DESCRIPTION. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. **DO NOT EXCEED THE SPACE PROVIDED.**

Mechanisms of Neuroprotection by C_{60} Derivatives

There is substantial evidence that oxidative injury plays a role in many types of cytotoxic insults, and that the role of free radical damage may be especially prominent in neurological disease states, such as stroke, trauma and neurodegenerative disorders such as Parkinsons disease. Recently, we reported that water-soluble derivatives of C_{60} were highly effective neuroprotective agents capable of rescuing cortical neurons from a broad range of insults, including excitotoxicity induced by NMDA or AMPA, apoptosis produced by growth factor withdrawal or application of Aβ(1-42), and neuronal death following oxygen-glucose deprivation. In addition, intraperitoneal administration of these compounds to a mouse model of familial ALS delayed both the onset of motor deterioration and death. Electron paramagnetic resonance spectroscopic studies confirmed that these water-soluble malonic acid derivatives of C_{60} retain the potent free radical scavenging capabilities of native C_{60}, with the ability to eliminate both hydroxyl and superoxide radicals.

Based on these observations, we believe that neuroprotection provided by these antioxidants reflects their ability to scavenge not only hydroxyl radical but superoxide radical ($O_2^{\bullet-}$) at concentrations in the micromolar range. This application proposes to test the specific hypothesis that superoxide radical scavenging by C_{60} derivatives is a critical determinant of neuroprotective efficacy by C_{60} derivatives. We plan to study mechanisms of neuroprotection by generating C_{60} derivatives substituted with similar functional groups, but possessing differing degrees of $O_2^{\bullet-}$ scavenging ability. The rank-order potency of derivatives will be compared for both neuroprotection and superoxide scavenging to see if these properties correlate. In addition, using *Sod2*^{m1ucsf} mice, which have a specific impairment in the ability to eliminate mitochondrial $O_2^{\bullet-}$, we will test the protective efficacy of these agents on neurons cultured from these mice, and in neonatal mice.

The broader goals of this proposal are: 1) to develop these molecules as tools to study the role of $O_2^{\bullet-}$ as both signaling molecule and neurotoxin, 2) to continue exploration of the contribution of mitochondrial $O_2^{\bullet-}$ to neuronal cell death, 3) and to begin to define structure-function relationships for these promising compounds as a necessary step towards pre-clinical trials.

PERFORMANCE SITE(S) *(Organization, city, state)*

All research will be performed at Washington University, St. Louis, MO.

KEY PERSONNEL. See instructions on Page 11. *Use continuation page s as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
Laura L. Dugan, MD	Washington University	PI
Tien-sung (Tom) Lin	Washington University	Collaborator
Anne Cross	Washington University	Collaborator
Eva Lovett	Washington University	Research Associate
Robert Bart, MD	Washnigton University	Research Associate
Bei-wen Ma, MD	Washington University	Technician
David Holtzman, MD PhD	Washington University	Consultant
Steven Rothman, MD	Washington University	Consultant
E. Gene Johnson, PhD	Washington University	Consultant
Charles Epstein, MD PhD	University of California, San Francisco	Consultant
Richard Smith	Pacific Northwest National Laboratory	Consultant

RESEARCH GRANT
TABLE OF CONTENTS

*Type density and size must conform to limits provided in Specific Instructions on page 10.

Appendix *(Five collated sets. No page numbering necessary for Appendix)*
Number of publications and manuscripts accepted or submitted for publication *(Not to exceed 10)*: 6
Other items (list):

√ Check if Appendix is Included

BUDGET JUSTIFICATION

A. PERSONNEL

1. Laura L. Dugan, MD: Principal Investigator (50% effort, 0% salary until 2/1/99; 50% salary 2/1/99-duration of grant). The Principal Investigator will spend 50% of her time on the proposed studies. Salary support is available for 100% of her salary through 1/31/99 (CIA, and non-Federal sources), and for 30% of her salary from 2/1/99-1/31/00. Funds are requested for 50% of the PI's salary beginning 2/1/99 through the duration of the grant. The PI will direct in vitro and in vivo studies on C_{60} derivatives, will work with Dr. Lin and the chemist, Eva Lovett, to develop additional C_{60} derivatives, and will coordinate and perform analyses of C_{60} metabolites and free radical chemistry of C_{60} derivatives. She will also assist in and direct the in vitro and in vivo experiments on the *Sod2* -/- mouse.

2. Eva Lovett: Research Associate (100% effort, 100% salary (year 1-2), 35% effort, 35% salary (year 3-5). Eva Lovett is an experienced synthetic chemist who has successfully synthesized and purified specific isomers of malonic acid esters and acids of C_{60}. She is experienced in the use of NMR techniques and will be responsible for continued production of malonic acid-C_{60}, and for devising and carrying out synthetic schemes for other C_{60} derivatives. She will work with Dr. Lin in studies on the interaction of these derivatives with lipid membranes. 100% of her time is expected to be necessary for the first two years of the project for synthesis and characterization of C_{60} derivatives. Her level of commitment to this project will be reduced to 35% for years 3-5; during this period when she will focus on analysis of metabolites.

3. Tien-sung (Tom) Lin, PhD: Collaborator (10% effort, 10% salary). Dr. Lin and Dr. Dugan have been collaborating for 4 years on various aspects of free radical biology in CNS injury. Professor Lin has extensive experience using electron paramagnetic resonance spectroscopy to study free radical chemistry, and will work with Dr. Lovett to carry out aqueous/spin-trapping and low temperature EPR experiments to characterize radical scavenging specificity and potency of various derivatives. His laboratory will also be responsible for EPR studies with spin-labeled lipids to examine membrane interactions of C_{60} derivatives.

4. Anne Cross, MD: Collaborator (No salary requested). Dr. Cross' research focuses is on muliple sclerosis and white matter injury. She will work with the PI to establish oligodendroglial cultures, and will assist in neuroprotection studies on oligo cultures.

5. Robert Bart, MD: Research Associate (100% effort, 0% salary). Dr. Bart will be responsible for analysis of free radical reactions in cell cultures, and brain slice preparations. He will also assist with in vivo experiments in *Sod2* -/- mice. He is currently paid (100%) from clinical revenues in the Department of Pediatrics, so no salary is requested. In year 3, it expected that Dr. Bart will be in the process of setting an independent research focus, and will shift his time away from this project. Salary is requested for a research associate (see below) to continue the analysis of radical reactions and neuroprotection by C_{60} derivatives. Dr. Bart will remain affiliated with the project to provide advice on the in vivo aspects of the study.

6. Research Associate -TBN (100% effort, 100% salary, Years 2-5) A research associate is requested to continue studies initiated by Dr. Bart in years 1-2, with the assumption that in year 2, transition and training will occur.

7. Bei-wen Ma: Research technician (100% effort, 100% salary). Dr. Ma currently prepares and maintains primary neuronal and astrocyte cultures, performs neurotoxicity and neuroprotection studies on cell cultures, performs Western immunoblots, DNA purification and analysis, and assists with biochemical analysies of ROS. She will continue these duties full-time for the proposed project.

8. Research technician-TBN (100% effort, 100% salary). A second technician is requested to specifically work with the *Sod2* - colony, to perform genotyping, to prepare and maintain cultures from these animals, and

to perform preliminary characterization of these cultures immunocytochemically and pharmacologically, with a specific focus on glutamate receptors and anti-oxidant systems. Dr. Dugan will work closely with the technician to develop, optimize, and characterize neuronal cultures from these mice. It is likely that such cultures, especially as methods are being developed, will be labor-intensive. Therefore, a full-time position is requested.

9. Scott Schweikart: Student lab assistant (20% effort, 0.50 appointment). Scott is a talented undergraduate with 1½ years of experience in animal husbandry of transgenic mice (including the ALS G93A Gurney H1 mice, SOD WT overexpressers, and AT -/- mice). He will breed the *Sod2* -/- mice, identify animals by ear-clipping, and obtain samples for genotyping by the research technician.

10. Gene Johnson, PhD (Unpaid Consultant). Dr. Johnson will provide his considerable expertise in mechanisms of cell death to the project, and will assist the PI in studying neuroprotection by C_{60} derivatives in superior cervical ganglion neurons deprived of NGF.

11. David Holtzman, MD,PhD (Unpaid Consultant). Dr. Holtzman will provide assistance to the project on the proposed *in vivo* experiments with neonatal *Sod2* knockout mice, and will work with the PI to determine the most reliable method for assessing neuronal rescue in these mice.

12. Charles Epstein, MD (Unpaid Consultant) Dr. Epstein and colleagues will provide founder mice for the *Sod2* knockout colony, and will provide guidance on using these mice for *in vitro* and *in vivo* studies.

13. Richard Smith, PhD (Unpaid Consultant) Dr. Smith is director of the Laboratory Mass Spectroscopy Research Resource at the Pacific Northwest National Laboratory, a facility equipped with unique mass spectrometry instumentation. At Dr. Smith's request, the PI previously submitted a collaborative proposal to use their facility to study specific aspects of the chemistry C_{60} derivatives that could not be studied by conventional mass spectrometry instruments.

B. EQUIPMENT

A Perkin Elmer thermocycler is requested. The PI currently uses a thermocycler in Dr. Dennis Choi's laboratory, but this instrument is already heavily used. To allow adequate genotyping of *Sod2* - mice and the mouse pups used for cultures, an independent instrument is requested. Year 1. $9000

C. SUPPLIES

1. Cell cultures
   - A. Serum, media, sterile plasticware, growth factors, other disposable supplies $3000
   - B. Cover slips and coverslip 35 mm dishes (for confocal), poly-D-lysine and laminin for coatings, specialty gas tanks for low-O_2 incubator $3000

2. Reagents required for C_{60} synthesis
   - A. C_{60}, reactants for side group additions, column packing material, glassware, etc. $9000
   - B. HPLC supplies for Waters HPLC to allow purification and identification of C_{60} derivatives (HPLC columns and maintenance supplies, solvents) $800
   - C. Preparation/usage fees for characterization of compounds by NMR and mass spectroscopy (e.g. NMR usage fee is $40.00/h) $2500

3. Supplies for EPR studies on free radical scavenging and biomembrane interactions (quartz flat cells, spin-traps, spin-labels, replacement of EPR klystron q 500 hours) $2000

4. Confocal microscopy reagents (fluorescent probes, share of laser replacement costs)	$1500
5. Slice preparation and ROS measurement reagents (salicylate, buffers, gas tanks, etc.)	$500
6. HPLC supplies/maintenance for Hewlett-Packard system (salicylate oxidation products)	$1200
7. Genotyping supplies (Taq polymerase, reagents for DNA extraction /purification, PCR reagents)	$3000
8. Miscellaneous reagents and supplies	$1500
TOTAL	$28000

ANIMAL CARE COSTS

Cage costs and per diems (animals in the *Sod2* mouse breeding colony are expected to be 100-150 mice (20-40 cages))	$8000

SPECIFIC AIMS

We recently reported that water-soluble derivatives of the fullerene (C_{60}) molecule are antioxidants with surprising neuroprotective properties *in vitro*, and activity *in vivo*. A unique feature of these compounds is their ability to eliminate both superoxide anion ($O_2^{\bullet-}$), as well as hydroxyl radical ($^{\bullet}OH$), at low (micromolar) concentrations. Based on our findings, we believe that the ability of these molecules to detoxify $O_2^{\bullet-}$ is responsible for the greater degree of neuroprotection they afford compared to other antioxidants we have studied. **We propose the specific hypothesis that superoxide radical scavenging by C_{60} derivatives is a critical determinant of neuroprotective efficacy by C_{60} derivatives.** We plan to test this idea by generating C_{60} derivatives with different degrees of $O_2^{\bullet-}$ scavenging ability, and comparing their neuroprotective properties. In addition, using SOD2-deficient (*Sod2*^{m1ucsf}) mice, which show impaired elimination of mitochondrial $O_2^{\bullet-}$, we will test the protective efficacy of these agents in neuronal cultures, and in neonatal -/- mice. Three specific aims detail our approach to testing our hypothesis:

Aim 1 Synthesize and characterize C_{60} compounds with increasing numbers of malonic acid additions (decreasing radical scavenging), and aminoamido and polyethyleneglycol (PEG) derivatives.

