UNIVERSITY OF CALIFORNIA, SAN DIEGO

Peripheral Neuropathy Caused by DCA-induced Oxidative Stress

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology

by

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The Thesis of Charlie Liou is approved, and it is acceptable

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Dedication

I dedicate this Thesis to my parents, sisters, and my friends, for their supports.

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ABSTRACT OF THE THESIS

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Professor Nigel Calcutt, Chair

Dichloroacetate (DCA) is water chlorination by product. Initial toxicology studies suggested that DCA can be carcinogenic, but the dosage found in water is much lower than hazardous. DCA activates pyruvate dehydrogenase and facilitates pyruvate metabolism to acetyl-CoA. DCA's ability to activate mitochondria and lower lactic level has attracted attention and it has been proposed as a therapeutic agent to treat lactic acidosis, mitochondrial diseases

and cancer. However, peripheral neuropathy has been reported in patients received DCA treatment. Peripheral neuropathy includes distal numbness, decreased nerve conduction velocity, thermal hypoalgesia, and allodynia. The underlying mechanism of DCA-induced peripheral neuropathy is unclear. We established animal models that mimic DCA-induced peripheral neuropathy to investigate the mechanism. Oxidative stress is observed in animals treated with DCA so we have examined the efficacy of known antioxidants. We found that ellagic acid can prevent DCA-induced peripheral neuropathy and that DHA has transient protective effects. In contrast, lipoic acid appeared to worsen DCA-induced peripheral neuropathy while fisetin appeared to have no effects. We have also begun to explore two pathways that may be involved in DCA-induced oxidative stress. Results from δ-ALA and 2-CP pathway are inconclusive and both studies are currently on-going. Oxidative stress in peripheral neurons from these animals will be measured. Finally, we investigated a potential cause of painful neuropathy in DCA treated rats. There was no difference in expression of spinal KCC2 transporters between control and DCA treated rats.

1 INTRODUCTION

1.1 Dichloroacetate as water contaminant

Dichloroacetate (DCA) is found in drinking water as a consequence of water purification methods. The first recorded use of water chlorination in the U.S. was by Chicago's Union Stockyards in September, 1908 (1). Since then, chlorination has become the most widely used and cost efficient way to provide clean water source (2). However, concerns have been raised that these disinfectants could produce harmful by-products (3). For example, reaction between chlorine and natural organic materials (NOM) leads to production of trihalomethanes, haloacids, and haloacetonitrilies as halogenated disinfection by-products (DBPs) (4)(5-8). Multiple studies have explored the toxicity of these DBPs (9) (4). DCA has received particular interest due to its high carcinogenic potency (10)(11). Some studies show that DCA is harmless and can be used as a chemotherapeutic drug (12, 13) (14) while other studies demonstrate that it can act as a hepatocarcinogen in rodents (11, 15-17). However, while the concentration of DCA in drinking water varies between studies (3)(5) (4), it is generally many orders of magnitude below the clinical dosage (18, 19).

1.2 The physicochemical property of DCA

Sodium DCA is odorless, bitter-tasting white compound that has a molar mass of 151 g/mol. Its pKa is 1.26 (20). It can be easily dissolved in water at 100 mg/ml and forms a colorless solution with a pH of approximately 7.

1.3 DCA metabolism

Less than 5% of DCA administrated to healthy volunteer is detected in urine unchanged or as its metabolites (21, 22). This suggests that DCA is rapidly metabolized and absorbed by the body. It has been shown that DCA can be concentrated in various tissues after administration (23). DCA is metabolized to glyoxylate, oxalate, and carbon dioxide. DCA is also biotransformed to glyoxylate by glutathione S-transferase zeta (GSTz) (24). Interestingly, DCA also rapidly inactivates GSTz which reduces the clearance rate of DCA and prolongs its activity (25). The glyoxylate derived from DCA is oxidized to oxalate by lactate dehydrogenase (26). Conversion of glyoxylate to carbon dioxide is catalyzed by α-ketoglutarate:glyoxylate carboligase (27). Oxalate inhibits gluconeogenesis from lactate (28, 29). DCA does not compete with pyruvate for coenzyme A and it is not converted to dichloroacetyl CoA by perfused rat heart (30).

1.4 DCA and pyruvate dehydrogenase.

Activation of pyruvate dehydrogenase by DCA has been recorded in *vitro* and *vivo*. Pyruvate dehydrogenase is the rate-limiting enzyme that oxidizes pyruvate to acetyl-CoA via pyruvate decarboxylation. Pyruvate dehydrogenase is regulated by end product inhibition, substrate activation, and reversible phosphorylation (31-33). With available ATP, pyruvate dehydrogenase kinase phosphorylates and inactivates pyruvate dehydrogenase, while pyruvate dehydrogenase phosphatase reverses this inhibition by dephosphorylation. DCA stimulates pyruvate dehydrogenase activity by inhibiting pyruvate dehydrogenase kinase and prevents inactivation by the kinase (34). Activation of pyruvate dehydrogenase by DCA is observed in liver, kidney, and other organs except the testis and small intestine (35-38). DCA can increase

pyruvate dehydrogenase complex activity more than one way. Beside inhibition of pyruvate dehydrogenase kinase, repeated drug administration of DCA to healthy rats increases pyruvate dehydrogenase activity (39). As mentioned above, DCA inactivates GSTz and therefore reduces its clearance rate. This auto-inhibition of DCA metabolism explains why repeated DCA administration can increase pyruvate dehydrogenase activity over time.

1.5 Physiological effects of DCA.

By changing activity of the pyruvate dehydrogenase complex, DCA has profound effects on pyruvate level in the bloodstream. Pyruvate is the key substrate required for gluconeogenesis. Oral administration of DCA reduces hyperglycemia in diabetic animals (20, 40). DCA reduces hyperglycemia by increasing glucose metabolism or inhibiting glucose production. It has been shown that DCA stimulates glucose oxidation in cardiac and skeletal muscle in healthy and diabetic animals (30, 41). In healthy human, DCA increases peripheral glucose oxidation and decreases glucose storage in liver (42)(41)(41). Another way DCA reduces hyperglycemia is by inhibiting glucose production. The DCA metabolite oxalate inhibits glucose synthesis by inhibiting pyruvate carboxylase, a rate-determining enzyme in gluconeogenesis (36, 43). Available glucose in the bloodstream is closely related with pyruvate and lactate levels. Glucose undergoes glycolysis and is broken down to pyruvate. Pyruvate is an important molecule used for energy production. During oxidative phosphorylation, pyruvate dehydrogenase converts pyruvate to acetyl-CoA and acetyl-CoA enters Kreb cycle to generate ATP. Electron acceptor NADH has to be recycled to NAD^+ in order to continue glycolysis. During aerobic conditions, NADH donates its electron to oxygen in electron transport chain. In contrast, under anaerobic conditions, NADH donates its electron to pyruvate to generate NAD^+. This

affects lactate levels in the system. By receiving the electron, pyruvate is reduced to lactic acid. Lactic acid can be oxidized and converted back to pyruvate in muscle. Lactic acid can also enter the Cori cycle for gluconeogenesis in liver.

DCA also directly affects lactate levels. Pyruvate is in equilibrium with lactate and alanine in our body. DCA can decrease lactate concentration systemically within 60 minutes of drug administration (21). One proposed mechanism that DCA can reduce lactate concentrations is by shifting the equilibrium between pyruvate and lactate. DCA activates pyruvate dehydrogenase which facilitates the breakdown of pyruvate to acetyl-CoA. Activated pyruvate dehydrogenase thus shifts cellular respiration from glycolysis towards Kreb cycle. Production of pyruvate is reduced due to this shift and more pyruvate is broken down to acetyl-CoA and metabolized in mitochondria. This decreases pyruvate concentration. Since pyruvate and lactate are in equilibrium, lactate is converted to pyruvate to maintain the equilibrium. As a result, overall lactate concentration is reduced. Another mechanism for DCA to reduce blood lactate levels is by inhibiting lactate production. DCA activates pyruvate dehydrogenase via kinase inhibition. It has been shown that activated pyruvate dehydrogenase decreases production of lactate and alanine in peripheral tissues (44, 45).

1.6 DCA as a therapeutic agent

DCA has been proposed as a therapeutic agent to treat lactic acidosis. Lactic acidosis occurs when mitochondria are unable to meet the energy demand during hypoxia and cells have to rely on glycolysis for energy production. Excess pyruvate produced by glycolysis is converted to lactic acid, which acidifies tissue and blood. Lactic acidosis also occurs in a variety of diseases,

including those caused by genetic mutations in mitochondria that impair mitochondrial function such as mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS). The proposed mechanism of action by which DCA may counter lactic acidosis is that DCA activates the pyruvate dehydrogenase complex and accelerates metabolism of pyruvate and lactate to acetyl-CoA, therefore reducing lactate levels in blood. The use of DCA to treat lactic acidosis has provided mixed results in clinical trials. One study showed that 3 months of DCA treatment at 25-50 mg/kgevery 12 hours to 3 MELAS patients successfully lowered their serum lactate levels and concurrently reduced headache and abdominal pain in these patients (46). Another study showed that DCA helped reduced arterial blood lactate level in 7 out of 11 patients with lactic acidosis (47). In contrast, one study showed inconclusive results when DCA was given to patients with lactic acidosis (48) while a 6-month study of 25 hyperlactatemia patients between 0.9 to 19 years old who were treated with DCA at 12.5 mg/kgevery 12 hours for 6 months showed no significant differences in Global Assessment Treatment Efficacy (GATE) scores (49). A 24-month study with 15 MELAS patients who received DCA at 25 mg/kg/day also found no significant difference in average GATE scores between treatment and placebo groups but was terminated prematurely due to severe peripheral neuropathy (50). Indeed, regardless of the conflicting results regarding the efficacy of DCA to relieve lactic acidosis, most studies of DCA treatment reported that patients experienced some form of peripheral neuropathy such as the nerve conduction velocity slowing seen in 12 patients who received oral DCA treatment at 50 mg/kg/day for 12 months (51). A young adult with hypercholesterolemia who received DCA daily at 50 mg/kg for four months experienced mild weakness in muscle and decreased nerve conduction velocity, which was reversed in several weeks after cessation of treatment (52). This

neurotoxicity restricts both the maximal dose of DCA that can be used and slow the duration of treatment, and may impede use of DCA as a viable therapeutic agent. The main focus of my research project is to identify the mechanism of DCA-induced neuropathy.

1.7 DCA toxicity

The single dose LD_{50} of sodium DCA in most species is 1 to 5 g/kg by mouth or 0.5 to 1 g/kg by intravenous or intraperitoneal routes (20, 53). In *vitro*, DCA binds to glutathione S-transferases from rat liver, which decreases its clearance rate and can prolong its activity in *vivo* (54). Studies have demonstrated that DCA can increase carcinomas in male B6C3F mice, male F334 rats, and female B6C3F mice at a daily dose of 80-800 mg/kg (55-57). It has also been suggested that unpurified DCA can be mutagenic, but purified DCA have failed to demonstrate any mutagenic effects (58-60) and DCA has a good safety profile in clinical studies. The clinical trials described above suggest that DCA can cause peripheral neuropathy and there have been a number of studies that have attempted to replicate this finding in animal models. Edema and demyelination in CNS were observed when rats were exposed to high doses of DCA (61) and chronic dosing exceeding 1 g/kg lead to weight loss (62). Animals that received DCA for several weeks also showed reversible hind limb weakness, decreased nerve diameter and decreased peripheral nerve conduction velocity (61, 63, 64). These studies suggest that DCA neurotoxicity in rodents incorporates damage to both the CNS and PNS.

The Calcutt laboratory has recently begun to develop and characterize rodent models of DCA-induced peripheral neuropathy that lack the CNS damage, as the latter is not noted during clinical use. DCA was given by oral gavage at doses of 50-500 mg/kg daily to both juvenile and

adult rats. Juvenile rats did not develop any nerve disorders after 16 weeks of DCA treatment. In contrast, adult rats developed tactile allodynia and a dose-dependent motor nerve conduction velocity (MNCV) slowing after 8 weeks of treatment (65). A dose-dependent reduction in mean axonal diameter of the sciatic nerve from adult rats was also observed. Axonal caliber is a major determinant of conduction velocity in myelinated fibers (66) and is likely to contribute to MNCV slowing in DCA-treated rats. Aside from these functional and structural indices of large fiber neuropathy, DCA-treated rats also developed loss of sensation to a heat stimulus (thermal hypoalgesia) that reflects dysfunction of small unmyelinated sensory fibers. This model of DCA-induced neuropathy therefore shows both sensory and motor nerve dysfunction (65).

