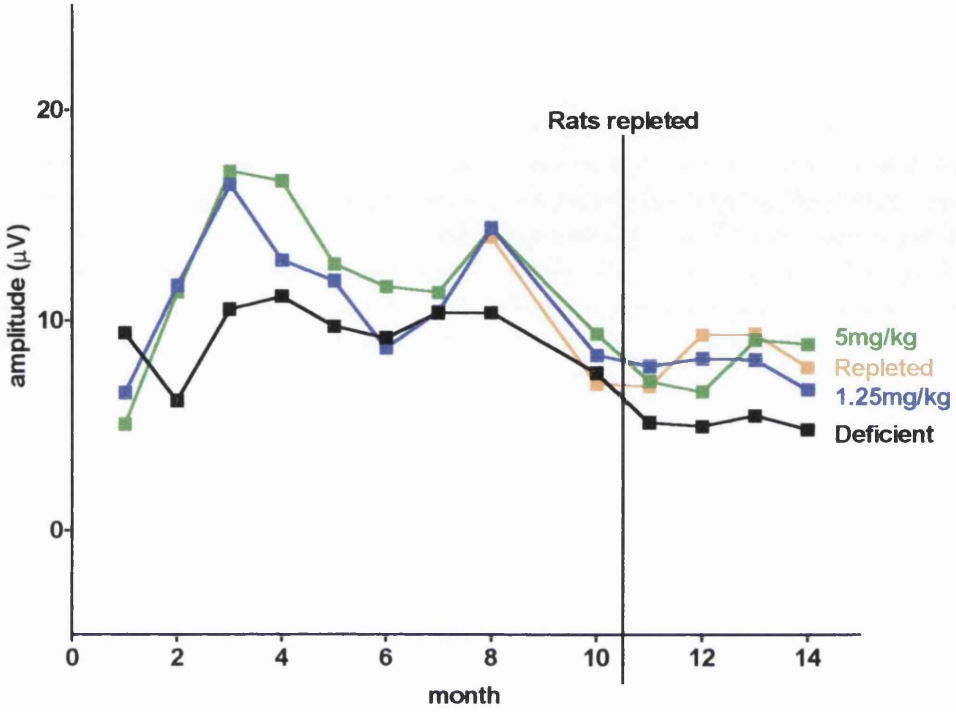
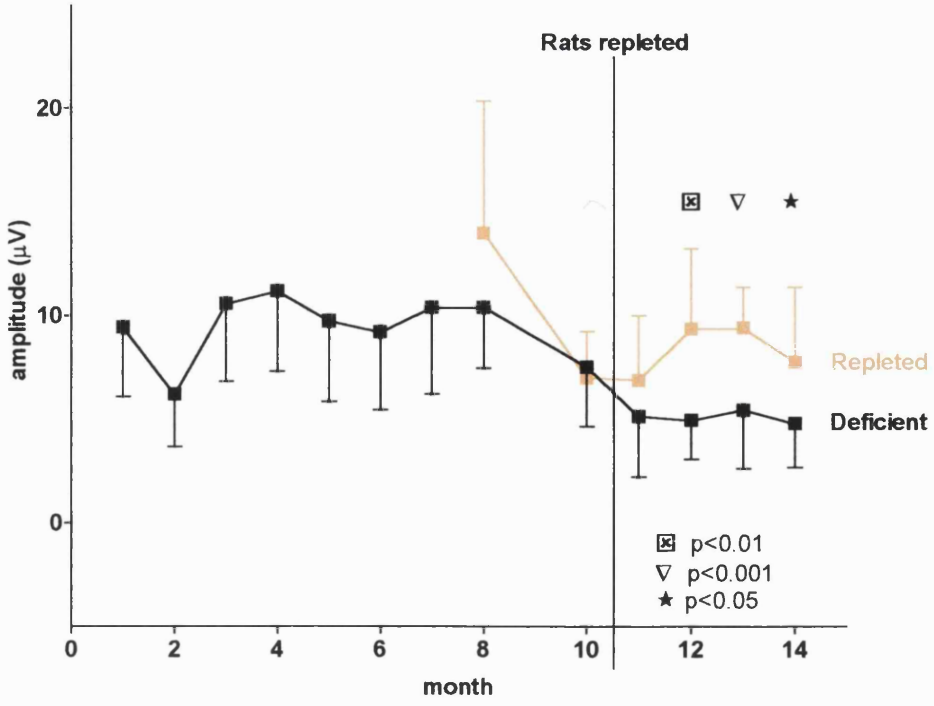


Figure 7.19 Group mean cortical SEP amplitudes +/- 1SD - deficient v repleted (upper limb)



7.4.3 Visual function: dim (blue-filtered) flash stimuli

ERG latencies

The mean ERG b wave latencies, recorded in response to dim (blue-filtered) flash stimuli, for the deficient, 1.25mg/kg, 5mg/kg and repleted groups are shown in Figure 7.20. The deficient group had a consistently significantly longer latency than the repleted group after month 12 (80.50 +/- 14.79 compared with 63.69 +/- 6.95ms respectively, $P<0.05$) as shown in Figure 7.21, whereas no significant differences were found when the 1.25mg/kg group was compared with the repleted group. There were no consistently significant differences between the repleted and 5mg/kg groups.

ERG amplitudes

Figure 7.22 shows the group mean ERG peak-to-peak amplitudes after dim flash stimuli for the deficient, 1.25mg/kg, 5mg/kg and repleted groups. No statistically significant differences were found between the four groups at any time point during the course of the study.

Figure 7.20 Group mean ERG b wave latencies (dim blue flash)

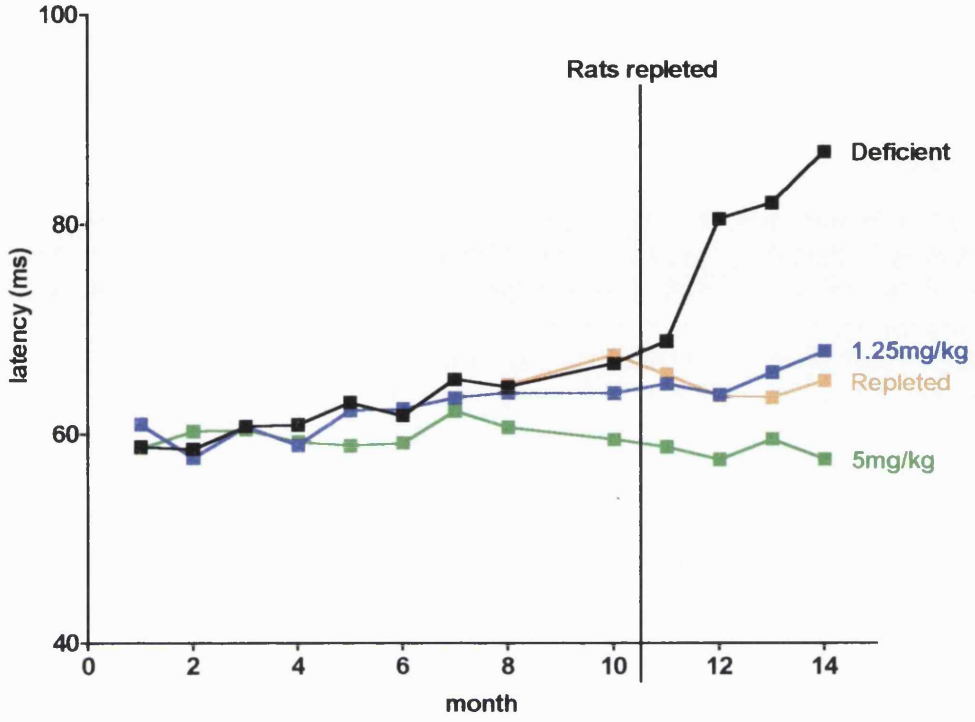


Figure 7.21 Group mean ERG b wave latencies +/- 1SD
- deficient v repleted (dim blue flash)

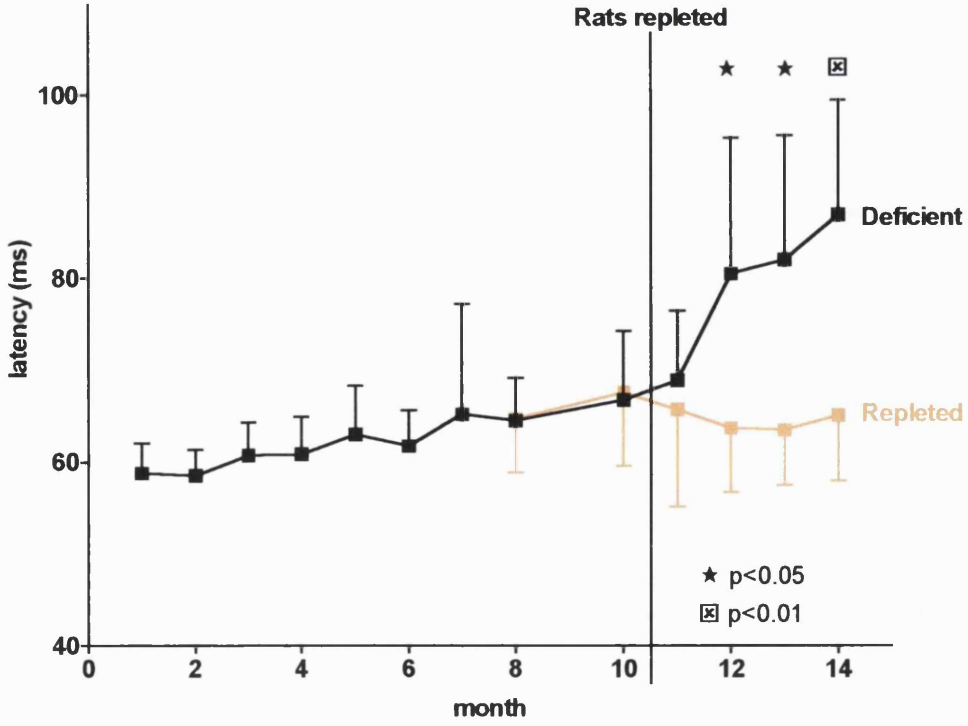
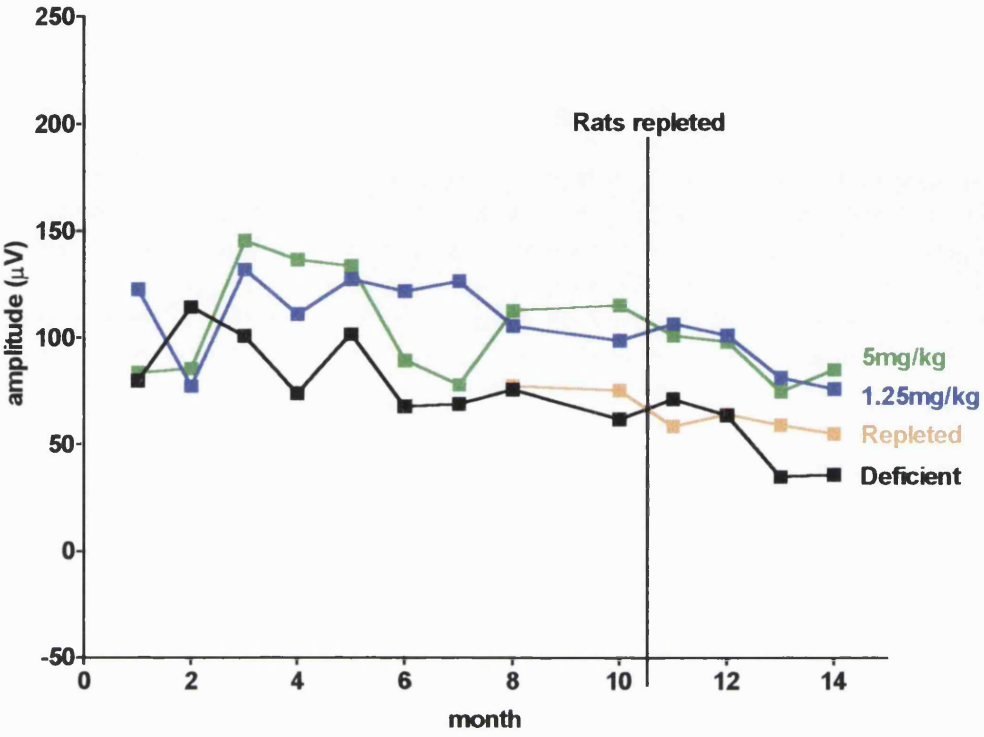


Figure 7.22 Group mean ERG peak-to-peak amplitudes (dim blue flash)



VEP latencies

Figure 7.23 shows the group mean VEP onset latencies after dim flash stimuli for the deficient, 1.25mg/kg, 5mg/kg and repleted groups. The repleted group had a significantly longer VEP latency (32.43 +/- 2.69ms) than the 1.25mg/kg (29.10 +/- 1.39ms, $P<0.01$) and 5mg/kg (29.97 +/- 2.81ms, $P<0.05$) at month 10, but there were no consistently significant differences between these groups after repletion. The deficient rats had consistently significantly longer onset latencies than the repleted group from month 12 (34.58 +/- 3.28 compared with 30.75 +/- 3.61ms respectively, $P<0.05$) as shown in Figure 7.24.

VEP amplitudes

No significant differences were found when the VEP peak-to-peak amplitudes of the repleted group were compared with those of the deficient, 1.25mg/kg or 5mg/kg groups (Figure 7.25).

Figure 7.23 Group mean VEP onset latencies (dim blue flash)

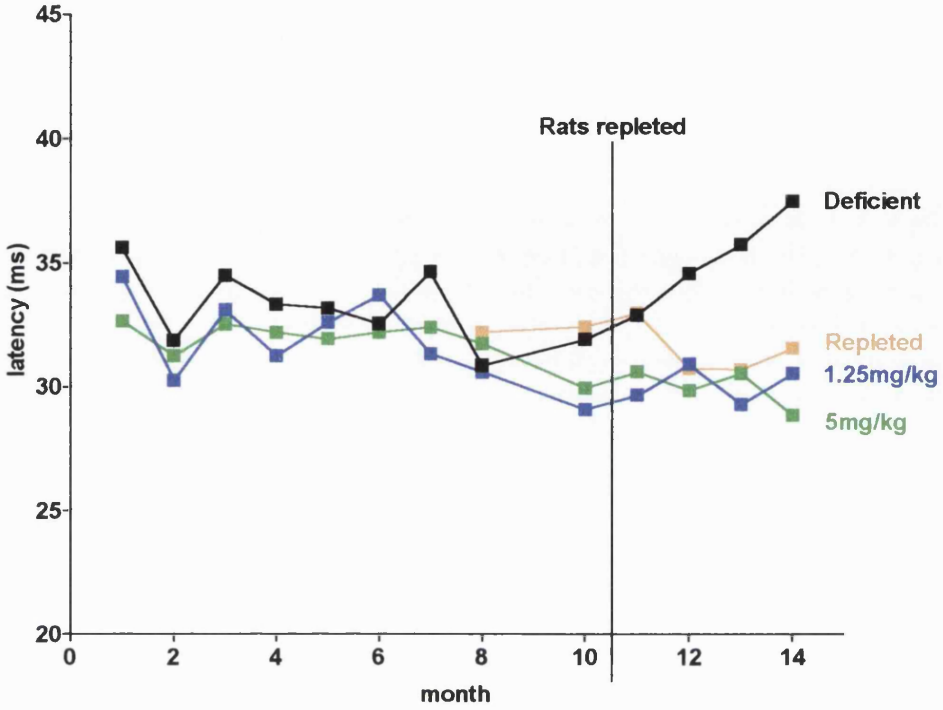


Figure 7.24 Group mean VEP onset latencies +/- 1SD
- deficient v repleted (dim blue flash)

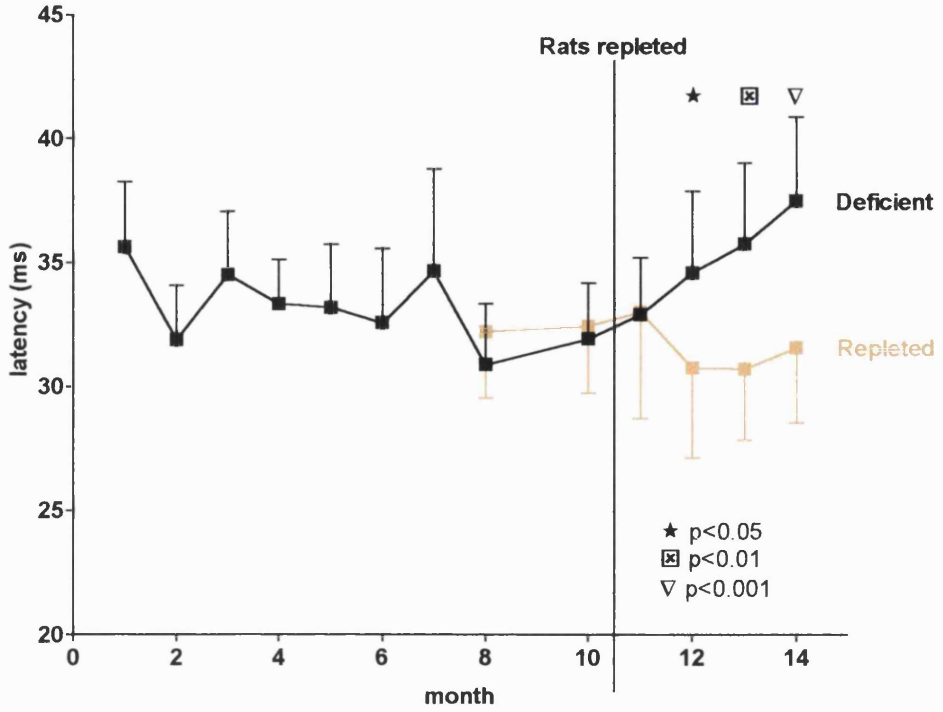
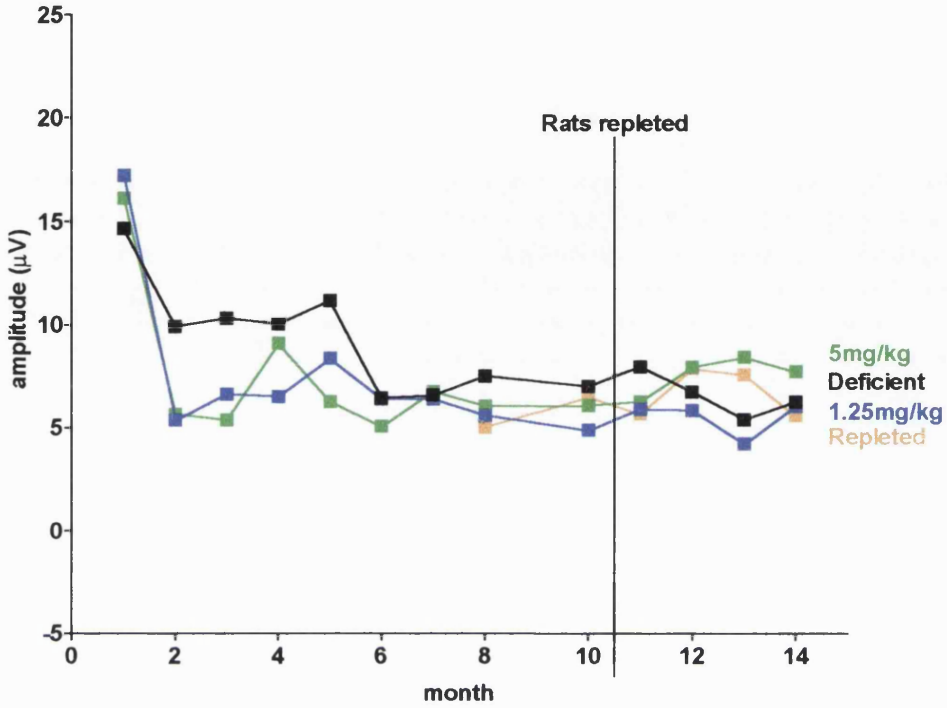


Figure 7.25 Group mean VEP peak-to-peak amplitudes (dim blue flash)



7.4.4 Visual function: Bright white flash stimuli

ERG latencies

Figure 7.26 shows the group mean ERG b wave latencies, recorded in response to bright white flash stimuli. The ERG latencies of the repleted group increased until month 11, but then remained relatively constant for the rest of the study. There were no significant differences at any time point, when the repleted group was compared with either the 1.25mg/kg or deficient animals. The repleted group was, however, consistently significantly different from the 5mg/kg group after 10 months as shown in Figure 7.27.

