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13. ABSTRACT (Maximum 200 Words)

The initial three years of this project determined the contributions of bioenergetic defects and oxidative stress to neurodegeneration in Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS), as previously reported. The current report period includes the first eleven months of work on the Consortium project "Mitochondrial Free Radical Generation in Parkinson's Disease", assessing *in vivo* whether mitochondria are the source of free radical generation in animal models of Parkinson's disease (PD). Studies in the first three years of this grant generated several exciting novel observations of presymptomatic energetic abnormalities in both HD and ALS models. In this period of the study, we have identified abnormalities in cerebral ATP generation in another mouse HD model (R6/2 mice), first evident around the age of symptom onset, and exacerbated as the phenotype progresses. We have also commenced studies examining the relationship between mitochondrial complex I inhibition and free radical-mediated oxidative damage. Using *in vivo* approaches we are optimizing dose and time-course studies after intracerebral administration of rotenone and pyridaben. We found increased lipid peroxidation and induction of the stress-response marker heme oxygenase-1 shortly after rotenone administration, concomitant with complex I inhibition. These observations will be characterized further in the next year of study.

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4. INTRODUCTION

The goal of the original three-year proposal was to gain insight into the roles of mitochondrial energy metabolism and oxidative stress in the etiology of neuronal degeneration in Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS). The development of mutant mouse lines (expressing human mutant transgenes or mutations in mouse homologues), that mimic aspects of human diseases, provide novel opportunities to assess the temporal progression of pathological changes over the course of disease development in an *in vivo* animal model. Experiments aimed to determine the relative contributions and sequential order of bioenergetic dysfunction and oxidative damage to cell death processes in two different mutant mouse models of HD (*Hdh* knock-in mice; R6/2 mice) and one transgenic (Tg) mouse model of familial ALS (G93A SOD1 over-expressors) (White et al, 1997; Mangiarini et al., 1996; Gurney et al., 1994). We found that one of these mouse lines (R6/2 HD mice) develop a diabetic profile that interfered with cerebral glucose use assays. This necessitated substituting another transgenic line that similarly expresses a fragment of mutant human huntingtin (N171-82Q mice; Schilling et al., 1999) in some experiments. The use of mitochondrial toxins also allows us to model in animals the cerebral pathogenic sequelae induced by naturally occurring agents that are potentially extremely hazardous to humans. Further studies in this proposal used 3-nitropropionic acid (3-NP), a toxin that produces brain lesions and symptoms in humans resembling those seen in HD following systemic exposure. In 2002, this proposal was extended by the addition of a second project, subtitled "Mitochondrial Free Radical Generation In Parkinson's Disease". This project continues the theme of investigating the interactions between energy metabolism and oxidative stress in the etiology of neurodegenerative disorders, and is a sub-project of a Consortium of investigators. The aims of this project are to try to ascertain *in vivo* whether inhibition of a component of the mitochondrial respiratory chain (complex I), implicated in the pathogenesis of Parkinson's disease (PD), induces pathogenesis via free radical generation, and whether the mitochondria are the initial source of these free radicals. These are questions that are somewhat easier to address by *in vitro* approaches (as in other Consortium projects), given the extreme technical difficulties of discretely measuring purely mitochondrial events *in vivo*. Therefore we are taking an indirect approach, by measuring the time-course and nature of oxidative events caused by toxic insults directed specifically against mitochondrial molecules. Results may give insight into sites for drug targeting in PD.

The original Specific Aims for the five years of study are:

<u>**Year 1:** October 1998 - September 1999</u>

1) a) Measurement of local rates of cerebral glucose use ($lCMR_{glc}$) in *Hdh* knock-in mice expressing CAG repeat lengths found in HD patients (48 CAG repeats, Hdh^{Q50}), relative to $lCMR_{glc}$ in wild type littermates (7 CAG repeats, Hdh^{Q7}).
   b) Assessment of any gene dosage effect: Homozygous vs. heterozygous animals.
2) Measurement of electron transport chain activities in *Hdh* knock-in mice with disease-length and normal-length CAG repeats.

3) Measurement of $lCMR_{glc}$ in the G93A Tg mouse model of ALS overexpressing human mutant SOD1. Analysis of the temporal progression of $lCMR_{glc}$ changes, at 50, 90 and 120d of age.
4) Measurement of electron transport chain enzyme activities in G93A ALS mice at 60 and 120d.

NB: *The laboratory moved from Massachussetts General Hospital, Boston, MA, to Weill Medical College of Cornell University in New York, NY. This resulted in a delay in funding during grant transferal (September 1999 to August 2000), and in re-establishing mutant mouse colonies in the New York.*

Year 2: June 2000 – May 2001

5) Measurement of the effects of increasing CAG repeat number on $lCMR_{glc}$ in *Hdh* knock-in mice.
6) Measurement of $lCMR_{glc}$ in the R6/2 mouse model of HD. Analysis of the temporal progression of any glucose use changes by measurement at 42d, 56d and 84d of age.
7) Measurement of electron transport chain enzyme activities in R6/2 HD mice.
8) NMR assessment of cerebral lactate levels in *Hdh* and R6/2 mice.

Year 3: June 2001 – May 2002

9) Measurement of cerebral levels of oxidative damage markers (protein carbonyls; nDNA OH^8dG; hydroxyl radical production; nitrotyrosine levels) in *Hdh* knock-in and R6/2 HD mouse models.
10) Measurements of oxidative damage markers in G93A FALS Tg mice.
11) HPLC measurement of cerebral energy metabolite levels in HD *Hdh* and R6/2 Tg mice.
12) Measurement of $lCMR_{glc}$ in a rat model of 3-nitropropionic acid neurotoxicity.

Consortium Project: **Mitochondrial Free Radical Generation In Parkinson's Disease**
Year 4: June 1st 2002 – May 31st 2003
Research Goals

13) Characterization of the regional and temporal development of complex I inhibition in specific brain regions at time points after administration of subunit-specific complex I inhibitors to rats:
(i) Stereotactic unilateral intracerebral injection of rotenone, DCCD, or pyridaben into the region of the substantia nigra of anesthetized rats; and vehicle into the contraleteral hemisphere. Rats will be sacrificed and brain tissue harvested at multiple time-points post-injection (1h, 6h, 24h, 7d).
(ii) Spectrophotometric measurement of complex I activity, citrate synthase activity (a marker for mitochondrial number), and protein levels in post-mortem tissue from the striatum, nigra, cortex and cerebellum.

14) Commence characterization of the regional and temporal development of cerebral oxidative damage after administration of rotenone, DCCD and pyridaben to rats.
(i) HPLC measurement of oxidative damage markers at time-points before and after complex inhibition (time-points and regions determined in 1) by:
HPLC detection of brain levels of DNA damage product 8-hydroxy-deoxyguanosine (8-OHdG),
(ii) HPLC and immunohistochemical detection of lipid peroxidation marker malondialdehyde in affected brain regions.