Recent studies have also developed mouse models of DCA-induced neuropathy. DCA was given to mice at 0.25, 50, 100, 500, and 1000 mg/kg via oral gavage. MNCV slowing was observed in mice that received 1000 mg/kg /day DCA developed MNCV slowing within 4 weeks of onset of treatment along with paw thermal hypoalgesia (Calcutt, unpublished observations). The mouse therefore offers a more rapid onset of both motor and sensory nerve dysfunction after DCA treatment

1.8 Mechanisms of DCA induced peripheral neuropathy

The mechanisms by which DCA causes peripheral neuropathy are not known. However, one plausible mechanism is suggested by the observation that DCA treated rats showed elevated levels of the oxidative stress markers malodialdehyde and 4-hydroxynonenal in the peripheral nerve after 8 weeks of daily DCA at 500 mg/kg (65). Oxidative stress contributes to nerve damages in a variety of diseases (67). We have therefore proposed that oxidative stress

could cause indices of peripheral neuropathy in DCA-treated rodents. Further, as it is also unclear how DCA treatment might induce oxidative damage to peripheral nerve, we have proposed that at least two mechanisms are possible:

1. DCA activates pyruvate dehydrogenase, which facilitates breakdown of pyruvate to acetyl-CoA in mitochondria. More acetyl-CoA enters Kreb's cycle and more reactive oxygen species (ROS) are produced by thein electron transport chain during oxidative phosphorylation, leading to a shift in the endogenous antioxidant-ROS balance.

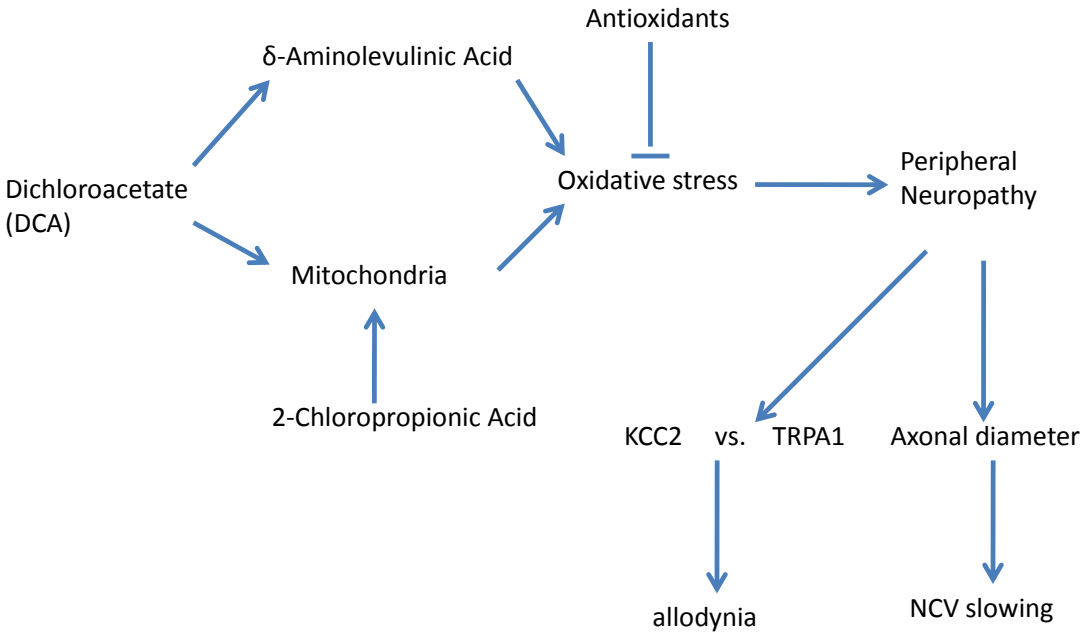
2. DCA enhances production of the heme precursor and pro-oxidant δ-ALA (68).

1.9 Aims:

The experiments presented in this thesis investigate the role of oxidative stress in mediating DCA-induced peripheral neuropathy by:

1. Determining whether antioxidants can prevent DCA-induced neuropathy in rodents.

2. Determining the underlying mechanisms by which DCA causes oxidative stress, with a focus on two potential pathways:


Antioxidants
δ-Aminolevulinic Acid
Dichloroacetate
(DCA)
Oxidative stress
Peripheral
Neuropathy
Mitochondria
2-Chloropropionic Acid
KCC2
vs.
TRPA1
Axonal diameter
allodynia
NCV slowing

2 MATERIALS AND METHODS

2.1 ANTIOXIDANT TREATMENT

I have used a variety of known antioxidants to test the hypothesis that preventing oxidative stress in DCA-treated rodents can also prevent peripheral neuropathy:

2.1.1 Lipoic acid

Lipoic acid (1,2-Dithiolane-3-pentanoic acid) was first discovered in 1937(69) but was not successfully isolated until 1951 (70). Lipoic acid can be synthesized by plants and animals (71) and is found in both the membrane and cytosol due to its solubility in water and lipid (72). 200 mg of lipoic acid in aqueous solution has an estimated 38% R form and 28% S form (73).

Lipoic acid is rapidly absorbed when administrated orally with a Tmax of 0.5 to 1 hour (74) and is capable of crossing the blood brain barrier(75).

Lipoic acid is an important cofactor for pyruvate dehydrogenase (E1) in the pyruvate dehydrogenase complex, which catalyze oxidative decarboxylation of pyruvate during aerobic metabolism (76). Lipoic acid is reduced to dihydrolipoic acid intracellularly by mitochondrial dihydrolipoamide dehydrogenase and cytosolic glutathione reducatse (77)(78)(75)(75). Lipoic acid is metabolized in the liver (79).

Although lipoic acid itself has some antioxidant effects, such suppressing formation of hydrogen peroxide and reactive hydroxyl radical (80), it is more valuable in its ability to recycle other oxidized radical scavengers such as glutathione, ascrobate (vitamin C), and tocopherol (vitamin E) (81). It was later shown that dihydrolipoic acid is responsible for recycling other antioxidants. Lipoic acid was found to be able to prevent symptoms of vitamin E deficiency (82).

Dihydrolipoic aicd has more antioxidant properties than lipoic acid due to its highly reactive dithiol structure. Dihydrolipoic acid is capable of scavenging peroxyl radical directly in either aqueous or membrane, whereas lipoic acid cannot (83). It is also a strong reductant that can regenerate oxidized antioxidants (84).

Free-radical damage can be induced by transition metals by catalyzing decomposition of hydroperoxides and generation of toxic hydroxyl radicals. Some studies speculate that metal chelating activity of lipoic acid is what contributes to its antioxidant activity (85). This could explain the beneficial effects of lipoic acid in heavy metal poisoning (86, 87).

In addition to antioxidant properties, lipoic acid also recruits the glucose transporter-4 to plasma membrane, thereby increasing glucose uptake via an insulin-stimulated pathway (81). Studies have shown that lipoic acid can prevent some forms of peripheral neuropathy (88, 89).

2.1.2 Ellagic acid

Ellagic acid, 2,3,7,8-Tetrahydroxy-chromeno(5,4,3-cde)chromene-5,10-dione, is a polyphenol antioxidant found in vegetables and fruits. Ellagic acid is a structural component of plant cell walls and its hydrolizable tannin form called ellagitannins is present in cell membranes (90). High concentrations of ellagic acid can be found in pomegranate and berries such as strawberries, raspberries, cranberries, and grapes (91, 92). Ellagic acid has many beneficial effects in our body and it has been reported that ellagic acid show antioxidant, anti-inflammatory and anticarcinogenic activities in mammalian systems (93, 94).

The anticancer activity of ellagic acid may involve inhibiting formation of carcinogenic molecules (95), or preventing carcinogens from binding DNA to achieve antimutagenic effects

(96). There are also a number of mechanisms by which ellagic acid can act as an antioxidant. For example, ellagic acid can scavenge ROS directly (97) or remove ROS indirectly by stimulating endogenous antioxidant enzymes such as superoxide dismutase, catalase or glutathione transferase (98, 99). However, some studies have found that ellagic acid has no effects on catalase or superoxide dismutase activities in mice (100). Another way for ellagic acid to exert its antioxidant property is by reducing activity of pro-oxidative enzymes. In a study using mice, ellagic acid in drinking water decreased NADPH- and ascrobate-dependent lipid peroxidation (101). Similarly, there was significantly reduced lipid peroxidation and increased glutathione and total antioxidant status in human serum that were treated for 12 months pomegranate juice which containing 121 mg/L of ellagic acid (102). Together, these antioxidant effects prompted us to investiage the ability of ellagic acid to prevent oxidative stress caused by DCA.

2.1.3 Fisetin

Fisetin, 3,7,3',4'-tetrahydroxyflavone, is a flavinoid, one of a series of low molecular weight polyphenolic compounds that can be found in fruits, vegetables, and plants. Flavonoids have antioxidant, anti-inflammatory, and anti-caricinogenic effects in *vitro* (103-105). However, the mechanism of action for flavonoids remains unclear. Initial studies suggest that flavonoids exert their antioxidant ability by directly donating hydrogen thus stabilizing free radicals (105, 106). Flavonoids can protect neurons from oxidative stress more effectively than ascorbate (107). However, the ability of flavonoids to inhibit tumor growth via apoptosis or down-regulate pro-inflammatory cytokines suggests that flavonoids are more than a simple scavenging antioxidant (104, 108, 109). Flavonoids can interact with several intracellular signaling pathways. Flavonoids inhibit nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) production, a

transcription factor that regulates pro-inflammatory genes expression (110, 111). Flavonoids also modulate mitogen-activated protein kinase (MAPK) signaling cascades (112)(110-112)(110-112). Interactions between flavonoids and mitochondrial ATPase, protein kinase A, and protein kinase C have also been observed (113-115). Some flavonoids are capable of interacting with rat $GABA_A$ receptors binding sites in rat brain (116).

Fisetin exhibits its anti-inflammatory ability in multiple ways. Fisetin down-regulates NFκB (117). Fisetin can inhibit IL-4 and IL-13 production in human basophils and may alleviate allergy (117). In human mast cells, fisetin inhibits gene expression and production of IL-1, IL-4, IL-6, IL-8, and tumor necrosis factor-α (TNF- α) (118). It was reported that fisetin can suppress lipopolysaccharide (LPS)-induced microglial activation and inhibit gene expression of COX-2 (119). Some view COX-2 as an important anti-cancer target because COX-2 produces prostaglandin H_2 (PGH_2), which is converted to prostaglandin E_2 (PGE_2) by prostaglandin E_2 synthase. PGE_2 stimulates cancer progression (120). Inhibition of COX-2 can prevent PGE_2 production and slow cancer progression. This may explain fisetin's anti-cancinogenic ability (121, 122).

There are conflicting results on fisetin's ability to protect neurons from oxidative stress. Fisetin plays a similar role as α -tocopherol (vitamin E) in isolated microsomes from rats as a chain-breaking antioxidant and assist glutathione in scavenging free radicals (123). On the other hand, fisetin did not show any neuroprotective properties in the 6-hydroxydopamine rat model for Parkinson's disease. (124). Fisetin can increase basal glutathione level by 30-80% and block hydrogen peroxide toxicity to protect cultured mouse hippocampus cells from oxidative stress-

induced death (125). It appears that there is emerging evidences to support fisetin as viable antioxidant.

2.1.4 Docosahexaenoic acid

Docosahexaenoic acid (DHA) is an omega-3 fatty acid that is rich in brain, especially in neuronal cell membrane and retinols (126). DHA is essential for early stage brain development (127) and is highly conserved in neuronal membranes. DHA deficit during development has been associated with decrease learning, loss in spatial memory and olfactory based learning (128, 129). The sperm is another cell that has enriched DHA in its membrane. It has been shown that DHA is retained by membrane phospholipid in neuronal cells and unaffected by low dietary intake (130, 131). It takes two generations of low DHA diet to see a 50-80% decline of DHA in brain and retinal (132).

DHA plays an important role in the nervous system. Phosphatidylserine is a negatively charged phospholipid in cell membrane that is responsible for many signaling pathways and interacts with protein kinase (133, 134). A high content of DHA in cell membrane increases phosphatidylserine synthesis whereas depletion of DHA leads to a decrease if phosphatidylserine in membranes (135-137). Thus, changes in the DHA concentration affect phosphatidylserine in cell membrane which in turn can impact intracellular signaling pathways.