ERG amplitudes

The group mean peak-to-peak amplitudes of the ERG are presented in Figure 7.28. The ERG amplitudes of the repleted group remained relatively constant from 10 months. When the repleted group ERG amplitudes were compared with the other groups, no consistently significant differences were found.

Figure 7.26 Group mean ERG b wave latencies (bright white flash)

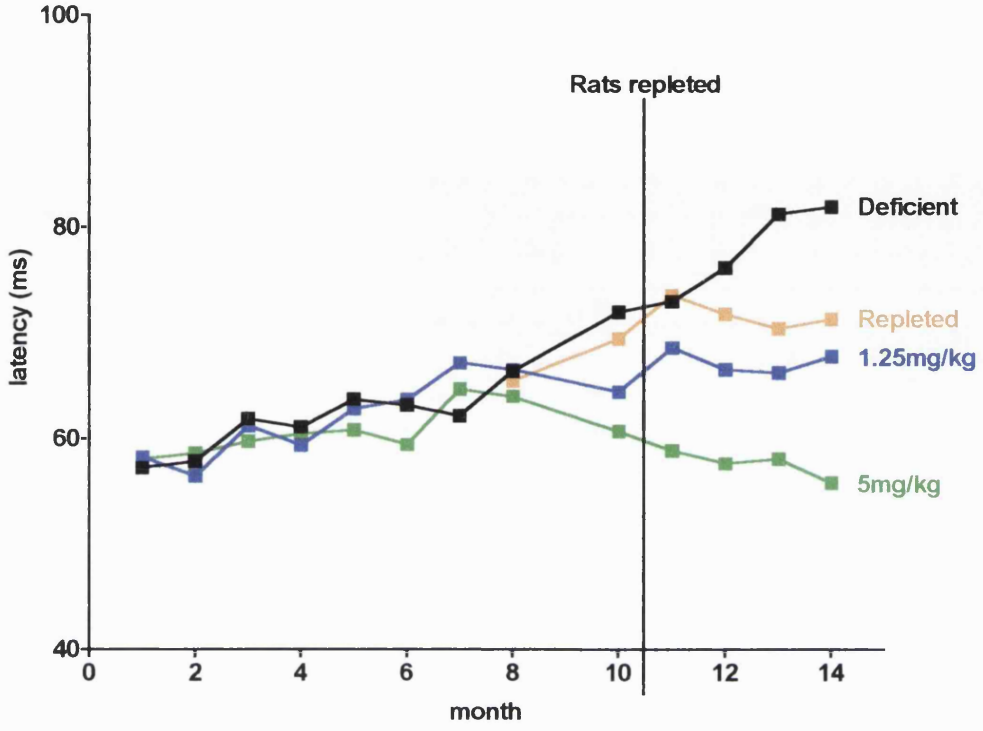


Figure 7.27 Group mean ERG b wave latencies +/- 1SD
- 5mg/kg v repleted (bright white flash)

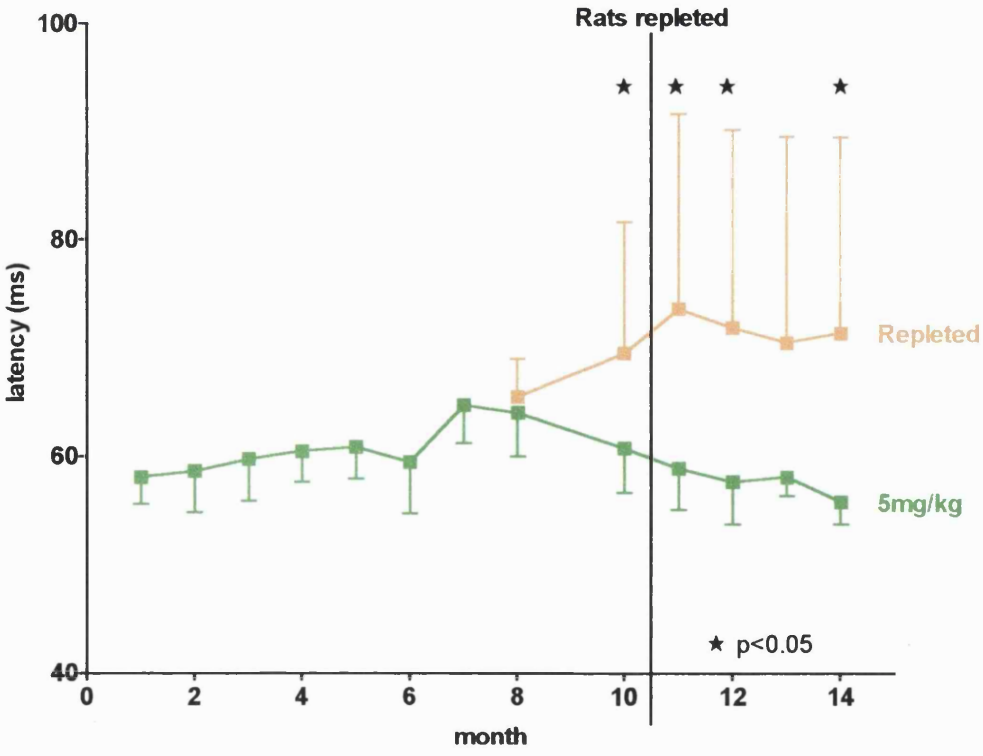
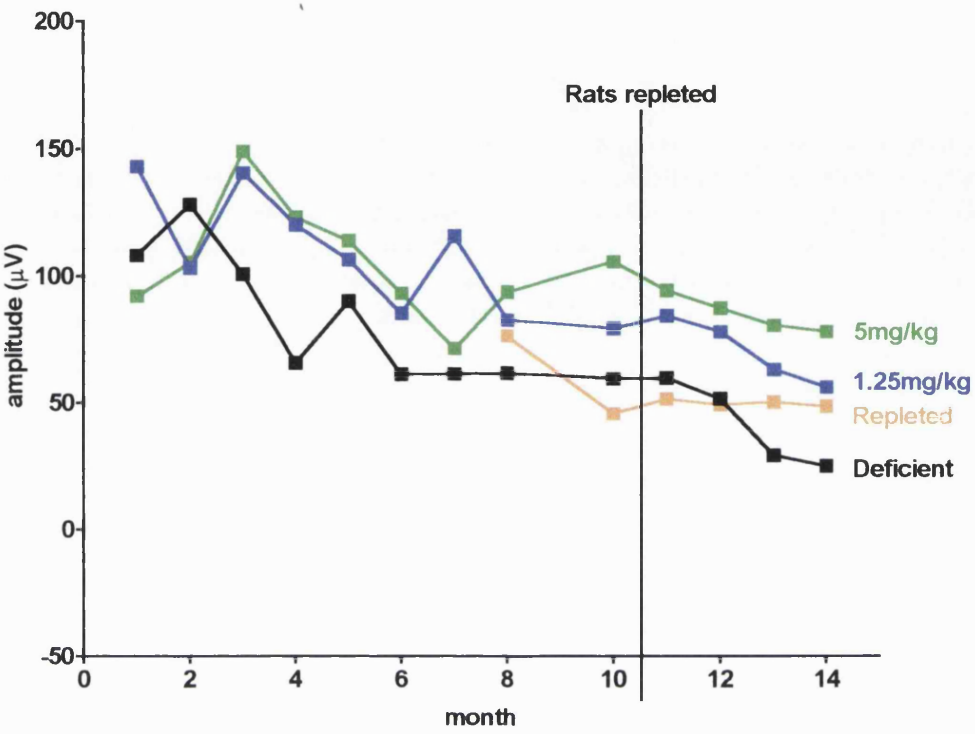


Figure 7.28 Group mean ERG peak-to-peak amplitudes (bright white flash)



VEP latencies

The group mean VEP onset latencies, recorded after white light flash stimuli, are shown in Figure 7.29. For all the groups, the latencies were similar and relatively constant until month 10, when those of the deficient and repleted groups tended to increase. After month 10, the repleted group remained relatively constant, whereas the latencies of the deficient group continued to increase, becoming significantly longer ($P<0.05$) than the repleted group at month 13 (33.63 +/- 3.12 compared with 30.08 +/- 3.25ms respectively, Figure 7.30). The VEP onset latencies of the 1.25mg/kg and 5mg/kg groups remained constant for the duration of the study, and no consistently significant differences were observed when these two groups were compared with the repleted group.

VEP amplitudes

The mean VEP peak-to-peak amplitudes of the various groups are shown in Figure 7.31. There were no consistently significant differences between the groups at any time point.

Figure 7.29 Group mean VEP onset latencies (bright white flash)

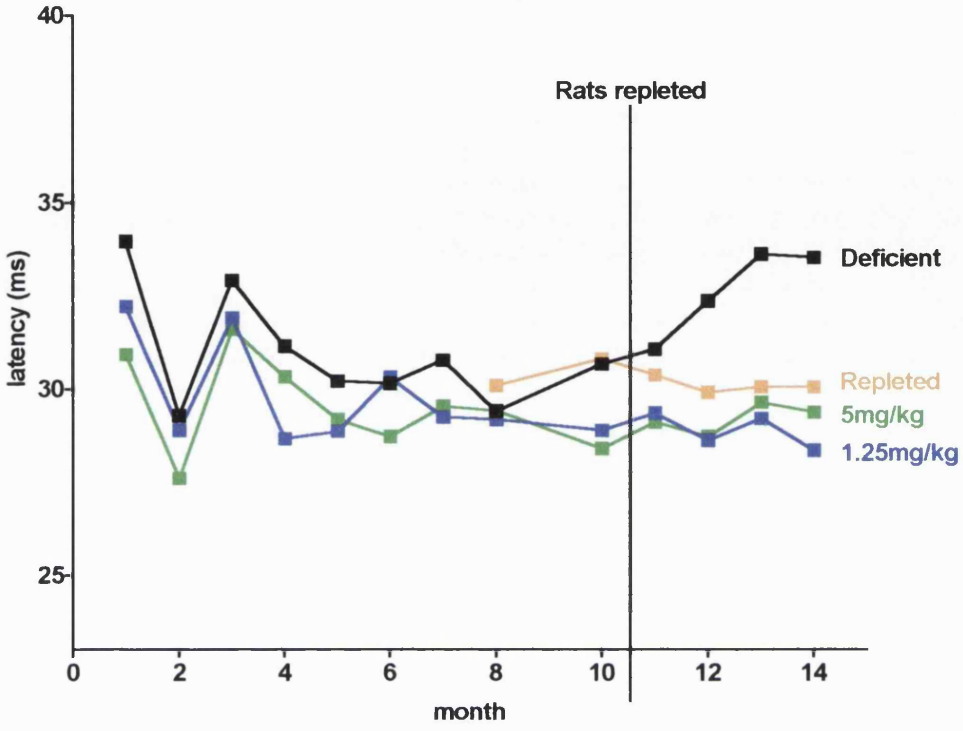


Figure 7.30 Group mean VEP onset latencies +/- 1SD
- deficient v repleted (bright white flash)

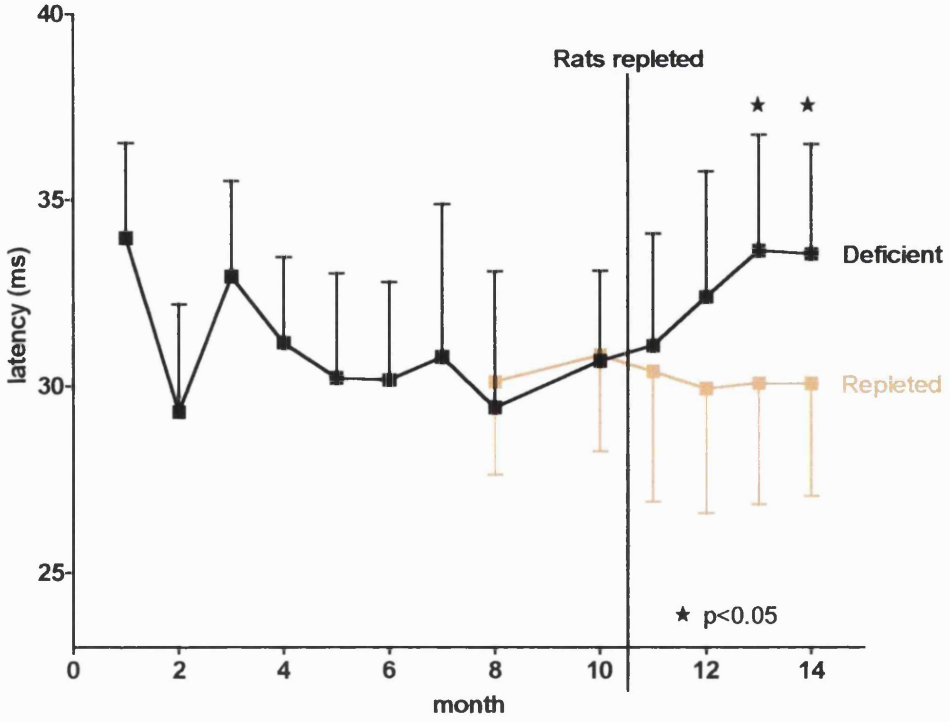
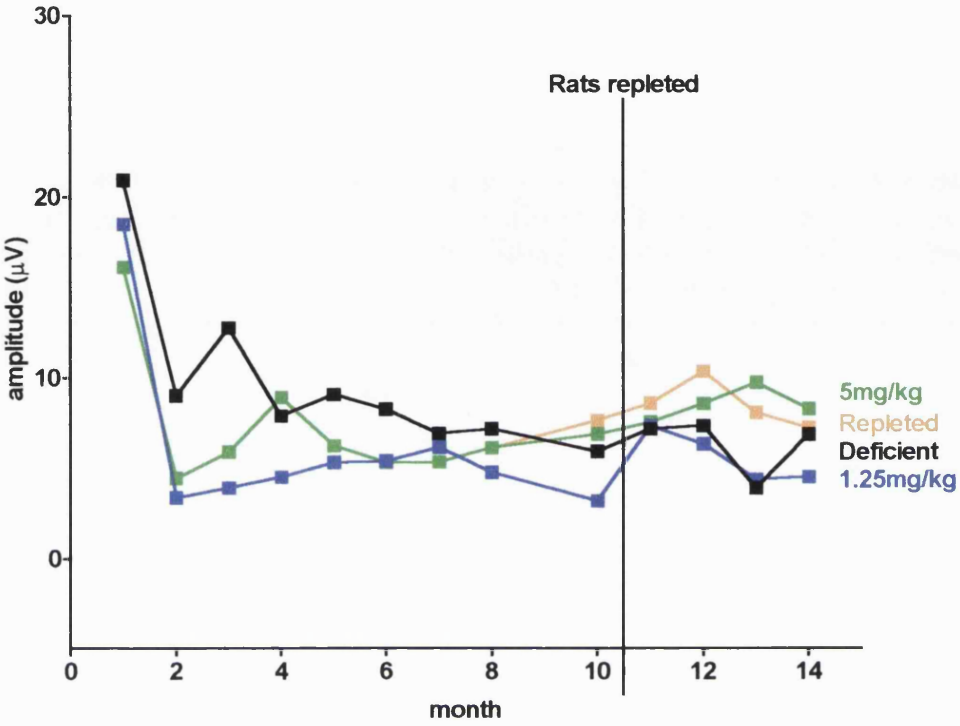