Year 5: June 1st 2003 – May 31st 2004
14) Continued.... Completion of oxidative damage studies begun in year 1 (Objective 14)

15) Assessment of levels of free radical markers in striatal ECF microdialysates before and after inhibiting sub-unit activity, at time-points elucidated in (i), by:
(i) HPLC detection of hydroxyl ($OH^{\cdot}$) radical levels in microdialysate samples from striatum; by measuring the extent of conversion of salicylate (i.p. injection) to DHBA by $OH^{\cdot}$, DHBA detected in dialysate.
(ii) HPLC detection of microdialysate levels of DNA damage product 8-hydroxy-deoxyguanosine (8-OHdG); and
(iii) Immunocytochemical localization of 8-OHdG throughout brains of inhibitor-treated rats.

Note on May 2003 Annual Report:

The grant period covered by this annual report is May 1 2002- April 30th 2003. Therefore, this period covers the *end of the "YEAR 3"* study period (covering "original" grant submission SOW); *and* the *first 11 months of the Consortium project SOW* (here termed "YEAR 4").

This annual report briefly summarizes the findings of Years 1 to 3 of the grant period, previously reported in detail, and then concentrates on novel findings between May 1 2002 - April 30th 2003. This period covers work from:

(i) SOW Year 3 not covered in the last report period – namely findings from Objective 11; and

(ii) First year of the Consortium SOW ("Year 4" – *NB:* an incomplete SOW period is covered by this report period).

5. BODY

A. *Overview of Original Proposal Project*

The pathogenetic mechanisms in both Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) are still unclear, however, in both cases *in vivo* and *in vitro* studies implicate the involvement of bioenergetic defects in the disease process (for review see Browne and Beal, 2000; Menzies et al., 2002). Attempts to ascertain the role of energetic dysfunction in pathogenesis have been greatly enhanced by the development of several different transgenic mouse lines replicating aspects of each disorder. HD models express the huntingtin gene mutation underlying HD, an abnormal expansion of the polyglutamine (Q) domain in huntingtin protein, encoded by an expanded triplet (CAG) repeat in exon 1 of the huntingtin gene. Familial ALS (FALS) models express some of the multiple Cu/Zn superoxide dysmutase (SOD-1) mutations that occur in approximately 25% of familial ALS patients. These models allow novel determinations of metabolic parameters over the life-span of the animals, before, during and after onset of pathological changes and disease symptoms. HD mouse lines differ in terms of the site of mutant gene incorporation, CAG repeat length, copy number, and promoter used, and these differences are reflected in the phenotypes of the mice generated. Studies in this project utilize *Hdh* CAG knock-in mice (White et al., 1997), R6/2 transgenic mice overexpressing an N-terminal fragment of human mutant huntingtin, including a 145 polyglutamine repeat stretch (Mangiarini et al., 1996), and N171-82Q mice also expressing an N-terminal fragment of human mutant huntingtin, but with a shorter (82Q) polyglutamine repeat stretch (Schilling et al., 1999). The ALS mouse model employed is the G93A model (Gurney et al., 1994), overexpressig a human SOD-1 glycine to alanine mutation. Studies utilized both *in vivo* and *in vitro* experimental approaches in mutant mice and in rats, to investigate parameters of cerebral energy metabolism (*in vivo* cerebral glucose use measurement by [^{14}C]-2-deoxyglucose autoradiography; NMR lactate imaging; *in vitro* spectrophotometric oxidative phosphorylation enzyme assays; HPLC detection of metabolites), and to investigate the generation of DNA and protein oxidative damage products, and free radical generation (HPLC detection; immunohistochemistry).

Studies in the first three years of this grant generated several exciting novel observations of presymptomatic energetic abnormalities in both HD and ALS models. Firstly, we found that cerebral glucose utilization is markedly elevated in the forebrain of mice expressing the HD mutation. Most interestingly, this hypermetabolism occurs prior to any evidence of pathologic changes or symptoms in these animals. Further, we have recapitulated this observation in *two* distinctly different HD mutant mouse models: Hdh^{Q92} "knock-in" mice expressing a mutant expansion of the disease-causing CAG repeat in the murine homologue *HD* gene (92 CAGs) (White et al., 1997); and N171-82Q mice expressing a fragment of human huntingtin gene containing a mutant CAG repeat length (82 CAGs) (Schilling et al., 1999). Further experiments in mice expressing different CAG repeat lengths suggest that this effect is CAG length-dependent, and shows a gene dosage effect. Another point of interest is

that glucose use changes are not restricted to brain regions susceptible to degeneration, ie. the striatum. This observation supports suggestions that striatal neurons are especially vulnerable to metabolic stress. Increases in glucose uptake prior to any pathologic changes suggest increased glucose demand to fulfill cells' functional requirements, perhaps due to impaired metabolic enzyme activity, uncoupling of mitochondria, and/or increased dependence on glycolysis. We have therefore commenced studies to examine the functional capacities of components of the glucose metabolic pathway in these HD mice, in search of potential sites of dysfunction. Initial studies suggest that ATP synthesis is sub-optimal in N171-82Q mice, but we found that activities of mitochondrial enzyme complexes II and IV (known to be defective in late stage HD) are normal in these animals. We have also found evidence of increased oxidative damage to DNA and lipids in a third transgenic mouse model of HD (R6/2 line), although preliminary results suggest the changes occur relatively late in the disease progression.

In addition to mouse lines expressing the HD mutation, another approach to model the disease is to directly inhibit mitochondrial enzyme complex II. Impaired activity of this enzyme occurs in late stage HD (Browne et al., 1997), and experimental or environmental exposure to an agent that specifically inhibits this enzyme (3-NP) produces pathological lesions and phenotypic changes similar to HD (Ludolph et al., 1992). In studies in the rat, we found striatal neurons to be selectively vulnerable to 3-NP toxicity despite widespread complex II inhibition throughout the brain. However, in contrast to presymptomatic changes in genetic models, significant reductions in glucose use following 3-NP intoxication coincided with neuronal loss in this mouse line. Therapeutic approaches using pro-energy agents such as creatine have been shown (in other studies) to be neuroprotective in this lesion model (Andreassen et al., 2001). We believe this model provides a useful approach for further elucidation of regional vulnerability in HD.