DHA can have neuroprotective effects. After a prolonged incubation, DHA is slowly incorporated into phosphatidylserine and overall phosphatidylserine content is increased in membranes of cultured neurons. DHA exhibits its protective effects by inhibiting neuronal apoptosis (138)(138, 139)(138, 139). Caspase-3 has been shown to mediate mammalian

apoptosis. Cells enriched with DHA have lower caspase-3 mRNA levels (139). This suggests that neuronal cells accumulate DHA to ensure survival. DHA also has anti-inflammatory activities. Natural killer cell activity is inhibited by DHA and the production of pro-inflammatory cytokines IL-1 and TNF-α are significantly reduced (140). It has been reported that DHA can promote apoptosis in cancer cells (141), although it is unclear how DHA can selectively target different cells for pro- or anti-apoptosis. Nonetheless, it is likely that DHA exerts its neuroprotetive ability by inhibiting production of pro-inflammatory cytokines and apoptotic factors in neuronal cells.

As a polyunsaturated fatty acid containing double bonds, DHA is susceptible to peroxidation. A study showed that healthy men who received 6 week of DHA treatment had increased lipid peroxidation (142). Rats fed fish oil for 3 weeks also had greater phospholipid hydroperoxide accumulations in plasma, liver, and kidney (143). 4-Hydroxyhexenal is a highly reactive cytotoxic molecule that is produced when DHA is oxidized (144). It is a specific product from DHA peroxidation and can be used as a biomarker. DHA also induces oxidative damage in bone marrow DNA (145).

In our study we chose DHA as a potential drug to counter DCA induced neuropathy because DHA is an important component for the neuronal membranes and could assist neurons against DCA-induced oxidative stress.

2.2 PRO-OXIDANT TREATMENT

I also treated normal rats with other agents in order to mimic the effects of DCA on peripheral nerve and determine the involvement of these metabolites in the pathogenic mechanism of DCA induced neuropathy:

2.2.1 δ -aminolevulinic acid

δ –aminolevulinic acid (δ-ALA) is the first compound in the heme synthesis pathway (146). It has been shown that children with the mitochondrial disorder MELAS who are treated with DCA have elevated δ -ALA in their urine (147). DCA is dechlorinated by glutathione S-transferase, which is identical to maleyacetoacetate isomerase, the penultimate enzyme in the tyrosine catabolic pathway catalyze the isomerization of maleylacetoacetate to fumarylacetoacetate (148). DCA binds and depletes maleyacetoacetate isomerase, causes accumulation of succinylacetone. Succinylacetone competitively inhibits δ-aminolevulinate dehydratase and leads to accumulation of δ-ALA (68).

δ -ALA is a known structural analog of γ-aminobutyric acid (GABA) and interaction with the GABA receptor has been reported (149). Some have suggested that the potentially reactive keto group on the molecule makes δ-ALA capable of damaging GABA receptors, mitochondria, and other tissues (150-152). GABA is an inhibitory neurotransmitter. By damaging GABA receptors, the inhibitory signals are diminished. δ -ALA's interaction with the GABA receptors could explain DCA-induced allodynia. Co-administration of antioxidant with δ-ALA can reduce CNS toxicity, linking its pro-oxidant property to the CNS damage (153, 154).

As δ -ALA can induce oxidative damage in rat CNS (155) and damage GABA receptors, we investigated whether we can reproduce DCA-induced neuropathy by administrating δ -ALA to the rodents.

2.2.2 2-Chloropropionic acid

2-chloropropionic acid (2-CP) has very similar chemical composition to DCA, having one less chloride. Both DCA and 2-CP can activate pyruvate dehydrogenase complex (156). 2-CP differs from DCA because it does not get metabolized to glyoxylate or oxalate (157, 158). We have used 2-CP to activate the pyruvate dehydrogenase complex and generate excess free radicals via the electron transport chain without concurrent generation of glyoxylate or oxalate (159). Using 2-CP allows us to examine whether production of free radicals from mitochondria is sufficient to cause neuropathy. 2-CP-induced toxicity has previously been reported (63) and animals that received 2-CP at 750 mg/kg/day developed ataxia and necrosis in the cerebellum (160, 161). This 2-CP-induced cerebellar necrosis could be prevented by a non-competitive NMDA receptor antagonist MK-801, suggesting NMDA receptor mediated mechanism (162). (162, 163)(162, 163)Reduction of glutathione concentration in brain and liver after 2-CP administration has also been reported. Vitamin E provides incomplete but significant protection against 2-CP induced neurotoxicity (164). Increased GABA concentrations in the cerebellum after 2-CP administration have also been reported (163).

Both 2-CP and DCA can activate pyruvate dehydrogenase and produces excess free radical. However, 2-CP does not get metabolized to glyoxylate or oxalate and it does not cause the accumulation of δ -ALA. Therefore, 2-CP allows us to investigate whether DCA-induced δ -

ALA accumulation or excess free radical production from the electron transport chain in mitochondria underlies DCA-induced neuropathy.

2.3 MEASURING PERIPHERAL NEUROPATHY

I used a mouse model of DCA-induced neuropathy to focus on efficacy of potential therapeutic against measures of degenerative neuropathy such as MNCV slowing (large fiber dysfunction) and thermal hypoalgesia (small fiber dysfunction). Later studies using a rat model of DCA-induced neuropathy allowed me to also study mechanisms of painful neuropathy and collect tissue for biochemistry assays. A combination of behavioral and physiological assays were used to measure nerve disorders in rodents:

2.3.1 Tactile withdrawal threshold

Rats were removed from their cages and placed in a plastic chamber on top of a wire mesh grid suspended over a table. Rats could move freely within the plastic chamber and they were allowed 15 minutes to acclimatize before the experiment. Paw withdrawal thresholds were assessed by using calibrated nylon von Frey filaments with the following logarithmic sizes: 3.84, 4.08, 4.31, 4.56, 4.74, 4.93, and 5.18, representing and applied force of between 0.41 and 15 g (165). Starting with the 4.31 filament, each filament was orientated to the center of the hind paw and pressure applied until the filament bowed for 5 times to produce a constant and reproducible force. This force is proportional to the fiber diameter and inversely proportional to the length. A positive response was recorded when the rat flinched its paw away from the filament, or lifted up and licked its paw. If the rat did not respond to the filament, a negative response was recorded. After a positive response, the next lightest filament was used. If a

negative response was recorded, the next heaviest filament was used. The process was carried out until 4 responses were measured after the first change in response, or when the maximum or the minimum filament of the series was reached.

The 50% paw withdrawal threshold was calculated using this equation: 50% withdrawal threshold (g) = $(10^{(x_f + k\delta)})/10{,}000$, where x_f = the logarithmic value of the final von Frey filament applied, k = a tabular value from Chaplan et al., 1994 and δ = the mean difference in log units between the filaments used. A paw withdrawal threshold less than 5g is, by convention, considered as tactile allodynia.

2.3.2 Thermal response latency

Rats were placed in a plastic chamber on top of thermal testing apparatus (UARD, San Diego, CA, USA) and allowed 15 minutes to acclimatize to the glass surface, which was warmed to 30°C. A mobile heat source was maneuvered below the center of the hind paw and turned on. Once turned on, a timer was activated and the heat source warmed the glass at a rate of 1 °C/s. Withdrawal of the hind paw triggered a movement sensor, causing the timer to stop and shutting off the heat source. To prevent any tissue damage, the heat source was programmed to automatically cut off after a maximum of 20 seconds. Each paw was measured 4 times with a minimum of 5 minutes intervals. The first measurement represented a conditioning exposure, and the median of the final 3 values was used to represent the value for that paw.

2.3.3 Nerve conduction velocity

Nerve temperature was maintained at 37 °C with a heat lamp and warm-water pad. A PowerLab 4/30 (AD Instruments) was used to deliver suprathreshold, monophasic electrical

stimuli (50 V amplitude and 50 µs duration) to an electrode inserted initially at the sciatic notch and then the ankle. Another electrode was inserted behind the neck of the rat to serve as an electrical ground. A second pair of needle electrodes was placed in the interosseous muscles of the ipsilateral foot of the rat, to record evoked responses via Neuro Amp (AD Instruments) and Scope 4 installed on a computer. The procedure was repeated 3 times. The distance between stimulation sites with extended hindlimb was measured. Motor NCV, expressed in m/s was calculated by dividing the distance between stimulation sites at the sciatic notch and ankle by the median latency differences between M wave responses evoked from the ankle and sciatic notch.

2.3.4 Formalin-evoked flinching

50 µl of 0.5% formalin solution was injected sub-dermally into the hindpaw dorsum of rats. After injection, rats were placed in an observation chamber and allowed to move freely. Flinching behavior was counted in 1 min blocks every 5 minutes for one hour.

2.3.5 Biochemistry tests

Spinal cord was obtained after rats were anaesthetized and decapitated. Lumbar segments were collected and kept on ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5%Triton X, protease inhibitor cocktail) and homogenized before centrifugation (14,000g). The supernatant was incubated in 37˚C water bath for 30 minutes in Laemmli LDS sample buffer (Invitrogen, Carlsbad, CA, USA). 15 µg of protein was separated on 4-12 % SDS-PAGE Bis-Tris gels (Novex, Invitrogen, Carlsbad, CA, USA) and immunoblotted on nitrocellulose membrane. Membrane was incubated with anti-KCC2 (1:2000) or anti-actin

(1:5000), followed by anti-rabbit or anti-mouse secondary antibody (1:20,000). Blots were developed using an ECL western-blotting protocol (Enhanced Chemiluminescence, Lumilight Roche Applied Science, Indianapolis, IN, USA). Immunoreactivity was quantified by densitometric scanning using Quantity One software (BioRad, San Diego, CA, USA). The intensity of each band was adjusted by calculating the ratio between KCC2 to actin.

2.3.6 Statistical Analysis

When there are only two groups, two-tailed unpaired t test were used to for statistical analysis. When there are more than two groups, we used one-way ANOVA and Tukey's post-hoc test for statistical analysis.

3 RESULTS

Various antioxidants were tested for effects against DCA-induced peripheral neuropathy. Peripheral neuropathy was assessed by behavioral (tactile withdrawal threshold and thermal withdrawal latency) and electrophysiological (motor nerve conduction velocity: MNCV) tests.

3.1 EFFICACY OF ANTIOXIDANT

3.1.1 Ellagic Acid in mice

Swiss Webster mice that received daily DCA at 1 g/kg via oral gavage for four weeks had significantly ($P < 0.001$) reduced MNCV compared to control mice. Mice that received DCA also developed thermal hypoalgesia ($P < 0.001$) compared to control mice. Co-administration of ellagic acid at 75 mg/kg/day with DCA to mice for four weeks completely prevented the DCA-induced MNCV deficit and thermal hypoalgesia ($P < 0.05$ vs. DCA-treated mice: Figure 1).

3.1.2 Lipoic Acid in mice

Swiss Webster mice that received daily DCA at 1 g/kg via oral gavage for four weeks had significantly ($P < 0.05$) reduced MNCV compared to the control mice. Mice that received DCA also developed thermal hypoalgesia ($P < 0.05$). Co-administration of lipoic acid at 10 mg/kg/day or 100 mg/kg/day with DCA to mice for four weeks showed no ability to prevent the DCA-induced MNCV deficit or thermal hypoalgesia and indeed mice that received lipoic acid with DCA had a tendency to slower MNCV than mice that received DCA alone (Figure 2).

3.1.3 Docosahexaenoic Acid and fisetin in mice

Swiss Webster mice that received docosahexaenoic acid (DHA) twice per day via oral gavage at 50 mg/kg had partially ($P > 0.05$) reduced MNCV compared to control mice at the end of eight week study (Figure 3). A separate group of mice were fed with fisetin diet. Mice consumed 3.5 g of 500 ppm fisetin diet per day. For a mouse that weighs 25 g, this is equivalent to 70 mg/kg/day of fisetin. There were no difference in MNCV between control mice and control mice that consumed fisetin diet.

Swiss Webster mice that received daily DCA at 1 g/kg via oral gavage for eight weeks had significantly ($P < 0.001$) slower MNCV compared to the control mice. Mice that received both DHA and DCA had significantly ($P < 0.05$) faster NCV than mice that received DCA alone (Figure 4). Fiseitin diet exhibited no effects on preventing NCV deficit caused by DCA. We failed to observe significant thermal hypoalgesia in DCA treated mice in this study.

3.1.4 DHA in rats

Sprague Dawley rats received daily DCA at 500 mg/kg via oral gavage for 14 weeks. DCA treatments prevented rats from gaining weight ($P < 0.05$ vs. control: Figure 5). Rats that received DCA also showed limb weakness and lost the sensory (H) wave of the electromyogram by eighth week of the study. Rats that received DCA developed a MNCV deficit compared to control rats by week 8 ($P < 0.05$). Rats that received DHA at 50 mg/kg every 12 hours along with DCA appeared to have faster, though not statistically significant, NCV compared to rats that received only DCA, suggesting that DHA might have some partial effects combating DCA-induced MNCV slowing. By week 14, both DCA alone and DCA with DHA groups exhibited MNCV slowing and

the MNCV values showed no difference between these two groups. Animals that received DHA alone appeared to be developing a MNCV deficit between weeks 8 and 14, although this decrease was not statistically significant at week 14. Rats received DCA treatment for 14 weeks developed thermal hypoalgesia compared to control rats ($P < 0.05$).