Figure 7.31 Group mean VEP peak-to-peak amplitudes



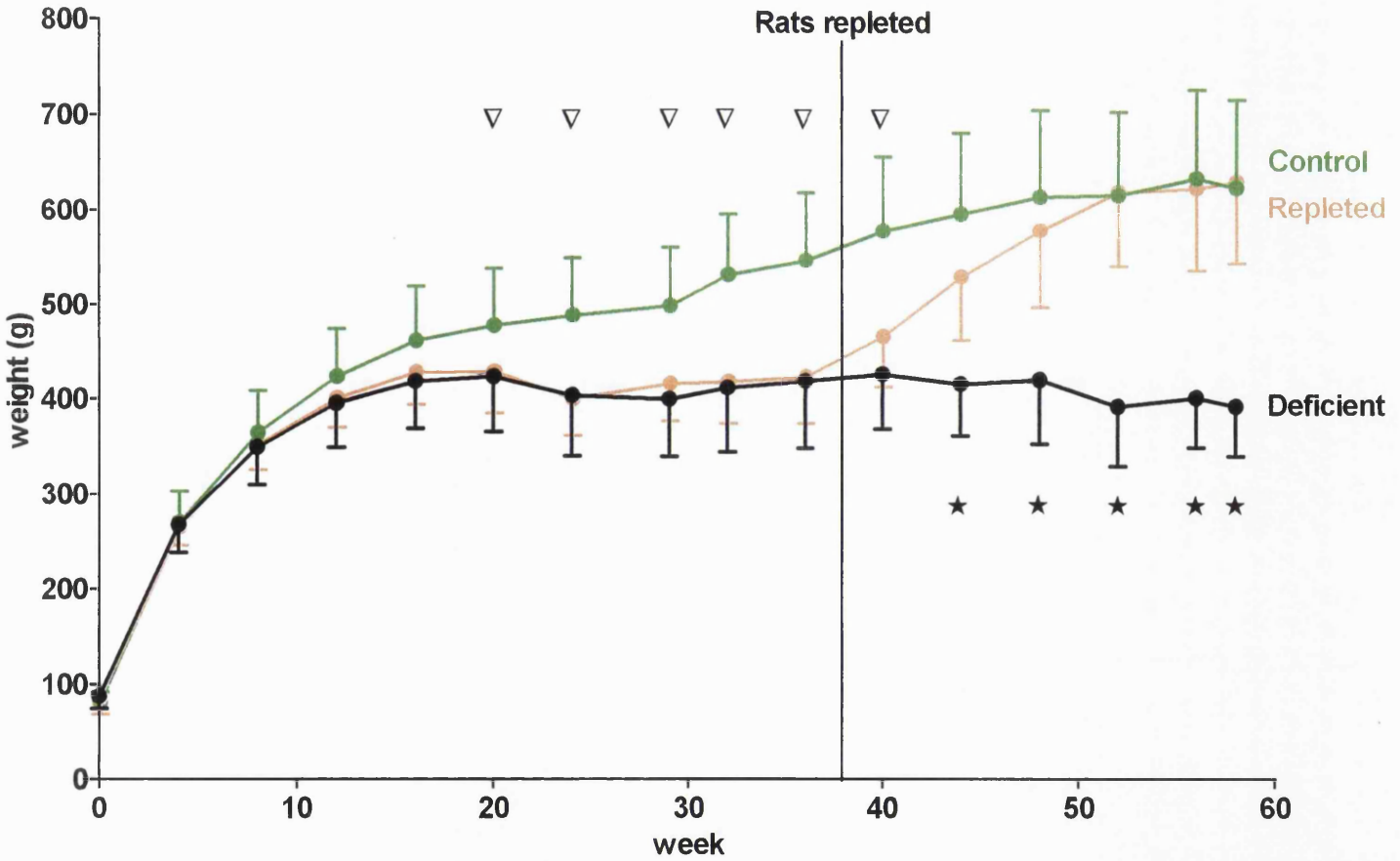
STUDY II

7.5 Growth and condition of rats

The rats were weighed weekly, and the mean weights (n=15) of the deficient, repleted and 5mg/kg (RRR-α-tocopheryl acetate) groups plotted at monthly intervals are shown in Figure 7.32. For the first 16 weeks, the animals in all the groups gained weight rapidly. After this time, the rate of gain slowed in the 5mg/kg group while there was no further increase in mean weight in the deficient group. The repleted group rats also stopped gaining weight after week 16, and were significantly lighter than the 5mg/kg group after 20 weeks (429 +/- 43.8 compared with 478 +/- 61.0g respectively). The repleted group was switched from the deficient to the 36mg/kg all-rac-α-tocopheryl acetate diet at week 38. After repletion, the animals started to regain weight and became significantly heavier than the deficient group after 44 weeks (529 compared with 415g respectively). By week 52 of the study, the group mean weight of the repleted group was similar to that of the 5mg/kg group (619 +/- 78.3 compared with 616 +/- 87.6g respectively).

After 16 weeks on the tocopherol deficient diet, the general condition of rats in both the deficient and repleted groups began to deteriorate. Their coats became greasy and the fur matted. This was accompanied by an increase in porphyrin secretion, which gave the fur a pink tinge. The first symptoms of neurological dysfunction became

Figure 7.32 Study II Growth curves



▽ Repleted group significantly ($P<0.05$) different from 5mg/kg group
★ Repleted group significantly ($P<0.05$) different from deficient group

apparent after 43 weeks, when 3 of the repleted group presented with an abnormal gait and slightly impaired balance. These features continued until the end of the study (week 59), but did not appear to worsen. This is in contrast to the deficient group, in which four rats showed signs of neurological problems at week 43 and by the end of the study, the 10 remaining animals of the group had severely abnormal gait, balance and muscle wastage.

7.6 Electrophysiology results

7.6.1 Lower limb SEPs

Peripheral conduction velocities

Figure 7.33 shows the group mean peripheral CV for the deficient, 5mg/kg and repleted groups. There were no significant differences found between the groups at any point during the study.

Central conduction velocities

The group mean central CV from the deficient, 5mg/kg and repleted groups are shown in Figure 7.34. The deficient and repleted groups had similar central CVs before repletion of the latter group, after which the CV increased in the repleted group but decreased in the deficient. When compared with the deficient group, the repleted group had a consistently significantly greater central CV after 11 months of the study

Figure 7.33 Group mean peripheral conduction velocities (lower limb)

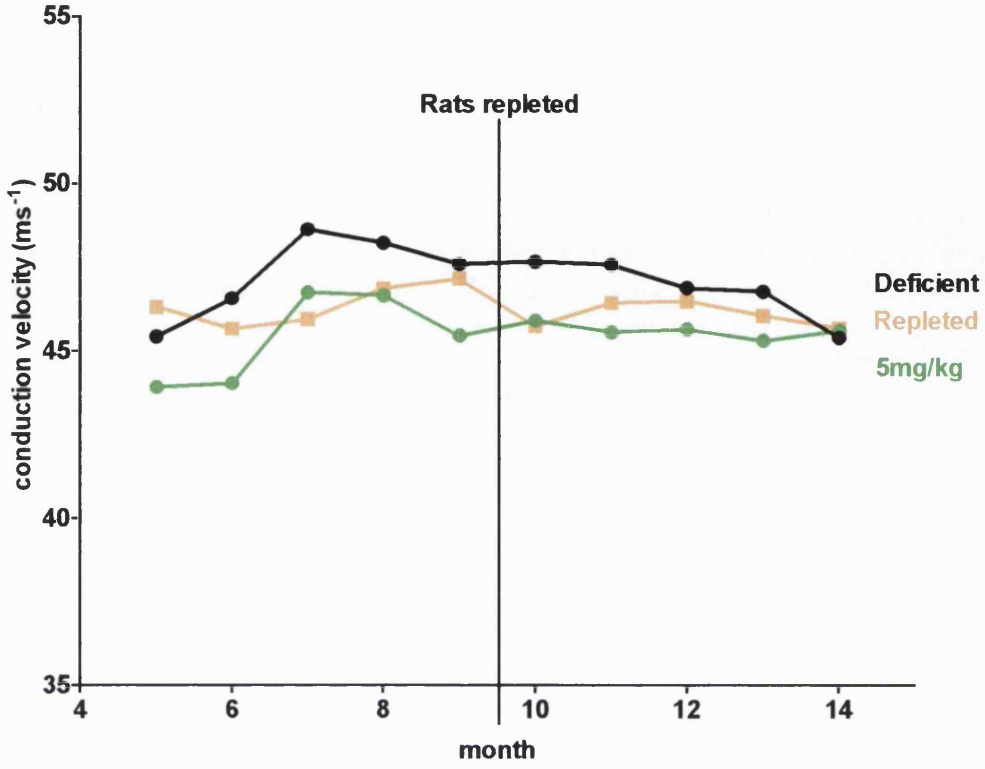


Figure 7.34 Group mean central conduction velocities (lower limb)

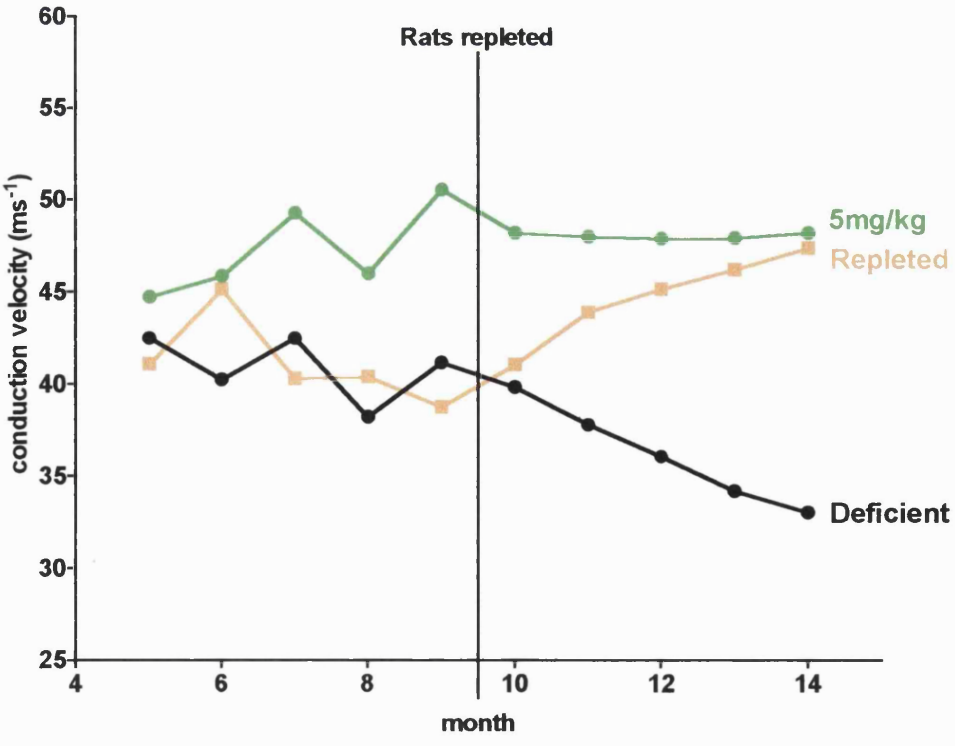


Figure 7.35 Group mean central CV +/- 1SD - deficient v repleted (lower limb)

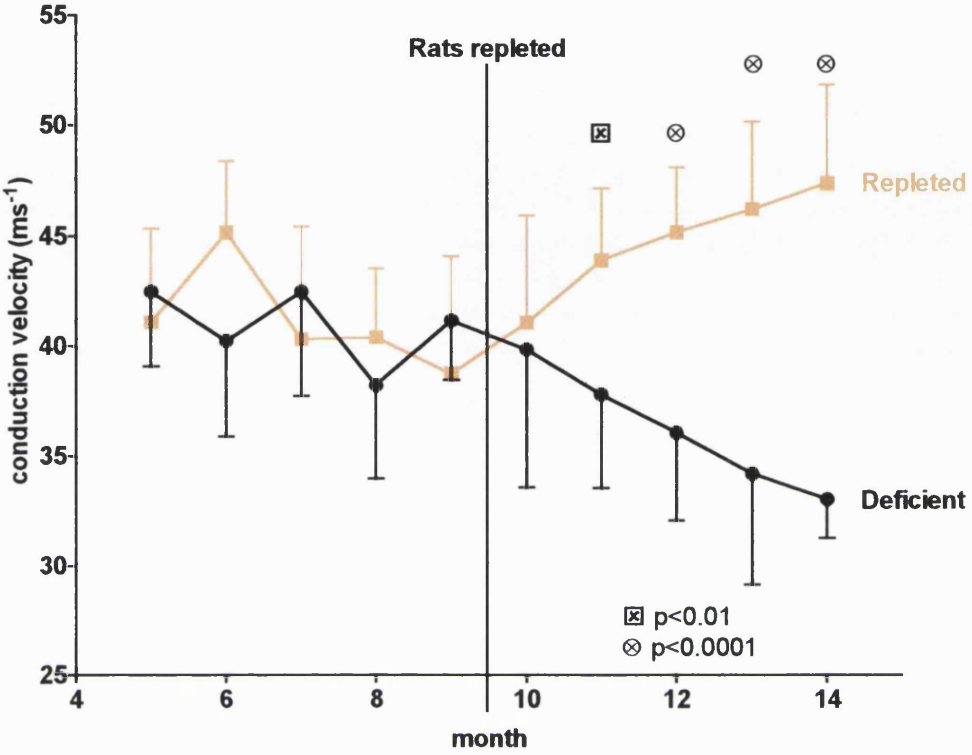
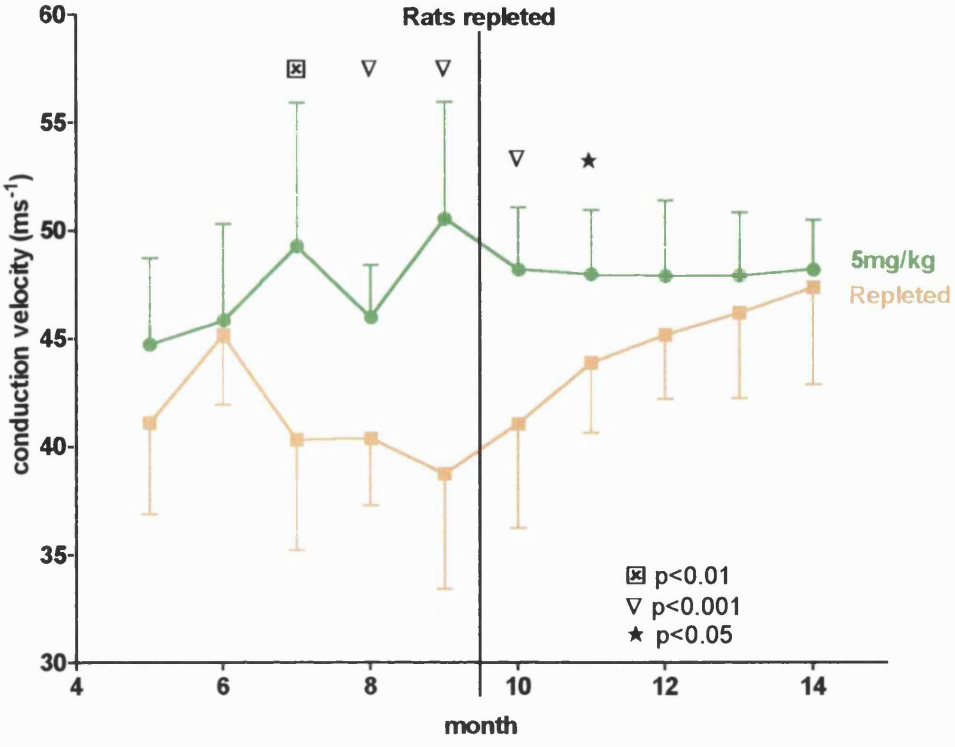


Figure 7.36 Group mean central CV +/- 1SD - 5mg/kg v repleted (lower limb)



(Figure 7.35). Rats fed a diet containing 5mg/kg RRR-α-tocopheryl acetate were found to have a consistently significantly greater central CV than the repleted group from month 7 to 11 (Figure 7.36). After this point, there were no significant differences between the two groups.

SEP amplitudes

The group mean peak-to-peak amplitudes of the SEPs recorded at the level of the spinal cord (L5), in response to tibial nerve stimulation, are presented in Figure 7.37. The 5mg/kg group maintained a relatively constant amplitude throughout the study, whereas up to 8 months the amplitudes of the deficient and repletion groups tended to decrease. After 8 months, the deficient rats had relatively constant amplitudes for the remainder of the study, whereas those of the repleted group began to increase after repletion, becoming significantly larger than those of the deficient group after 13 months (5.71 +/- 1.93 compared with 4.09 +/- 0.99μV respectively, $P<0.05$) as shown in Figure 7.38. The repleted animals were found to have significantly lower SEP peak-to-peak amplitudes than those fed a diet containing 5mg/kg RRR-α-tocopheryl acetate at 8, 9 and 10 months ($P<0.05$, Figure 7.39), whereas from month 11, no significant differences were found between the repleted and 5mg/kg groups.

Figure 7.40 shows the group mean peak-to-peak amplitudes of SEPs recorded at the level of the somatosensory cortex following lower limb nerve stimulation. The cortical amplitude of the repleted group decreased from month 4 to 7, and then became stable until month 9. After the rats were changed from a tocopherol-deficient diet to one

containing 36mg/kg all-rac-α-tocopheryl acetate at week 38, an increase in SEP amplitudes was observed. They became significantly larger than those of the deficient animals after 14 months only (2.99 +/- 0.60 compared with 1.83 +/- 0.91μV, $P<0.05$). Cortical SEP peak-to-peak amplitudes recorded in the repleted group were significantly smaller than those recorded in the 5mg/kg group at 7, 9, 10 and 11 months, but no statistically significant differences were observed between the groups after this time point (Figure 7.41).