A. Determine purity and structure of derivatives by NMR and mass spectroscopy.

B. Assess free radical scavenging by electron paramagnetic resonance (EPR) spectroscopy.

C. Determine lipid bilayer interactions/membrane intercalation using EPR with spin-labeled lipids.

D. Refine tools for analyzing C_{60} derivatives and their metabolites in biological tissues, using HPLC with fluorescence coupled with UV/vis detection.

Aim 2 Define the relationship between $O_2^{\bullet-}$ scavenging and neuroprotective efficacy of C_{60} derivatives by establishing dose-response curves for derivatives generated by experiments outlined in Aim 1. The rank-order of $O_2^{\bullet-}$ scavenging and neuroprotection should be similar to support our hypothesis.

A. Produce excitotoxic injury in cortical neuronal cultures by exposure to NMDA, AMPA, and oxygen-glucose deprivation, and to oligodendroglia in culture by application of AMPA. Cell death will be quantified by LDH release (neurons) and loss of galactosidase-C positive cells (oligos).

B. Induce apoptosis of cortical neurons by serum withdrawal, and in superior cervical ganglia (SCG) neurons by NGF deprivation. Apoptotic death will be assessed by Annexin V staining and propidium iodide fluorescence/ cell counts.

C. Determine "biological" free radical scavenging in cultured neurons and oligodendroglia, using oxidation-sensitive fluorescent dyes with confocal microscopy, biochemical measures including intracellular salicylate oxidation and TBARS formation, and peroxyl radical aasay by EPR spectroscopy.

Aim 3 Assess neuroprotective efficacy of C_{60} derivatives in SOD2 deficient neurons and oligodendroglia. If $O_2^{\bullet-}$ scavenging contributes importantly to neuroprotection by C_{60} derivatives, these compounds should rescue cells in which the primary defect is impaired detoxification of $O_2^{\bullet-}$.

A. Establish dose-response curves in neuronal and oligo cultures from SOD2 deficient mice at baseline, and after exposure to excitotoxic and apoptotic insults, as outlined in Aim 2, A and B.

B. Determine efficacy of free radical scavenging in SOD2 deficient cultures, looking at indices of free radical formation using methods oulined in Aim 2B, with a specific focus on $O_2^{\bullet-}$ handling.

C. Deliver C_{60} derivatives to *Sod2* -/- (*Sod2*^{m1ucsf}) mouse pups orally. Survival will be compared to untreated *Sod2* -/- pups. We will also look for indices of oxidative damage to brain by assessing nitrotyrosine levels, salicylate oxidation, and fluorescent dye oxidation in treated and untreated animals.

Long-term Goals The broader goals of this proposal are: 1) to develop these molecules as tools to study the role of $O_2^{\bullet-}$ as both signaling molecule and neurotoxin, 2) to continue exploration of the contribution of mitochondrial $O_2^{\bullet-}$ to neuronal cell death, 3) and to begin to define structure-function relationships for these

promising compounds as a necessary step towards pre-clinical trials.

B. BACKGROUND AND SIGNIFICANCE

Chemistry and antioxidant properties of water-soluble C_{60} derivatives

Buckminsterfullerene, the third known form of pure carbon after graphite and diamond, was discovered by Smalley, Kroto, and Curl in 1985, a discovery for which they received the Nobel Prize in Chemistry in 1996. The original fullerene molecule (C_{60}) is a sphere consisting of interlocking hexagons (20) and pentagons (12) in the exact pattern of a soccer ball. Because fullerenes possess a highly conjugated double bond system, which imparts characteristics of both metals and superconductors to them, they also possess the ability to accept multiple radical additions per C_{60} molecule - a "radical sponge" (Krusic et al, 1991a,b). Native C_{60} is only soluble in a limited number of biologically unattractive solvents, such as toluene. Recently, however, techniques for synthesizing a variety of water-soluble C_{60} derivatives have been described; in addition to the polyhydroxyl (Chiang et al., 1992, 1993) and malonic acid derivatives (Lamparth and Hirsch, 1994; Hirsch et al., 1994) we have studied, methods for synthesizing amino and amido derivatives (An et al., 1993; Isaacs and Diederich, 1993; Yamago et al., 1993; Maggini et al., 1994; Skiebe and Hirsch, 1994), peptide (Prato et al., 1993; Toniolo et al., 1994), oligonucleotide (Boutorine et al., 1994) adducts, and sugar derivatives (Vasella et al., 1992) have been reported. Krusic et al (1991a) reported the addition of > 30 methyl radicals to C_{60}, implying that placing a limited number of functional groups on the C_{60} sphere to confer water-solubility might not abolish its radical scavenging properties.

Role of mitochondrial ROS and CNS disease

Reactive oxygen species (ROS) have been implicated in the pathogenesis of central nervous system damage in the setting of trauma (Braughler and Hall, 1989), ischemia-reperfusion (Flamm et al, 1982; Kontos and Wei 1983; Chan et al 1987; Siesjo et al, 1989), and have been suggested to contribute to certain neurodegenerative disorders (Halliwell, 1992; Beal, 1992). Excitotoxic injury to neurons and other neural cell types contributes to tissue injury in many of the same CNS insults (Choi, 1988; Matute et al, 1997). Support for the idea that glutamate receptor activation might be one of the triggers which initiates oxidative damage has been provided by observations that antioxidant approaches can attenuate glutamate receptor-mediated neurotoxicity (Dykens et al, 1987; Monyer et al, 1990; Chan et al, 1990; Yue et al, 1993), and by direct detection of ROS generation *in vitro* (Garthwaite et al, 1988; Lafon-Cazal et al, 1993; Reynolds and Hastings, 1995; Dugan et al, 1995; Bindokas et al, 1996) and *in vivo* (Schulz et al, 1995; Lancelot et al, 1995).

Mitochondria may be an important source of ROS in brain subjected to acute insults such as ischemia-reperfusion (Hasegawa et al, 1993; Piantadosi and Zhang, 1996) or trauma, or in certain neurodegenerative diseases (Olanow, 1990; Beal, 1992). Since the first demonstration of ROS production in mitochondria by Chance and Ohniso (1971), extensive work on isolated mitochondria has demonstrated that inhibition of the respiratory chain by mitochondrial poisons, such as antimycin A or cyanide, can elicit mitochondrial ROS production (Nohl and Hegner, 1978). Dykens (1994) observed hydroxyl radical production by mitochondria exposed to elevated [Ca^{2+}] and [Na^{+}], raising the possibility that alterations in cellular homeostasis involving these ions might participate in mitochondrial ROS formation. In addition to elevated Ca^{2+}, fatty acids, released by the action of phospholipases on membrane lipids during CNS injury (Bazan, 1977) may uncouple mitochondrial electron transport from ATP production (Lazarawicz et at, 1972; Wotjtczack, 1976; Hillered and Chan, 1988). Elevation of $[Ca^{2+}]_i$, in addition to inducing generation of ROS, may also result in abrupt loss of the mitochondrial membrane potential associated with the calcium-dependent opening of an inner membrane pore (Gunter et al., 1994).

Once formed, mitochondrial ROS may not only increase the cellular free radical "burden", with consequent damage to cell components, but may inhibit mitochondrial enzymes, leading to a feed-forward loss of mitochondrial energy production. Exposure of isolated mitochondria to oxygen radicals in the presence of elevated calcium specifically inhibits complex I (Malis and Bonventre, 1985; Cleeter et al, 1992; Dykens, 1994), the main entry-point of electrons into the electron transport chain. Reduced activity at this site may diminish electron transport and result in loss of mitochondrial membrane potential and impaired mitochondrial energy production. Depletion of energy stores may be a key event en route to cell

death not only by direct effects on cellular homeostasis, but by enhancing both excitotoxicity (Novelli et al, 1988) and free radical-mediated injury (Dugan and Choi, 1993).

Sod2 knockout mice

Mice deficient in SOD2 (MnSOD) due to deletion of the *Sod2* gene have been generated independently by two groups (Li et al, 1995, Sod^{m1ucsf}, deletion in exon 3; Lebovitz et al, 1996, $Sod2^{m1BCM}$, exon 1, 2). Heterozygous mice from both strains show no overt pathology, but homozygous Sod^{m1ucsf} pups die within ~ 1 week after birth, with dilated cardiomyopathy, fatty liver, but no gross pathological changes to CNS (Li et al, 1995). Homozygous $Sod2^{m1BCM}$ mice, in contrast live for up to 3 weeks of age, by which time gross morphological damage to mitochondria and neurodegenerative changes (brainstem, basal ganglia) were observed. However, both strains of mice exhibited biochemical evidence of mitochondrial respiratory impairment, including elevated lactate levels, and inhibition of aconitase and succinate dehydrogenase activity within the first week of life. Although it is not clear why the lifespan of the two strains differ, Lebovitz et al (1996) reported a small elevation in Cu,Zn-SOD (SOD1) levels in their mice, suggesting that a compensatory mechanism not observed in the UCSF mice may contribute to the longer survival of the $Sod2^{m1BCM}$. Regardless, it appears that both strains develop early mitochondrial damage which continues to progress in the $Sod2^{m1BCM}$ mice.

C_{60} derivatives and neuroprotection

Water-soluble C60 derivatives have proven to be broadly-effective neuroprotective agents in vitro, and in preliminary experiments in FALS mice, appear to have in vivo neuroprotection, as well. Based on our work with these compounds, we have formulated the idea that superoxide radical scavenging by the C60 sphere is the critical feature that confers the high degree of neuroprotection observed with these compounds. Several relevant observations ar detailed below:

1. Both malonic acid and polyhydroxyl derivatives reduce NMDA neurotoxicity, with the C3 compound providing the greatest degree of protection of any agent we have studied short of direct receptor blockade. However, they are not NMDA (or AMPA) receptor antagonists, and do not reduce NMDA-induced calcium entry. Results from our laboratory and others suggest that NMDA receptor activation causes mitochondrial radical ($O2^{\bullet-}$and $^{\bullet}OH$) production (Reynolds and Hastings, 1995; Dugan et al, 1995; Bindokas et al, 1996)

2. C_{60} derivatives also reduce excitotoxicity produced by AMPA, protecting both cortical neurons (Dugan et al, 1996; 1997b) and oligodendroglia (Dugan, Althomson and Goldberg, unpublished observations).

4. C_{60} derivatives block apoptotic degeneration in cortical neurons after serum withdrawal (Dugan et al, 1997b), SCG neurons after NGF deprivation (Putcha, Johnson and Dugan, unpublished), cortical neurons exposed to $A\beta_{1-42}$(Dugan et al, 1997b), and hepatoma cells exposed to TGFβ (Huang et al, 1997).

5. All derivatives studied have been excellent free radical scavengers, capable of scavenging both $^{\bullet}OH$ and $O_2^{\bullet-}$, eliminating $^{\bullet}OH$ at ~ 5μM, and $O_2^{\bullet-}$ at 40 μM. There are relatively few non-enzymatic radical scavengers which interact with $O_2^{\bullet-}$ to any great extent, with nitroxide and nitrone spin-trapping agents among the few exceptions. However, the relatively low affinity of spin-traps for superoxide requires them to be used in the mM range. For EPR studies, they are typically used at 50-100 mM. We found that malonic acid derivatives of C_{60} (40 μM) were able to out-compete the spin-trapping agent, DMPO (100 mM), for $O_2^{\bullet-}$, implying a uniquely avid affinity of C_{60} derivatives for $O_2^{\bullet-}$.

6. Polyhydroxy derivatives, which required ~ 200 μM to eliminate superoxide radical in EPR experiments were less effective than *C_3*, which scavenged a similar amount of $O_2^{\bullet-}$ at ~ 40 μM, in protecting neurons from excitotoxicity, especially against fulminant NMDA-induced neuronal cell death. Thus, it is possible that $O_2^{\bullet-}$ scavenging relates fairly closely to neuroprotective efficacy, since the ED_{50} for neuroprotection by the polyhydrodroxy compound against NMDA-induced death was ~ 500 μM, but was only ~ 50 μM for the *C_3* compound.

6. In a pilot study administering a malonic acid derivative by intraperitoneal pump to FALS mice (SOD1 G93A H1 mice, Gurney et al, 1993) these agents appear to possess *in vivo* activity, delaying both the onset of motor deterioration and death by over 1 week.