In another mouse model of a neurodegenerative disorder, the G93A mouse model of familial ALS (overexpressing human mutant Cu/Zn superoxide dismutase, SOD-1), we also found a pattern of early metabolic changes that precede the first observations of neuronal pathology (mitochondrial disruption at ~70 days) and symptom onset (~100 days). Studies revealed reduced glucose utilization in brain and spinal cord at 60 days of age, concomitant with increased mitochondrial complex I activity in these mice. Further, depletions in brain and spinal cord ATP levels were evident as early as 30 days of age. Elevated free radical generation is also evident in the cortex by 90 days (earliest time-point examined to date). In this fALS model, cells appear to have reduced capacity for glucose uptake before symptom onset, but the rate of complex I activity is elevated, perhaps in an attempt to increase electron transport and boost falling ATP production.

Results in mutant mouse models of both ALS and HD clearly demonstrate the early involvement of metabolic changes in the sequence of events initiated by expression of the mutant disease gene, prior to pathologic changes, symptom onset and cell death. The nature of the metabolic changes seen differs between the HD and ALS models, suggesting that the nature of triggering events set in motion by the gene defect may vary in these disorders. While it still remains to elucidate the exact pathway from

mutant gene expression to induction of metabolic changes, these studies have underscored the importance of energy metabolism defects to disease development, and may impact on therapeutic approaches in the future.

Summaries of the outcomes of each of the research objectives of this oroginal project follow, in chronological order of the annual Aims.

YEAR 1

1. The finding that cerebral glucose use is not significantly altered in *Hdh*^{Q50} CAG knock-in mice at 4 months of age, relative to levels in wild type animals; and that no gene dosage effect is seen in *Hdh*^{Q50} mice (48/48 vs. 48/7 CAG repeats).
2. The finding of impaired activities of complexes II-III and IV of the electron transport chain in *Hdh*^{Q50} and *Hdh*^{Q92} mouse brain cerebellum at 4 months of age (preceding symptom onset and NII formation).
3. The finding that aconitase activity is increased in the cerebellum of *Hdh*^{Q50} (48/48) mice at 4 months of age.
4. The finding that cerebral glucose use is reduced in several forebrain regions in the G93A transgenic mouse model of FALS at 60 days of age – a time point preceding the onset of the first pathological changes in these mice.
5. The finding of increased complex I activity in the forebrain of G93A mice at 60 days of age, indicating impaired mitochondrial energy metabolism consistent with the defect seen in FALS A4V patients with a SOD1 mutation, which precedes onset of symptoms and pathological changes.

YEAR 2:

6. The finding that cerebral glucose use is significantly increased in *Hdh*^{Q92} CAG knock-in mice at 4 months of age, relative to levels in wild type animals, and relative to levels in *Hdh*^{Q50}mice (Year 1 results).
7. The finding that cerebral glucose use in *Hdh*^{Q92} CAG knock-in mice shows a gene-dosage effect, with homozygote (90/90 CAG repeat) mice showing increased glucose use elevations than heterozygote (90/7) mice. In fact, the magnitude of glucose use increases in 90/90 mice is approximately double levels in 90/7 mice.
8. The finding that alterations in cerebral glucose use in *Hdh*^{Q92} mice is evident at 4 months of age in these animals (the earliest time-point investigated). These changes precede pathological or behavioral changes in *Hdh*^{Q92} mice. (NB: *Hdh*^{Q92} mice do not develop a movement disorder, unlike *Hdh*^{111} mice and other HD transgenic mouse models). There is evidence that mutant huntingtin protein (*htt*) is translocating to the nucleus at this time-point, but neuronal intranuclear inclusions (NII) are not evident until 15 months of age in this model (Wheeler et al., 2000). Aggregate

formation occurs faster in *Hdh*^{Q111} mice, hence we are currently examining the same parameters of energy metabolism in these animals.

9. The finding that presymptomatic increases in glucose utilization also occur in multiple forebrain regions in another transgenic HD mouse line, N171-82Q mice expressing a mutant human HD fragment with an 82 polyglutamine repeat. Taken together, findings suggest that metabolic compromise may be an early event in the pathophysiology associated with the expression of mutant huntingtin protein. Glucose use elevations suggest that cells may be attempting to increase glycolytic ATP production, or increase substrate feed into mitochondrial energetic pathways to compensate for a metabolic stress or blockade. The exact mechanism has yet to be elucidated.
10. The finding that R6/2 transgenic HD mice develop a diabetic profile, with onset at 7-8 weeks of age (around the time of movement disorder symptom onset).
11. Observations that metabolic enzymes which show impaired activity in late-stage HD patients (reduced complex II-III and IV in post-mortem brain) do not show evidence of altered activities in pre-symptomatic R6/2 HD mouse brains (whole tissue homogenate preparations; 3.5 and 8 week-old mice). Some alterations are evident in late-stage (12 week-old) R6/2 mice. However, the nature of the metabolic enzyme activities we detected did not correlate with observations in symptomatic HD patients (Browne et al., 1997), or with previously reported alterations in homogenate samples from 12 week old R6/2 mice (Tabrizi et al., 2000). It is possible that use of whole brain homogenate preparations is masking any subtle region-specific changes occur, for example in the striatum of R6/2 mouse brains. Therefore we are currently repeating these assays in striatal preparations from these mice.

YEAR 3:

12. The finding of increased lactate production in symptomatic HD mice, suggesting abnormal energy metabolism at this stage of the disorder in R6/2 mice. This is consistent with lactate elevations seen in symptomatic HD patients.
13. Increases in multiple oxidative damage markers (OH8dG, hydroxyl radical, F2 isoprostaglandins, and hydroxynonenol) in symptomatic R6/2 and N171-82Q HD mouse models.
14. Findings of increased oxidative damage to protein (carbonyl detection), but not lipid (malondialdehyde) in the G93A ALS model. Earliest effects occurred in symptomatic mice (110d).
15. The finding of reduced ATP levels in R6/2 HD mouse forebrain, and to a lesser extent in cerebellum
16. The observation that cerebral glucose utilization in striatum does not appear to be significantly altered prior to lesion formation induced by 3-NP administration (in rats).

B. *Overview of Consortium Proposal Project*

The overall goals of the studies in this grant are to gain insight into the roles of mitochondrial energy metabolism and oxidative stress in the etiology of neuronal damage and death in neurodegenerative disorders. This study is Project III of a Consortium consisting of four projects (Project I, G. Fiskum PI, Grant # 17-99-1-9483; Project II, T. Sick PI; and Project IV, I. Reynolds PI, Grant # 17-98-1-8628). Projects comprising this consortium will use different *in vitro*, *ex vivo*, and *in vivo* approaches to elucidate the specific roles of mitochondria and reactive oxygen species (ROS) in the pathogenesis of Parkinson's disease (PD).