3.1.5 Formalin-evoked flinching in DCA rats

Beside tactile withdrawal threshold, thermal response latency, and nerve conduction velocity, we also measured formalin-evoked flinching in these rats. 50 µl of 0.5% formalin solution was injected sub-dermally into the hindpaw dorsum of control and DCA only rats. Formalin-evoked flinching behaviors were counted at 5 minutes block for an hour. We observed that rats treated with DCA flinched more than control rats in both phase I ($P < 0.01$ vs. control) and phase II ($P < 0.01$ vs. control: Figure 10).

3.2 TREATING WITH PRO-OXIDANT

3.2.1 δ-ALA in rats

δ-ALA was dissolved in phosphate-buffered saline, and the pH was adjusted to 7 with sodium hydroxide. Sprague dawley rats received δ-ALA via intraperitoneal (i.p.) injection every other day at 40 mg/kg during first 12 weeks of the study. The dosage was doubled to 80 mg/kg after week 12. At week 8, there seems to be a trend in our tactile withdrawal threshold data suggesting that animals received δ-ALA were developing allodynia, but this trend disappeared in week 12 (Figure 7), and resurfaced in week 16, after doubling the dosage to 80 mg/kg for the last 4 weeks. Our thermal response latency data showed that animals received δ-ALA had statistically significantly lower thermal response threshold at week 8, suggesting that animals

received δ-ALA were developing thermal hyperalgesia, but this difference disappeared in week 12 and 16 (Figure 6-8). Our NCV data showed no difference between control and δ-ALA treated group at weeks 8, 12, or 16.

3.2.2 2-CP in rats

Sodium hydroxide was used to adjust 2-CP pH to 7. The solution was further diluted with dH_2O. One group of Sprague Dawley rats received daily DCA at 500 mg/kg via oral gavage, while another group of sprague dawley rats received 50 mg/kg of 2-CP per day via oral gavage. The 2-CP group received 500 mg/kg and 100 mg/kg of 2-CP for one day. Several rats were found dead the next day and more rats were sick, forcing us to lower the dosage to 50 mg/kg to keep the remaining rats alive. After 8 weeks of treatment, rats that received DCA developed a significant ($P < 0.05$ vs. control) MNCV deficit compared to the control rats (Figure 9). MNCV measured in 2-CP treated rats was not different from that of control rats. Thermal latency response tests showed that rats that received DCA developed significant ($P < 0.01$ vs. control) thermal hypoalgesia at week 8, whereas paw thermal latency in 2-CP treated rats was normal. Neither DCA treated rats nor 2-CP treated rats showed tactile allodynia at week 8.

3.3 PROTEIN EXPRESSION

3.3.1 KCC2 transporters expression

Spinal cord segment was obtained from rats that received 14 weeks of daily DCA treatment at 500 mg/kg. Rats treated with DCA appeared to have more KCC2 protein expression compared to control rats, but the difference wase not significant (Figure 11).

4 DISCUSSION

4.1 ANTIOXIDANTS

We used our recently established mouse model that mimics the DCA-induced peripheral neuropathy seen in humans to screen multiple antioxidants in combined treatment with DCA to assess their ability to counter DCA-induced oxidative stress. We chose mice as our animal model because their neuropathy progressed faster than rats. Smaller quantities of drugs are also required due to shorter study time and the smaller animal size. Once we found an antioxidant that showed some effects, the study was repeated in rats to obtain more samples for biochemistry studies.

Nerve conduction velocity is determined by axonal diameter and reduction of axonal diameter had been found in DCA-treated rats (65). In our mouse model, mice that received DCA at 1 g/kg/day for four weeks developed slowing of motor nerve conduction velocity (MNCV). Mice that received daily ellagic acid at 75 mg/kg along with 1 g/kg of DCA did not develop MNCV slowing compared to the mice who received DCA alone. This data suggests that ellagic acid can prevent DCA-induced peripheral neuropathy. It is possible that the efficacy of ellagic acid is mediated by its antioxidant capacity, as ellagic acid can scavenge ROS directly and stimulate endogenous anti-oxidative enzymes activity (98, 99). However, we cannot test this hypothesis in mice, as not enough nerve tissue was available for standard biochemical assays of lipid oxidative damage. We have therefore begun a parallel experiment in rats treated with DCA and ellagic acid. Preliminary data indicates that ellagic acid again prevented MNCV slowing in DCA-treated rats and this was associated with prevention of oxidative damage to lipids in the nerve and spinal cord (Calcutt and Stacpoole, preliminary data). This offers supportive data to our

hypothesis that the efficacy of ellagic acid is mediated by anti-oxidant properties. Current studies in the laboratory are aimed at developing an assay for nerve oxidative stress that can be applied to the small amounts of tissue available from mouse nerve.

Lipoic acid was chosen as our next antioxidant because lipoic acid has demonstrated its ability to protect peripheral nerve from damage in diabetic rodents (166, 167). Lipoic acid is a FDA approved drug and can potentially be administrated to MELAS patients right away. At 10 mg/kg and 100 mg/kg, lipoic acid was co-administrated with 1 g/kg DCA daily in mice and it was not able to prevent DCA-induced NCV slowing after 4 weeks. Indeed, mice that received lipoic acid with DCA had a slower MNCV than mice that received only DCA. This result suggests that lipoic acid might be worsening DCA-induced peripheral neuropathy. We speculat that since lipoic acid is an important cofactor for pyruvate dehydrogenase(76), and DCA is known to activate pyruvate dehydrogenase, lipoic acid supplementation might assist activation of pyruvate dehydrogenase by DCA and therefore enhanced rather than inhibit oxidative stress arising from increased ETC flux. Again, we await development of micro-assays for lipid oxidative stress markers to test this hypothesis.

The neuroprotective properties of DHA and fisetin were explored in an eight week study in DCA-treated mice. DHA is an antioxidant and also an important component of neuronal membranes (126). DHA can be obtained through fish oil supplements and can be used in clinical trials directly without further safety studies while fisetin can assist glutathione in scavenging free radicals (123).

Mice fed diet containing 500 ppm fisetin consumed an average of 3.5 g of fisetin diet per day per mouse. That is equivalent to 70 mg/kg/day of fisetin. Fisetin was without effect on DCA-induced MNCV slowing. Control mice that received 50 mg/kgevery 12 hours of DHA for 8 weeks had significantly slower NCV than untreated control mice. This suggests that DHA was damaging peripheral nerves of normal mice. However, when DHA was co-administrated with 1 g/kg DCA daily, DHA prevented DCA-induced NCV slowing at the end of 8 week study. Thus, DHA damaged peripheral neurons when administrated to normal mice but showed neuroprotective ability when co-administrated with DCA.

Since DHA was able to prevent the DCA-induced MNCV deficit in our mouse model, we were prompted to repeat the experiment in rats, as rats can provide more tissue for future biochemical studies of oxidative stress. In our previous studies we have found that rats developed neuropathy more slowly than mice but required half of the DCA dosage. Sprague Dawley rats that received daily DCA at 500 mg/kg for 8 weeks had significantly slower MNCV compared to control rats. Rats that received DHA at 50 mg/kg every 12 hours with daily DCA during the same period appeared to have a faster MNCV compared to DCA alone group, though this difference was not significant. This result suggested that DHA was exhibiting some neuroprotective ability during the first eight weeks. There was no difference between control rats and control rats received DHA after 8 weeks of treatment. The study was extended to 14 weeks to see if DHA was capable of preventing DCA-induced MNCV slowing over a longer period of time. Unfortunately, the partial protective effects by DHA observed in week 8 disappeared at week 14. At week 14, rats that received DHA along with DCA had MNCV values that were not different from rats that received only DCA and both were significantly slower than control rats.

This result showed that DHA failed to prevent NCV slowing caused by DCA-induced oxidative stress after 14 weeks. In addition, rats that received only DHA for 14 weeks showed a trend toward MNCV suggesting the onset of the neuropathic properties of DHA in control rats that we had previously noted in DHA-treated control mice.

The apparently neurotoxic effects of DHA on normal mice and rats were surprising. One possible reason for this may be that it has been shown that endogenous ROS are required for cell signaling (168). If DHA was scavenging a lot of ROS, it might be neutralizing the endogenous ROS required for cell signaling and cause unexpected damage, whereas the same dose of DHA in animals undergoing DCA-treatment would be directed at neutralizing oxidative stress. The time course of initially helpful and subsequently harmful effects of DHA in DCA-treated rats may also derive from the chemical structure of DHA. DHA is a polyunsaturated fatty acid and the abundant double bonds make DHA susceptible to oxidation. Neuronal cells that were exposed to DHA slowly incorporated DHA into cell membrane (130, 131). During the initial weeks of the study, DHA may have acted as an antioxidant and scavenged ROS produced by DCA while DHA was simultaneously being incorporated and accumulated in neuronal cell membranes. After 8 weeks of accumulation, enough DHA may have been incorporated in the neuronal membrane for it to become a target for lipid peroxidation (142).

To test this hypothesis, a future experiment can be carried out using higher dose of DHA with the same dosage of DCA. It will provide insights on whether accumulation of DHA can cause NCV slowing. If the accumulation of DHA causes NCV slowing, higher dosage of DHA should lead to faster accumulation of DHA, and MNCV slowing should appear sooner. This experiment will

allow us to test if higher dosage of DHA can provide more protection against DCA-induced oxidative stress, or if high dose of DHA accelerates neurotoxicity.

In summary, our antioxidants screening studies have shown that ellagic acid can prevent DCA-induced MNCV slowing in both mice and rats and fisetin had no effects. Lipoic acid had no effects on DCA-induced peripheral neuropathy and tended to exaggerate the neuropathy. DHA had some partial protective effects after 8 weeks of treatment but appeared to exaggerate MNCV slowing by 14 weeks. It has been shown that DCA causes oxidative stress and we have demonstrated that some antioxidants can prevent DCA-induced peripheral neuropathy.

4.2 PRO-OXIDANTS

The next step of my study was to determine the mechanism of DCA-induced oxidative stress that has been reported in DCA-treated rats (65). There are two potential pathways that I have begun to investigate. The first hypothesis is that DCA-induced oxidative stress is caused by accumulation of the heme precursor δ-ALA. DCA binds and depletes GSTz/MAAI enzyme (24), leading to accumulation of succinylacetone. The accumulation of δ-ALA is the direct result of δ-aminolevulinate dehydratase inhibition by succinylacetone (68). δ-ALA has pro-oxidant properties. Oxidative stress has been reported after 2 weeks of δ-ALA treatment at 40 mg/kg of δ-ALA via i.p. injection every other day (169). Moreover, δ-ALA is a known structural analog of GABA and can damage GABA receptors. GABA receptors are responsible for inhibitory signals in spinal cord. It has been shown that DCA treated animals developed allodynia (65). δ-ALA's interaction with GABA receptor may therefore be a possible source of allodynia. To test if δ-ALA contributes to DCA-induced oxidative stress, we have begun to treat rats with δ-ALA. There was

no significant difference in behavior or electrophysiological test results between control and δ-ALA treated rats after 12 weeks using a dose previously reported to cause oxidative damage after 2 weeks (169)(68). The dosage was therefore increased to 80 mg/kg after week 12 to investigate if a higher dosage would have more profound effects. There was no difference in test results between control and δ-ALA treated animals after 16 weeks. We intend to sacrifice the animal to determine whether δ-ALA treatment has caused any oxidative stress to the nerve. If it has, then δ-ALA-induced oxidative stress cannot be a cause of peripheral neuropathy and may not be the mechanism by which DCA causes neuropathy. If we cannot detect oxidative damage, then the study should be repeated at higher doses.