These results suggested that the free radical scavenging ability of the C_{60} unsaturated carbon sphere, itself, is responsible for the neuroprotective properties of the derivatives. Although side groups confer attractive solubility characteristsics, and could be added to achieve additional effects, they are not the critical determinant of cytoprotection (both polyhydroxy and malonic acid derivatives were effective). Further, based in part on their unique potency against NMDA toxicity, an injury we and others believe involves mitochondrial $O_2^{\bullet -}$ generation, and the fact that they are able to scavenge $O_2^{\bullet -}$ at concentrations in the low/mid micromolar range, we believe that $O_2^{\bullet -}$ scavenging by these molecules confers much of their neuroprotective properties.

Significance

As impaired free radical homeostasis becomes implicated in an ever-increasing list of neurological diseases states, the need for tools to study, and hopefully to treat, this component of CNS injury becomes increasingly important. While it is too early to foresee whether C_{60} derivatives will eventually be useful clinically, they appear to provide a unique and powerful tool to assist in the study of physiological and pathological roles of free radical in the CNS. Therefore, the broader goals of this proposal are: 1) to develop these molecules as tools to study the role of $O_2^{\bullet -}$ as both signaling molecule and neurotoxin, 2) to continue exploration of the contribution of mitochondrial $O_2^{\bullet -}$ to neuronal cell death, 3) and to begin to define structure-function relationships for these promising compounds as a necessary step towards pre-clinical trials. The results of the proposed experiments should determine whether mitochondrial $O_2^{\bullet -}$ scavenging by C_{60} derivatives plays an important role in their neuroprotective effects, may suggest that mitochondrial $O_2^{\bullet -}$ contributes to neuronal injury in a variety of insults, and would further support the concept that scavenging of mitochondrial $O_2^{\bullet -}$ might be an attractive therapeutic target in many neurological disease states .

C. PRELIMINARY STUDIES

To date, we have studied two water-soluble fullerene derivatives, polyhydroxylated C_{60} and hexacarboxylated C_{60} compounds as neuroprotective agents. Both classes of compounds are excellent free radical scavengers, and are neuroprotective against both excitotoxic and apoptotic insults. Results for polyhydroxyl C_{60} are reported in Dugan et al, 1996 (attached), and for malonic acid derivatives of C_{60} in Dugan et al, 1997 (attached). Head-to-head comparison of neuroprotection by C_3 vs several other antioxidants against NMDA excitotoxicity is shown in Table 1. The C_3 derivative provided near-complete protection against NMDA-induced neuronal death, with other agents providing 0-20% protection.

Tris-malonic acid derivatives of C_{60}, $[C_{63}(COOH)_2)_3]$ have been synthesized and purified (Hirsch, 1990) in the PI's laboratory to give regioisomers with C_3 or D_3 symmetry (Fig. 1). The purity of each isomer was confirmed by ^{13}C NMR. The spectrum from the potassium salt of the C_3 acid synthesized by E. Lovett is shown in Fig. 2. As reported for the native C_{60} molecule (Krusic, 1991), C_{60} derivatives retain potent free radical scavenging properties. EPR spectroscopy with DMPO as the spin-trapping agent was used to determine the concentrations at which the C_3 and D_3 compounds were able to effectively eliminate a defined amount of $^{\bullet}OH$ or $O_2^{\bullet -}$. Both isomers effectively abolished the $^{\bullet}OH$ and $O_2^{\bullet -}$ signals at 4 μM ($^{\bullet}OH$) and 40 μM ($O_2^{\bullet -}$), as demonstrated by EPR spectroscopy (Fig. 3). In comparison, the polyhydroxy C_{60} derivatives, which are more highly substituted than the tris malonic acid compounds (C_3 or D_3), were unable to reduce the superoxide signal from xanthine/ xanthine oxidase at concentrations < ~ 200 μM (data not shown), and were less effective and less potent neuroprotective agents. This suggest that the degree of substitution to the fullerene sphere may determine radical scavenging potency, an observation which we will exploit to allow correlation of the radical reactivity with neuroprotection of different C_{60} derivatives. *In vitro* studies in murine cortical cell cultures demonstrated that C_{60} derivatives are highly effective in reducing excitotoxic neuronal death produced by NMDA or AMPA (Fig. 4 and Table 1), as well as neuronal death induced by oxygen-glucose deprivation (Fig. 5), an injury mediated by both NMDA and AMP/kainate receptors. $^{45}Ca^{2+}$ tracer experiments have confirmed that these derivatives do not act as NMDA receptor antagonists (Fig. 4C).

Cultured superior cervical ganglion neurons have been reported to generate reactive oxygen species (ROS) early in the process of apoptotic neuronal death induced by growth factor deprivation (Greenlund et al, 1995; Dugan et al, 1997a), and anti-oxidants provide partial protection (Greenlund et al, 1995). Cortical neurons in glial-

deficient culture also demonstrate production of reactive oxygen species 6-8 h after serum withdrawal (Fig. 6). Both apoptotic death and enhanced production of ROS were inhibited by addition of the C_3 compound (Fig. 6).

FALS mice (Gurney G1 strain) were used for a pilot trial *in vivo* of the malonic acid C_{60} derivatives. At 9 weeks of age, Alzet mini-osmotic pumps (28 day, 6 µl/day, 15 mg/kg/day) containing carboxy C_{60} (the mixed isomers) or saline were implanted intraperitoneally. The pumps were replaced at 13 weeks of age. Videotaped assessments of open field walking were performed and scored using the Bresnahan scale for spinal cord injury (Basso et al, 1995). As reported by Gurney and colleagues (1993), these animals develop motor symptoms by approximately 90 days of age, and are moribund between 125 and 145 days of age. The group treated with carboxy C_{60} showed a 7-8 day delay in death (Fig. 8) with a $p = 0.12$. They also demonstrated some delay in onset of symptoms (not shown). A number of littermates who lacked the mutant *sod1* gene were also given two months of carboxyfullerene therapy, and showed no adverse effects - they were as active as littermates, and weights were similar (within genders) for treated and untreated WT animals and for FALS mice before they became symptomatic.

In collaboration with Dr. Steve Rothman, consultant on this project, we have established techniques to evaluate production of ROS by brain slices exposed to various insults, employing salicylate oxidation with slice perfusion, and using oxidation-sensitive compounds such as dihydrorhodamine 123 (DHR) and dihydroethidium (DHE) with confocal fluorescence microscopy. DHR loading is rapid and is easily discerned from tissue autofluorescence (Fig. 8 A, B), and increased fluorescence from dye oxidation is observed after application of ethacrynic acid (Fig. 8 C, D), which impairs detoxification of endogenously-generated ROS by depleting intracellular glutathione (Hockenberry et al, 1993). Using perfusion of slices with artificial CSF (ACSF) containing salicylic acid, we observed that reintroduction of oxygen and glucose after oxygen-glucose deprivation to slices produces an early increase in the salicylate oxidation product, 2,3-DHB (Fig. 8 E) consistent with increased ROS in ischemia-reperfusion injury reported by many groups. We observed a similar increase in human slices (not shown). We have also used dihydroethidium oxidation as a means to detect intracellular superoxide generation. Cultures with dihydroethidium in the medium show enhanced fluorescence from dye oxidation 40 minutes after exposure to NMDA (Fig. 9 A,B). It appears that individual cells may produce different levels of superoxide in response to the same stimulus (Fig. 9C). As an additonal measure of one index of oxidative damage, lipid peroxidation, we have used EPR spectroscopy to detect peroxyl ($LOO^{\bullet}$) lipid radicals in mouse brain liposomes (Fig. 10); the C_3 isomer was more effective than the D_3 isomer at protecting lipids from radical attack., consistent with our previous finding that C_3 penetrates lipid membranes to a greater extent than D_3.

Table 1 Neuronal death following 10 min exposure to NMDA is relatively resistant to neuroprotection by antioxidants: comparison of the efficacy of C_3 with other antioxidants. Mixed cortical cultures were exposed to NMDA for 10 min in the presence of antioxidant compounds, and cell death assessed at 24 h by LDH release. The antioxidants studied have been reported to be neuroprotective by in other systems, or against less fulminant types of neuronal injury. A range of concentrations for each antioxidant were tested; the maximal protection and the concentration at which it was achieved are listed.

Antioxidant	Maximal reduction in neuronal death (%), Mean ± SD	Concentration providing best protection (µM)
C3	91 ± 8	100
α-tocopherol (Na succ)	22 ± 12	100
PBN	15 ± 7	300
U74006F	10 ± 18	3
Trolox	8 ± 17	100
U74500A	8 ± 15	3
Ubiquinone	0	NA

D. EXPERIMENTAL PLAN

General methods

Synthesis and characterization of water-soluble malonic acid, PEG, and amino C_{60} derivatives

Synthesis of malonic acid C_{60} derivatives will be carried out as described (Lamparth and Hirsch, 1994; Hirsch et al., 1994; Dugan et al, 1997). Briefly, diethyl bromomalonate is added to a solution of C_{60} in toluene, followed by addition of 1,8-diazobicyclo[5,4,0]undec-7-ene (DBU), which results in a color change from violet to dark red. After stirring for 4 days, solvent is removed in vacuo and the blackish residue chromatographed on silica gel (270-230 mesh) using toluene-hexane (1:1 by volume) as eluent. The unreacted C_{60} is obtained first followed by a brown band which corresponds to the diester. The eluent is changed to toluene-hexane (4:1), and several bands corresponding to tetraester isomers are obtained. The eluent is changed again to toluene-hexane (9:1), and a red band elutes, corresponding to the D_3 isomer as the major component. Toluene (100%) is then used to elute three bands; the third band corresponds to semi-pure C_3. Additional bands containing hexa- and octaesters are eluted later. The fractions containing semi-pure C_3 or D_3 regioisomers of the malonic acid tris-adduct, and the higher order adducts are rechromatographed on silica gel (230-400 mesh) using toluene as the mobile phase. NaH is added to the purified samples of isomers, and the mixture refluxed for 1 h. After the heating source is removed, MeOH (5 ml) is added immediately to quench the reaction. Red powder soon precipitates and is collected by centrifugation. The powder is washed with toluene and hexane, then dissolved in water to which HCl (4 M) has been added. The precipitate is collected by centrifugation, redissolved in MeOH, and the solvent is then removed in vacuo to give powdery, pure isomer acid. Because the above reaction generates little hexa-malonic acid adduct, a template-based synthesis (Lamparth et al, 1995) which is reported to yield >50% of the hexa$C_{66}(COOH)_{12}$ will be used.

Identification of each isomer, and evaluation of structure and purity of each fraction will be performed as follows. Thin layer chromatography (TLC) is used to screen fractions coming off the column for degree of substitution and general purity. Fractions which appear to contain a single isomer visually are then evaluated by NMR, and are then hydrolyzed to acids if they appear pure. NMR on the resulting acid is then done to confirm purity. More detailed characterization of the structure of C_{60} derivatives and oxidation products will be determined by mass spectroscopic analysis. These will be carried out at the Washington University Resource for Biomedical and Biorganic Mass Spectrometry (Director, Michael Gross) using conventional mass spectrometry with negative ion analysis (increased sensitivity for negatively charged groups such as carboxylic acids), and electrospray mass spectrometry. In addition, we have a collaborative agreement with the Pacific Northwest National Laboratory (Director, Richard Smith), which is equipped with a 12 Tesla Fourier transform ion cyclotron resonance, FITCR, spectrometer with both electrospray and matrix-assisted laser desorption ionization, MALDI, sources, which may be used to characterize metabolites of C_{60} derivatives if the products are either complex, or scarce.

We also plan to synthesize aminoamido and PEG derivatives of C_{60}. Because of the uncontrollable nature of most additions to C_{60}, a majority of derivatization methods described to date do not result in a predictable or defined set of products. Therefore, to generate chemically specific amino and PEG derivatives, we plan to capitalize on our experience with the Bingel reaction, and take advantage of the limited and defined products of the malonic acid derivatization procedure described above. We will first synthesize aminoamido or polyethylene glycol (PEG) derivatives of malonic acid, and will then use the synthetic strategy described above for native malonic acid to produce the final aminoamido- or PEG C_{60} derivatives. An alternative approach for synthesizing amino C_{60} derivatives will be by direct addition of bis secondary amines, piperazine, N,N'-dimethylethylene diamine, or N,N'-Bis(2-hydroxyethyl)ethylene diamine, as described by Kampe et al, 1993.