Project III specifically addresses the contribution of mitochondrial complex I to ROS generation *in vivo*, by measuring oxidative damage markers in rat brain after inhibiting activity of specific complex I subunits. Oxidative damage and mitochondrial dysfunction, specifically reduced activity of NADH:ubiquinone oxidoreductase (complex I) of the electron transport chain, are well characterized components of Parkinson's disease (PD) etiology. *In vivo* studies show that complex I inhibition (eg. by MPTP/MPP+ or rotenone) in the brain can result in region-specific neuropathologic changes resembling PD. *In vitro* studies implicate mitochondria as a major source of free radicals mediating oxidative damage and pinpoint a number of the >40 complex I subunits as candidate sites for free-radical production. An important step in understanding the mechanism of region-specific cell damage in PD is to determine *in vivo* whether there is a direct link between abnormal mitochondrial function and the generation of ROS in the disease.

We aim to approach this question by manipulating the activities of different complex I subunits within mitochondria, using intracerebral delivery of subunit-specific complex I inhibitors in rats. Markers of free radical generation will then be measured *in vivo* by microdialysis, and in post-mortem tissue. We intend to test inhibitors with different specificities for the ND1 (mitochondrially encoded) and PSST subunits of complex I. By this approach we will determine if selectively altering functional components of mitochondrial complex I affects complex I activity and free radical generation. By limiting the intervention to a mitochondrial component, and by measuring ROS production shortly after the mitochondrial insult, we will ascertain if generated ROS derive from mitochondria rather than other cellular origins.

NADH-ubiquinone oxido-reductase (complex I) is the first and largest enzyme complex of the mitochondrial respiratory chain. The overall function of complex I is to transfer one pair of electrons from NADH to flavin mono-nucleotide (FMN), and ultimately to ubiquinone (UQ), whilst simultaneously pumping hydrogen ions out of the mitochondrial matrix into the inter-membrane space. Complex I has a molecular mass of approximately 900-1000 kDa and comprises at least 43 subunits (Triepels et al., 2001), 7 of which are encoded by mitochondrial (mt) DNA (ND1-ND6, ND4L), and the remainder by nuclear (n) DNA. Subunits are organised into an L-shape structure, consisting of a hydrophobic membrane arm embedded into the inner mitochondrial membrane (IMM) and a hydrophilic peripheral arm aligned perpendicular to the IMM and directed into the mitochondrial

matrix. The peripheral arm is comprised of 2 fractions; a flavoprotein (FP) where electron transfer begins, and iron-sulphur (Fe-S) clusters (N), several of which act as redox groups that facilitate electron transfer. Binding sites for NADH and FMN are found on the peripheral arm. The membrane arm is comprised of at least 24 nDNA-encoded subunits , the 7 mtDNA subunits, and possibly two Fe-S clusters (Okun et al., 1999).

The electron carrier NADH enables entry of electrons into complex I. There are two well defined binding sites for NADH in eukaryotes, 51kDa and 39kDa subunits. It is believed that 39kDa subunit is required to maintain stability of the redox group X and thus facilitate electron transfer (Schulte et al., 2001). Electron input occurs via the FMN prosthetic group together with Fe-S clusters (Rasmussen et al., 2001). The 'catalytic core' of complex one is comprised of the PSST, TYKY, NUOD, ND1 and ND5 subunits (Schuler et al., 2001). PSST is a 23-kDa subunit containing one binding site for Fe-S cluster N2. It plays a vital role in electron transfer by functionally coupling N2 to CoQ (Schuler et al., 1999). The binding of CoQ is the final stage in electron transfer via complex I and an important function of the membrane arm. The actual quinone binding site has not been equivocally proven, and different studies suggest it is encoded by the ND1 and ND4 proteins (Triepels et al., 2001), or a hydrophilic 49-kDa/NUOD subunit located at the interface of the peripheral and membrane arms (Darrouzet et al., 1998). The TYKY subunit is proposed to bind two tetranuclear Fe-S clusters (N6a and N6b) that form the novel redox groups found in complex I. TYKY is part of a special class of 8Fe-ferredoxins and works as an electrical driving unit for the proton pump (Rasmussen et al., 2001).

The original Consortium project SOW cited using rotenone (ND1 and PSST affinities), pyridaben (PSST subunit specific), and dicyclohexylcarbodiimide (DCCD; ND1 subunit specific) to selectively inhibit complex I subunit function. However, it has been necessary to make some modifications to this aim in the first year of study. Due to the extreme toxicity of DCCD to humans, combined with its ease of systemic penetration (via skin, eyes, inhalation etc), and the nature of the survival animal experiments employed in this study (facilitating exposure to potentially toxic animal excretions), we deemed it unsafe for experimenters to be exposed to this agent under normal laboratory conditions. Therefore we are currently concentrating on studies utilizing rotenone and pyridaben. While there is some overlap in the targets of these inhibitors in terms of complex I binding sites, rotenone shows greater specificity for the ND1 subunit, and pyridaben for the PSST subunit (Schuler et al., 1999). We have recently obtained a third complex I inhibitor for use in these studies, Piericidin A (the kind gift of Dr. Thorsten Friedrich, Albert-Ludwigs-Universitaet, Freiberg Germany). Whilst previous studies suggest that piericidin A also binds within the large complex I pocket for CoQ binding, with some overlap with the rotenone binding site (Okun et al., 1999), a recent study suggests that the two agents differentially affect semiquinone function, and therefore may have distinct binding domains (Magnitsky et al., 2002). Piericidin A is structurally similar to CoQ and appears to prevent CoQ

reduction by dehydrogenase in both complexes I and II (succinoxidase). The sensitivity of complex I for piericidin A is however much greater than complex II, with complete inhibition at 0.036 nmole/mg of mitochondrial protein (Magnitsky et al., 2002). This observation highlights another potential problem inherent to all studies using compex I inhibitors – namely that they are not completely selective for only complex I. We aim to circumvent this problem by comparing and contrasting the effects of multiple different complex I inhibitors.

C. Experimental Results, May 1st 2002-April 30th 2003.

1) Original Grant SOW, Objective 11

HPLC measurement of cerebral energy metabolite levels in HD *Hdh* and R6/2 Tg mice.

(i) *Measurement of Cerebral Metabolite Levels in HD Mouse Brains*

R6/2 Mice: HPLC was used to measure levels of the energy metabolites ATP, ADP, AMP, creatine and phosphocreatine in brain tissue in brain tissue of R6/2 HD mice at 4 weeks of age (presymptomatic), 7 weeks of age (onset of initial symptoms of disease; tremor and weight loss), and 12 weeks of age (symptomatic; reduced weight, atrophy of striatum and striatal neurons, tremor, impaired motor performance on a rotarod apparatus). Results were compared with levels in wild-type littermates. Metabolite levels in cerebral cortex, striatum and cerebellum of 12 week and 7 week-old mice are presented in Tables 1 and 2.