Figure 7.37 Group mean L5 SEP peak-to-peak amplitudes (lower limb)

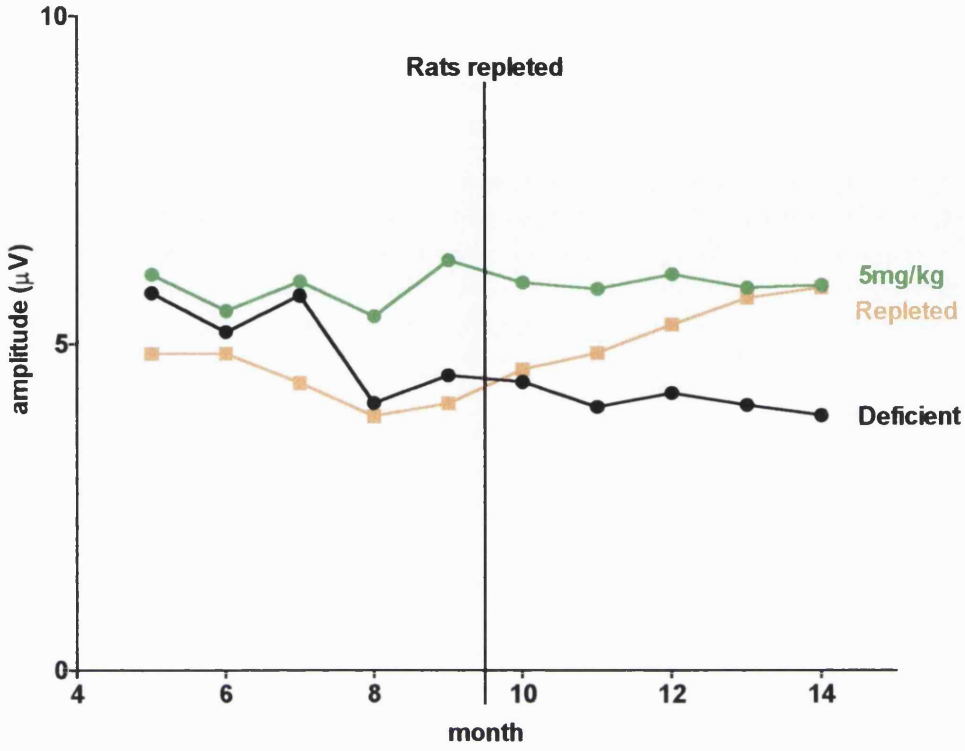


Figure 7.38 Group mean L5 SEP peak-to-peak amplitudes +/- 1SD - deficient v repleted (lower limb)

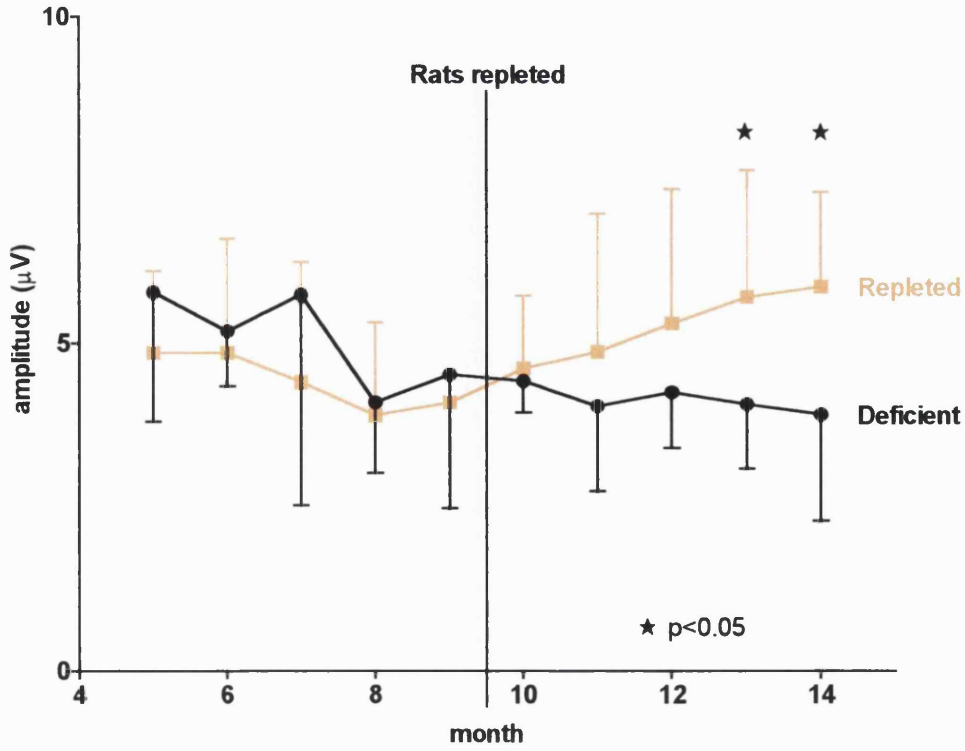


Figure 7.39 Group mean L5 SEP peak-to-peak amplitudes +/- 1SD - 5mg/kg v repleted (lower limb)

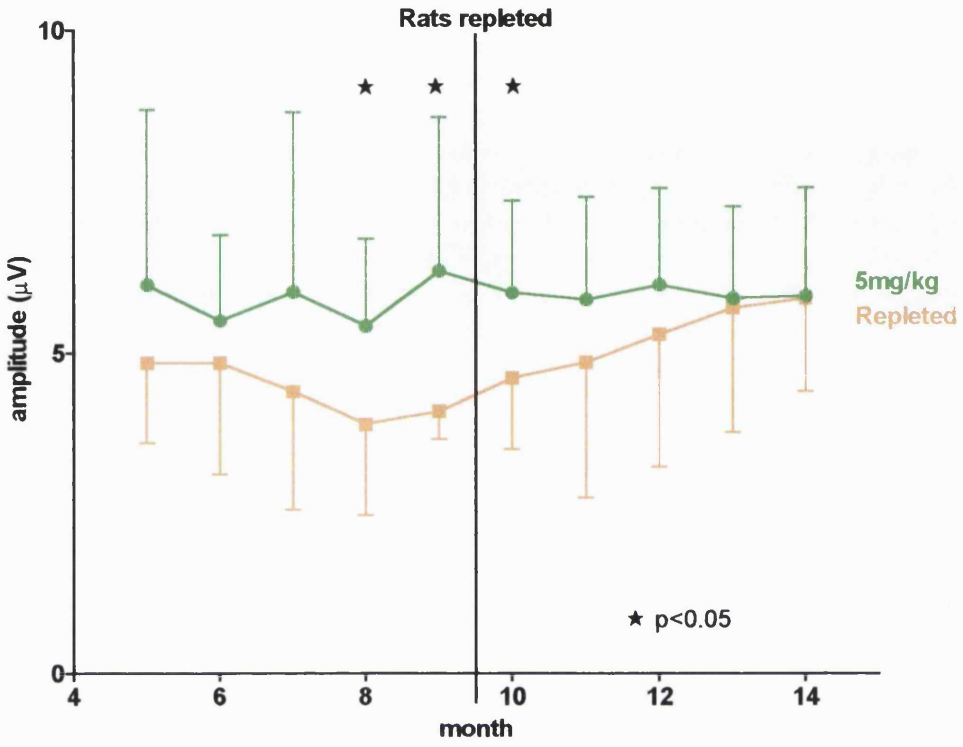


Figure 7.40 Group mean cortical SEP peak-to-peak amplitudes (lower limb)

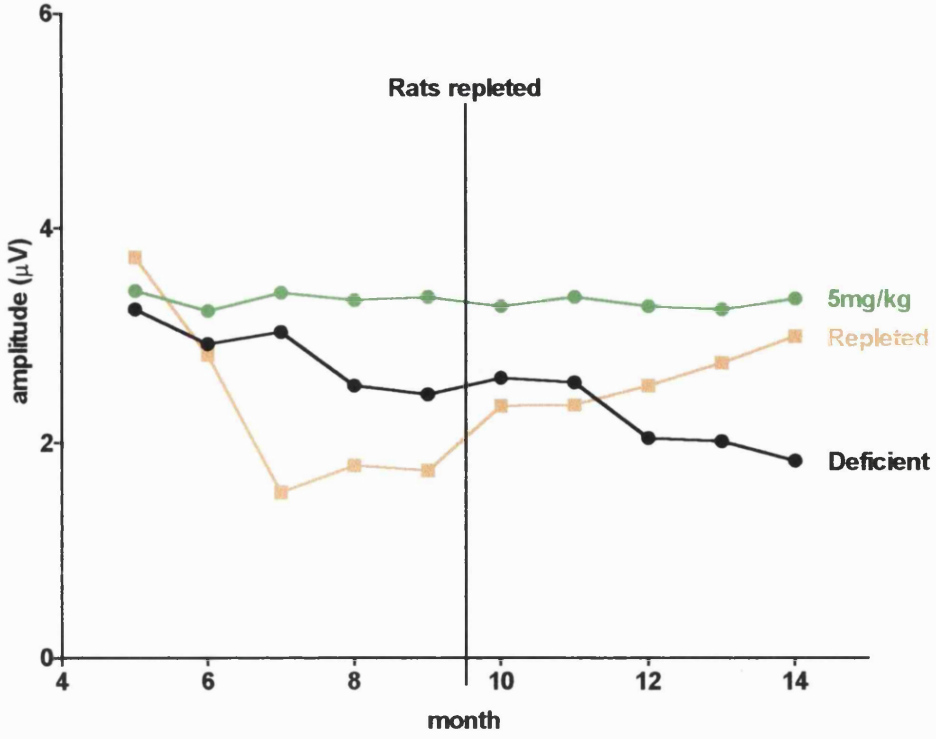
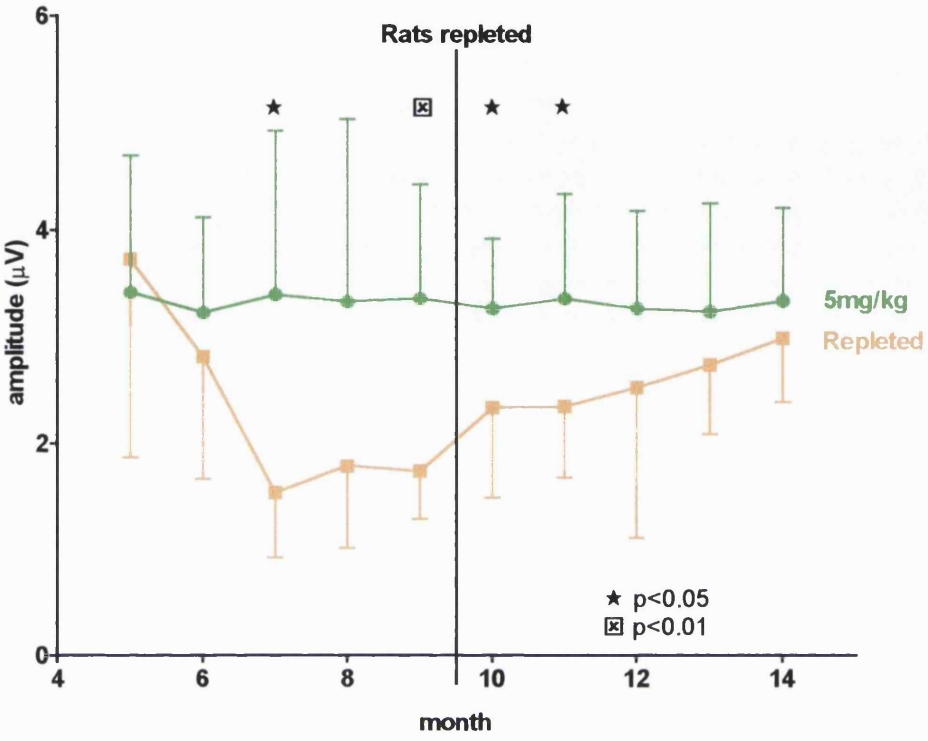


Figure 7.41 Group mean cortical SEP peak-to-peak amplitudes +/- 1SD - 5mg/kg v repleted (lower limb)



7.6.2 Visual function (bright white flash)

ERG latencies

The group mean ERG b wave latencies recorded from rats in the deficient, repleted and 5mg/kg groups following bright white flash stimulation are shown in Figure 7.42. The ERG latencies of the deficient animals became consistently significantly ($P<0.05$) longer than those of the repleted group after 12 months (Figure 7.43). The repleted group showed a significant increases in ERG b wave latencies compared with the 5mg/kg rats from 8-10 months after which there were no significant differences (Figure 7.44).

ERG amplitudes

Figure 7.45 shows the group mean ERG peak-to-peak amplitudes recorded in the deficient, repleted and 5mg/kg RRR-α-tocopheryl acetate groups. Prior to repletion, the deficient and repleted groups gave similar ERG amplitude results. After the repleted group were changed to a diet containing 36mg/kg all-rac-α-tocopheryl acetate (month 9), the ERG amplitudes increased while those of the deficient rats continued to decrease. The mean ERG peak-to-peak amplitudes of the repleted group became consistently significantly larger than those of the deficient group after 11 months (Figure 7.46). When compared to the 5mg/kg group, the ERG amplitudes of the repleted group were significantly decreased from 8-11 months (Figure 7.47), after which there were no significant differences in amplitudes between the two groups.

Figure 7.42 Group mean ERG b wave latencies (bright white flash)

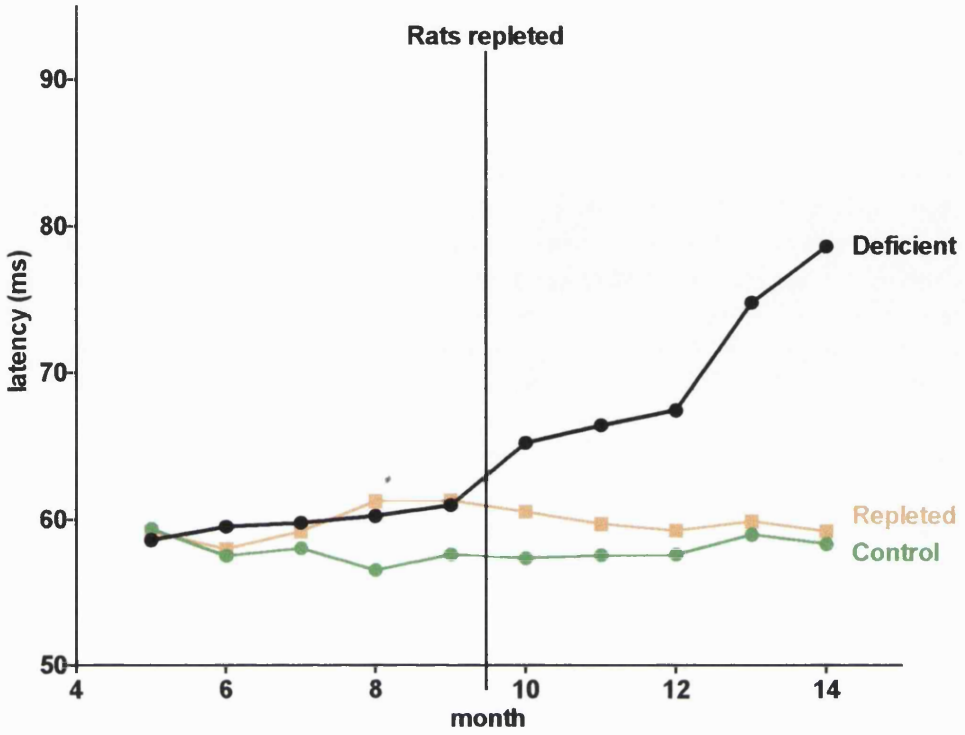


Figure 7.43 Group mean ERG latencies +/- 1SD - deficient v repleted (bright white flash)

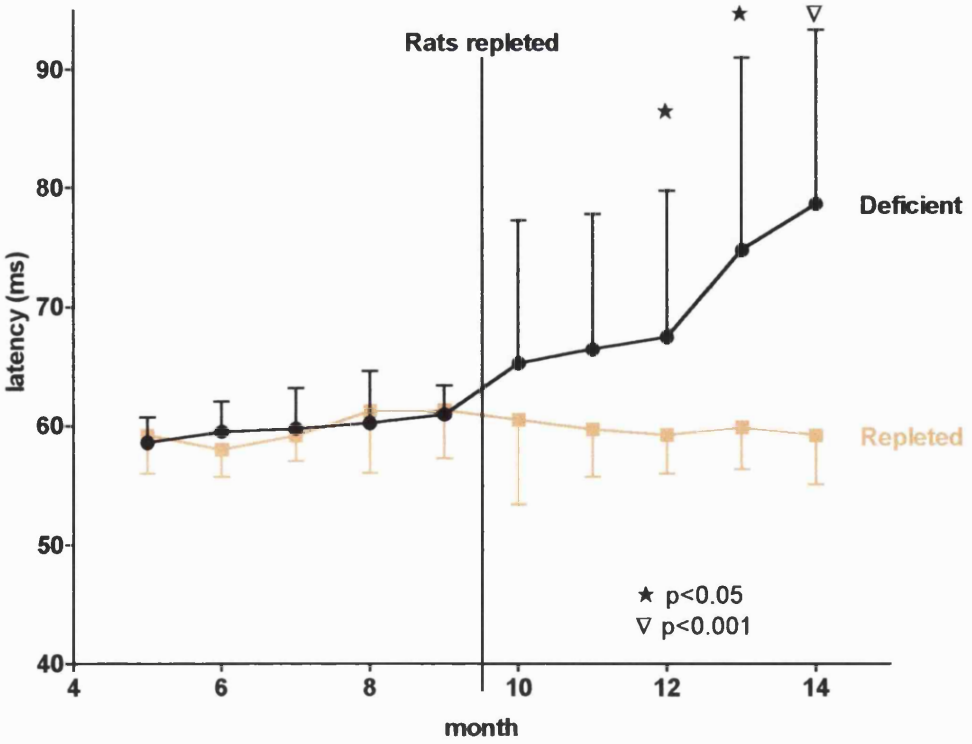


Figure 7.44 Group mean ERG latencies +/- 1SD - 5mg/kg v repleted (bright white flash)

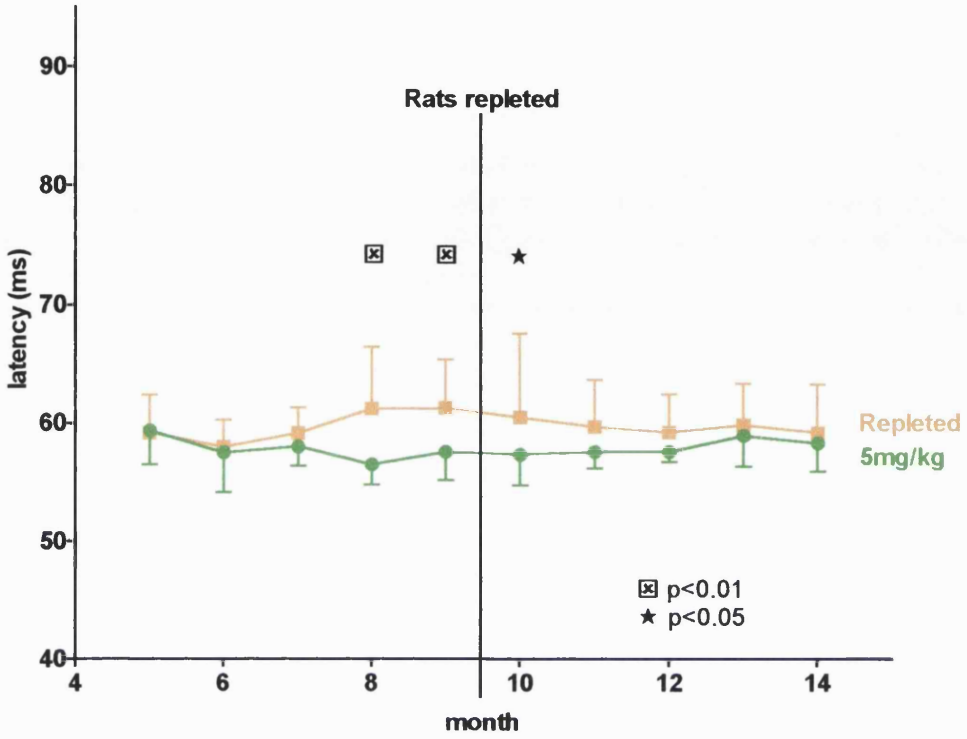


Figure 7.45 Group mean ERG peak-to-peak amplitudes (bright white flash)