Electron paramagnetic resonance (EPR) spectroscopy: radical reactivity

The reactivity of malonic acid, amino and PEG fullerene derivatives with oxygen radicals ($O_2^{\bullet-}$, $^{\bullet}OH$) will be assessed by EPR spectroscopy using spin-trapping agents, 5,5-dimethyl-1-pyrolline-N-oxide (DMPO) or N-*tert*-butyl-α-phenylnitrone (PBN). Superoxide radical is generated by xanthine oxidase (10 mU/ml) plus xanthine (25 μM), and hydroxyl radical is produced by 100 μM H_2O_2 with 10 μM Fe^{2+} via the

Fenton reaction. Samples containing spin-trapping agents (100 mM), C_{60} derivatives (0-100 μM), and either xanthine/xanthine oxidase, or H_2O_2 /Fe are loaded into a quartz flat cell (60 x 10 x 0.25 mm) and analyzed on a Bruker 200 X-band spectrometer, using EPR settings: power = 1.6 mW, modulation = 1 G, field modulation = 100 Hz, R.G. = $3.2x10^5$.

The ability of C_{60} derivatives to scavenge lipid peroxides ($LOO^{\bullet}$) will be examined by exposing liposomes made from mouse brain lipids to H_2O_2, in the presence of C_{60} derivatives (0-100 μM). EPR settings are the same as those described above. No spin-trapping agents are used. Assessment of endogenous levels of peroxyl radical will be performed on lipids from cultures or brain by placing frozen brain (frozen in liquid nitrogen and powdered by crushing in a cold mortar) or cell cultures (frozen in liqiud nitrogen, then scraped off the culture dish) into the quartz flat cell as a frozen powder. Samples take approximately 2 minutes to thaw, at which time, EPR analysis is performed using the settings above.

Low temperature (77^oK) EPR will also be performed on C_{60} derivatives exposed to xanthine/xanthine oxidase or to H_2O_2 /Fe to determine whether oxidation produces a paramagnetic (radical) species. EPR settings for these experiments will be: microwave power = 0.64 mW, frequency = 9.4437 GHz, modulation amplitude = 6.3 G, and modulation frequency = 100Hz.

Membrane interactions of fullerene derivatives.

We will evaluate the degree of membrane interaction of C_{60} derivatives, comparing regioisomers of the series of malonic acid adducts with increasing degrees of substitution, using spin-labeled lipids and EPR analysis as described (Dugan et al, 1997). Mouse brain lipid extracts (Hara and Radin, 1979) are aliquoted into test tubes. Spin-labeled lipids, 5-doxyl or 16-doxyl ketostearic acids, are added at a ratio of 1:50, and dried under N_2 (Hubbell and McConnell, 1968; Devoust et al, 1983). Tris saline (25 mM, pH 7.4), with or without C_{60} derivative, is added to each tube. Compounds will be tested at concentrations of 10-300 nmol (1:30-1:1 compared to lipids). Settings for the EPR are: power = 1.6 mW, modulation = 1 G, field modulation = 100 Hz, R.G. = $3.2x10^5$.

Extraction and separation of C_{60} derivatives from biological samples.

Malonic acid derivatives will be extracted from cells or brain tissue employing a method similar to that currently in use in the PI's laboratory to extract salicylate oxidation products from cells and tissues (see *"Intracellular salicylate oxidation"*, below). Briefly, cells treated with C_{60} derivatives will be rinsed twice with cold PBS. The PBS is completely aspirated and 1 M acetic acid is added (0.3 ml/well). Cultures are frozen (-20^oC), thawed, refrozen, and thawed again. The extract is removed to a text tube, and ethyl acetate (0.5 ml) is added. After the samples are vortexed, and allowed to stand at 4^o C overnight, the upper ethyl acetate phase is transferred to a second tube. The initial tube is re-extracted for 2 h, the upper phases are combined, and the extract dried under nitrogen. We have determined that C_3 extracts quantitatively into the ethyl acetate phase. Extraction of C_{60} from brain will be carried out by a modification of the above procedure. The brain will be removed from mice after sacrifice and perfusion with cold PBS. Chilled brain will be placed in 0.1 M perchloric acid (1 g/ 3 ml) and homogenized using a Branson tissue disruptor. After an equal volume of chilled acetic acid (1 M) is added to the brain homgenate, the sample will undergo 4-5 more passes of the disruptor. An equal volume of ethyl acetate is added, and the sample is vortexed and placed at 4^oC in the dark overnight. Samples are then extracted twice as described above for culture extracts.

A commonly-used method to enhance the sensitivity and specificity of detection for functional groups, such as carboxylic acids, is derivatization of the group with a fluorophore or chromophore. Derivatization of carboxyl groups with fluorescent tags can be achieved using an extensive variety of reagents, each with specific advantages and problems (reviewed in Mukherjee and Karnes). Pre-column derivatization and HPLC fluorescence detection has been reported for the 1-bromoacetylpyrene conjugate of bile acids (Kamada et al, 1983). This fluorescent reagent is attractive because its excitation and emission λ (Ex 370 nm, Em 440 nm) can be used with an in-line HPLC fluorescence detector. An alternative derivatizing reagent, 1-pyrenyldiazomethane (PDAM) is available from Molecular Probes (Eugene, OR), and is

reported to allow detection of carboxyl-containing compounds at concentrations less than 1 pmol (Staffeldt et al, 1991). We will test both of these reagents to determine which provides the easiest and most effective derivatization of each of the ~ 10 malonic acid isomers, and will determine derivatization efficiency using pure standards.

After derivatization, cell culture or tissue extracts will be injected into the HPLC, and separated on a silica gel normal phase column (Zorbax SIL, Phenomenex), using a method designed to resolve a broad range of lipid species (Dugan et al, 1986). A gradient from hexane:2-propanol (HIP, 3:2) to HIP with 5.5% water will separate compounds ranging from non-polar lipids, such as cholesterol, through fatty acids, to the more polar phospholipids. Detection of C_{60} derivatives will be performed by scanning UV/vis diode array detector in series with a fluorescence detector. For a given malonic acid derivative, we expect to observe absorbance maxima at ~ 300 nm, and another between 400 - 550 nm (depending on the specific isomer), and a fluorescence peak that co-elutes with this absorbance pattern.

Cell Cultures:

Standard neuronal cultures.

Neocortical cell cultures will prepared from fetal (E15) Swiss-Webster mice (Simonson) as described previously (Dugan et al, 1995a). Cortical hemispheres are dissected away from the rest of the brain and placed in trypsin (0.25%, Gibco) for 15 min. The cortices are briefly centrifuged, the trypsin removed and the hemispheres resuspended in plating medium, which consists of media stock (MS: Eagle's Minimal Essential Media minus L-glutamine, Gibco 11430-022) with 20 mM glucose, 26.2 mM $NaHCO_3$), 5% fetal calf serum, and 5% horse serum (Hyclone) for pure neuronal cultures (<0.5% astrocytes). For neuron-astrocyte co-cultures, the same media, supplemented with L-glutamine (2 mM) is used. After trituration, cell suspensions are diluted and plated onto a pre-existing bed of mouse cortical astrocytes in 24-well Primaria culture plates or 35 mm culture dishes (Mat-Tek) possessing an oval cut-out sealed by a glass cover slip (for mixed cultures), or onto poly-D-lysine (PDL): laminin-coated 35 mm dishes or 24-well plates (for pure neuronal cultures). Cells are also grown on 25 mm glass coverslips coated with PDL-laminin, for use in a coverslip holder for the confocal microscope. Mixed cultures are fed biweekly with growth medium (media stock with 10% horse serum, 2 mM L-glutamine), until the final feeding at day 11 or 12 in vitro, when cultures are fed with media stock supplemented with 2 mM L-glutamine. Cells are used for experiments after 12-17 days in culture.

Single embryo dissections for Sod2 null mice

A protocol to prepare cultures from single embryo dissections is currently being used in the PI's laboratory to prepare neuronal cultures from FALS mice, and will be used to establish cultures from *Sod2* embryos. Mouse embryos (E15) from *Sod2* +/- matings are dissected as described above, except that each pup is dissected with separate instruments and placed in individual tubes for trituration. The tail and a hindlimb are removed and used for subsequent genotyping. Dissociated cortices from each pup are plated separately into coverslip dishes or into 24-well culture plates.

Oligodendroglial cultures.

Newborn mice are anesthetized, decapitated and the cerebral hemispheres isolated with removal of all meninges. The hemispheres are minced and cells dissociated by trituration. Cells are incubated in T75 flasks undisturbed for 4 days at 37^{o}C, 5% CO_2, then fed with complete medium [F12/DMEM containing insulin (5 mg/ml), transferrin (50 mg/ml), selenite (30 nM), and biotin (10 nM)] and every other day thereafter for 8 days (Bottenstein et al, 1990). Cultures are shaken on day 8 -12 on an orbital shaker at 220 rpm overnight at 37^{o}C, and the suspended detached oligodendrocytes are removed. Cells are then placed on hydrophobic Petri dishes at 37^{o}C for 3 h, to allow microglial cells to attach. Unattached oligodendrocytes are centrifuged and resuspended in complete medium with 10mg/ml polyornithine. Cells at 4 weeks in culture are >90% galactocerebroside positive.

Brain slices.

Mice are killed using 95 % inspired CO_2, and the brain rapidly harvested and chilled, and mounted on an 3% agar disk using SuperGlue®. The agar disk is glued to the vibratome dish, which contains cold artificial cerebrospinal fluid (ACSF: 200mM sucrose, 30 mM NaCl, 5mM KCl, 9mM $MgCl_2$ ($6H_2O$), 1mM $MgSO_4$, 10 mM glucose, 20 mM HEPES, 3 mM $NaHCO_3$, 1 mM NaH_2PO_4.). Brain slices (300 μM) are cut by vibratome, and placed in a holding chamber with ACSF aerated with 95% O_2 / 5% CO_2. After a 60 minute equilibration period, each slice is transferred to a 35 mm culture dish with oval cutout, sealed by a coverslip. A weighted nylon mesh is used to hold the slice in place, and the dish is filled with 3 ml ACSF containing dihydrorhodamine (20 μM). DHR fluorescence (Ex λ 488 nm, Em λ > 515 nm) is visualized on an inverted confocal microscope (Noran Odyssey) using a 60 X (1.2 NA) water immersion objective.

Injury Paradigms.

Brief exposure to N-methyl-D-aspartate (NMDA) is carried out as described (Dugan et al, 1997b). The culture media is exchanged twice with HEPES, bicarbonate-buffered balanced salt solution (HBBSS), and then NMDA (200 μM) is added alone or with C_{60} derivative for 10 min. Exposure is terminated by exchanging the medium four times with MS. The cells are returned to the 37°C (5% CO_2) incubator for 24 h, when injury is assessed by determination of LDH release (see below). To determine whether C_{60} derivatives alter block NMDA receptor-mediated calcium entry, cells are exposed to NMDA for 10 min in the presence of tracer $^{45}Ca^{2+}$ (0.5 μCi/well; NEN, Boston, MA), and the amount of intracellular $^{45}Ca^{2+}$ determined as previously described (22). Slowly triggered AMPA receptor-mediated excitotoxicity is induced by the addition of 8-10 μM AMPA (plus 10 μM MK-801: Merck, Sharp and Dohm, Essex, PA, included to block activation of NMDA receptors by released endogenous glutamate) with or without C_{60} derivatives for 24 hr. Combined oxygen, glucose deprivation is performed as described (Goldberg and Choi, 1993). Cultures are placed in an anoxic chamber (O_2< 2mm Hg), and the media exchanged three times with a balanced salt solution (BSS_0) lacking glucose and oxygen. After 45-60 min, the cells are returned to oxygen, glucose-containing medium and placed in the normoxic culture incubator until assessment of cell death at 24 and 48 h.