Results demonstrate that by 12 weeks of age, towards end-stage of their short lifespans (13-17 weeks), ATP levels in the cortex of R6/2 mice are markedly reduced (~3-fold), relative to levels in wild-type mice (Table 1). In contrast, levels of all other metabolites measured were significantly increased in the cerebral cortex. In the cerebellum, metabolite fluctuations resembled those in the cortex, but only changes in creatine and phosphocreatine (PCr) reached statistical significance. In the striatum, phosphocreatine (PCr) levels were similarly significantly increased, but creatine, AMP and ADP levels reduced. ATP levels showed a trend towards decrease in R6/2 striatum, relative to levels in wildtypes. In 7 week-old mice on the verge of symptom onset, few alterations in metabolite levels were evident (Table 2). PCr again showed a significant elevation in cortex, and trends to increase in the other regions. ADP was significantly reduced in striatum. No other alterations were detected. In younger, 4 week-old presymptomatic mice (n= 6-8 per group), no significant alterations in any parameter were detected (data not shown).

Table 1: Energy Metabolites in 12 week-old Symptomatic R6/2 and Wt mice

	CORTEX		STRIATUM		CEREBELLUM	
	Wt n=5	R6/2 n=6	Wt n=5	R6/2 n=6	Wt n=5	R6/2 n=6
Creatine	49.4 ± 2.7	68.5 ± 1.6 **	73.8 ± 3.2	64.2 ± 3.7 *	55.7 ± 6.0	84.2 ± 7.7 *
PCr	15.1 ± 0.6	39.3 ± 2.0 **	2.8 ± 0.3	4.0 ± 0.5 *	11.2 ± 1.7	26.4 ± 5.5 *
AMP	2.7 ± 1.1	8.0 ± 0.1 **	5.3 ± .3	3.8 ± 0.5 *	4.7 ± 1.0	3.9 ± 0.5
ADP	3.1 ± 0.4	5.8 ± 0.3 **	12.5 ± 0.7	8.4 ± 0.6 **	3.9 ± 0.8	6.3 ± 0.9
ATP	15.8 ± 1.4	4.7 ± 0.5 **	13.2 ± 1.1	11.5 ± 0.9	9.3 ± 1.9	5.3 ± 1.2

Metabolite levels (nmol/mg protein, mean ± SEM) in 12 week-old R6/2 mice, and littermate wild-type (Wt) controls. *$p< 0.05$, ** $p< 0.001$, significant difference relative toWt levels (Student's unpaired t-test).

Table 2: Energy Metabolites in 7 week-old R6/2 and Wt mice

	CORTEX		STRIATUM		CEREBELLUM	
	Wt n=5	R6/2 n=6	Wt n=5	R6/2 n=6	Wt n=5	R6/2 n=6
Creatine	65.2 ± 3.3	72.3 ± 2.7	68.1 ± 2.6	70.3 ± 1.4	61.5 ± 2.8	57.8 ± 4.1
PCr	15.6 ± 1.8	22.5 ± 2.4 *	7.9 ± 1.8	9.5 ± 1.7	11.6 ± 3.3	14.9 ± 3.5
AMP	4.7 ± 0.5	3.7 ± 0.8	4.6 ± 0.7	3.1 ± 0.5	5.7 ± 1.1	5.7 ± 1.1
ADP	7.8 ± 0.7	6.6 ± 0.6	9.2 ± 0.4	7.7 ± 0.5 *	6.2 ± 0.3	5.8 ± 0.5
ATP	8.9 ± 0.5	10.3 ± 1.0	11.9 ± 0.8	13.0 ± 1.0	5.1 ± 0.6	5.1 ± 0.8

Metabolite levels (nmol/mg protein, mean ± SEM) in 7 week-old R6/2 mice, and littermate wild-type (Wt) controls. *$p< 0.05$, significant difference relative to Wt levels (Student's unpaired t-test).

Discussion: Results suggest that generation of ATP is hindered in the cortex of symptomatic12-week old R6/2 mice. Elevations in cellular levels of AMP and ADP may reflect an inability to generate ATP in cells, leading to a build up of precursors. Increases in levels of other metabolites, phosphocreatine and creatine, may implicate that at this late stage in the disorder, cells are using alternative energy sources. Alternatively, energy demand in this region may be reduced. Further studies are required to determine the significance of these increases in creatine and phosphocreatine. Metabolite alterations are less clear cut in striatum – an intriguing observation, given that the striatum is the primary site of metabolic alterations and cell loss in HD. However, this discrepancy may be in part due to the mouse model utilized, since the R6/2 mice show little evidence of actual cell loss or other metabolic alterations in the striatum (although mitochondrial morphological changes have been reported, Yu et al., 2003). Also, there is mounting evidence that functional alterations in cortical neurons may play a role in inducing alterations in the striatum in HD; thus cortical metabolic changes may precede striatal

alterations in this model. These results add to existing evidence of the involvement of metabolic defects in the disease pathogenesis in mutant mouse models. (NB: It has not been possible to measure glucose use in R6/2 mice, due to their diabetic status).

***(ii) Hdh* Mice:** We are currently conducting similar assays in presymptomatic (4 month old) and symptomatic (18 month-old) Hdh^{Q111}mice (results have been delayed due to the long period required to breed mice and age them to 18 months). However, in the interim we have conducted studies assessing mitochondrial ATP synthesis and oxygen consumption (Respiratory Control Ratios, RCR), in freshly extracted forebrain mitochondria from 4 month-old Hdh^{Q111}mice. This time-point coincides with the age at which we previously detected significant increases in cerebral glucose utilization in forebrain cortical regions and striatum in these mice (reported in years 2 and 3 of grant). Experiments used the Clark Oxygraph apparatus with glutamate/malate substrate for respiratory rate calculations, and the luciferase-luciferin electrode apparatus for ATP synthesis (Manfredi et al., 2002).

Results demonstrate a slight trend towards reduced respiratory control ratio in homozygote Hdh^{Q111}mice, compared with wildtype Hdh^{Q20}mice (Hdh^{Q111} = 7.9 ± 1.5, Hdh^{Q20} = 9.3 ± 1.1; mean ± SEM, n= 7/group). However, ATP synthesis was slightly elevated in the Hdh^{Q111}mice (Hdh^{Q111} = 376 ± 38 nmol ATP/min/mg/protein, Hdh^{Q20}= 305 ± 25 nmol ATP/min/mg/protein; mean ± SEM, n= 7/group; p = 0.14, unpaired t-test). The small magnitude increase in ATP synthesis is perhaps consistent with the increased uptake of the energy substrate glucose in the brain of these mice at 4 months of age, putatively required to overcome an energetic stress. Studies are currently being extended to older animals.