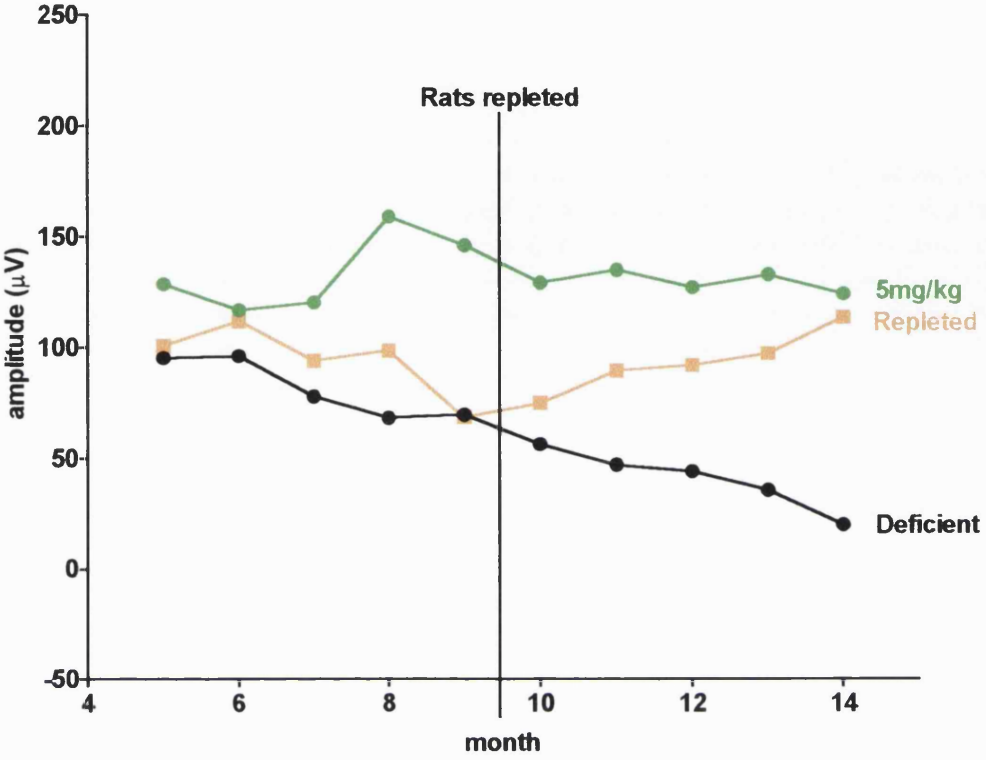


Figure 7.46 Group mean ERG peak-to-peak amplitudes +/- 1SD - deficient v repleted (bright white flash)

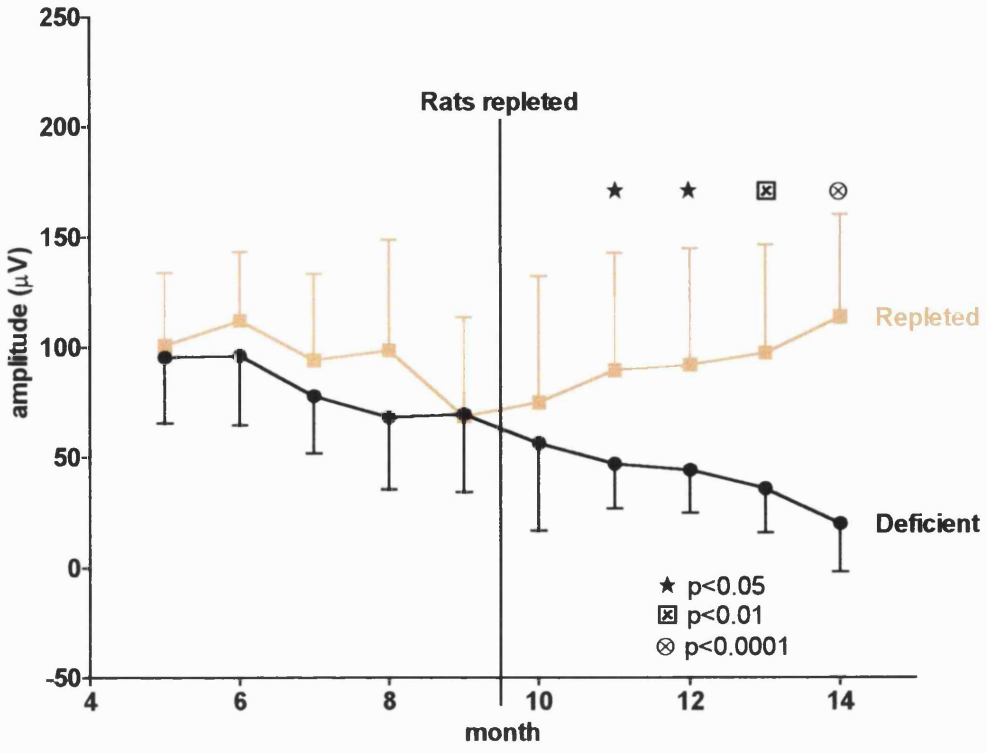
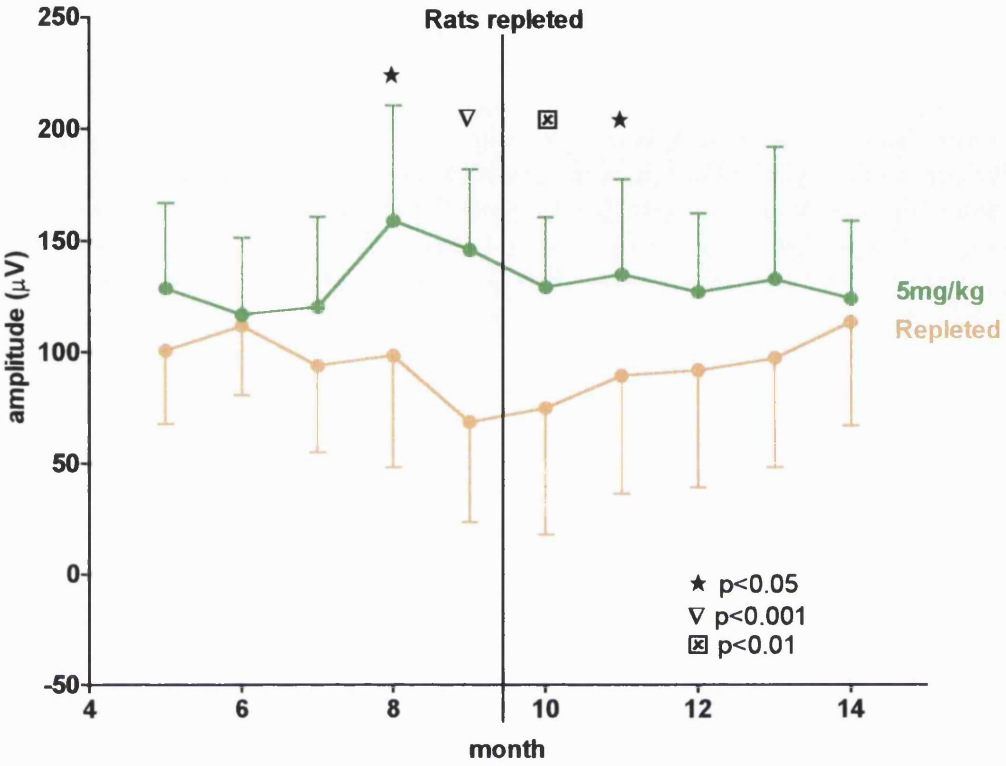


Figure 7.47 Group mean ERG peak-to-peak amplitudes +/- 1SD
- 5mg/kg v repleted (bright white flash)



VEP latencies

The group mean VEP onset latencies, recorded in response to white flash stimuli, are shown in Figure 7.48. The repleted group gave consistently significantly longer latencies than the 5mg/kg group from months 6-11, after which no significant differences were observed between the two groups (Figure 7.49). There were no consistently significant differences between the deficient and repleted groups at any time point during the study.

VEP amplitudes

Group mean VEP peak-to-peak amplitudes recorded from rats in the deficient, repleted and 5mg/kg groups are shown in Figure 7.50. After month 10, mean amplitudes decreased in both the deficient and repleted groups but stayed relatively constant in the 5mg/kg group. Rats in the repleted group were found to have a consistently significantly smaller mean VEP peak-to-peak amplitude than those fed a 5mg/kg RRR-α-tocopheryl acetate diet after 11 months (Figure 7.51). There were no statistically significant differences between the deficient and repleted groups at any point during the study.

Figure 7.48 Group mean VEP onset latencies (bright white flash)

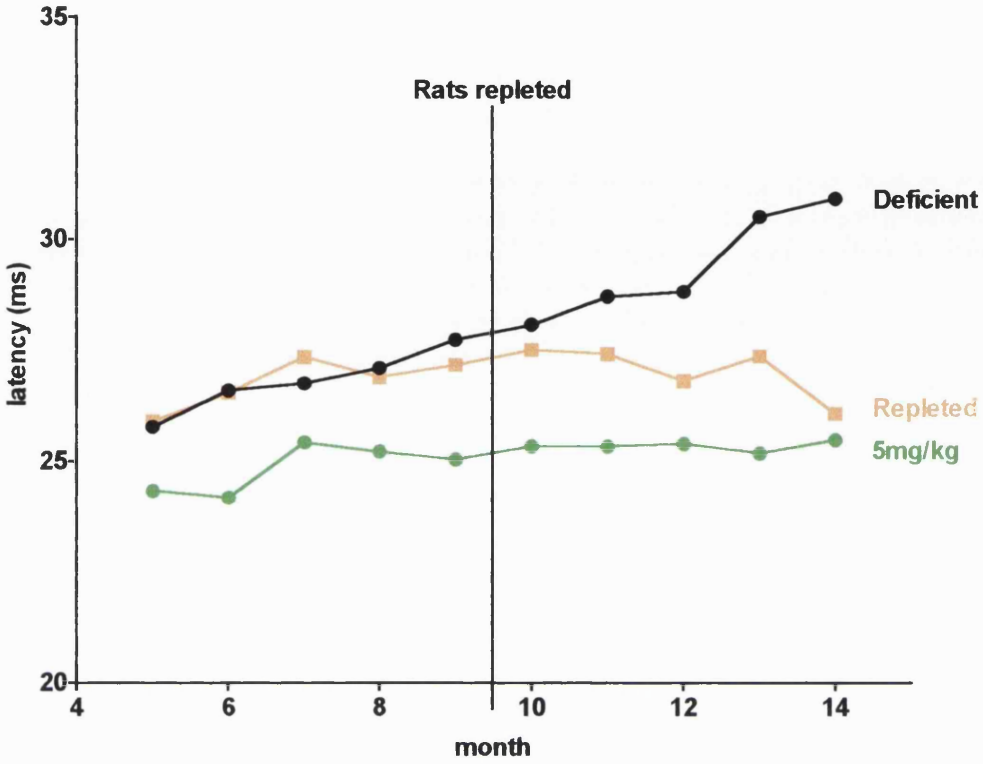


Figure 7.49 Group mean VEP onset latencies +/- 1SD - 5mg/kg v repleted (bright white flash)

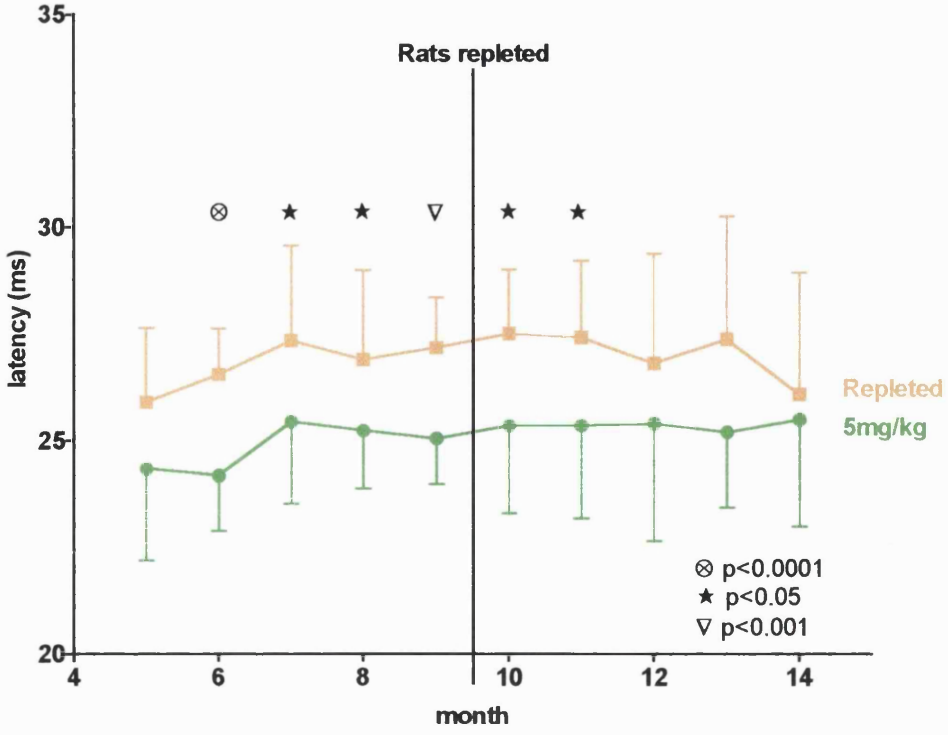


Figure 7.50 Group mean VEP peak-to-peak amplitudes (bright white flash)

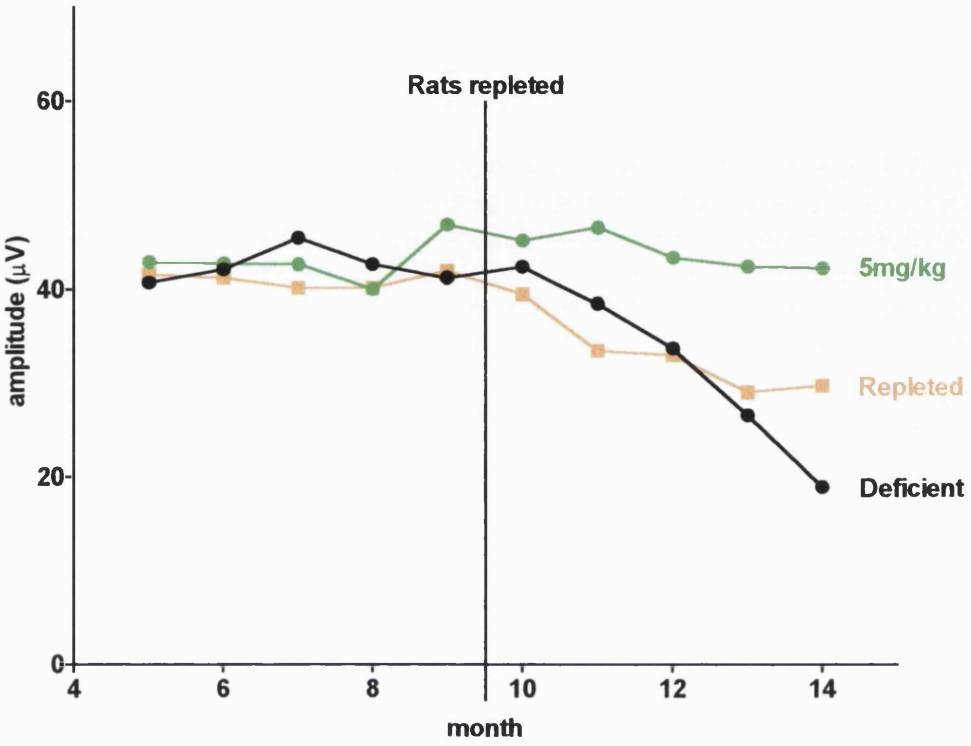
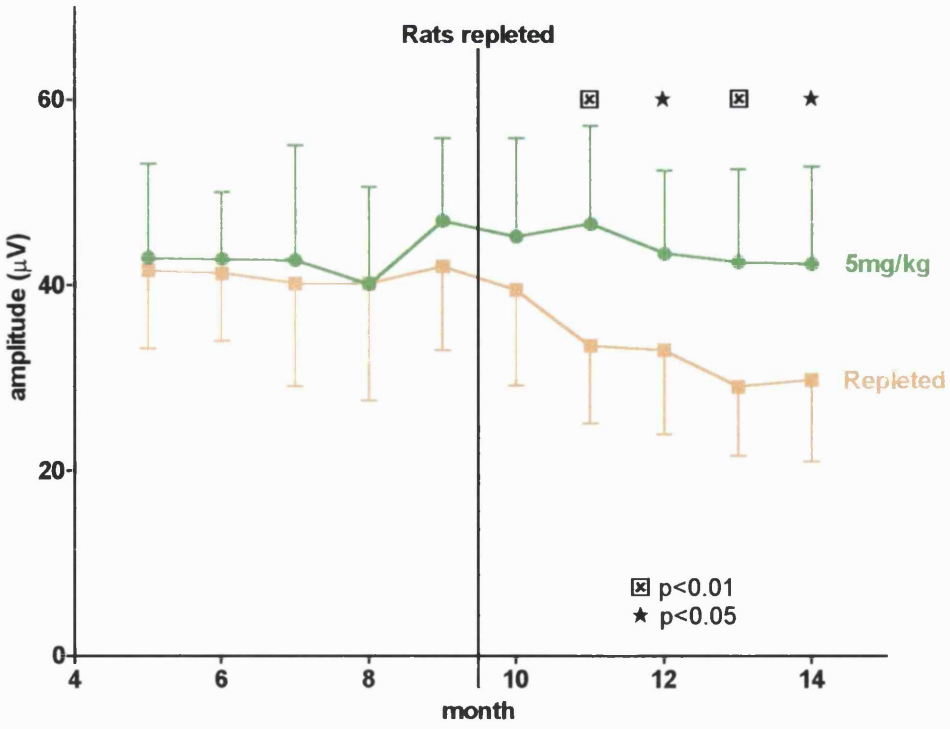


Figure 7.51 Group mean VEP peak-to-peak amplitudes +/- 1SD - 5mg/kg v repleted (bright white flash)



7.7 Tissue α-tocopherol concentrations

At the end of the study (14 months), the concentration of α-tocopherol was determined in tissues from six rats from each repleted group (study I and study II) and compared with the concentrations obtained in the 36mg/kg group (study I, chapter 4). At this point, the repleted group from study I had received the deficient diet for 42 weeks and the 36mg/kg all-rac-α-tocopheryl acetate diet for 18 weeks. The repleted group in study II received the 36mg/kg diet for 22 weeks, following 38 weeks of tocopherol deficiency. Figure 7.52 shows the α-tocopherol concentrations in plasma and non-neural tissues (liver, gastrocnemius muscle, heart and testis) and Figure 7.53 the concentrations in neural tissues (sciatic nerve, thoracic cord, cerebral cortex and eye). When compared with the 36mg/kg group, the repleted group I had significantly lower α-tocopherol concentrations in plasma ($P<0.01$), testis ($P<0.0001$), sciatic nerve ($P<0.01$) and thoracic spinal cord ($P<0.05$). There were no significant differences between repleted group II and the 36mg/kg group in any of the tissues tested, except for the liver, where the repleted group had significantly increased ($P<0.05$) concentrations.