To induce apoptosis, pure neuronal cultures containing less than 1 % astrocytes are deprived of serum on day *in vitro* (DIV) 7 by exchanging the medium with serum-free MS (Dugan et al, 1995a; Koh et al, 1995). Washed controls are returned to medium with serum. Cell death is assessed at 24 h and 48 h by propidium iodide staining/cell counts, LDH release, morphology, and Annexin V staining (Martin et al, 1996). Caspace inhibitors ZVAD and YVAD will be used as positive controls (Barge et al, 1997; Medema et al, 1997) as one confirmation that injury is due to caspace-dependent apoptosis.

Superior cervical ganglion neurons are deprived of nerve growth factor (NGF) by exchanging the medium three times with MEM without serum or NGF (Martin et al, 1988). Anti-NGF (1:500) antiserum is added with the last exchange. Control cultures are returned to NGF. Apoptotic cell death is quantified by staining with propidium iodide and cell counting.

Spontaneous death of neuronal and oligo cultures from *Sod2* knockout mice will be followed by adding propidium iodide (10 μg/ml) to the cultures, and performing sequential counts, allowing an assessment of nuclear morphology as well as cell death. Since propidium iodide can be maintained on cultures for several days without effects on viability, and shows little increase in nonspecific fluorescence with prolonged application, this will allow each culture well to serve as its own "density control" and will also minimize the number of cultures necessary for these experiments. The number of cells rescued by glutamate receptor antagonists, or caspace inhibitors (YVAD, ZVAD) will examine the mechanism behind this spontaneous death. Annexin V staining (see next section) will also be performed as another index of apoptotic neurodegeneration.

Assessment of cell death

Cell death in cortical cultures will be assessed by both qualitative and quantitative measures. Morphology after excitotoxic or apoptotic insults will be examined by phase contrast microscopy and under Nomarski optics on the confocal microscope. Annexin V staining, an indicator of the early

transmembrane movement of phosphatidylserine from inner to outer membrane leaflet observed during apoptosis, and associated with caspace activation (Martin et al, 1996), will complement other morphological assessments of apoptosis. Neuronal death will be determined quantitatively by assay of lactate dehydrogenase (LDH) released into the bathing medium (Koh and Choi, 1987), and by manual counts of cells which no longer exclude propidium iodide (10 mM for 7 min). Generally, 8 consecutive 10 X fields are counted across the diameter of the culture well. The appearance of propidium iodide nuclear staining (showing large aggregates of condensed DNA, and sometimes, the presence of DNA in membrane-delimited blebs - apoptotic bodies) has correlated well with other features of apoptosis, including the ability of caspace inhibitors to block cell death, in these cultures (Gottron et al, 1997).

Annexin V staining is performed with an R&D Systems kit, using a modification developed by the PI in conjunction with the manufacturer, to adapt their standard procedure for FACS analysis to fluorescence microscopy. 100 μl of annexin V reagent (=solution A) is added to 1 ml of 1 X buffer in an Eppendorf tube. Cells grown in 35 mm dishes with coverslip cutouts are gently rinsed twice with 2 ml PBS at room temperature. The PBS is removed, and 200 μl of solution A is added. The dishes are placed in the dark for 10 min. 800 μl of 1 X binding buffer is added to each dish, and staining is imaged by confocal microscopy, using FITC settings (Ex λ 488, Em λ> 515 nm).

Loss of oligodendroglia exposed to AMPA, or due to spontaneous death in *Sod2* null cultures, will be determined as loss of Gal-C positive cells, compared to the number in untreated controls. Gal-C staining is performed on fixed cells using rat anti-galactocerebroside (1:1200; marker for oligodendrocytes, Sigma).

Measurement of neuronal free radical handling: cultures and slices

Confocal Fluorescence Microscopy.

Dihydrorhodamine 123 (Molecular Probes, Eugene, OR) stock (10 mM) is prepared in DMSO, and stored at -20^{o}C until use. Cultures are washed into HEPES, bicarbonate-buffered balanced salt solution (HBBSS, containing, in mM, 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1.8 $NaPO_4$, 12 HEPES, 25 $NaHCO_3$, 5.5 D-glucose, pH 7.40), plus 15 μM dihydrorhodamine, and returned to the 37^{o}C, 5% CO_2 incubator for 30 min. After loading with dihydrorhodamine, initial cellular fluorescence is imaged using a laser scanning confocal microscope (Noran Odyssey), with an argon-ion laser coupled to an inverted microscope (Nikon Diaphot) equipped with a 60x water-immersion objective (Nikon Plan Apo, N.A. 1.2) (Dugan et al, 1995b; 1997a). The excitation λ is 488 nm, and fluorescence is monitored at λ > 515 nm. Laser intensity is attenuated to less than 5% of maximum illumination, and laser exposure was limited to brief image acquisition intervals. Frame-averaged confocal images are digitized at 640 x 480 pixels using microcomputer-based imaging software (MetaMorph, Universal Imaging). For analysis of fluorescence pixel intensity, regions of interest are selected to included neuronal cytoplasm (excluding the nucleus).

Dihydrorhodamine (DHR) is a lipophilic molecule which enters cells easily, and which may partition to some extent into lipid membranes. In our hands, it appears to detect mitochondrial production of reactive oxygen species with greater sensitivity than it detects cytosolic ROS production (Dugan and Lobner, unpublished observation), possibly reflecting its concentration in membranes. Non-fluorescent dihydrorhodamine is oxidized to fluorescent rhodamine 123, primarily by hydroxyl radical; DHR shows little direct reactivity with $O_2^{\bullet-}$ (Henderson et al, 1993; Royall and Ischoropoulos, 1993). Rhodamine 123 is positively charged and, following the Nernst equation, will concentrate in mitochondria (membrane potential, Ψ, ~ -180 mV) (Farkas et al, 1989).

Dihydroethidium, in contrast, reacts with $O_2^{\bullet-}$ to generate ethidium, which then binds to nuclear and mitochondrial DNA, showing a substantial increase in fluorescence on binding (Bindokas et al, 1996). Dihydroethidium appears to be diffusely present in all cell compartments, but moves to DNA after oxidation. The use of each of the probes allows some specificity for the type of radical detected ($^{\bullet}$OH vs. $O_2^{\bullet-}$). In addition, while we believe that dihydrorhodamine detects primarily mitochondrial ROS in the cells and under conditions we have studied to date, we routinely use mitochondrial inhibitors (rotenone, antimycin A, oligomycin, FCCP) to confirm the mitochondrial source for any ROS signal we observe.

Intracellular salicylate oxidation

Cultures are loaded with salicylic acid (1mM) for 4 hours in MEM with 20 mM D-glucose (Lobner et al, *Submitted*; attached). The medium is exchanged with medium lacking salicylate, and drugs are then added in MEM/glucose containing 100 μM salicylate for a designated period of time. To terminate the exposure, cells are washed twice with cold PBS, which is removed completely, and 1 M acetic acid is added. Cultures are frozen at -20^{o}, then thawed (to break open cells). After refreezing, they are thawed again, and the acetic acid extract removed to a text tube. Ethyl acetate is added, the samples vortexed and allowed to stand at 4^{o} C overnight. The upper, ethyl acetate, phase is transferred to a second tube. The initial tube is re-extracted for 2 h, the upper phases combined, and dried under nitrogen. The dried extract is then redissolved in 0.1 N HCl, and injected onto the HPLC. Salicylate oxidation products, the 2,3- and 2,5-dihydroxybenzoic acids, as well as salicylate, are separated on a C_{18} ODS column using 30 mM citric acid (brought to pH 4.7 with glacial acetic acid) plus 3% methanol. Compounds are measured by electrochemical detection using a Waters 464 ECD with glassy carbon electrode set at 600 mV, comparing unknowns to retention times and peak area responses of commercial standards. Salicylate is detected by absorbance at 234 nm.

Thiobarbituric acid reactive substances (TBARS)

The ability of C_{60} derivatives to protect membrane lipids from peroxidative damage will be evaluated using two complementary methods: assay of TBARS and EPR analysis of lipid peroxyl radicals. We have not been successful in our attempts to use a third commonly-employed measure of lipid peroxidation, spectrophotometric detection of conjugated dienes, and believe that this technique is too insensitive for use in our culture system. Lipids for TBAR assay will be extracted from cultures or brain using the method of Hara and Radin, 1978. Hexane:2-propanol (3:2) is added to cultures, and pipetted over the cells several times to extract the lipids. Lipids from brain or spinal cord tissue are extracted by homogenizing tissue in cold hexane:2-propanol (1 mg/18 ml). TBARS are determined by taking an aliquot of lipid extracts from cultures or tissue which is dried under nitrogen, and adding 2.0 ml of TBA-TCA solution (15% w/v trichloroacetic acid TCA, 0.375% w/v thiobarbituric acid TBA, 0.25 N HCl plus 0.02%) (Anderson et al, 1994). After vortexing, samples are heated in a boiling water bath for 15 min in test tubes covered with marbles. When cool, the precipitate is removed by brief centrifugation (1000 x g for 5-10 min), and the samples read at 532 nm (in a Perkin-Elmer LS50B Fluorescence Spectrometer) against a reagent blank. To prevent peroxidation of lipids during the assay procedure, 0.02% butylated hydroxytoluene is added to both the TCA-TBA-HCl reagent and to any wash or perfusion solutions (usually cold PBS for both cultures and for perfusion of mice prior to removal of brain for lipid extraction) to minimize artifactual production of TBARS during processing.

Nitrotyrosine (NT) Immunocytochemistry

NT staining of tissue will be performed in collaboration with Dr. Anne Cross (collaborator); these methods are routinely used in her laboratory. Sections (8 mm) are cut and air dried and fixed in 1% paraformaldehyde for 5 min, followed by cold 100% ethanol. Sections are blocked (3% normal serum from host of secondary Ab) in phosphate-buffered saline containing 2% nonfat dry milk and 2% bovine serum albumin for 1 h at RT. In some cases, sections are permeabilized (0.3% Triton-X-100). Separate sections are incubated at 4^{o}C for 16 h with a rabbit antiserum to to L-nitrotyrosine (1:500) or normal rabbit serum (control), or rabbit antiserum to murine iNOS (1:200; generated to the carboxy terminal 21 amino acids of murine iNOS, as described in Cross et al, 1997, in press; attached), and together (for double-immunostaining) with rat anti-glial fibrillary acidic protein (GFAP-a marker for astrocytes; 10 mg/ml, Zymed), mouse monoclonal anti-Mac-1 (a marker for macrophages and microglia, Dako), mouse anti-human CD3 (a marker for T cells, Dako) or rat anti-galactocerebroside (1:1200; marker for oligodendrocytes, Sigma). The antiserum to nitrotyrosine was generated by immunizing a rabbit with nitrated keyhole limpet hemocyanin, as described (Cross et al, 1997) (generated at Searle), and is always applied in the presence of excess L-tyrosine (10 mM, Sigma, St. Louis). Sections have also been

immunostained successfully using anti-NT antisera from Upstate Biotechnology (1:100). As a control for specificity of staining, 10 mM 3-nitro-L-tyrosine (Sigma) will be used to competitively block staining. NT immunoreactivity will be localized using a Cy3-conjugated donkey anti-rabbit IgG (1:300; Jackson ImmunoResearch). GFAP and GC immunoreactivity are localized using a fluorescein (DTAF)-conjugated affinity purified donkey anti-rat IgG (1:100; Jackson ImmunoResearch). Slides are counterstained with hematoxylin, examined under epifluorescence and photographed using a Nikon Labophot-2. Dr. Cross has also developed a method whereby the avidin-biotin-chromagen (ABC) technique can be used sequentially with fluorescence for double-staining, to improve sensitivity of detection for rare or less avid primary antibodies.

Breeding/identification of Sod2 knockout mice

A mouse colony will be established from founders provided by Dr. Charles Epstein and colleagues at the University of California, San Francisco (Li et al, 1995). Their experience with these mice suggests that homozygotic mice on a CD-1 background live for approximately 1 week after birth (Lebowitz et al, 1996; Huang et al, 1997), and thus would be the best strain to attempt to use for in vivo experiments. Animals will be identified by ear-punching. Genotyping will be done by PCR of genomic DNA extracted from a small segment of tail, using techniques currently in place in the PI's laboratory. Optimal sequences for the PCR primer sets will be obtained from Dr. Epstein.

In vivo neuroprotection in Sod2 -/- animals.