2) Mitochondrial Free Radical Generation In Parkinson's Disease

Year 4: June 1st 2002 – May 31st 2003

Original Research Goals:

13) Characterization of the regional and temporal development of complex I inhibition in specific brain regions at time points after administration of subunit-specific complex I inhibitors to rats:

(i) Stereotactic unilateral intracerebral injection of rotenone, DCCD, or pyridaben into the region of the substantia nigra of anesthetized rats; and vehicle into the contraleteral hemisphere. Rats will be sacrificed and brain tissue harvested at multiple time-points post-injection (1h, 6h, 24h, 7d).

(ii) Spectrophotometric measurement of complex I activity, citrate synthase activity (a marker for mitochondrial number), and protein levels in post-mortem tissue from the striatum, nigra, cortex and cerebellum.

14) Commence characterization of the regional and temporal development of cerebral oxidative damage after administration of rotenone, DCCD and pyridaben to rats.

(i) HPLC measurement of oxidative damage markers at time-points before and after complex inhibition (time-points and regions determined in 1) by:
HPLC detection of brain levels of DNA damage product 8-hydroxy-deoxyguanosine (8-OHdG),

(ii) HPLC and immunohistochemical detection of lipid peroxidation marker malondialdehyde in affected brain regions.

NB: The Goals of this study have been modified in the past year by 1) Removal of DCCD as a test substrate, as previously discussed, and 2) Change of location of the injection site of the inhibitors. Preliminary studies were performed in 10 rats to optimize the localization of stereotactic injections directly into the substantia nigra (SN). These proved to result in a great deal of inter-animal variability (due to the small size of this brain region), and produced substantial mechanical damage thoughout the overlying brain regions. Since the primary aim of this study does not require selective targeting of dopaminergic nigro-straital projections, it was decided to target injections to the striatum – a larger brain region that allows more reproducible lesions without affecting multiple other brain regions.

Results

Objective 13: The first requirement of this aim was to find an optimal dosing regime for the inhibitors to be tested. We have commenced studies with rotenone and pyridaben.

Rotenone induces selective degeneration in dopaminergic nigro-striatal projections following systemic administration (either intravenous via the jugular veing, sub-cutaneous injection, or intraperitoneal) (Betarbet et al., 2000; Alam and Schmidt et al., 2002, Sherer et al., 2003, Antkiewicz-Michaluk et al., 2003). These effects are associated with widespread decreases in complex I activity throughout the brain (Hoglinger et al., 2003), but require chronic low dose administration paradigms for generation of these selective lesions (Antkiewicz-Michaluk et al., 2003). In this study we want to identify oxidative damage paradigms as soon after the complex I inhibition is induced as possible, to try to localize these events to the mitochondria, rather than measuring the downstream repercussions of long-term energetic compromise affecting ROS production in other cellular compartments. Therefore we opted for a direct intracerebral injection administration paradigm for the complex I inhibitors.

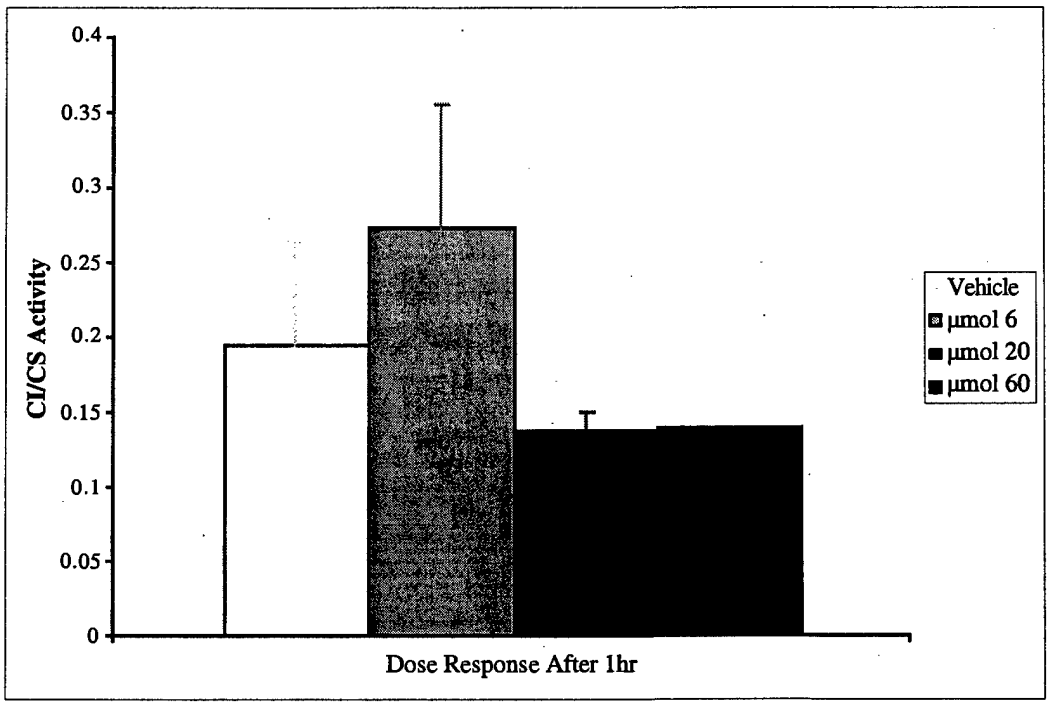
All experiments utilize male Lewis rats, 250-300g at the commencement of studies. For stereotactic drug injection into the brain, rats are anesthetized with a cocktail of ketamine (100mg/kg) and xylazine (10mg/kg). Rats are placed in a Kopf stereotactic frame, and body temperature maintained at 37°C by means of a heating pad.

(i) Dose-response Curve for Rotenone: Complex I Inhibition 1 hour Post-injection

A dose range of 6, 20 and 60 μmol rotenone was selected for the initial experiments, based on reported effective doses in studies using other routes of administration. Rotenone (6, 20, 60 μmol) or vehicle (DMSO/polyethylene glycol cocktail) was injected bilaterally into the striata of rats (3μl volume, injected over 10 min, plus 5 min lag time before needle is removed; n=4-5/group). After one hour animals were sacrificed by Na-pentobarbital overdose. The striata, cortex and cerebellum were dissected from the brain and rapidly frozen on dry ice. Mitochondrial extracts were prepared from brain regions by repeated centrifugation and exposure to a percoll

gradient (modification of Lai and Clark, 1979). Complex I activity was measured in cortex and striatal mitochondria by spectrophotometric assay of the rate of oxidation of NADH at 340nm (Hatefi, 1978). Values were corrected for protein content, determined by the method of Bradford (1976), and for variable amounts of mitochondria in the assay preparations, by measuring activity of the mitochondrial matrix enzyme citrate synthase (rate of oxidation of DTNB at 412nm; Shepherd and Garland, 1969). Results for striatal tissue are demonstrated in Figure 1. Doses of 20 and 60 µmol rotenone produced 25% reductions in complex I activity in the striatum (compared with levels in vehicle-treated rats, $p>0.05$).