Figure 7.52 Alpha-tocopherol concentrations in non-neural tissues

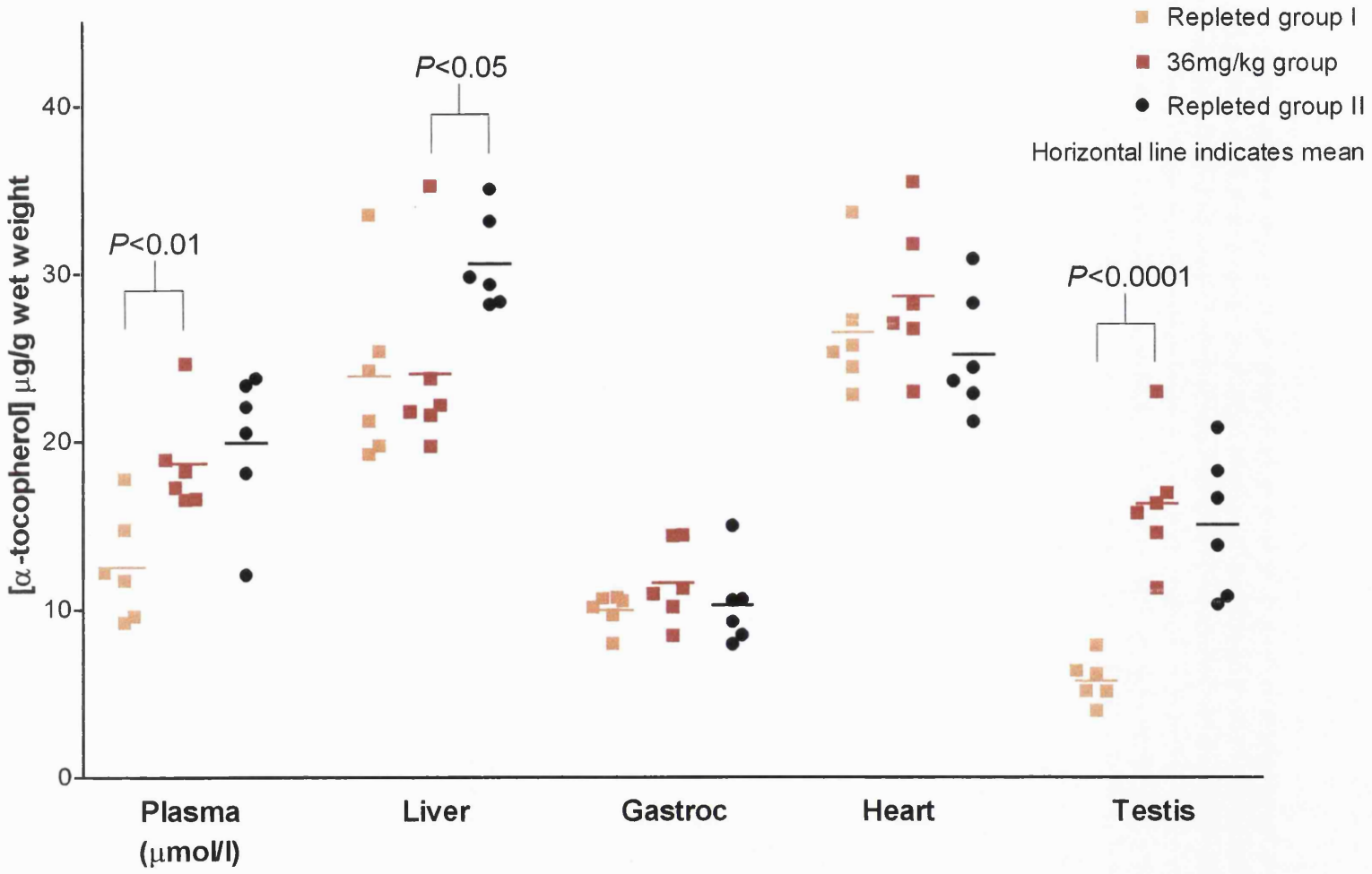
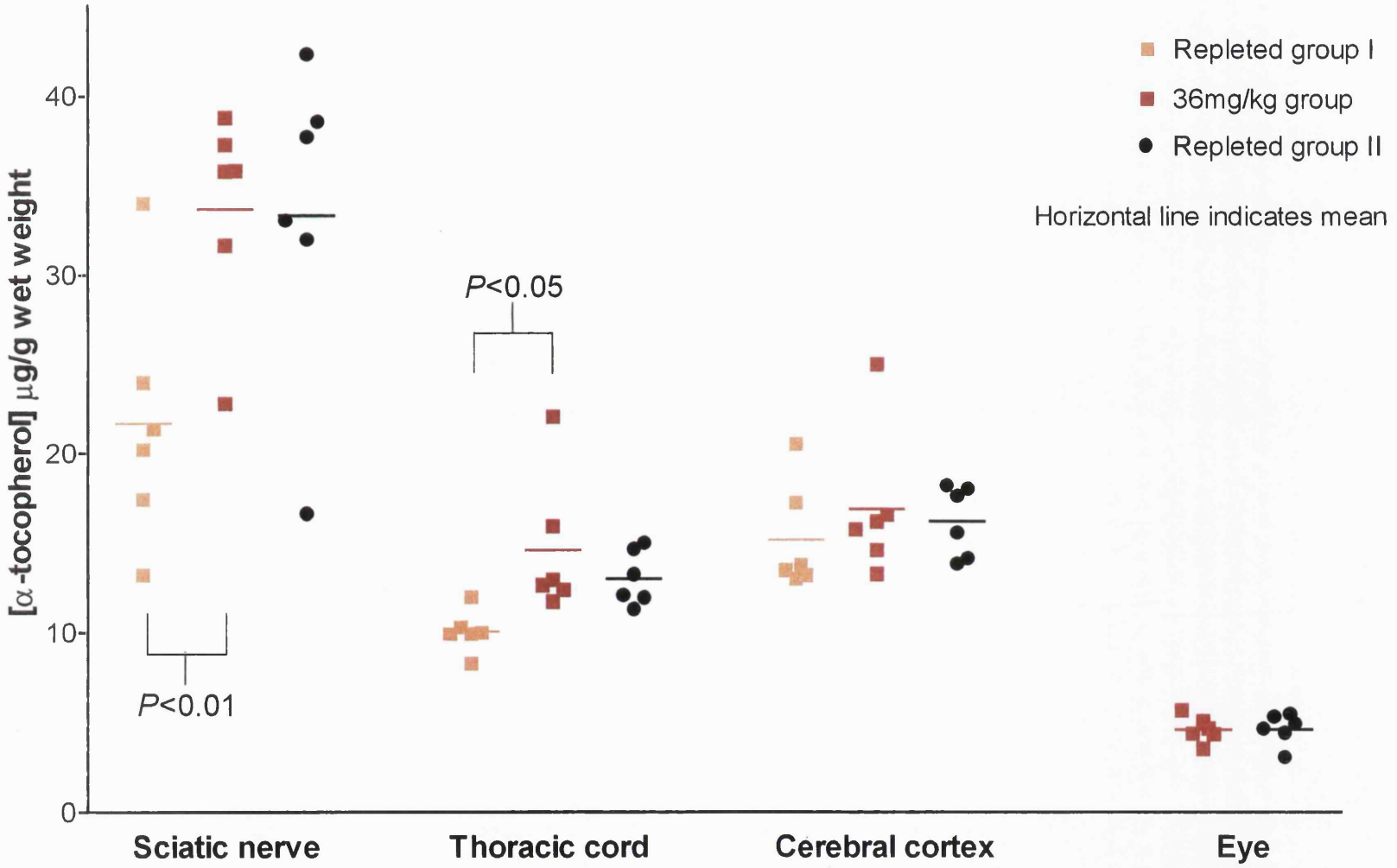


Figure 7.53 Alpha tocopherol concentrations in neural tissues



7.8 Discussion

The current studies were undertaken to investigate the effects of repletion on the growth and general condition of the rats and on the neurological signs associated with vitamin E deficiency. The two studies differed in the duration of deficiency and repletion, but gave essentially similar results.

The repleted rats in both studies showed rapid 'catch up' growth and their weights at the end of the studies were similar to the 5mg/kg groups.

The physical changes seen in the deficient rats (poor coat condition, muscle wasting, and varying degrees of ataxia) were similar to those reported previously (Goss-Sampson *et al*, 1990, 1988; Towfighi 1981; Machlin *et al*, 1977). In both studies, neurological changes started to develop in the animals in the repleted group at the same time as in the deficient rats. This would be expected since both groups were receiving the same diet deficient in α-tocopherol. Repletion with 36mg/kg all-rac-α-tocopheryl acetate, however, halted the progression of the physical neurological changes in all but one of the rats in which they had been observed. Furthermore, in contrast to the deficient animals, neurological symptoms did not develop in the remainder of the repleted group.

The electrophysiological results obtained from the repleted groups until the point of repletion were, as expected, similar to those recorded in the deficient groups. There tended to be an increase in the latencies and decrease in the amplitudes of SEPs, VEPs

and the ERGs. After repletion, the electrophysiological responses tended to become more like the 5mg/kg group and less like the deficient group. For example, the lower limb central CVs of the repleted group in study II increased and was no longer significantly different from that of the 5mg/kg group after 11 months. The CVs of the deficient group, in contrast, continued to decrease for the remainder of the study. Despite the inherent variability of the various electrophysiological parameters, these trends were consistent.

Deficient, repleted and 5mg/kg groups in study I gave similar results for lower limb SEP amplitudes at both L5 and the cerebral cortex. In study II, however, the SEP amplitudes of the repleted group were significantly smaller than those of the 5mg/kg group after 8 months (recorded at L5) and after 7 months (at cortex), but increased from month 10 resulting in no significant differences between the repleted and 5mg/kg groups by the end of the study. Possible reasons for the differences between the two studies have been discussed in Section 6.2.3.1.

The latencies of the ERGs and VEPs of the deficient and repleted groups in both studies increased from month 8. After repletion, however, the latencies of the ERGs and VEPs of the repleted group in study I, and those of the ERGs of the repleted group in study II, remained relatively constant. The VEP onset latencies of the repleted group in study II became shorter and were no longer significantly different from the 5mg/kg (control) group after month 12. These results indicated that repletion halted the retinal degeneration associated with vitamin E deficiency.

early. The VEP amplitudes of the repleted group in study II, however, continued to decrease even after repletion with vitamin E, suggesting that the post-retinal visual pathway may be more sensitive to vitamin E deficiency than the retina itself. In general, therefore, repletion of deficient rats with 36mg/kg RRR-α-tocopheryl acetate halted the deterioration of the visual electrophysiological parameters (VEPs and ERG). This agrees with the one other study of visual function in vitamin E repleted rats, carried out by Goss-Sampson *et al* (1998), in which VEPs and ERG were recorded at various time points over a year. After 30 weeks of deficiency, a group of rats was repleted with 40mg/kg α-tocopheryl acetate for 20 weeks. The animals were found to have normal retinal vitamin E and A concentrations after repletion, but the electrophysiological parameters continued to deteriorate throughout the period of repletion. A possible reason for the difference between the two studies is the size of the animals at the time of repletion. In the present studies, the mean weights of the repleted groups were 330g (study I) and 433g (study II), whereas Goss-Sampson's rats weighed approximately 500g when the group was repleted. The heavier animals would have contained extra fat deposits which, together with a deficiency of vitamin E, could have led to increased lipid peroxidation and neural degeneration. This degeneration, therefore, probably occurred earlier in the study of Goss-Sampson than in the current studies, and this may explain why no halt in the degradation of visual function was observed.

Repletion of deficient rats with 36mg/kg α-tocopheryl acetate led to an increase in tissue α-tocopherol concentrations. In study II, these concentrations were similar to those in the 36mg/kg group whereas the repleted group in study I had α-tocopherol

concentrations which were significantly lower for most of the tissues. A reason for this difference is that the repleted group in study I received the 36mg/kg diet for 18 weeks whereas the group in study II was repleted for 22 weeks, allowing more time for the normalisation of tissue α-tocopherol concentrations.

Apart from the study of Goss-Sampson *et al* (1998) discussed above, there have been very few other published reports on the effects of repleting vitamin E deficient animals. In a study by Nelson *et al* (1981), a group of 7 rhesus monkeys were maintained on a vitamin E deficient diet for 30 months. During the third year of deficiency, the monkeys suffered weight loss, generalised muscle weakness and anaemia. Vitamin E was undetectable in the serum and progressive central and peripheral nervous system lesions also developed. Sensory axons showed more marked degeneration than motor nerves, and the central nervous system was more severely affected than the peripheral. After 30 months of deficiency, two of the animals were repleted with oral doses of 100mg RRR-α-tocopheryl acetate three times a week for 2 months. After repletion, the serum vitamin E concentrations returned to normal and the muscle weakness and anaemia resolved. One monkey regained all the weight previously lost, and the other increased in weight by 12% over its previous maximum. The gross neuropathologic features were indistinguishable from those seen in the deficient animals. However, the repleted monkeys did not exhibit the ultrastructural changes indicative of early axonal injury (intra-axonal accumulation of membrane-bound vesicles and vacuoles) that were noted in the peripheral nerves of deficient animals. This may have signified a cessation of axonal degeneration (Nelson *et al*, 1981).

The effects of α-tocopherol repletion on neurological function have been studied more extensively in vitamin E deficient humans with conditions such as abetalipoproteinemia, cholestatic liver disease, other chronic fat malabsorptive disorders and ataxia with vitamin E deficiency. Patients with abetalipoproteinemia (discussed in section 1.7.1) have undetectable serum concentrations of α-tocopherol from birth (Muller *et al*, 1974). Muller and colleagues (1977, 1983) treated eight children with abetalipoproteinemia with large oral doses of vitamin E and followed their progress for up to 30 years. Five of the patients, who were treated from the age of 16 months, did not develop any of the neurological and retinal changes associated with abetalipoproteinemia. The remaining three children had displayed signs of neurological dysfunction before supplementation began, but in all cases vitamin E therapy either halted or reversed the progression of these signs.

The SEP results obtained in the present study agree with the findings of several studies of α-tocopherol repletion in deficient man. Five patients with abetalipoproteinemia were re-examined after up to 3 years of oral vitamin E and A therapy and four were found to have improved sensory conduction velocities (Brin *et al*, 1986). No patient worsened in condition and median nerve and cortical CVs returned to normal in two. Fagan and Taylor (1987) followed 5 patients for 4 years after commencing vitamin E therapy. Electrophysiological tests were performed every 6 to 12 months and improvements in VEPs were noted in one patient. VEPs of normal amplitude but increased latency were recorded in the other patients, but their condition did not deteriorate further. They also reported a normal peripheral SEP but abnormal central response in all 5 children. The cortical SEP remained stable in 3 of the patients during the 4 year study period, whereas fluctuations were noted in the other two. Muller *et al*

(1977) reported a halt in the retinal changes of a patient with abetalipoproteinemia, following vitamin E therapy. Twelve years after repletion began, the mild retinopathy which was present at diagnosis remained unchanged. Serial ERG and VEP studies in eight repleted abetalipoproteinemia patients, carried out by Brin *et al* (1986), also showed an arrest of the abnormalities.

Guggenheim *et al* (1982) measured nerve potentials in 4 children with chronic cholestatic liver disease. A decreased CV was recorded in the median nerve of one patient and sural nerve potentials were unobtainable. After treatment with large oral or intra-muscular doses of vitamin E, some improvement in the condition of 2 of the patients was seen, and there was no further deterioration in any of the patients. A low amplitude of the sural nerve compound sensory action potential (CSAP) was reported by Sokol *et al* (1985) in six out of seven children with vitamin E deficiency, associated with chronic cholestasis. After 10 to 12 months of vitamin E repletion, the CSAP amplitude had increased in four out of five patients who had shown clinical neurologic improvement. Perlmutter and colleagues (1987) observed a halt in the progression, but no improvement, of the neural symptoms associated with chronic cholestasis in 6 patients after vitamin E therapy. Partial improvement in oculomotor function was recorded in 4 of 11 cholestatic children given intramuscular injections of vitamin E after developing neurologic signs (Alvarez *et al*, 1985). A study carried out by Cynamon *et al* (1988) could not, however, demonstrate a consistent relationship between VEP latency and vitamin E status in 17 patients with chronic cholestasis.

The typical spinocerebellar syndrome associated with severe and chronic vitamin E deficiency has also been described in patients with extensive ileal resection (Harding *et*

al, 1982; Howard *et al*, 1982) and cystic fibrosis (Elias *et al*, 1981; Willison *et al*, 1985). Neurological sequelae are, however, rarely seen in patients with cystic fibrosis and tend only to occur as a result of added complications such as liver disease or intestinal resection. Improvement in neurological function following treatment with appropriate supplements of vitamin E has been reported in patients from all these groups (Elias *et al*, 1981; Harding *et al*, 1982; Howard *et al*, 1982; Willison *et al*, 1985).