C_{60} derivative will be administered orally to newborn mouse pups born to a cross between *Sod2* +/- parents. Because of the need to start treatment immediately, prior to genotyping, all pups in a litter will be given the same treatment, either C_{60} derivative in sugar water (20mM sucrose), or sugar water with food coloring to match the color of the derivative. One hundred μl of solution will be adminstered by syringe to each pup q 8 h. The dosing schedule will be guided by information obtained from experiments outlined in Aim 1, but will tentatively be 1 mg/kg/day, 5 mg/kg/day, and 15 mg/kg/day. Drug adminstration will be continued until tail-clipping and genotyping at ~ 18 days of age, and will be continued if any homozygous -/- animals are still surviving. Kaplan-Meier survival curves will be constructed for -/- animals in treated and untreated litters. Mean survival of treated and untreated litters will also be compared using t-test.

If survival is enhanced by C_{60} derivatives, we will examine the central nervous system of treated and untreated animals at two timepoints for neuroprotection Animals will be sacrificed at one week of age (just at the end of the normal lifespan of the homozygous null animals) or at death. Animals will be anesthetized with an overdose of inhaled halothane, and sacrificed with transcardial perfusion of 0.1 M PBS (pH 7.4), followed by 4% paraformaldehyde fixation. Brain and cord will be removed, and post-fixed in 4% para, followed by either sectioning and paraffin-embedding, or by sinking in sucrose solution. Paraffin sections will be re-cut (10 μm) and stained with H&E, Luxol fast blue, cresyl violet, and Bielschowski silver. Sucrose-permeated tissue will be processed for immunocytochemistry, using antibodies recognizing MAP-2, GFAP, and neurofilaments. Immunoreactivity will be visualized using either diaminobenzidine (with hematoxylin counterstain), or by secondary fluorescent antibody using confocal microscopy for cellular and subcellular resolution. In addition, paraffin-embedded tissue may be deparaffinized by washing twice with xylene. Other organs including heart and lung will also be removed from a limited number of animals and saved for future evaluation of non-CNS cytoprotection.

Assessment of *in vivo* ROS production in *Sod2* -/- mouse pups will be performed by determining salicylate oxidation in whole brain tissue after i.p. injection (Piantadosi and Zhang, 1996), and by determination of dihydroethidium oxidation after i.p. injection, as described by Kondo et al, 1997. Salicylate is adminstered (the highest concentration which is not lethal to the mice will have to be determined, but will be approximately 1 mg/kg), 1-2 h before sacrifice. Salicylate and oxidation products are extracted as described above, and evaluated by HPLC. Dihydroethidium (~ 5 mg/kg) will be adminstered i.p. and the animals sacrificed by overdose of inhaled halothane, and transcardially perfused and fixed, and the brains remooved and sectioned. Brain slices are then examined for ethium fluorescence

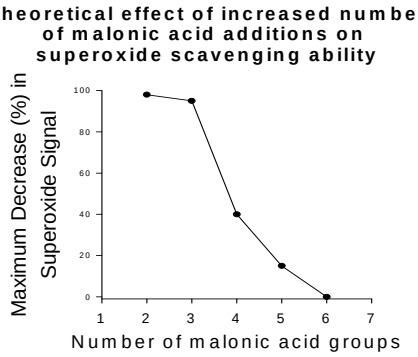
by confocal microscope as described above for experiments in live slices (ethidium settings: Em λ 488, Ex λ > 560).

Specific Experimental Plan (by Aim)

Aim 1 Synthesis and characterization of C_{60} derivatives, and refinement of tools to analyze C_{60} derivatives and metabolites in biological samples.

A. Synthesis and characterization of compounds.

1. Our first step will be to generate pure samples of malonic acid C_{60} derivatives bearing 2, 3, 4, or 6 malonic acid groups (corresponding to 4, 6, 8, or 12 carboxylic acid groups) per C_{60} molecule. Our current synthesis procedure generates bis- through tetra- adducts. Derivatives with 6 malonic acids will be synthesized by an alternative, template-driven protocol. Isomer purity of each adduct will be confirmed by NMR and thin layer chromatography (TLC). Approximately 10 isomers (1-3 isomers for each adduct, except the hexa-adduct (1)) will be synthesized and characterized. Samples will then be used for EPR analyses of radical scavenging properties, neurotoxicity studies, and as standards to continue developing methods to extract and quantify C_{60} derivatives in biological samples.



2. The free radical scavenging properties of these compounds for $^{\bullet}OH$ and $O_2^{\bullet-}$ will be evaluated by EPR, with special focus on the concentration at which they eliminate a standardized amount of $O_2^{\bullet-}$. We have chosen to use an enzymatic source of $O_2^{\bullet-}$ (xanthine oxidase with xanthine as substrate), since inorganic superoxide anion (as KO_2, for example) produces an extremely rapid and short-lived superoxide signal that is difficult to work with. It is expected that compounds with a higher degree of substitution will lose radical scavenging potency; the expected pattern is shown to the left. This information will allow us to determine whether the concentration at which neuroprotection occurs correlates well with the concentration at which each derivative eliminates $O_2^{\bullet-}$. Dose-response curves for malonic acid derivatives with 2-4, or 6 additions be determined.

3. We will also determine whether products of radical addition to the derivatives have unpaired electrons (i.e. are paramagnetic). Products which are paramagnetic would have the potential to become prooxidants, which might counterbalance any neuroprotection achieved from free radical scavenging (or other mechanism). Although to date, preliminary studies have not detected paramagnetic species as metabolites of these compounds (T. Lin, unpublished data), it is possible that isomers with more than 3 malonic acid groups may react differently due to steric considerations or loss of the conjugated double bond system. Therefore, a more detailed study using low temperature (77^{o} K) EPR will be performed on derivatives exposed to H_2O_2 or xanthine/xanthine oxidase (without spin-trap) to look for paramagnetic species.

4. Structures of the malonic acid derivatives will be detemined by mass spectroscopy, using electrospray mass spectroscopy, and negative ion conventional mass spectroscopy. Most sample analyses will be performed at the Washington University Mass Spectroscopy Resource Center.

5. The ability of regioisomers of the bis- tris-, tetra-, or hexa-malonic acid adducts of C_{60} to intercalate into lipid bilayers will be compared using EPR with spin-labelled lipids. The ability of regioisomers to limit lipid peroxidation in mouse brain liposomes will be assessed using EPR analysis of $LOO^{\bullet}$, and determination of TBARS.

6. We have previously observed neuroprotection by two chemically unrelated C_{60} derivatives - polyhydroxy and malonic acid C_{60}. However, both types of compound may be negatively charged at neutral pH. Therefore, we have chosen to generate an aminoamido derivative, which should have positively charged groups, to determine if side group characteristics affect neuroprotection. In addition, a PEG C_{60} derivative will also be synthesized. PEG has been added to proteins and compounds to increase their half-life and solubility. Because PEG-ated compounds have been tested in animal models and now human clinical trials, PEG-C_{60} is likely to be useful to allow comparison of the efficacy of C_{60} vs. other PEG-ated neuroprotective agents, comparing similar functional groups.

B. Refinement of techniques to extract and separate C_{60} compounds from biological tissues.

1. Our preliminary extraction procedure, using a 2-phase system with ethyl acetate, appears to give quantitative recovery of the C_{60} derivative in the solvet phase. We will more thoroughly determine whether other lipophilic molecules, such as lipoproteins, steroids, or membrane components, might interfere with the extraction.

2. HPLC separation of the malonic acid or amino C_{60} derivatives from biological samples will use techniques currently in place in the PI's laboratory. A lipid separation procedure which separates both neutral and phospholipids, will be coupled to pre-column deivatization of the carboxyl groups by a fluorescent tag (1-bromoacetylpyrene or PDAM) for malonic acid derivatives. Derivatized C_{60} compounds eluting from the HPLC will be detected by fluorescence detector, and real-time UV/vis scanning of samples as they come off the HPLC. The *C_3* tris malonic acid regioisomer has an absorbance maximum at 530 nm; this maximum differs between isomers and may assist in identification. In addition, the native C_{60} sphere has absorbance in the 220-240 nm range, due its highly inconjugated double bond system. The fluorescence derivatization will enhance our sensitivity of detection, and the combination of fluorescence detection and UV/vis in series will increase specificity for C_{60} derivatives. Samples will be collected off the HPLC, and eveluated by TLC and NMR for purity and for general identitiy. Mass spec will be run on selected samples to confirm identification and structure. Previously characterized standards will be used to calibrate the fluorescence and UV/vis response, and to allow a standard curve to be generated for each compound.

We note that several groups have reported quantitation of C_{60} derivatives from plasma and tissue samples, and do not expect that the approach that we propose here should pose substantial difficulties (res).

3. Using the procedures characterized in section 2, we will assay levels of C_{60} derivatives in plasma and brain of animals given derivatives i.p. and p.o. Two sets of control expriments would be useful at this point - administration of radio-labelled derivative, and assessment of levels of derivatives in tissue from other organs (liver, kidney, muscle, spleen) to begin to determine the pharmacokinetics of C_{60} derivatives. Because of the expense involved in generating a ^{14}C-labeled derivative, we would approach this after a fairly extensive assay with non-labeled compounds was complete. No funds were requested for this specific experiment; we plan to 1) seek funding from private sources for the synthesis of the radiolabelled compounds, or 2) obtain ^{14}C-labeled compound from an outside source (MERCorp).

4. Analysis of metabolic products of C_{60} derivatives will then be performed by collecting peaks off the HPLC, and performing mass spec analyses of the products. Extracts from various tissues will be compared.

5. The pharmacokinetics of C_{60} derivatives will be further assessed using the radiolabeled derivative. Depending on the data obtained with biological uptake of non-labelled compounds,^{14}C-labeled malonic acid or amino derivatives will be prepared, and administered To determine whether minor products of the derivatives are being missed, all fractions off the HPLC will be collected and assessed by β-counting. In addition, tissue extracts from animals treated with the radio-labeled C_{60} derivative will be counted after extraction to detect non-extracted label.

Aim 2. To establish the relationship between neuroprotective efficacy of C_{60} derivatives and their potency as $O_2^{\bullet-}$ scavengers, and to determine whether the ability of a derivative to intercalate into lipid membranes further modifies neuroprotection. Our general approach will be to establish the rank-order potency of each derivative as a $O_2^{\bullet-}$ scavenger and a neuroprotective agent to see if the rank orders

match. Determination of intracellular levels of compounds, using techniques optimized in the experiments detailed in Aim 1, will allow additional refinement of comparisons between the concentration at which a compound provides neuroprotection vs. $O_2^{\bullet}$ radical scavenging. Assays of intracellular superoxide and hydroxyl radical scavenging be fluorescence (DHR, DHE), biochemically (salicylate oxidation), and by assessing targets of radical attack (lipid peroxidation by TBARS and EPR spectroscopy will also assist in correlating "biological" scavenging effectiveness with neuroprotection. We will also compare neuroprotection by pairs of isomers with similar side group substitution, but differing degrees of membrane lipid interaction, to see if this property further correlates with neuroprotection. This will not require additional experiments, since we will obtain data on membrane interactions fro each isomer in experiments planned under Aim 1.

A. Determine protection dose-response curves for derivatives in excitotoxic and apoptotic insults, to allow a rank-order comparison with $O_2^{\bullet}$ scavenging.

1. Pure isomers of malonic acid (~10 compounds) will be tested for neuroprotective efficacy in cortical neuronal cultures exposed to excitotoxic insults (AMPA, oxygen-glucose deprivation, brief NMDA) or to serum deprivation, to trigger apoptosis. Dose-protection curves will be established for each compound against each insult. The concentration at which each compound produces toxicity when used alone will also be determined for each isomer. Cell death will be assessed quantitatively by measuring LDH efflux into culture medium, and by propidium iodide staining and cell counts, and assessed qualitatively by morphology and annexin V staining.

To determine whether these compounds produce a different profile of protection in another CNS cell type, oligodendroglia will be exposed to AMPA, and neuroprotection by derivatives assessed. Cell death will be quantified by counting remaining Gal-C positive cells, compared to the number in untreated controls.

2. As a second, well-characterized model of apoptosis, superior cervical ganglion cells will undergo NGF deprivation as described (Johnson et al, 1993). Neuroprotection dose-response curves for C_{60} derivatives will be established apoptotic insult.