Figure 1: Complex I activity in Striatum 1h post-injection.



Data presented as mean ± SEM (nmol/min/mg protein/CS) $p> 0.05$, ANOVA. N=4-5/group

The level of complex I inhibition induced by rotenone was more pronounced in the cortex, shown in Table 3. It is postulated that this effect (which was greater than that anticipated) may be due to back-flow of rotenone up the needle tract. Alternatively, it may reflect transport back to cortex via cortico-striatal nerve terminals, although this seems unlikely given the relatively short period following rotenone injection (1h).

Table 3: Complex I Aactivity in Cortex 1h Post-Intrastriatal Injection.

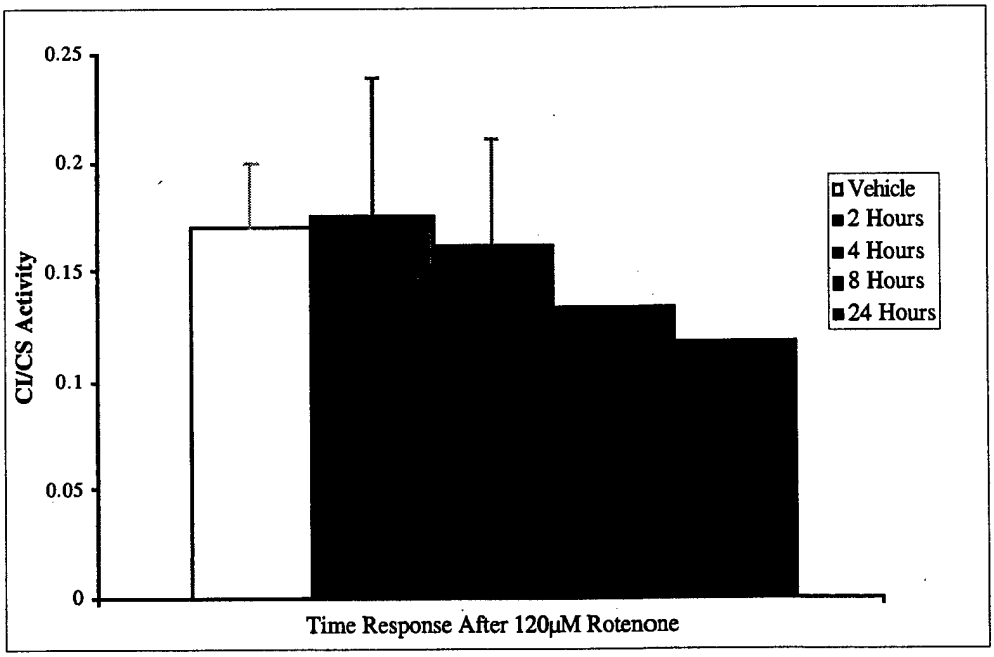
Rotenone (µmol)	Complex I (nmol/min/mg protein/CS)	% Inhibition
0 (Vehicle)	0.22 ± 0.11	-
6	0.16 ± 0.07	27
20	0.18 ± 0.10	18
60	0.09 ± 0.04 *	59

Data presented as mean ± SEM. * $p< 0.05$, ANOVA followed by Student's unpaired t-test. N=4-5/group.

(ii) Time Course of Rotenone Effects: Complex I inhibition

We were dissatisfied with the magnitude of complex I inhibition in the striatum following 60µmol rotenone injection, and therefore conducted pilot experiments with a higher dose, 120µmol. This dose produced an approximate 3 fold reduction in complex I activity in mitochondrial prearations from striatum, in pilot studies assessing effects at 0, 2, 4, 8 and 24h pst-injection. Therefore we chose to use this dose in subsequent experiments. Experiments were expanded to larger group sizes, and results are shown in Figure 2. Complex I activity was not markedly altered up to 4 hours post-injection, but showed a reduction at 8 and 24 hours post-injection.

Figure 2: Time course of Complex I activity in Striatum After Rotenone Injection.



Data presented as mean ± SEM (nmol/min/mg protein/CS) $p> 0.05$, ANOVA. N=4-5/group

Ongoing Experiments For Objective 13, and problems encountered:

(a) We are currently analyzing data from experiments using an alternative route of rotenone administration, namely intracerebroventricular administration, in an attempt to rapidly generate striatal lesions without inducing the mechanical damage inherent with stereotactic injections.

(b) We have encountered many problems with the variability of complex I measurements using the spectrophotometric assay approach. This has proved to be extremely time consuming, and tissue consuming, and is also prone to problems associated with rapid and accurate dissection of brain regions. Therefore we have performed further dose-response studies with rotenone, cut brains into coronal cryostat sections, and are currently processing these sections for histochemical and autoradiographic assessments of complex I activity, according to the methods of Jung et al. (2002) and Higgins and Greenamyre (1996), respectively.

(c) We are concurrently running experiments elucidating optimum dosing regimes for pyridaben. Experiments are at the stage of measuring complex I, protein and citrate synthase activities/levels.

(d) Cerebellar tissue from each experiment has been dissected and stored. We will analyze cerebellar tissue only from experiments yielding reportable complex I alterations in the striatum (lesion target). Nigral tissue could not be accurately and rapidly dissected from the rats (and is also of less interest since the injection site has been changed to the striatum), however we will be able to detect any complex I changes in this region using the histochemical and autoradiographic approaches.

Objective 14. (This goal is to be carried out over both years of the consortium project).
The research goals of this objective were to use HPLC and immunohistochemical approaches to characterize the nature of any oxidative damage arising after intra-striatal inhibitor injections, and to assess the time course of oxidative damage.

(i) Oxidative Damage Markers After Rotenone Administration

HPLC Studies: We have commenced these studies after rotenone administration in rats. Tissue has been collected from rats that received bilateral injections of rotenone (6, 20, 60 120 μmol) or vehicle (1 hour post-injection), and at 0, 2, 4, 8 and 24 h post 120μmol rotenone. Tissue from striatum, cortex and cerebellum is currently being processed for HPLC assessment of 8-hydroxydeoxyguanosine (OH8dG, DNA damage marker) and malondialdehyde (lipid peroxidative damage marker).