Patients with ataxia with vitamin E deficiency (AVED) present with low serum vitamin E levels and neurological abnormalities, but show no generalised fat malabsorption (see section 1.7.3). Vitamin E therapy in these patients has also been shown to either prevent further deterioration or improve neurological status (Burck *et al*, 1981; Harding *et al*, 1982; Sokol *et al*, 1988; Rayner *et al*, 1993).

The results in the vitamin E deficient rat are, therefore, consistent with results in vitamin E deficient man following supplementation with the vitamin. They, therefore, confirm that the vitamin E deficient rat is a good model for studying deficiency in man.

Chapter 8

Conclusions and future work

Vitamin E (α-tocopherol) is essential for the normal structure and function of neural tissues and the retina in both man and animals. The current project was undertaken to define the minimum requirements of vitamin E necessary to prevent the neurological features of deficiency and to compare the biological activities of synthetic (all-rac-) and natural (RRR-) α-tocopheryl acetate in neural tissues and the retina.

Non-invasive electrophysiological techniques were used to measure visual and somatosensory evoked potentials, and the electroretinogram, longitudinally in anaesthetised rats, receiving various dietary amounts of vitamin E. Anaesthetic agents can affect these electrophysiological parameters, so a pilot study was carried out to find an agent with minimal and reproducible effects. From the results obtained, a mixture of the opioid fentanyl/fluanisone and midazolam was chosen for use in subsequent studies. After 14 months of electrophysiological recording, various neural and non-neural tissues were harvested from the rats for biochemical analyses. Methods for the determination of α-tocopherol and malondialdehyde (an index of lipid peroxidation) by HPLC were established, to investigate the relationship between these biochemical parameters and electrophysiological function.

There were differences between the results obtained in the two studies, which could be accounted for by problems of diet storage in study I, different growth rates and variability of results leading to differences in statistical significance. In general, however, a dietary intake of little or no vitamin E tended to cause increases in the latencies, and hence decreases in conduction velocities (CVs), of the SEPs and decreases in their amplitudes. Increases in latencies and decreases in amplitudes were also observed in the ERG and VEP responses of rats fed little or no vitamin E. These

results are similar to those reported in man, validating the rat as a good model for studying vitamin E deficiency in man.

The results of the present studies indicated that a dietary intake of 1.25mg/kg all-rac-α-tocopheryl acetate provided marginal protection against the neural and retinal degeneration associated with vitamin E deficiency. They also showed that 0.75mg/kg RRR- and 1.0mg/kg all-rac-α-tocopheryl acetate tended to have similar biological activities in the nerves and retina of rats. This meant that the respective biological activities of all-rac and RRR-α-tocopheryl acetates in the neural system were similar to those found using the classical rat foetal resorption assay.

A linear relationship was shown between dietary intake and tissue concentrations of α-tocopherol up to 5mg/kg diet, and also between tissue and plasma concentrations, indicating that plasma tocopherol levels gave a good indication of tissue vitamin E status. These conclusions could assist in the clinical treatment of disorders associated with chronic vitamin E deficiency and monitoring of the efficacy of treatment.

Neural tissues retained α-tocopherol more effectively than non-neural tissues. There were even detectable concentrations found in the neural tissues of rats which had been fed, from the age of 3 weeks, a diet totally deficient in vitamin E for 14 months. This was obviously vitamin E obtained from the dam during gestation and suckling. It would therefore be of interest to find out if the rats from the other dietary groups retained some α-tocopherol from the mother. Since the experimental diets contained deuterated α-tocopherol, whereas the rat dams were fed diets containing undeuterated

α-tocopherol, it is proposed to measure the relative proportions of the two substances using gas chromatography/mass spectrometry. As α-tocopherol is retained by neural tissues, it took a long time (at least 7 months) for the neurological signs of deficiency to manifest. It would be helpful to shorten this time, since experimental animals are expensive to maintain and research projects are often funded for a limited period. One way this could be achieved is by feeding the rats a diet high in polyunsaturated fatty acids (PUFA). This would lead to increased substrate for lipid peroxidation, resulting in increased concentrations of lipid peroxides which could attack cell membranes throughout the body. This damage would occur virtually unchecked in the absence of vitamin E, and therefore accelerate the appearance of the signs of deficiency.

The present study also investigated the effects of repleting chronically deficient animals with vitamin E. Repletion led to rapid 'catch up' growth and halted the progression of the physical neurological changes. There was no further deterioration in any of the electrophysiological parameters measured after repletion, and there was definite improvements in the central CVs, ERG and VEP latencies and ERG amplitudes of the repleted group in study II. This suggests that the time of onset of repletion was important, i.e. improvements in function occurred only if treatment was started sufficiently early. It would have been interesting to monitor the repleted rats for longer, in order to ascertain whether the period of vitamin E deficiency had any long-term effects. It would have also been of interest to investigate lipid peroxidation in tissues before and after repletion. The results of the present study agree with studies conducted in man with severe deficiency as a result of abetalipoproteinemia, other fat malabsorptive conditions and AVED, thereby confirming the validity of the rat model.

Very little is known about the mechanism of action of vitamin E in neurological tissues, particularly at a molecular level. This is because it is currently a difficult and time-consuming process to deplete vitamin E from tissues and cause deficiency symptoms in experimental animals. Recently, however, a knockout mouse model has been established which lacks the cytosolic α-tocopherol transfer protein (Jishage *et al*, 2001). Using this model, it was shown that the concentration of α-tocopherol in the circulation was regulated by the expression levels of α-tocopherol transfer protein and that the α-tocopherol transfer protein was necessary for the normal development of placental labyrinthine trophoblasts, with embryos of these mice dying at mid-gestation (Jishage *et al*, 2001). Further studies, using this knockout mouse model, should provide more detailed information regarding the mechanisms involved in the neurological sequelae associated with severe and chronic vitamin E deficiency.

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Comparison of the effects of four anaesthetic agents on somatosensory evoked potentials in the rat

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Summary

Electrophysiological techniques provide an objective and non-invasive measure of neurological function. In order to undertake detailed evoked potential studies in rats on repeated occasions, it is necessary to find an appropriate anaesthetic agent which has minimal and reproducible effects on the parameters to be studied and also has a minimal effect on the general welfare of the animals. In this study we compared the effects of four common anaesthetic agents (ketamine–xylazine, medetomidine, isoflurane and fentanyl/fluanisone–midazolam) on somatosensory evoked potentials (SEPs) in rats following electrical stimulation of the fore- and hind-paw. Fentanyl/fluanisone–midazolam was found to be well tolerated by the animals and to have, in general, the least deleterious effect on SEPs. For example, the response recorded at the level of the somatosensory cortex (P1), following forelimb stimulation, appeared on average 1.80 ms earlier with fentanyl/fluanisone–midazolam than with the other agents and the peak-to-peak amplitude (CI to CII) of the response recorded at the cervical (C3) level was on average 5.86 μV greater with fentanyl/fluanisone–midazolam. Fentanyl/fluanisone–midazolam is, therefore, recommended as the anaesthetic of choice for longitudinal studies of SEPs in the rat.

Keywords Somatosensory evoked potentials; rat; anaesthesia

Somatosensory evoked potentials provide an objective and non-invasive electrophysiological technique to measure neurological function. An SEP is the electrical response of the nervous system produced by activation of a peripheral nerve, and consists of a series of peaks or waves, which are characterized by their polarity, latency and amplitude. SEPs can be useful in repeated longitudinal studies in the same animal, but anaesthetic agents can significantly affect their size and timing (Angel & Gratton 1982, Sloan *et al.* 1990, Thornton *et al.* 1992). In order to carry out repeated periodic studies, it is therefore necessary to find an agent which has the least deleterious effects on SEPs, and also on the general welfare of the animal.

Anaesthesia comprises a number of components including drug-induced loss of consciousness or hypnosis and analgesia, with different anaesthetic agents having different hypnotic and analgesic properties. The SEP is influenced by the hypnotic properties of an anaesthetic (Thornton *et al.* 1992) and is, therefore, likely to be affected to varying extents by different anaesthetic agents. Ketamine–xylazine and pentobarbitone have been previously compared in the rat and the former was found to have a lesser effect on SEPs (Goss-Sampson & Kriss 1991). However, ketamine–xylazine causes an increase

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in blood pressure and muscle tone, and severe respiratory depression which can sometimes be fatal (Flecknell 1996). In addition, the high dose rates required for surgical anaesthesia lead to a slow recovery.

A study was, therefore, undertaken to find an appropriate anaesthetic and the effects of the following four agents on the latencies and amplitudes of SEPs were compared:

(1) ketamine–xylazine—an injectable agent;
(2) medetomidine—an injectable agent which is reversed by atipamezole;
(3) isoflurane—a gaseous anaesthetic;
(4) fentanyl/fluanisone–midazolam—an injectable agent which is partially reversed by buprenorphine.

Ketamine–xylazine has been previously shown to cause a dose-dependent increase in latency and decrease in amplitude of cortical SEPs (Angel & Gratton 1982, Goss-Sampson & Kriss 1991). The effects of medetomidine on SEPs do not appear to have been previously investigated. Isoflurane has consistently been shown to increase the latencies and decrease amplitudes of cortical SEPs (Sebel *et al.* 1986, Thornton *et al.* 1992). These changes are similar to those reported for other volatile anaesthetics (Thornton *et al.* 1992). As far as we are aware there have been no previous studies which have compared the effects of inhalation and injectable anaesthetic agents on cortical SEPs. Fentanyl, given alone to human adults, had no effect on the latency or amplitude of median nerve SEPs (Loughnan *et al.* 1987). Midazolam has been reported to cause a small increase in latency and decrease in amplitude of cortical SEPs in humans (Sloan *et al.* 1990). There have been no studies on the effects of a fentanyl/fluanisone and midazolam anaesthetic regimen on SEPs in rats.

Materials and methods

Experimental animals

Conventional male Wistar rats (250–320 g) were obtained from B&K Universal Ltd, UK and housed in groups of two or three animals per cage. The animals were kept at a controlled temperature of 21 ± 1°C, relative humidity of 55 ± 5% and a 12/12 h light/dark cycle. Fresh drinking water and pelleted diet (Rat Maintenance Diet RM1, Special Diet Services, UK) were provided *ad libitum*.

The rats were arbitrarily assigned to one of four groups ($n = 8$), with each group receiving one of the following anaesthetic regimens:

(1) A combination of 90 mg/kg ketamine (VETELAR, Parke-Davis Veterinary, UK) and 10 mg/kg xylazine (ROMPUN, Bayer, UK), mixed in the same syringe, and administered intraperitoneally (i.p.).
(2) Medetomidine (DOMITOR, SmithKline Beecham, UK) administered at 0.3 mg/kg intramuscularly (i.m.) with atipamezole (ANTISEDAN, SmithKline Beecham, UK) given at 1 mg/kg i.p. to reverse the anaesthesia.
(3) Isoflurane (Abbott Laboratories, UK) administered in oxygen at a flow rate of 2 l/min via an induction chamber (IMS, UK). A combination of 5% isoflurane was used to induce anaesthesia and 2% for maintenance.
(4) A combination of fentanyl/fluanisone (HYPNORM, Janssen, UK) and midazolam (HYPNOVEL, Roche, UK) was administered. One part Hypnorm (fentanyl 0.315 mg/ml; fluanisone 10 mg/ml) plus one part Hypnovel (5 mg/ml) were mixed in the same syringe with two parts water and administered by injection at a dose of 2.7 ml/kg i.p. Buprenorphine (TEMGESIC 0.3 mg/ml, Reckitt & Coleman Products, UK) was injected at 0.1 mg/kg i.m. to aid recovery.

The drug concentrations chosen were the minimum required to produce surgical anaesthesia in the rat (Flecknell 1996). The depth of anaesthesia was regularly assessed by testing behavioural responses to pedal and palpebral (corneal) stimulation. Rectal temperature was maintained throughout between 35°C and 37°C using a heated blanket. Each rat was given 3 ml saline i.p. to correct for fluid loss during the procedure.

The animals were weighed before being anaesthetized and on each of the following 5 days. The time taken to induce a surgical level of anaesthesia (i.e. loss of corneal and pedal withdrawal reflexes) and to regain the

righting reflex were recorded and any adverse effects on the rats were noted.

Electrophysiology

Identical inducing and recording procedures were carried out in the four groups of rats, and all recordings were completed within 20 min from induction of anaesthesia. Subdermal platinum needle electrodes were used to stimulate peripheral nerves and record lower and upper limb SEPs.

Following electrical stimulation of the tibial nerve at the right tarsus, the lower limb SEPs were recorded from an electrode inserted between the 5th and 6th lumbar vertebrae (L5) and one inserted subcutaneously at the scalp over the contralateral somatosensory cortex. These active electrodes were referred to electrodes inserted at L2 and at the caudal tip of the frontal bone (snout), respectively. The recording derivation was such that a positivity at the active electrode produced a downward deflection.

Following median nerve stimulation at the right carpus, upper limb SEPs were recorded subcutaneously at the 3rd cervical vertebra (C3) and at the scalp over the contralateral somatosensory cortex, both referred to a subdermal electrode at the caudal tip of the frontal bone.

An earth electrode was inserted into the left hind foot for both lower and upper limb recordings. A constant current stimulus (between 1 and 2 mA) of 0.1 ms duration was delivered at the rate of 3 s^{-1}. Responses were averaged over a 30 ms period which included a 1.5 ms pre-stimulus interval. The amplifier band pass was set between 1 Hz and 3 kHz and 128 responses were averaged for each run. Runs were duplicated to confirm consistency. A Medelec Sensor was used to average the responses, which were then transferred to a computer and stored on disk for later analysis.

The latencies and peak-to-peak amplitudes of the selected SEP components from each group of rats were compared using one-way analysis of variance (ANOVA) across the groups and the significance of difference between mean values was calculated by the Student–Newman–Keuls test (SPSS 6.0 statistical software program, SPSS Inc.) with $P < 0.05$ being regarded as significant.

These experiments were performed under appropriate personal and project licences issued by the Home Office.

Results

Induction and duration of anaesthesia

Mean induction and recovery times are presented in Table 1. Anaesthesia with isoflurane gave the shortest times when compared to the other anaesthetics (mean induction and recovery times 3.33 and 5.33 min respectively). Medetomidine had the longest induction time (10.38 min) but recovery was rapid after the antidote had been given (8.00 min). Ketamine–xylazine and fentanyl/fluanisone–midazolam had similar induction times (6.00 and 4.90 min respectively), but recovery from ketamine–xylazine was much slower (103.63 compared with 29.78 min).

Ketamine–xylazine, isoflurane and fentanyl/fluanisone–midazolam all produced good levels of surgical anaesthesia for the duration of the procedure and no responses to pedal or corneal stimulation could be elicited. Medetomidine produced a deep level of anaesthesia in five of the eight rats, but the remaining three animals

Table 1 Induction and recovery times for each anaesthetic ($n = 8$ in each group)

	Induction time (min)		Recovery time (min)	
Anaesthetic agent	Mean	SD	Mean	SD
Ketamine–xylazine	6.00	(1.15)	103.63	(32.08)
Medetomidine	10.38	(3.74)	8.00	(2.20)
Isoflurane	3.33	(0.78)	5.33	(1.15)
Fentanyl/fluanisone–midazolam	4.90	(0.74)	29.78	(10.74)

responded when the needle electrodes were inserted into the feet, although they had no pedal or corneal reflexes.

Weight loss

Rats anaesthetized with either medetomidine or ketamine–xylazine showed a decrease in weight (means of 4.1 and 2.5 g respectively) on the first or second day after the procedure and then gained weight steadily. Animals which received fentanyl/fluanisone–midazolam showed a decrease in weight (mean 4.5 g) on the second or third day after the procedure, and then gained weight steadily. Rats anaesthetized with isoflurane did not show consistent loss of weight during the 5 days following the procedure.

Somatosensory evoked potentials

For each anaesthetic agent, consistent responses were recorded at the cervical (C3) and cortical regions after stimulation of the median nerve at the wrist. The mean onset latency at C3 was significantly different ($P < 0.02$) when the groups were compared using ANOVA (see Table 2). The onset latency at C3 was significantly shorter with ketamine–xylazine than isoflurane ($P < 0.05$). The mean latencies for all components of the cortical response were longest in the rats which received the ketamine–xylazine combination. There were significant differences between the groups in mean cortical onset ($P < 0.05$) and P1 ($P < 0.001$) latencies. Animals anaesthetized with fentanyl/fluanisone–midazolam had significantly shorter cortical onset latencies than ketamine–xylazine (Fig 1). The P1 latencies were significantly shorter with fentanyl/fluanisone–midazolam compared with all the other agents and the latency with isoflurane was significantly shorter than with ketamine–xylazine. There were no significant differences in the CI and CII components of the cervical response, or in the N1 component of the cortical SEP when the anaesthetic groups were compared.