The second pathway by which DCA could cause oxidative damage to nerve is via activation of pyruvate dehydrogenase with a subsequent increase in mitochondrial activity. DCA inhibits pyruvate dehydrogenase kinase activity and keeps pyruvate dehydrogenase in an active state (31-33). This facilitates breakdown of pyruvate to acetyl-CoA. Acetyl-CoA is broken down for energy during oxidative phosphorylation inside mitochondria. During oxidative phosphorylation, oxygen is used as terminal electron acceptor in electron transport chain. However, the electron transport chain is not perfect and some ROS are produced. These ROS are normally neutralized by endogenous anti-oxidative enzymes. DCA increases the influx of acetyl-CoA in mitochondria and increases production of ROS in the system. It is therefore possible that excess ROS produced in mitochondria is the source of DCA-induced oxidative stress. However, DCA causes δ-ALA accumulation, which could be a contributor to oxidative stress. 2-CP is used to test this hypothesis. 2-CP activates pyruvate dehydrogenase complex (156), but it does not cause accumulation of δ-ALA as it has no effects on GSTz/MMAI. 2-CP therefore allows us to

investigate whether excess ROS produced from the mitochondria is the source of DCA-induced oxidative stress.

In my preliminary study, one group of rats received 500 mg/kg/day of DCA via oral gavage while another group of rats received 2-CP via oral gavage at 500 mg/kg/day. However, several animals that received 2-CP were found dead after a single treatment, and more rats appeared to be sick. 2-CP toxicity was more potent than initially expected. We were forced to lower 2-CP dosage to 50 mg/kg/day to continue the study.

After 8 weeks of treatment, animals that received 2-CP did not develop peripheral neuropathy that was detectable by our behavioral or electrophysiological tests. During the same time, DCA treated rats developed an MNCV deficit and thermal hypoalgesia. Tactile allodynia was absent in DCA treated rats, unlike what was found in previous studies (65). The study is continuing on for 12 weeks. This is an exploration study and it is likely that additional studies are required before we can determine the correct dosage for 2-CP. 2-CP has a LD_{50} of 750 mg/kg when received with water (63). We therefore have some room for exploration before the LD_{50} is reached.

4.3 DCA AND PAINFUL NEUROPATHY

As discussed above, DCA induces oxidative stress, and a degenerative neuropathy that presents as axonal atrophy, NCV slowing and thermal hypoalgesia. However, DCA also causes some pain symptoms such as allodynia (65). We were therefore interested in how DCA might cause pain and well as nerve degeneration. Formalin-evoked flinching test is a method used to measure hyperalgesia (increased response to a painful stimulus). The flinching behavior in

response to formalin injection usually has two phases. A high number of flinches occurrs during the first few minutes (phase I) and between 20-60 minutes (phase II) after formalin injection, with little or no flinching between the two phases. Flinching in phase I reflects initial primary afferent activity which is mediated by activation of TRPA1 channels (170). Phase II reflects spinal sensitization in response to the initial stimulus and is dependent on activation of NMDA receptors, as NMDA antagonist can completely prevent flinching in phase II but not phase I (171). The initial stimulus is required to initiate phase II and continued stimuli from the periphery are required to maintain phase II, as local anesthetics can stop phase II flinching (172). The formalin-evoked flinching test was carried out on control and DCA treated rats. Rats received daily DCA at 500 mg/kg for 14 weeks had significantly more flinching behaviors in both phase I and phase II in comparison to control rats. This suggests that DCA treated rats developed hyperalgesia, even though we failed to observe the allodynia with von Frey filaments.

It has been shown that hyperalgesia measured by the formalin-evoked flinching method in diabetic rats is caused by decreased expression of the KCC2 transporter in the spinal cord (173). KCC2 transporters are found on the membrane of post-synaptic neurons in the spinal cord and are responsible for regulating intracellular chloride concentration (174, 175) (Figure 12). KCC2 transporters pump chloride out of the cell to maintain low level of intracellular chloride (176). When GABA binds to post-synaptic $GABA_A$ receptors, a chloride channel opens to allow chloride inwards along its concentration gradient (177). This hyperpolarizes the cell and makes GABA an inhibitory neurotransmitter. In diabetic animals, there is a decreased expression of KCC2 transporters in spinal cords (173, 178). This leads to accumulation of chloride within the post-synaptic cell and high intracellular chloride level. When GABA binds to GABA receptors and

opens up the chloride channel, chloride leaves the cell and depolarizes the membrane potential (179). This increases the occurrences of action potential and GABA becomes an excitatory neurotransmitter. Hyperalgesia observed in diabetic animals in phase 2 can be explained by the reversed role of GABA and its loss of inhibitory property. As the response of DCA-treated rats to formalin was very similar to that of diabetic rats, we were prompted to investigate if hyperalgesia observed in DCA rats was mediated by change of spinal KCC2 expression.

DCA rats did not show spinal depletion of KCC2 and indeed had increased KCC2 transporters expression compared to control rats. This suggests that DCA-induced hyperalgesia in formalin-evoked flinching test is not mediated by KCC2 transporters. Flinching in phase I is carried out by activation of peripheral TRPA1 receptors. In future studies we would therefore like to investigate expression of TRPA1 channels in control rats compared to DCA treated rats.

4.4 SUMMARY

Oxidative stress is observed in animals treated with DCA (65). We have now demonstrated that treatment with some antioxidant can ameliorate DCA-induced peripheral neuropathy, with ellagic acid showing complete prevention, fisetin showing no effect and DHA showing a transient partial protection. This leads us to suspect that DCA-induced oxidative stress is the source of peripheral neuropathy, although we cannot yet exclude the possibility that these agents work by alternative mechanisms. In future studies, we hope to measure oxidative stress in the nerves of mice and rats treated with DCA, antioxidants or other pro-oxidants to help verify their mechanism of action. The mechanism by which DCA causes oxidative stress also need further study. We will continue to explore different doses of 2-CP to find one that will

activate enough pyruvate dehydrogenase to mimic DCA's effects on the enzyme. To determine the cause of painful neuropathy, western blots from TRPA1 channel expression could be carried out in nerve from control and DCA treated rats.

5 FUTURE STUDIES

5.5.1 Measuring oxidative stress in δ-ALA rats

Rats that received δ-ALA treatments will be sacrificed and their peripheral neurons will be examined for δ-ALA induced oxidative stress. We were not able to observe any peripheral neuropathy using our behavioral or electrophysiological tests after 16 weeks of δ-ALA treatments. If oxidative stress is absent in peripheral neurons, we will need to increase δ-ALA dosage and repeat the experiment. If oxidative stress is observed in peripheral neurons, this suggests that oxidative stress is not the source of the peripheral neuropathy and that ellagic acid prevents DCA-induced peripheral neuropathy through a non-oxidative stress related pathway.

5.5.2 Morphometric analysis of sciatic nerve

It has been shown that DCA treatment causes reduction in mean axonal diameter that is related to fiber frequency distribution (65). A portion of sciatic nerve will be used to measure myelinated fiber density, mean axonal diameter, myelin thickness, and myelin to axon ratios in control rats and rats that received DCA, δ-ALA and 2-CP. These results will help us correlate our behavioral and electrophysiological test results to nerve structures.

5.5.3 Small fiber count

During thermal response latency, the heat source was warmed at 1 °C/s and activated small C fibers. Rats that received DCA developed thermal hypoalgesia and this was prevented by ellagic acid treatment. The foot skin of these rats were collected and processed. Using morphometric analysis, we can determine if there was a loss of small C fibers in DCA treated

rats' foot skin compared to control rats, and if ellagic acid treatment was able to prevent the loss.

5.5.4 2-CP treatment

Rats that received 50 mg/kg of 2-CP will be sacrificed and protein assay will be done to determine pyruvate dehydrogenase activities in these rats. The result will be compared to rats that received 500 mg/kg of DCA. We will use this result as an indicator to decide future dosage required for effective 2-CP treatment.

5.5.5 TRPA1

Standard protocols for TRPA1 channels western blot will be developed. DCA treated rats showed increased flinching behaviors in phase I after formalin injection. It has been shown that phase I flinching observed in formalin-evoked flinching response is mediated by activation of TRPA1 channels (170). We would like to investigate if hyperalgesia observed in DCA treated rats is associated with changes in TRPA1 expression.

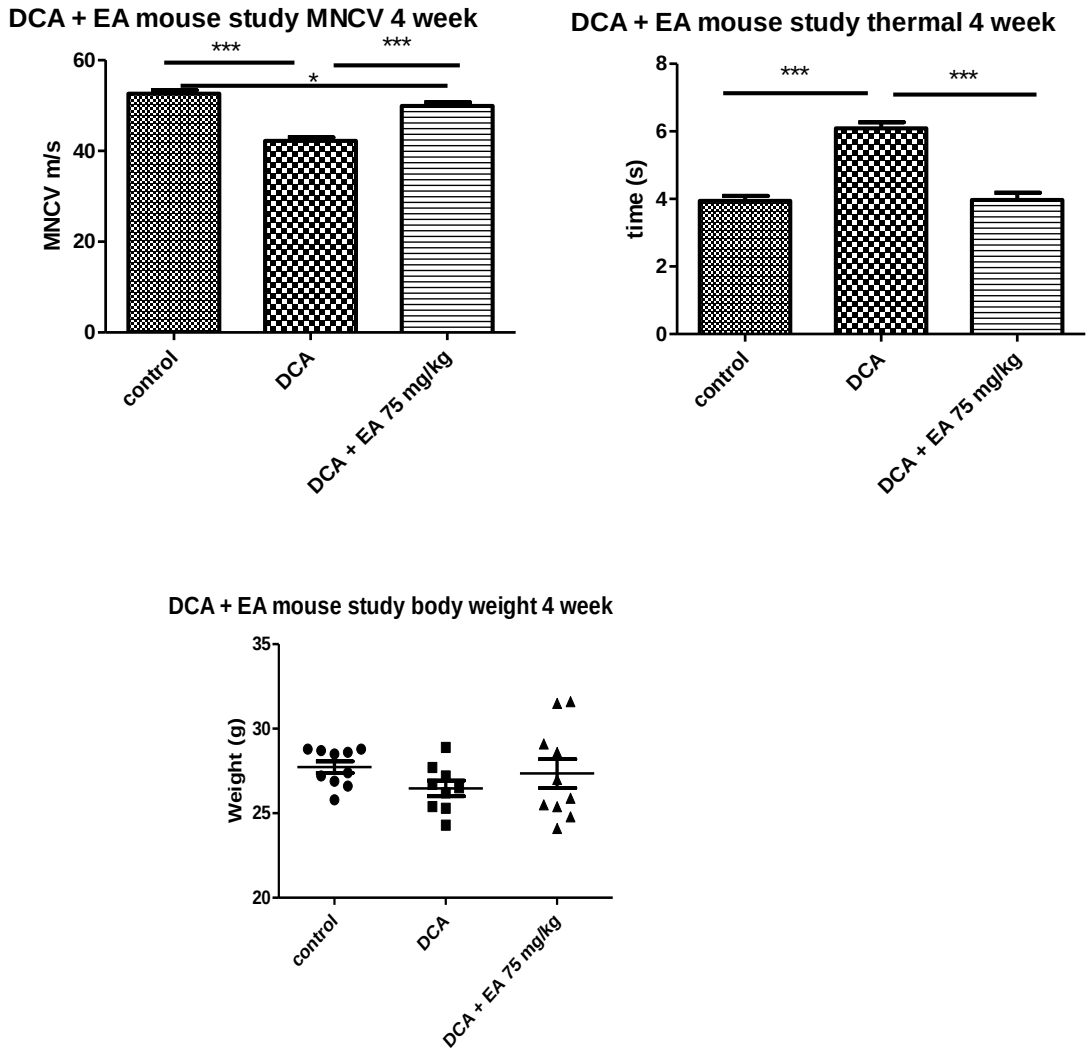


Figure 1: DCA mice received daily DCA at 1 g/kg via oral gavage. DCA + EA mice received daily DCA at 1 g/kg and ellagic acid at 75 mg/kg via oral gavage. Mice that received DCA had significantly slower MNCV compared to control mice ($P < 0.001$). Mice that received DCA developed thermal hypoalgesia compared to control mice ($P < 0.001$). Ellagic acid treatment completely prevented MNCV slowing and thermal hypoalgesia ($P < 0.001$). Data are group mean $\pm$ SEM.