B. "Biological" free radical scavenging. To complement the "test-tube" assessments of free radical scavenging outlined in Aim 1, we propose to use oxidation-sensitive dyes and a limited set of biochemical measures such as salicylate oxidation, TBARS, and peroxyl radical detection by EPR, to define "biological" antioxidant activity of these compounds in cultures exposed to the above insults.

1. Confocal microscopy of cultures exposed to the above insults will be performed with both DHE and DHR. Pharmacological inhibitors of mitochondria (rotenone, antimycin A, oligomycin) and other potential sources of ROS (^{G}N-Arg, for NOS, meclofenamate, for general arachidonic acid metabolism, diphenyliodinium (DPI) for NADPH oxidase, allopurinol, for xanthine oxidase) will be used to localize the source of the ROS signal.

2. Salicylate oxidation will be used in select experiments to assess predominantly cytosolic ROS production. Again, pharmacological agents will assist in identification of the source of any observed ROS.

3. TBARS and peroxyl radical will be determined in lipids extracted from cultures exposed to injury conditions in the presence or absence of C_{60} derivatives. We have specifically chosen to use EPR detection of peroxyl radical because of the relative insensitivity of the TBARS assay in our cultures (Dugan and Choi, unpublished). In the absence of peroxidases, lipid peroxyl radical has a relatively long half-life ~ 20-40 minutes. We have previously performed EPR/spin-trapping analysis of ROS from cultures (Dugan et al, 1995b), and believe we should have no problems perfoming the proposed cell culture analyses of peroxyl radical.

Aim 3 Assess neuroprotective efficacy of C_{60} derivatives in SOD2 deficient neurons and oligodendroglia, and determine whether C_{60} derivatives rescue *Sod2* -/- mice. To address this aim, we plan experiments similar to those described for Aim 2, using neuronal and oligodendroglial cultures from the *Sod2* knockout mice. An initial focus of this section will be on establishing neuronal and

oligodendroglial culures from these mice, which will then undergo neuroprotection studies and characterization of free radical handling. Concurrently, we expect to administer derivatives to neonatal mouse pups, looking for increased survival.

A. Determine protection dose-response curves for derivatives in excitotoxic and apoptotic insults, in SOD2-deficient cultures, to allow a rank-order comparison with $O_2^{\bullet}$ scavenging.

1. Although fibroblasts from *Sod2* null embryos have been grown in culture, they show decreased viabilty (Huang et al, 1997). Neuronal cultures are also likely to show decreased viability (C. Epstein, personal communication), but we believe that, since *Sod2* -/- pups from CD-1 mice live for over 1 week after birth, a culture protocol can be developed which will produce neuronal cultures which live for 12-14 days. We will use single embryo dissections as described above, and will test the following procedures for their ability to produce viable, long-lived neuronal cultures from these mice.

a. Grow cultures in a reduced O_2 environment. We will prepare neuronal cultures as described in the general methods section, but will maintain them in an incubator with 5% CO_2, and O_2 concentrations from 2-10%. A variable O_2 Heureaus incubator is in place in the PI's lab for use in these studies.

b. Grow cultures in a reducing environment, by including reducing compounds such as N-acteyl cysteine, dithiothreitol, or NADH.

c. Plate cultures on 25 mm glass coverslips, which are then inverted over previously-established WT astrocyte cultures. This would have two potential advantages. It would allow astrocytes to assist in scavenging superoxide radical, but still allow *Sod2* -/- neurons to be studied separately. It would also provide a decreased O_2 environment, as the coverslip limits O_2 diffusion to most of the cells on the coverslip. This could be combined with some decrease in O_2 in the incubator, as well.

2. To determine whether C_{60} derivatives inhibit spontaneous degeneration of these cultures, cultures will be grown in medium containing C_{60} derivatives from the time of plating. Since the genotype of a given culture will not be known until several days after plating, cultures from each pup in a dissection will be randomly assigned treament with either C_{60} compound, or vehicle with food coloring. The ability of C_{60} derivatives to protect *Sod2* -/- cells from spontaneous death may be a fairly good indicator of whehter $O_2^{\bullet}$ scavenging by C_{60} compounds correlates with neuroprotective properties.

3. To establish dose-response curves for protection by derivatives in SOD2-deficient cultures, selected experiments from those described for Aim 2 A will be reproduced in these. We may find a gene dose effect in vulnerability to excitotoxic

B. "Biological" free radical scavenging.

1. We plan a limited set of ROS analyses similar to those in Aim 2 part B, in cultures from these mice, focusing on 1-2 compounds which have the best correlation between $O_2^{\bullet}$ scavenging and neuroprotection, and 1-2 with a lesser degree of correlation. This would allow us to determine whether lack of protection was due to poor access to $O_2^{\bullet}$, for example, or whether our hypothesis is invalid.

2. If culture viability is not sufficient fro the proposed studies, we will use slices from 2-3 day postnatal mice. Mice will undergo tail-clipping of 2-3 mm of tail immediately after birth, and will be marked with indelible marker. After genotyping, slices (250-300 μm) will be prepared on a vibratome, and maintained in oxygenated Kreb's buffer, with the slices from each animals in a different dish. C_{60} derivatives will be added to some of the slices. Viability of slices will be determined by staining at 4-6 hours with propidium iodide (Goldberg et al, 1997). Cell counts of different brain regions will be performed. In addition, slices will be assessed for total propidium iodide fluorescence (a gross indicator of PI staining of dead cells) by placing slices in a fluorescence plate reader for total fluorescence per slice. We will also assess ROS by DHR or DHE oxidation with fluorescence confocal microscopy, and by perfusing slices with salicylate to measure oxidation products.

C. We will test the protective efficacy of these agents in neonatal *Sod2* -/- mice. In vivo studies will be performed to determine if oral adminstration of C_{60} derivatives will enhance survival of

homozygous mouse pups. One or two derivatives with the greatest degree of both neurprotective efficacy *in vitro* and $O_2^{\bullet -}$ scavenging potency will be tested. Because this *Sod2* strain appears to die of events related to its dilated cardiomyopathy, prior to onset of gross neurodegeneration, enhanced survival may not necessarily correlate with neuroprotection. Thus, we will look at indices of impaired free radical handling (nitrotyrosine levels, oxidation of DHE adminstered i.p. prior to sacrifice, level of lipid peroxyl radical and level of salicylate oxidation) in brain from treated and untreated pups, to determine whether C_{60} derivatives reduce markers of oxidative damage. Should survival be prolonged by the derivatives, examination of brain for loss of neurons will be done, with comparison of treated -/- animals, and untreated +/+ animals.

E.5. HAZARDOUS MATERIALS

No radioactivity is proposed for this project. Handling and storage of radioactive material will conform to all University rules and guidelines regarding the use of radiation. The only hazardous materials that will be used in these studies are ethyl acetate, methanol and propidium iodide (small amounts, ~10 ml). Storage and disposal will conform to established guidelines of Washington University, and pouring, filtering, and mixing will be performed in a fume hood.

F. HUMAN SUBJECTS

None.

G. VERTEBRATE ANIMALS

Brain tissue for cultures will be obtained from fetal mice at gestational age 15 for the purpose of cultivating brain neuronal cell cultures. These cell cultures will be used as model of brain to allow study mechanisms of neuronal injury that cannot be done *in vivo*. Among the advantages of these models, the use of cell cultures allows a large number of studies to done using a small number of animals. Mice are used as the lowest species with significant brain similarity to humans. Pregnant dams will be anesthetized with halothane and sacrificed by cervical dislocation. The fetal mice are removed and killed by rapid decapitation using sharp scissors. No experiments or painful procedures will be performed on the mice prior to sacrifice, and all animal care will follow the guidelines set forth by the Washington University Division of Comparative Medicine, following animal care protocols approved by the Animal Studies Committee.

Animal identification for the *Sod2* mouse colony will be performed by ear-punch of a numeric code and clipping a < 1 cm length of tail for DNA. Both are done under metaphane anesthesia. No other painful procedures are planned.

GENERAL TIMELINE

Year 1-2	**Year 3**	**Year4**	**Year 5**
Synthesis			
Generate malonic acid derivatives	Establish synthesis of C_{60} amino derivative	Establish synthesis of PEG derivative	Provide pure derivatives as needed
Chemical characterization			
EPR for radical reactivity; radical products COOH C_{60}		Same for amino, PEG derivatives	
Neuroprotection			
Compare malonic acid isomers against apoptotic and excitotoxic		injuries throughout	then *Sod2)*
Biological radical properties			
Imaging/ biochemistry	studies on WT cultures	Studies to continue	on *Sod2* ulltures
Sod2 null mice			
Establish colony	Develop cultures. Begin in vivo treatment	Neuroprotection and ROS assays	ROS assays.in vitro and in vivo

REFERENCES

An, Y.-Z., Anderson, J.L., Rubin, Y. (1993) Syntheses of a-amino acid derivatives of C60 from 1,9-(4-hydroxycyclohexano)-buckministerfullerene. *J. Org. Chem., 58: 4799-4801.*

Anderson DK, Dugan LL, Means ED, and Horrocks LA. (1994) Methylprednisolone and membrane properties of primary cultures of mouse spinal cord. *Brain Res*, 637:119-125.

Barge, RM, Willemze, Vanenabeele, P, Fiers, W, and Beyaert, R. (1997) Differential involvement of caspaces in apoptosis of myeloid leukemic acells induced by chemotherapy versus growth factor withdrawal. *FEBS Lett.* 409:207-10.

Bazan. NG Jr. (1970) Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain. *Biochem. Biophys. Acta* 218:1-10.

Beal, M. F. (1992). Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illness? Ann. Neurol. 31: 119-130.

Bindokas,VP, Jordan, J, Lee, CC, and Miller, RJ. (1996) Superoxide production in rat hippocampal neurons: Selective imaging with hydroethidine. J. Neurosci. 16:1324-1336.

Bottenstein JE, Hunter SF. Culture methods for oligodendrocyte cell lines and oligodendrocyte-type 2 astrocyte lineage cells. In: Methods in Neurosciences, Vol 2: Celll Culture. PM Conn, ed. Academic Press, Orlando, 1990 pp 56-75.

Boutorine, A.S., Tokuyama, H., Takasugi, M., Isobe, H., Nakamura, E., Helene, C. (1994) Fullerene-oligonucleotide conjugates: photoinduced sequence-specific DNA cleavage. *Angew. Chem. Int. Ed. Engl.* 33:2462-2465.

Braughler, J.M. and Hall, E.D. (1989) Central nervous system trauma and stroke. I. Biochemical considerations for oxygen radical formation and lipid peroxidation. *Free. Radic. Biol. Med.* 6:289-301.

Chan, P.H., Fishman, R.A., Longar, S., Chen, Yu, A. (1985) Cellular and molecular effects of polyunsaturated fatty acids in brain ischemia and injury. *Prog. Brain Res.* 63:227-235.

Chan, P.H., Chu, L., Chen, S.F., Carlson, E.J., Epstein, C.J. (1990) Attenuation of glutamate-induced neuronal swelling and toxicity in transgenic mice overexpressing human CuZn-superoxide dismutase. *Acta Neurochirurgica.* 51:245-247.

Chance, B and Oshino, N (1971) Kinetics and mechanisms of catalase in peroxisomes of the mitochondrial fraction. Biochem J 122:225-233.

Cino M, Del Maestro RF (1989) Generationof hydrogen peroxide by brain mitochondria: the effect of reoxygenation following postdecapatative ischemia. Arch Biochem Biophys 269:623-638.

Chiang, L.Y., Upasani, R.B., and Swirczewski, J.W. (1992) Versatile nitronium chemistry for C60 fullerene functionalization. *Amer. Chem. Soc*. 114:10154-10157.

Chiang, L.Y., Upasani, R.B., Swirczewski, J.W., Soled, S. (1993) Evidence of hemiketals incorporated in the structure of fullerols derived from aqueous acid chemistry. *Amer. Chem. Soc.* 114:5453-5457.

Choi, D.W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1:623-634.

Cleeter, MWJ, Cooper, JM, Schapira, AHV. (1992) Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium: evidence for free radical involvement. J. Neurochem. 58:786-789.