Following stimulation of the tibial nerve at the ankle, consistent responses were recorded for each group, at the lumbar (L5) region

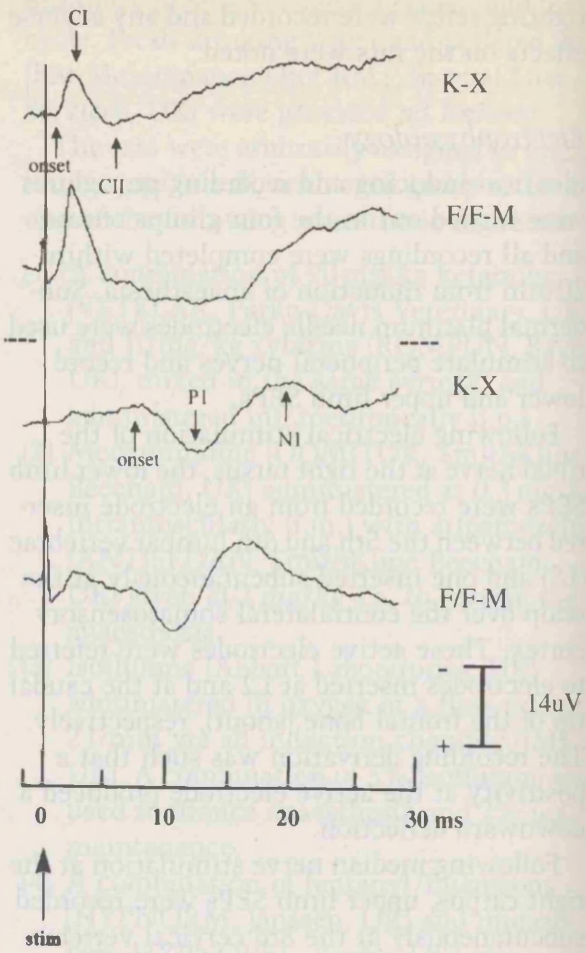

Fig 1 Averaged SEPs from two rats obtained during (a) ketamine–xylazine (K–X) and (b) fentanyl/fluanisone–midazolam (F/F–M) anaesthesia. Responses recorded at the cervical (C3) level (upper two traces) and over the contralateral somatosensory cortex (lower two traces), following forelimb stimulation. CI and CII represent the first and second cervical peaks respectively. P1 indicates the first positive peak and N1 the first negative peak. The solid line at time zero indicates the point of stimulus delivery. Note the larger amplitude of the cervical SEP and shorter latency of the cortical response during fentanyl/fluanisone–midazolam anaesthesia

of the spinal cord and cortex. Significant differences in mean lumbar onset ($P < 0.005$) and LI ($P < 0.05$) latencies were recorded between the groups. Fentanyl/fluanisone–midazolam gave significantly shorter L5 onset latencies than ketamine–xylazine and isoflurane and a significantly shorter LI latency than isoflurane (Fig 2). No significant differences were found when the LII components were compared. The mean cortical onset and P1 latencies were also found to be

Table 2 Latencies (ms) of somatosensory evoked potentials for each anaesthetic ($n=8$ in each group)

		Ketamine–xylazine		Medetomidine		Isoflurane		Fentanyl/fluanisone–midazolam		ANOVA
		Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)	*P* value
Forelimb SEPs										
C3	Onset	1.21	(0.12)	1.33	(0.10)	1.41	(0.16)†	1.30	(0.10)	< 0.02
	CI	2.57	(0.05)	2.66	(0.07)	2.70	(0.15)	2.62	(0.17)	NS
	CII	5.74	(0.46)	5.34	(0.20)	5.29	(0.87)	5.85	(0.40)	NS
Cortex	Onset	8.94	(0.91)*	8.38	(0.85)	8.50	(0.55)	7.69	(0.36)	< 0.05
	P1	12.28	(0.91)*	11.89	(0.82)*	11.09	(0.76)*†	9.95	(0.53)	< 0.001
	N1	17.49	(1.33)	17.15	(1.55)	15.47	(2.39)	15.47	(1.22)	NS
Hindlimb SEPs										
L5	Onset	1.58	(0.25)*	1.34	(0.24)	1.58	(0.42)*	1.10	(0.28)	< 0.005
	LI	2.29	(0.08)	2.26	(0.15)	2.45	(0.42)*	2.09	(0.22)	< 0.05
	LII	3.37	(0.19)	3.40	(0.18)	3.78	(0.77)	3.24	(0.46)	NS
Cortex	Onset	11.85	(0.88)*	12.82	(1.00)*	12.21	(0.71)*	9.25	(2.34)	< 0.001
	P1	15.98	(0.60)*	16.44	(0.77)*	15.24	(0.79)	14.33	(1.67)	< 0.005
	N1	20.53	(0.78)	20.17	(1.11)	19.75	(1.17)	19.85	(2.21)	NS

* Significantly different from fentanyl/fluanisone–midazolam ($P<0.05$)
† Significantly different from ketamine–xylazine ($P<0.05$)
NS = not significant

significantly different ($P<0.001$ and $P<0.005$ respectively) in rats anaesthetized with different agents (Fig 3). Significantly shorter cortical onset latencies were recorded from animals given fentanyl/fluanisone–midazolam when compared with the other three anaesthetics. It was also found that P1 latencies were significantly shorter with fentanyl/fluanisone–midazolam than with ketamine–xylazine or medetomidine. There were no significant differences in the N1 peak latencies.

The effect of different anaesthetic agents on mean SEP amplitudes is shown in Table 3. Isoflurane tended to depress the amplitude of both forelimb and hindlimb SEP components to a greater extent than the other agents. The cervical (C3 onset to CI and CI to CII) and

Table 3 Amplitudes (μV) of somatosensory evoked potentials for each anaesthetic ($n=8$ in each group)

		Ketamine–xylazine		Medetomidine		Isoflurane		Fentanyl/fluanisone–midazolam		ANOVA
		Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)	*P* value
Forelimb SEPs										
C3	Onset to CI	11.42	(3.15)	9.75	(3.39)	10.17	(2.70)*	15.18	(6.43)	< 0.05
	CI to CII	14.10	(2.93)*	12.66	(2.78)*	10.83	(3.39)*	18.39	(6.16)	< 0.002
Cortex	Onset to P1	8.21	(4.99)	7.52	(3.54)	5.41	(2.64)	6.29	(2.64)	NS
	P1 to N1	10.76	(7.59)	14.84	(7.67)	5.00	(2.16)*§	16.00	(5.61)	< 0.02
Hindlimb SEPs										
L5	Onset to LI	6.28	(2.37)	5.05	(1.77)	4.92	(2.15)	7.20	(3.28)	NS
	LI to LII	15.88	(3.56)	10.68	(5.34)	9.61	(4.10)	9.82	(6.07)	NS
Cortex	Onset to P1	7.25	(1.17)	3.22	(1.42)†	3.69	(1.38)†	4.01	(1.94)†	< 0.001
	P1 to N1	8.55	(4.24)	4.05	(1.94)†	3.30	(1.09)†	5.10	(1.92)†	< 0.005

* Significantly different from fentanyl/fluanisone–midazolam ($P<0.05$)
† Significantly different from ketamine–xylazine ($P<0.05$)
§ Significantly different from medetomidine ($P<0.05$)
NS = not signifcant

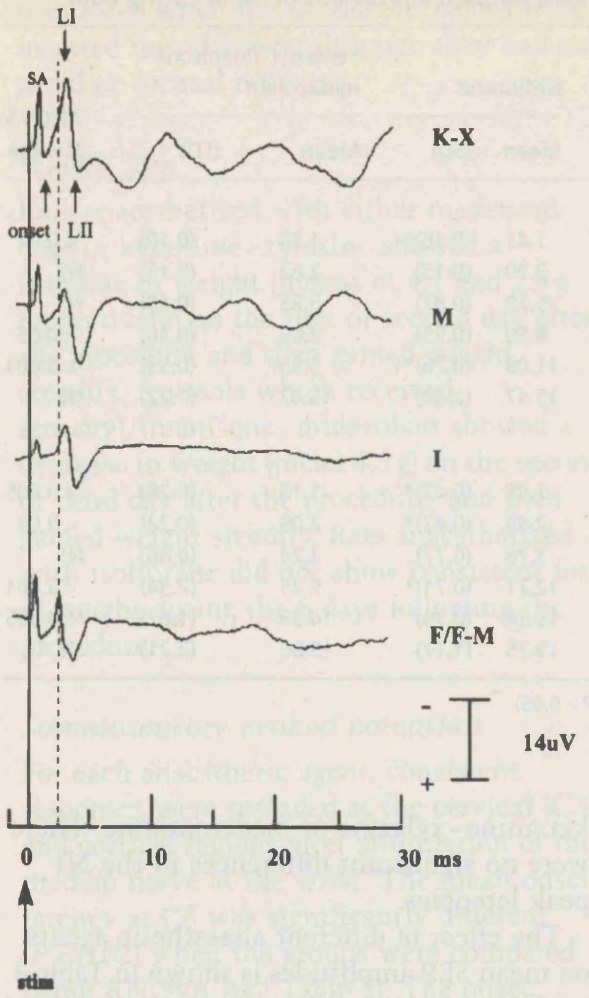

Fig 2 Averaged SEPs from four rats, recorded at L5 during anaesthesia with the four agents investigated. LI and LII represent the first and second peaks of the lumbar response and the solid line indicates the point of stimulus delivery. Note the earlier peak latency with fentanyl/fluanisone–midazolam compared to the other agents (indicated by the dotted line). The sharp peak (SA) preceding the lumbar SEP is a stimulus artefact. K–X = ketamine–xylazine; M = medetomidine; I = isoflurane; F/F–M = fentanyl/fluanisone–midazolam

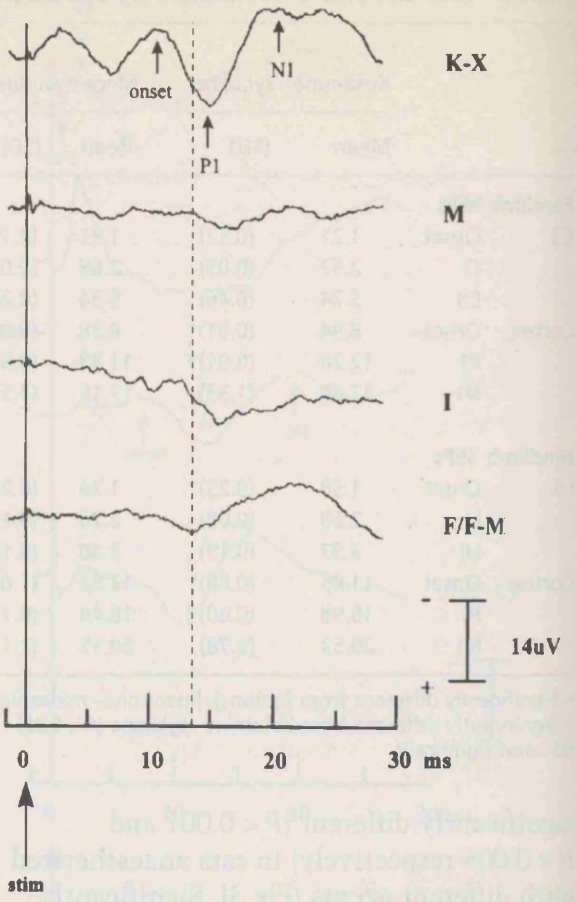

Fig 3 Comparison of averaged cortical SEPs from four rats, recorded following hindlimb stimulation. Note cortical SEP was of shorter latency during fentanyl/fluanisone anaesthesia (indicated by the dotted line). K–X = ketamine–xylazine; M = medetomidine; I = isoflurane; F/F–M = fentanyl/fluanisone–midazolam

cortical P1 to N1 responses showed significant differences in mean amplitude ($P < 0.05$, 0.002 and 0.02, respectively), whereas there were no significant differences in the amplitude of the cortex onset to P1 response. Following forelimb stimulation, fentanyl/fluanisone–midazolam gave significantly larger cervical CI to CII amplitudes than all the other agents and a larger C3 onset to CI amplitude than isoflurane. The cortical P1 to N1 wave amplitude was greater in rats given fentanyl/fluanisone–midazolam than those given isoflurane and was also greater in those given medetomidine than isoflurane. Following hindlimb stimulation there were no significant differences in amplitudes in the lumbar region, whereas significant differences were found between cortex onset to P1 ($P < 0.001$) and P1 to N1 ($P < 0.005$). Cortical (onset to P1 and P1 to N1) amplitudes were significantly higher in ketamine–xylazine anaesthetized animals, when compared with the other three drugs.

As part of a study to investigate the effect of different dietary intakes of vitamin E on neurological function, a group of control adult rats ($n = 12$) of similar weight to those assigned the different anaesthetic agents were studied longitudinally over 4 successive months. Table 4 shows the mean and

Table 4 Onset latencies (ms) of somatosensory evoked potentials recorded in adult rats during anaesthesia with fentanyl/fluanisone–midazolam on four successive months ($n = 12$)

	Age (months)							
	4		5		6		7	
	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)
Forelimb SEPs								
C3	1.38	(0.11)	1.36	(0.13)	1.39	(0.09)	1.40	(0.10)
Cortex	3.69	(0.58)	3.67	(0.35)	3.70	(0.42)	3.57	(0.25)
Hindlimb SEPs								
L5	1.29	(0.09)	1.22	(0.08)	1.27	(0.04)	1.25	(0.11)
Cortex	7.19	(0.54)	7.16	(0.78)	7.72	(0.78)	7.22	(0.89)

standard deviation of SEP onset latencies over a 4-month period. Responses were highly reproducible and there were no statistically significant differences between recordings.

Discussion

This study investigated the effects of four different anaesthetic agents on the latencies and amplitudes of SEPs in the rat. The early components of the SEP waves appeared to be more affected by the different anaesthetic agents than the later components.

In general, fentanyl/fluanisone–midazolam was found to have the least effect on the parameters measured, compared with the other anaesthetics tested. Midazolam, a water soluble imidazo–benzodiazepine, has previously been shown to produce only a small increase in the latency and decrease in amplitude of cortical SEPs (Sloan *et al.* 1990), and fentanyl given alone has been shown to have no effect (Loughnan *et al.* 1987). These results agree with the findings that indicate that the opioid fentanyl/fluanisone has a strong analgesic action (Thornton *et al.* 1992) and midazolam has mild hypnotic properties only (Sloan *et al.* 1990). It has been suggested (Thornton *et al.* 1992) that analgesia can affect structures situated between the medial lemniscus and the primary somatosensory cortex. Both the thalamus and primary cortical area appear to contribute to the generation of the N1 component (Chiappa, 1985), and this may account for fentanyl/fluanisone–midazolam having less effect on the latency of the P1 peak than the other agents, but not the N1 peak. Both of these previous studies were performed on humans, whereas the results of the present study show, for the first time, that this anaesthetic combination has little effect on SEPs in the rat. The data given in Table 4 showed that the latencies obtained with fentanyl/fluanisone–midazolam were consistent and reproducible, when the drug was used repeatedly over several months.

Isoflurane and medetomidine tended to have the greatest effect on the SEPs. In agreement with previous studies isoflurane increased the latencies and decreased the amplitudes of the responses (Sebel *et al.* 1986, Thornton *et al.* 1992), and we also found that medetomidine had similar effects. Basic pharmaco-physiological studies concerned with synaptic transmission have demonstrated that both drugs exert their effects by reducing the efficacy of pre- and post-synaptic transmission. Isoflurane predominantly affects glutamate-releasing neurones (Richter *et al.* 1977, Hablitz & Langmoen 1982), which results in a decrease in the degree of cortical activation by the primary afferent input. Medetomidine, a selective and specific full agonist of central and peripheral α_2-adrenoceptors, inhibits noradrenergic neurones in the locus coerulus (Virtanen 1989) causing a complete disruption of the signals passed from the ventrobasal thalamus to the cortex (Angel 1993).

Since a greater number of synapses have to be crossed to reach the higher regions of the tract, brainstem and cortical components are more affected than those generated more peripherally.

Ketamine is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist (Thomson *et al.* 1985, Yamamura *et al.* 1990) which binds to a site within the lumen of the NMDA-activated channel (MacDonald *et al.* 1987). Xylazine, like medetomidine, produces sedation by activation of central α_2-adrenoceptors in the locus coerulus (McGaraughty & Reinis 1993). The results of this study indicate that ketamine–xylazine had no significant effect on peripheral conduction. This observation is in agreement with earlier work carried out by Goss-Sampson and Kriss (1991), who suggested that ketamine–xylazine affects the sensory pathway beyond the level of the medial lemniscus. The cervical SEP response is associated with pre-synaptic activity at the level of the cuneate nucleus (CI) followed by post-synaptic activity at the level of the medial lemniscus (Claus *et al.* 1985). Central nerve conduction was affected by ketamine–xylazine, leading to an increase in cortical SEP latencies, although the amplitudes of the cortical SEPs were less affected. Changes in latency reflect the hypnotic component of anaesthesia (Thornton *et al.* 1992) and it appears that ketamine–xylazine may have a strong hypnotic effect, producing a profound loss of consciousness. Schubert and his colleagues (1990) found that earlier waves of the SEP were more resistant to the effects of ketamine and other anaesthetics. Our results, however, do not support this view as significant differences were found between the anaesthetics, when early wave latencies and amplitudes were compared.

When considering which anaesthetic agent would be the most appropriate for use in experimental longitudinal studies, it is necessary to find a balance between the general welfare of the animal, with respect to long-term deleterious effects, and the effects of the agent on SEPs which may confound the experimental variables studied. Rats which received ketamine–xylazine, medetomidine or fentanyl/fluanisone–midazolam lost weight in the days following the test procedure. This may have been caused by a decrease in food intake or by water loss as a result of evaporation from the respiratory tract. The former is a more likely explanation, since saline was administered to each animal to prevent a fluid deficit.

There were marked differences in the induction and recovery times between the groups. The route of administration of the anaesthetic agent affects the induction time, as this will dictate the rate of absorption. The differences in recovery times can be explained by the different rates of metabolism and tissue clearance of each drug.

Isoflurane, unlike the other anaesthetic agents, is gaseous which has the advantages that the animals do not require restraint and are thus subjected to less stress and, secondly, the depth of anaesthesia is easier to control. In addition, isoflurane is poorly soluble in blood and tissues, which aids in rapid recovery. However, isoflurane had a profound degrading effect on SEPs and was, therefore, rejected as a suitable anaesthetic for longitudinal studies of evoked potentials.

Medetomidine is an injectable anaesthetic agent, which is fast-acting and rapidly reversed with atipamezole. The reversible nature of this anaesthetic reduces the problems associated with a slow recovery, such as hypothermia. Our results, however, show that medetomidine severely affects SEPs (especially amplitude) and was, therefore, not considered suitable.

Ketamine–xylazine has been used previously in rats for this type of study (Goss-Sampson & Kriss 1991), since it was shown to have less effect on SEPs than pentobarbital. Indeed, the data presented here indicate that ketamine–xylazine has less deleterious effect than isoflurane or medetomidine. However, recovery was slow (mean 103.63 min) and external heat had to be applied to prevent hypothermia. The recovery time is somewhat longer than that reported by Goss-Sampson and Kriss (1991), who found that all the animals had recovered after 80 min, but we can offer no ready explanation for this difference. Ketamine–xylazine was not chosen for use in further electrophysiological studies because of the

deleterious effect on the welfare of the rats. In addition, the duration of anaesthesia provided by this agent was far longer than the 30 min needed to complete the experimental procedure.

The results of this study showed that fentanyl/fluanisone–midazolam generally had less effect on SEPs in the rat than the other anaesthetics tested. This combination produced surgical anaesthesia for up to 45 min and the effects of fentanyl can be reversed by administering buprenorphine. This reduces the recovery time and the risk of hypothermia. Furthermore, Flecknell (1996) stated that this anaesthetic combination was the agent of choice for rats. For these reasons fentanyl/fluanisone–midazolam was chosen as the most appropriate anaesthetic for longitudinal studies of evoked potentials in the rat. It is currently being used in detailed longitudinal studies of the effects of vitamin E deficiency in the rat.

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