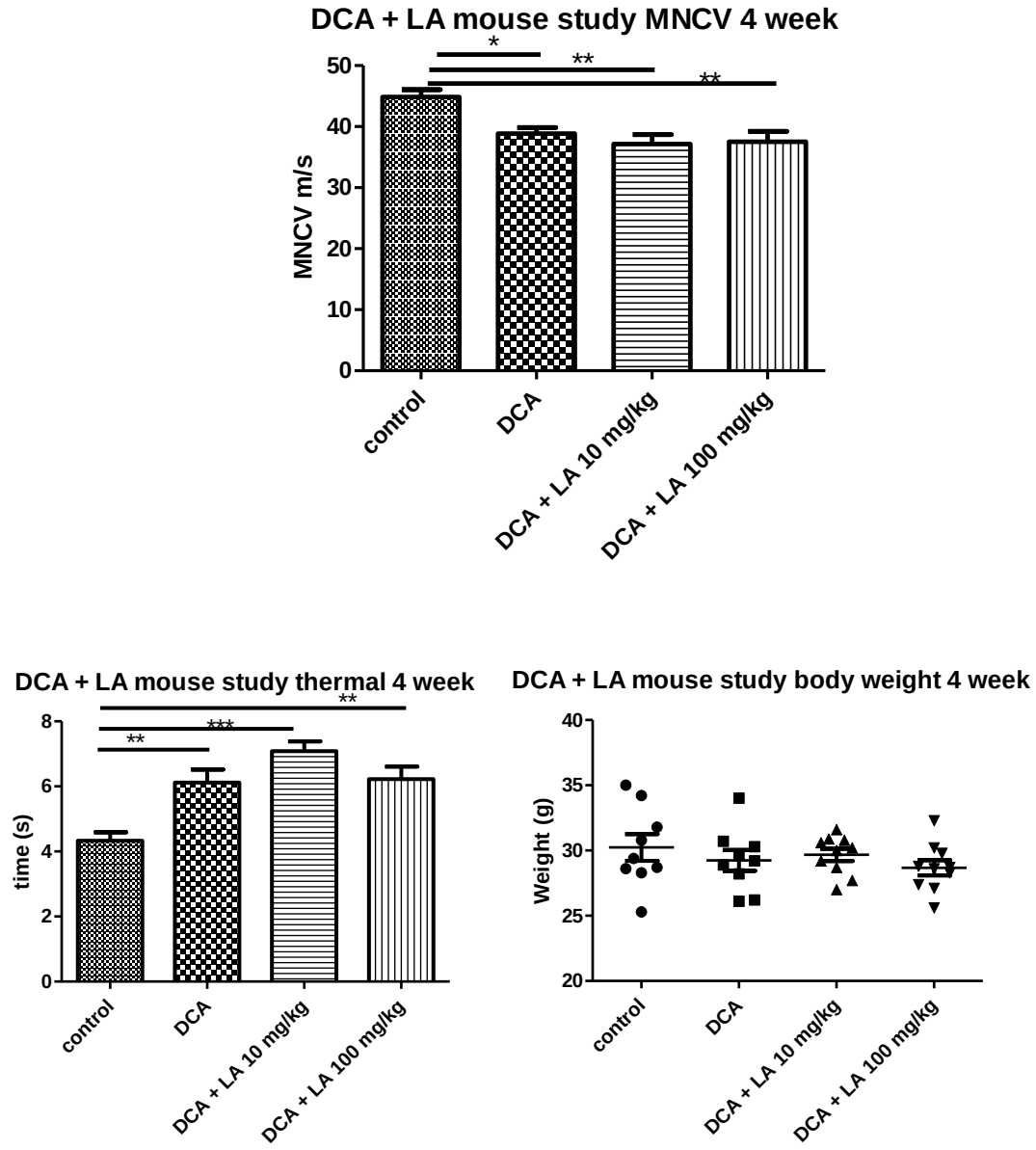


Figure 2: DCA mice received daily DCA at 1 g/kg via oral gavage. DCA + LA 10 mg/kg mice received daily DCA at 1 g/kg and lipoic acid at 10 mg/kg via oral gavage. DCA + LA 100 mg/kg mice received daily DCA at 1 g/kg and lipoic acid at 100 mg/kg via oral gavage. Mice that received DCA had significantly slower MNCV than control mice ($P < 0.05$). Mice that received DCA also developed thermal hypoalgesia ($P < 0.05$). Lipoic acid had no effects preventing DCA-induced MNCV deficit and thermal hypoalgesia. Data are group mean $\pm$ SEM.

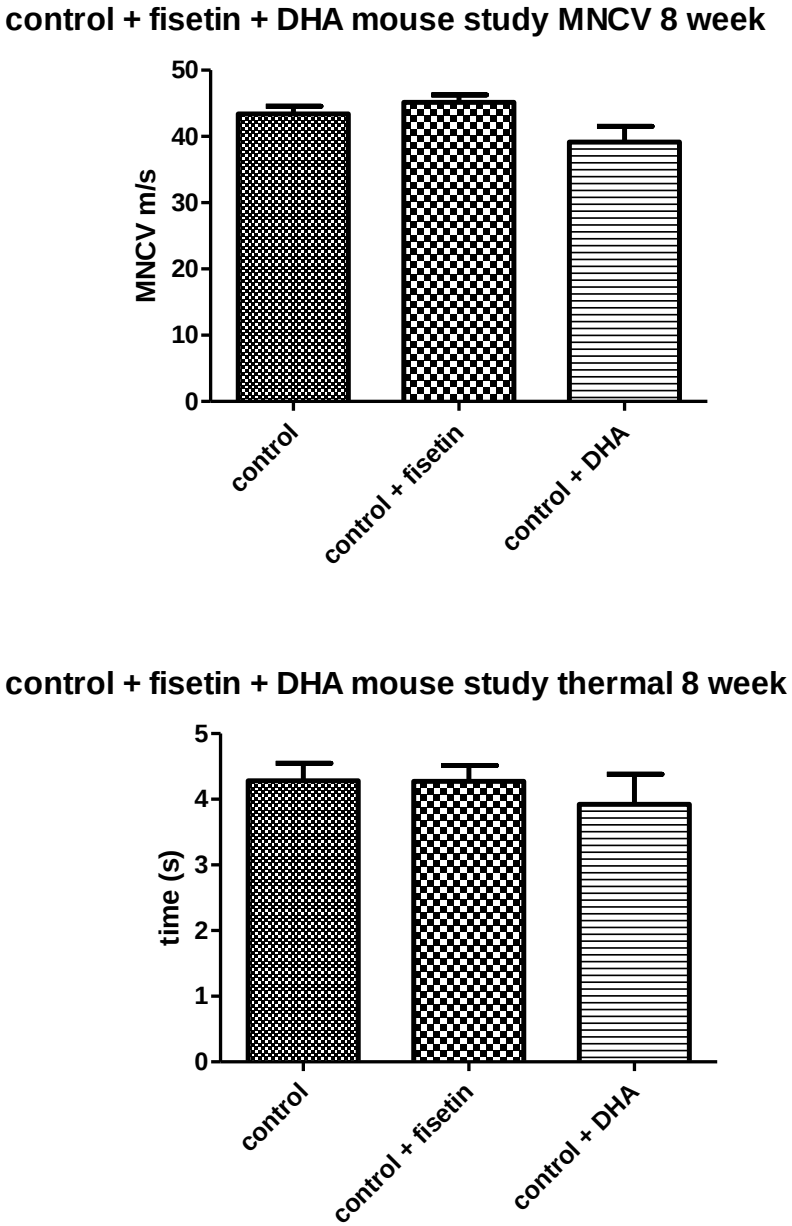


Figure 3: Control + DHA mice received DHA at 50 mg/kgevery 12 hours via oral gavage. Control + fisetin mice were fed 500 ppm fisetin diet. Each mouse consumed 3.5 g/day, which is equivalent to 70 mg/kg/day. Mice that received DHA had partially reduced MNCV compared to control mice, but it was not statistically significant ($P > 0.05$). There was no difference among groups in thermal response latency data. Data are group mean $\pm$ SEM.

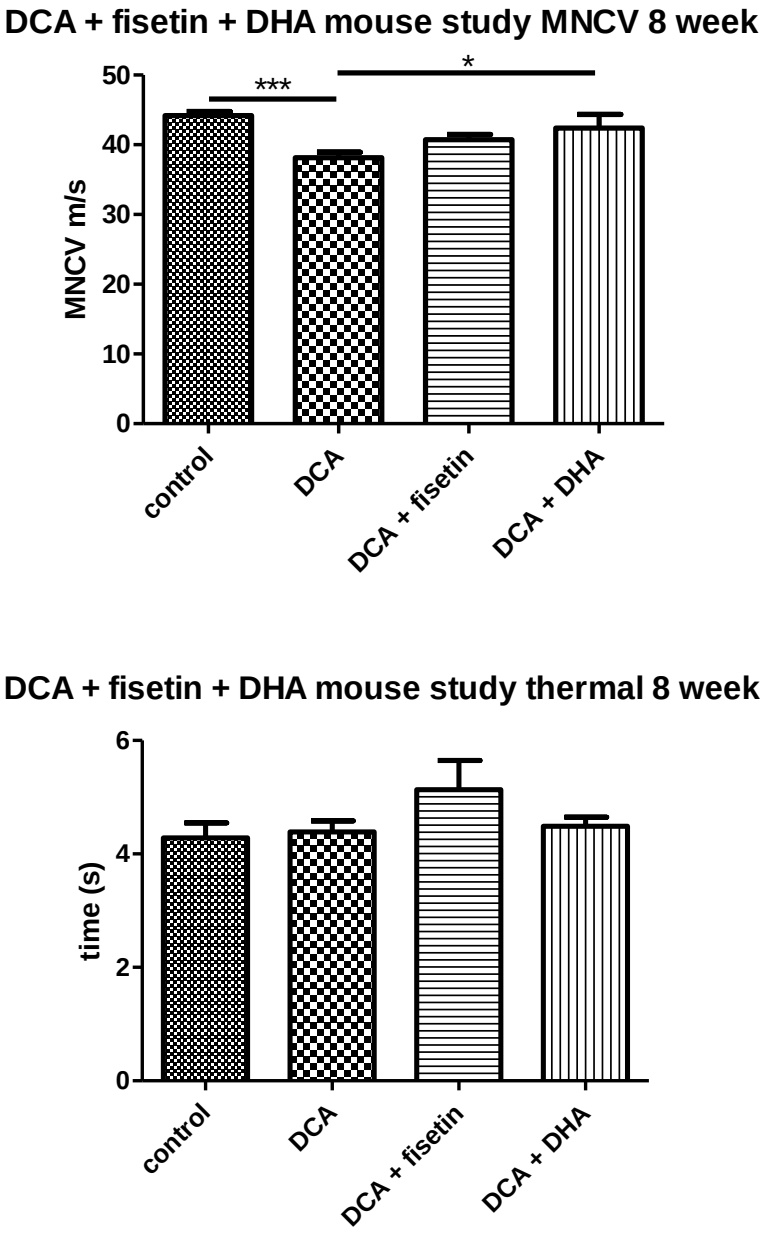


Figure 4: DCA mice received daily DCA at 1 g/kg via oral gavage. DCA + DHA mice received daily DCA at 1 g/kg and DHA at 50 mg/kgevery 12 hours via oral gavage. DCA + fisetin mice received daily DCA at 1 g/kg via oral gavage and were fed 500 ppm fisetin diet. Each mouse consumed 3.5 g/day, which is equivalent to 70 mg/kg/day. Mice that received DCA developed a MNCV deficit ($P < 0.001$) compared to control mice. Mice that received DHA and DCA had significantly ($P < 0.05$) faster MNCV compared to mice that received DCA alone. There was no difference in thermal response latency data. Data are group mean $\pm$ SEM.