Cross, AH, Manning, PT, Stern, MK, and Misko, TP. (1997) Evidence for the production of peroxynitrite in inflammatory CNS demyelination. *J. Immunol*., in press.

Devaust, J, Seigneuret, M, Herve, P, and Devaux, PF. (1983) Collisions between nitrogen-14 and nitrogen-15 spin-labels. 1. Lipid-lipid interactions in model membranes. *Biochem.* 22:3137-3145.

Dugan LL, Demediuk P, Pendley II CE and Horrocks LA. (1986) Separation of phospholipids by HPLC: All major classes, including ethanolamine and choline plasmalogens, and most minor classes, including lysophosphatidylethanolamine. *J. Chromatogr*. 378:317-327.

Dugan, L.L., Bruno, V.M.G., Amagasu, S.M., and R.G. Giffard, R.G. (1995a) Glial modulate the response of murine cortical neurons to excitotoxicity: glial exacerbate AMPA neurotoxicity. Submitted to *J. Neurosci.* 15:4545-4555.

Dugan, L.L., Sensi,S.L., Canzoniero, L.M.T., Handran, S.D., Rothman, S.M., Lin, T.-S., Goldberg, M.P. and Choi, D.W. (1995b) Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *J. Neurosci.* 15:6377-6388.

Dugan, L.L., Lin, T.-S., He, Y.-Y., Hsu, C.-Y. and Choi, D.W. (1995c) Detection of free radicals by microdialysis/spin trapping EPR following focal cerebral ischemia-reperfusion. *Free Rad. Res.* 23:27-33.

Dugan, LL, Gabrielsen, JK,Yu, SP, Lin,TS, Choi, DW (1996) Buckminsterfullerenol free radical scavengers reduce excitotoxic and apoptotic death of cultured cortical neurons. *Neurobiol. Dis.*3:129-135.

Dugan, LL, Creedon, DJ, Johnson, EM, and Holtzman, DM. (1997a) Rapid suppression of free radical formation by NGF involves the MAPK pathway. *Proc. Natl. Acad. Sci. U.S.A.* 94:4086-4091.

Dugan LL, Turetsky DM, Du C, Lobner D, Wheeler M, Almli R, Shen CKF, Luh TY, Choi DW and Lin TS. (1997b) Carboxyfullerenes as Neuroprotective Agents. Proc. Natl. Acad. Sci. U.S.A. 94:9434-9439.

Dykens, J.A., Stern, A., and Trenkner, E. (1987) Mechanism of kainate toxicity to cerebellar granule neurons in vitro is analogous to reperfusion tissue injury. *J. Neurochem.* 49:1222-1228.

Dykens JA (1994) Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca2+ and Na+: Implications for neurodegeneration. J Neurochem 63:584-591.

Farkas DL, Wei M, Febbroriello P, Carson JH, Loew LM (1989) Simultaneous imaging of cell and mitochondrial membrane potentials. Biophys J 56:1053-1069.

Flamm, E.S., Demopoulos, H.B., Seligman, M.L., Poser, R.G., and Ransohoff, J. (1978) Free radicals in cerebral ischemia. *Stroke* 9:445-447.

Garthwaite, J, Charles, SL, Chess-Williams, R. (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 336:385-388.

Goldberg M.P. and D.W. Choi. (1993) Combined oxygen and glucose deprivation in cortical culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. *J. Neurosci.* 13:3510-3524.

Goldberg, MP, Strasser, U, and Dugan, LL. (1996) Techniques for assessing neuroprotective drugs in vitro, in: "Neuroprotective agents and cerebral ischaemia", Cross, AJ, and Green, AR, eds., Academic Press, New York..

Gottron, F, Ying, H, and Choi DW. (1997) Caspase inhibition selectively reduces the apoptotic component of oxygen-glucose deprivation induced cortical neuronal cell death, *Mol. Cell. Neurosci.,* in press.

Halliwell, B. (1992) Reactive oxygen species and the central nervous system. *J. Neurochem.* 59:1609-1623.

Hara, A. & Radin, N.S. (1978) *Anal. Biochem.* 90, 420-427.

Hasegawa, K, Yoshioka, H, Sawada, T, Nishikawa, H. (1993) Direct measurement of free radicals in the neonatal mouse brain subjected to hypoxia: an electron spin resonance spectroscopic study. *Brain Res.* 607:161-166.

Henderson L, Chappell JB (1993) Dihydrorhodamine 123: a fluorescent probe for superoxide generation? Eur J Biochem 217:273-280.

Huang, L-Y, Luh, T-Y, Shen, CK-F, and Chou, C-K. (1997) Protective effects of Bcl-2 and carboxyfullerenes on TGF-β induced apoptosis. *J. Biol. Chem.*, in press.

Huang, T-T, Yasunami, M, Carlson, EJ, Gillespie, AM, Reaume, AG, Hoffman, EK, Chan, PH, Scott, RW, and Epstein, CJ. (1997) Superoxide-mediated cytotoxicity in superoxide dismutase-deficient fetal fibroblasts. *Arch. Biochem. Biophys.* 344: 424-432.

Hubbell, WL and McConnell, HM. (1968) Spin-label studies of the excitable membranes of nerve and muscle. *Proc. Natl. Acad. Sci. U.S.A.* 61:12-16.

Hirsch, A, Lamparth, I. and Karfunkel (1994) Fullerene Chemistry in three dimensions: isolation of seven regiosiomeric bisadducts and chiral trisadducts of C60 and di(ethoxycarbonyl)methylene. *Angew. Chem. Int. Ed. Engl.* 33: 437-438.

Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L., and Korsmeyer, S.J. (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75:241-251.

Isaacs, L. and Diederich, F. (1993) Structures and chemistry of methanofullerenes: a versatile route into N-[(methanofullerene)-carbonyl]-substituted amino acids. *Helv. Chim. Acta* 76: 2454-2464.

Kamada, S, Maeda, Tsuji, A. (1983) Fluorescence high-performance liquid chromatographic determination of free and conjugated bile adics in serum and bile using 1-bromoacetylpyrene as a pre-labeling agent. *J. Chromatogr.* 272:29-41.

Kampe, K-D, Egger, N, Vogel, M. (1993) *Angew. Chem. Int. Ed. Engl*. 32: 1174-1176.

Koh, J., and Choi, D.W. (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J. Neurosci. Meth.* 20:83-90.

Koh J, Gwag BJ, Lobner D, Choi DW (1995). Potentiated necrosis of cultured cortical neurons by neurotrophins. Science 268:573-575.

Kondo, T, Li, Y, Sato, S, Murakami, K, Copin, J-C, Huang, T-T, Epstein, CJ, and Chan, PH. (1997) Subcellular localization of superoxide anions following focal cerebral ischemia and reperfusion. *J. Cereb. Blood Flow Metab.* 17: 512.

Krusic, P.J., Wasserman, E., Keizer, P.N., Morton, J.R., Preston, K.F. (1991A) Radical reactions of C60. *Science* 254: 1183-1185.

Krusic, P.J., Wasserman, E., Parkinson, B.A., Malone, B. Holler, E.R., Keizer, P.N., Morton, J.R., Preston, K.F. (1991B) Electron spin resonance study of the radical reactivity of C60. *J. Am. Chem. Soc.* 113: 6274-6275.

Lamparth, I, Maichle-Mossmer, C, Hirsch. (1995) *Angew. Chem. Int. Ed. Engl*. 34: 1607-1609.

Lamparth, I. and Hirsch, A. (1994) Water-soluble malonic acid derivatives of C60 with a defined three-dimensional structure. *J. Chem. Soc., Chem. Commun.* 1727-1728.

Lancelot, E, Callebert, J, Lerouet, D, Revaud, ML, Boulu, RG, Plotkine, M. (1995) Role of the L-arginine-nitric oxide pathway in the basal hydroxyl radical production in the striatum of awake rats as measured by brain microdialysis. Neurosci. Lett. 202:21-24.

Lafon-Cazal, M., Pietri, S., Culcasi, M., and Bockaert, J. (1993) NMDA-dependent superoxide production and neurotoxicity. *Nature* 364:535-537.

Lebovitz, RM, Zhang, H, Vogel, H, Cartwright, J Jr, Dionne, L, Lu N, Huang, S, Matzuk, MM (1996) Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc. Natl. Acad. Sci .U. S. A.*. 93:9782-9787.

Li, Y, Huang, TT, Carlson, EJ, Melov, S, Ursell, PC, Olson, JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, et al. (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat. Genet.* 11:376-381.

Maggini, M., Scorrano, G., Bianco, A., Toniolo, C., Sijbesma, R.P., Wudl, F., Prao, M. (1994) Addition reactions of C60 leading to fulleroprolines. *J, Chem. Soc., Commun.* 335-336.

Martin, DP, Schmidt, RE, DiStefano, PS, Lowry, OH, Carter, JG, and Johnson, EM Jr. (1988) Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.*106:829-844.

Martin, SJ, Finucane, DM, Amarante-Mendes, GP, O'Brien, GA, and Green, DR. (1996) Phosphatidylserine externalization during CD95-induced apoptosis of cells and cytoplasts requires ICE/CED-3 protease activity. *J. Biol. Chem.* 271:28753-28756.

Medema, JP, Scaffidi, C, Kischel, FC, Shevchenko, A, Mann, M, Krammer, PH, and Peter, ME. (1997) FLICE is activatied by association with the CD95 death-inducing signaling complex (DISC). *EMBO J.* 16:2794-2804.

Matute, C, Sanchez-Gomez, MV, Martinez-Millan, L, and Miledi, R. (1997) Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes. *Proc. Natl. Acad. Sci. U.S.A.* 94:8830-8835.

Monyer, H., Hartley, D.M., Choi, D.W. (1990) 21-aminosteroids attenuate excitotoxic neuronal injury in cortical cell cultures. *Neuron* 5:121-126.

Mukherjee, PS, and Karnes, HT. (1996) Ultraviolet and fluorescence derivatization reagents for carboxyl ic acids suitable for high performance liquid chromatography: a review. *Biomed. Chromatogr.* 10:193-204.

Olanow, C.W. (1990) Oxidation reactions in Parkinson's disease. *Neurol.* 40:32-37.

Piantadosi ,CA, and Zhang, J. (1996) Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. *Stroke* 27:327-331.

Prato, M., Bianco, A., Maggini, M., Scorrano, G., Toniolo, C., Wudl, F. (1993) Synthesis and characterization of the first fullerene-peptide. *J. Org. Chem.* 58: 5578-5580.

Royall JA, Ischoropoulos H (1993) Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. *Arch Biochem Biophys.* 302:348-355.

Schulz, JB, Henshaw, DR, Siwek, D, Jenkins, BG, Ferrante, RJ, Cipolloni, PB, Kowall, NW, Rosen, BR, Beal, MF. (1995) Involvement of free radicals in excitotoxicity in vivo. J. Neurochem. 64:2239-2247.

Siesjo, B.K., Agardh, C.D., and Bengtsson, F. (1989) Free radicals and brain damage. *Cerebrovasc. Brain Metab. Rev*. 1: 165-211.

Staffeldt, B, Brockmoller, J, and Roots, I. (1991) Determination of S-carboxymethyl-cysteine and some of its metabolites in urine and serum by high performance liquid chromatography using fluorescent pre-column labeling. *J. Chromatogr.* 571:133-147.

Skiebe, A., Hirsch, A. (1994) A facile method for the synthesis of amino acid and amido derivatives of C60. *J. Chem. Soc. Commum.* 335-446.

Toniolo, C., Bianco, A., Maggini, M., Scorrano, G., Prato, M., Marastoni, M., Tomatis, R., Spisani, S., Palu, G., Blair, E.D. (1994) A bioactive fullerene peptide. *J. Med. Chem.* 37: 4558-4562.

Vasella, A.,Uhlmann, P., Waldraff, C.A.A./, Diederich, F, Thilgen, C. (1992) Fullerene sugars: preparation of enantiomerically pure, spiro-linked C-glycosides of C60. *Angew. Chem. Int. Ed. Engl.* 32: 1388-1390.

Yue, T.L., Gu, J.L., Lysko, P.G., Cheng, H.Y., Barone, F.C., Feuerstein, G. (1992) Neuroprotective effects of phenyl-t-butyl-nitrone in gerbil global brain ischemia and in cultured rat cerebellar neurons. *Brain Res.* 574:193-197.