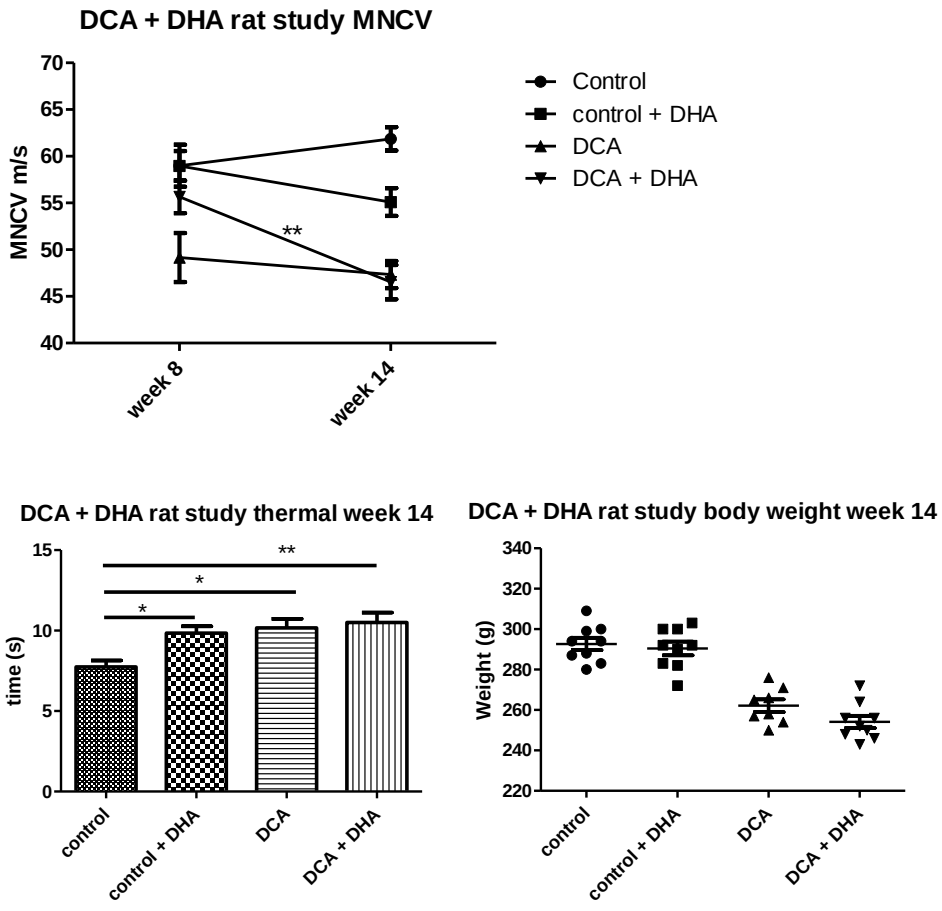


Figure 5: Control + DHA rats received daily DHA at 50 mg/kg. DCA rats received daily DCA at 500 mg/kg via oral gavage. DCA + DHA rats received daily DCA at 500 mg/kg and DHA at 50 mg/kg every 12 hours via oral gavage. Rats that received DCA developeda MNCV deficit compared to control rats ($P < 0.001$) after 14 weeks of treatment. Rats that received DCA developed thermal hypoalgesia compared to control rats ($P < 0.05$) after 14 weeks of treatment. Rats that received DHA treatment along with DCA showed no difference compared to rats that received DCA alone. Data are group mean $\pm$ SEM.

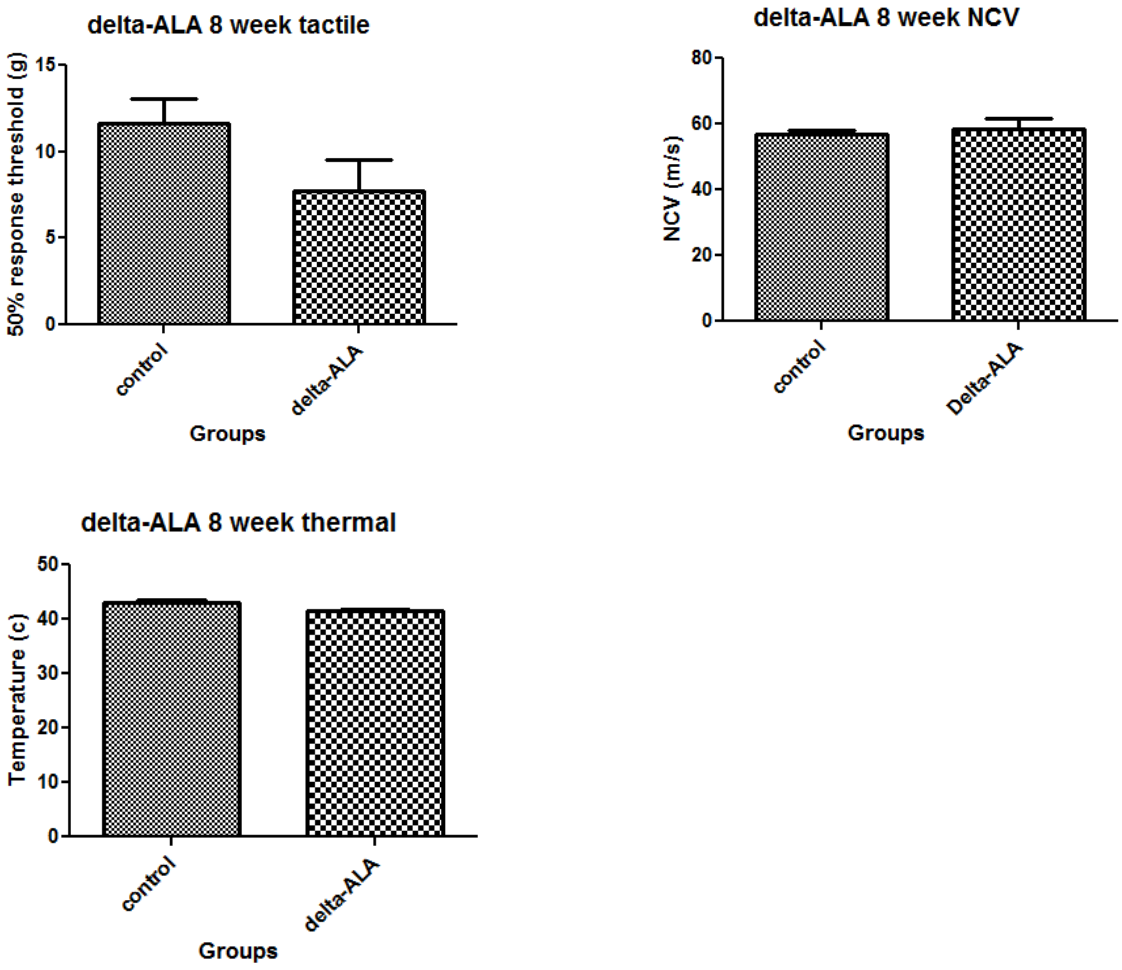


Figure 6: δ-ALA rats received δ-ALA via i.p injection at 40 mg/kg every other day. Data are group mean ± SEM.

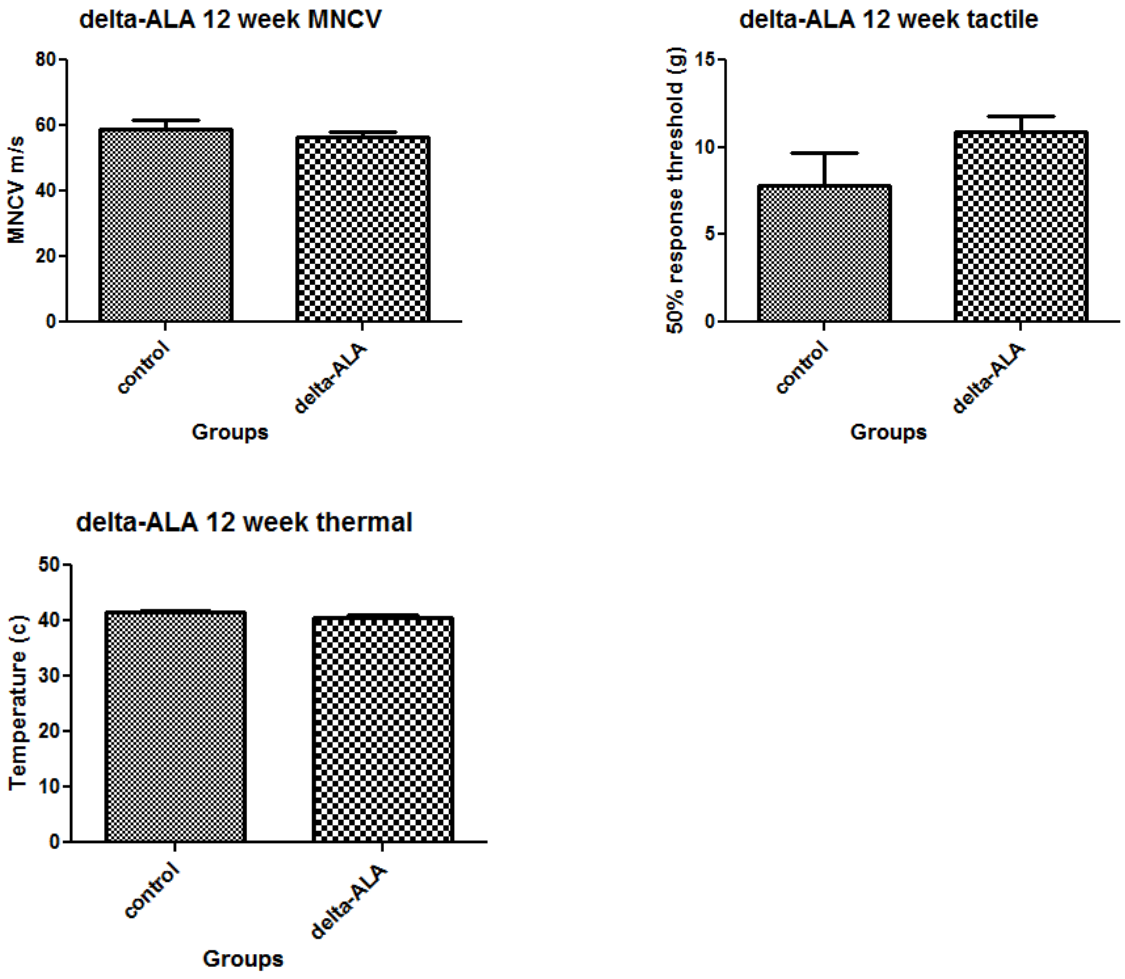


Figure 7: δ-ALA rats received δ-ALA via i.p injection at 40 mg/kg every other day. Data are group mean ± SEM.

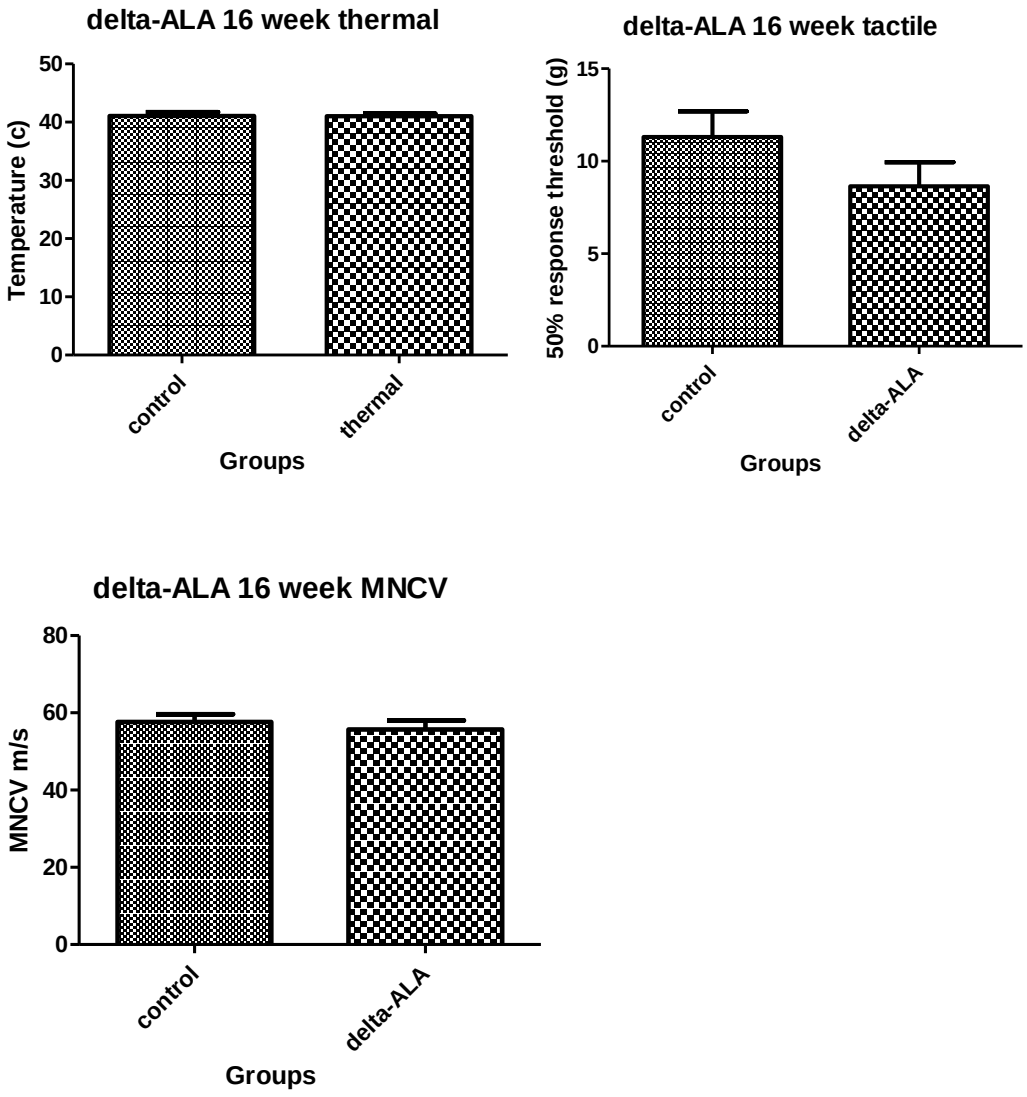


Figure 8: δ-ALA rats received δ-ALA via i.p injection at 80 mg/kg every other day. Data are group mean ± SEM.

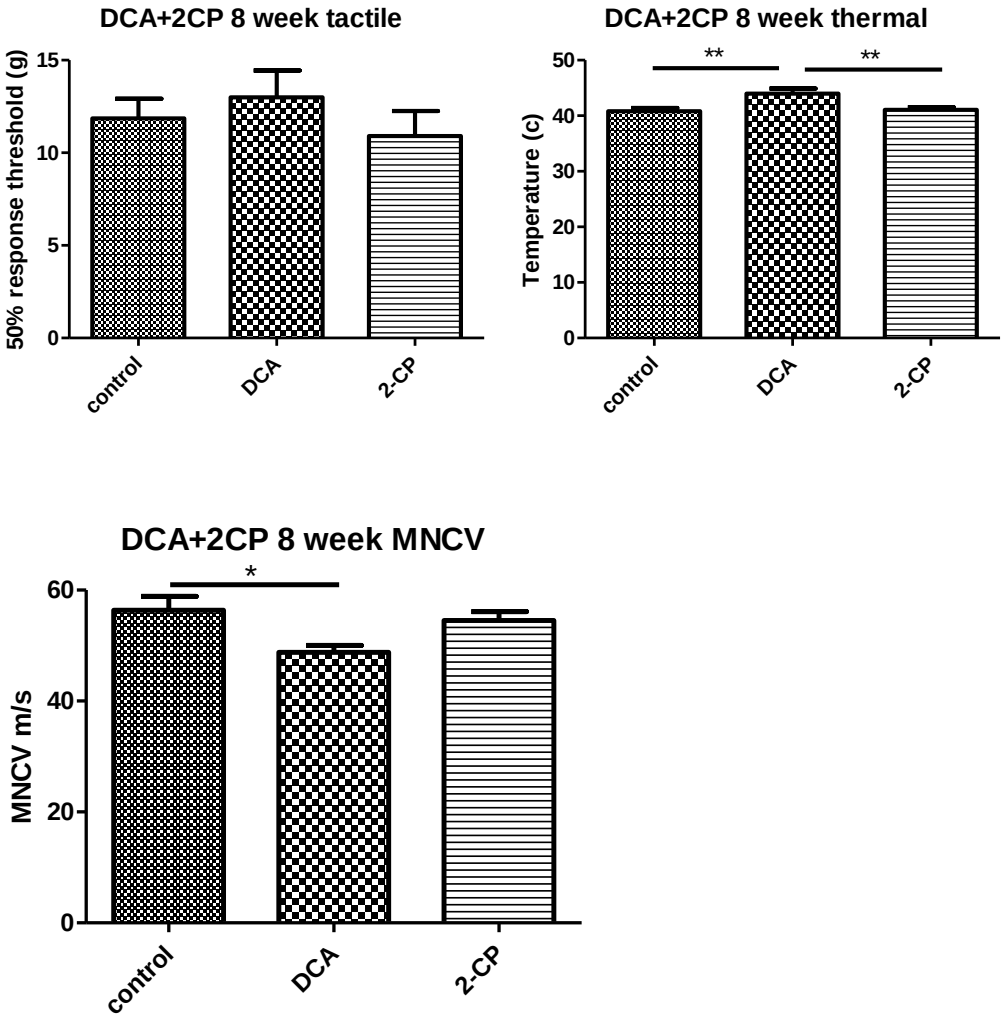


Figure 9: DCA rats received daily DCA at 500 mg/kg via oral gavage. 2-CP rats received daily 2-CP at 50 mg/kg via oral gavage. Rats that received DCA had significantly ($P < 0.05$) slower MNCV compared to control rats. Rats that received DCA developed thermal hypoalgesia ($P < 0.05$). Data are group mean $\pm$ SEM.

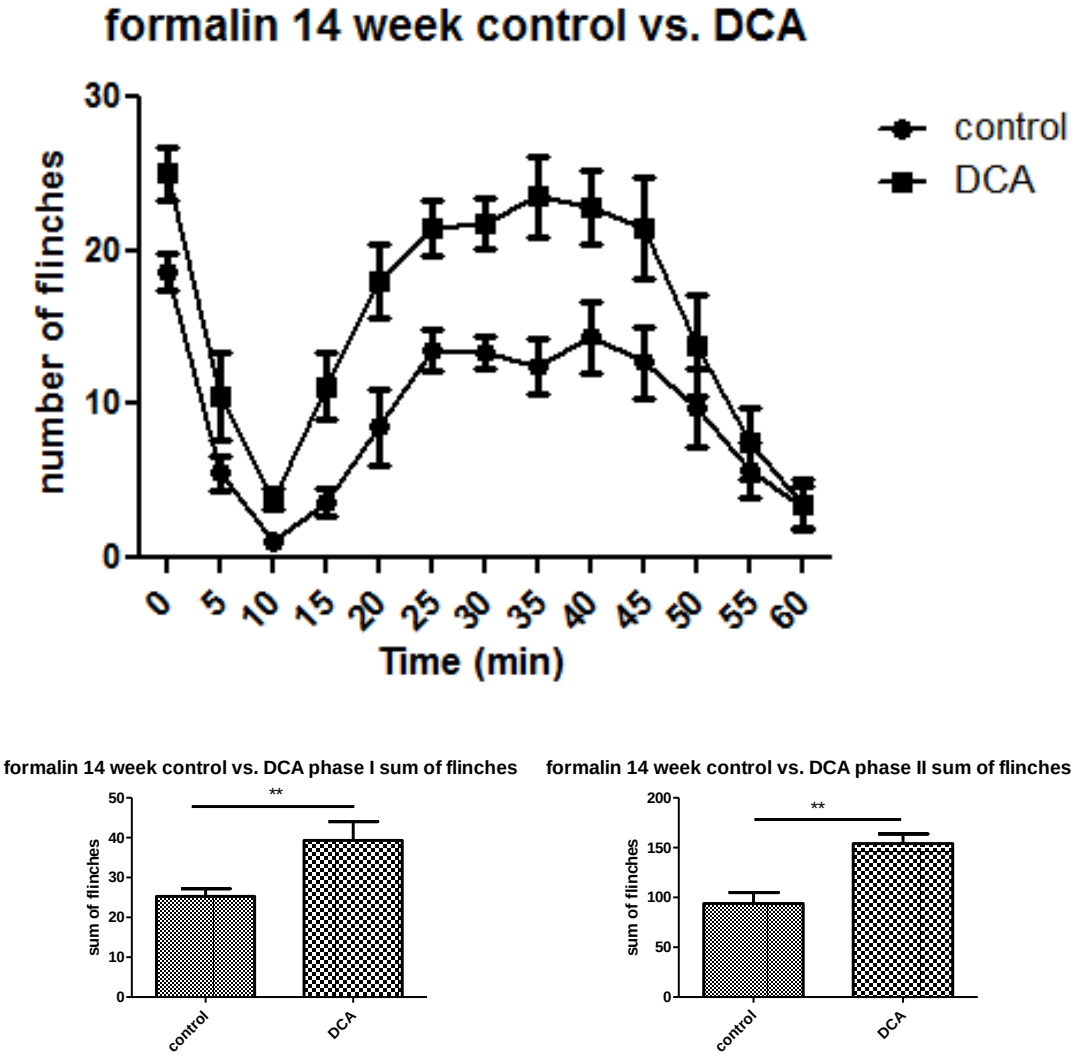


Figure 10: DCA rats received daily DCA at 500 mg/kg via oral gavage for 14 weeks. Rats that received DCA had significantly more flinching behaviors in both phase I and phase II ($P < 0.01$ vs. control). Data are group mean $\pm$ SEM.

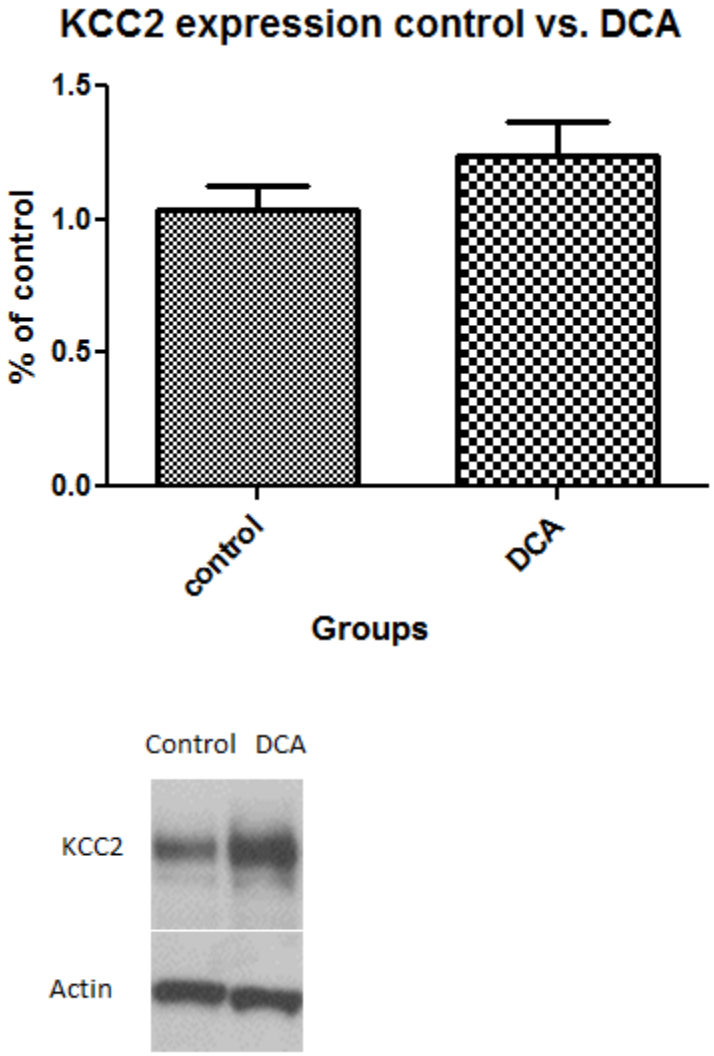


Figure 11: DCA rats received daily DCA at 500 mg/kg via oral gavage for 14 weeks. Representative lane from each group. Data are group mean ± SEM.

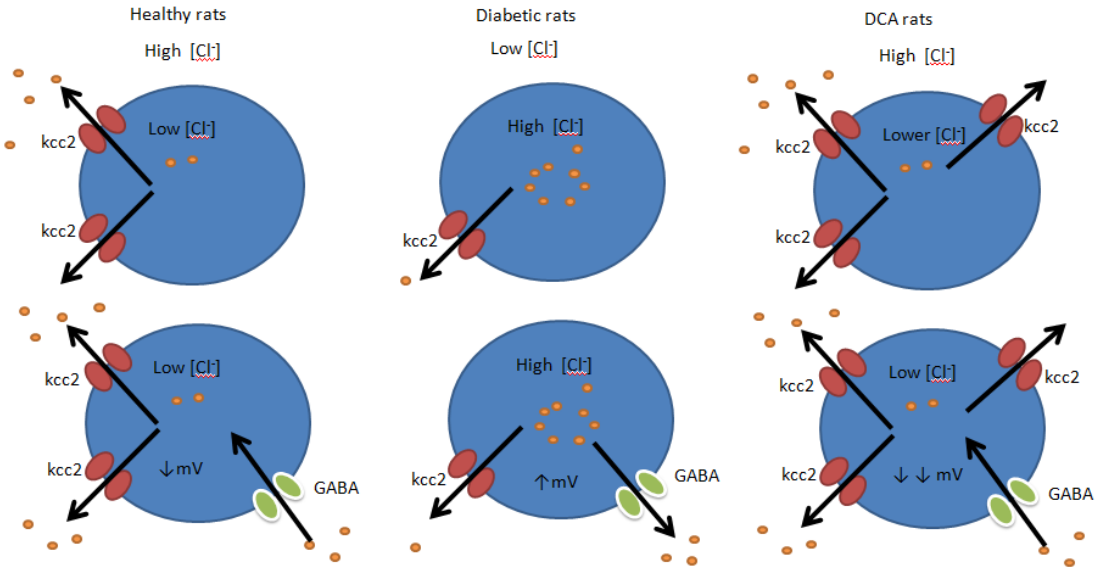


Figure 12: KCC2 transporters pump chloride out of the cell. GABA receptor is a chloride channel that allows chloride to flow freely across the membrane.

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