Immunohistochemical Studies: We have completed assessment of the effects of rotenone insult on levels of the lipid peroxidation markers malondialdehyde and 8-iso-prostaglandin F2, and on the oxidative stress response marker heme oxygenase. Experiments used immunohistochemical approaches at 8h and 24h after rotenone administration. Heme oxygenase-1 is a member of the stress-response protein superfamily that catalyzes the rate-limiting step in heme degradation in brain and other

tissues. We used a marker for inducible hemeoxygenase, HO-1. The HO-1 gene contains a heat shock element in its promoter region and is rapidly induced upon exposure to heme, metal ions, sulfhydryl compounds, UV light, and various pro-oxidants. Its metabolic products (carbon monoxide and bilirubin) have been shown to exert potent antioxidant and anti-inflammatory activities, and thus the HO pathway is a fundamental defensive mechanism for neurons exposed to an oxidant challenge. In the brain, astrocytes strongly express HO-1 in response to injury (Gonzales et al., 2002).

Rotenone (120μmol in 3μl) was unilaterally injected into one striatum in rats (as previously described), and vehicle (DMSO/PEG) into the contralateral hemisphere. Rats were euthanized by transcardial perfusion with 4% paraformaldehyde under Na-pentobarbital anesthesia, and fixed brains removed and cut into coronal cryostat sections. Adjacent serial sections through the striatum were then stained for:

a) *Malondialdehyde*, using rabbit antiserum against malondialdehyde-modified protein (kindly provided by Dr. Craig Thomas, Hoechst Marion Roussel) 1:1,000.
b) *8-iso-prostaglandin F2*, using rabbit anti-8-iso-prostaglandin F2 (Assay Designs, Inc., Ann Arbor, MI) 1: 1,000.
c) *Heme oxygenase-1*, using rabbit anti-heme oxygenase-1 (StressGen, British Columbia, Canada) 1: 3,000.

Results are shown in Figure 3. Increased staining for all three markers were evident in rotenone-injected striata, compared with vehicle-injected hemispheres in the same animals, both at 8h post-injection, and at 24. The pattern and approximate numbers of positively stained cells for each marker did not markedly differ between the two time points, suggesting that the maximal initial effect on oxidation may be achieved by 8 hours. Differences were apparent in the pattern and scope of staining with each of the markers. Malondialdehyde-positive cells were most prominent in the immediate vicinity of the lesion/injection site following rotenone. Cell morphologies suggest that most cells stained were neurons, but this must be verified by colocalizaion studies. Iso-prostaglandin F2 showed an intermediate level of staining, affecting more cells than malondialdehyde, over a larger area of striatum. Heme oxygenase-1 positive cells were the most abundant of all the markers examined. They were also present in the largest striatal volume. In addition, both glial and neuronal populations appear to stain positively for heme oxygenase-1.

We are currently performing co-localization studies to try to determine: a) whether specific neuronal populations are preferentially staining for these oxidation markers (eg. GABA, ACh), and b) to confirm the neuronal and glial localization of these markers. We are also conducting experiments to elucidate the earliest time-point oxidative events can be detected, relative to the complex I inhibitory insult.

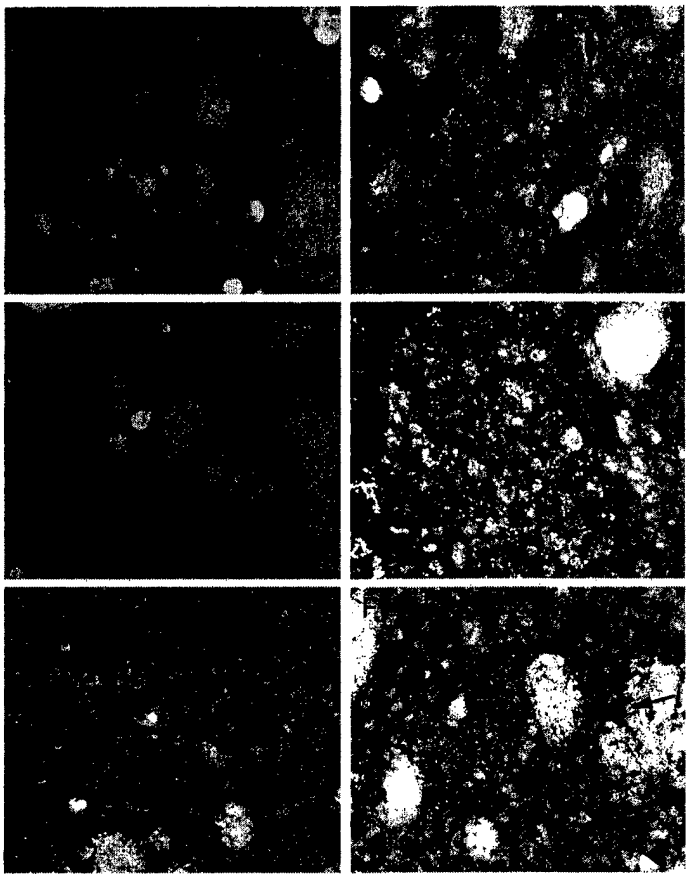


Figure 3
Malondialdehyde (A, B), Isoprostane (C, D) and Heme Oxygenase-1 immunostaining of the caudate adjacent to the injection site, 8 hours after vehicle (A, C, E) or rotenone (B, D, F) injection. Immunoreactive neurons (arrows) and glia (asterisks) are shown.

Discussion: During the first eleven months of this study, a great deal of time has been taken up by training the research technicians in the techniques used in this project, namely stereotactic surgery, microdialysis (to be used in year 2), histology, and HPLC. We have also been optimizing dosing paradigms and complex I measurement techniques for these studies. However, initial results show that whilst low-grade complex I inhibiton is occurring shortly after rotenone introduction into rat striata, a rapid oxidative response is being elicited, and is detectable in these animals.

6. KEY RESEARCH ACCOMPLISHMENTS

- Demonstration of reductions in ATP generation in affected brain regions in symptomatic HD mice (R6/2 model).
- Demonstration of elevations in other key phosphorylated energy metabolites over the course of phenotype generation in the R6/2 HD mouse model – most notably phosphocreatine.
- Determination of the time course of alterations in high-energy phosphates, over the life-span of R6/2 HD mice.
- Assessment of cerebral respiratory rates in the Hdh^{Q111} mutant moue model of HD – no alteration at a timepoint when cerebral glucose uptake is elevated (4 months of age).
- Assessment of ATP synthesis in the Hdh^{Q111} mutant moue model of HD – trend to increase at a timepoint when cerebral glucose uptake is elevated (4 months of age).
- Elucidation of optimum dosing paradigms and complex I measurements in a rat rotenone intrastriatal-injection model of PD-like neurodegeneration.
- Elucidation of the time-line of complex I inhibition following rotenone injection.
- Demonstration of early elevations in lipid peroxidation markers (malondialdehyde and 8-iso-prostaglandin F2) around the site of rotenone insult in rat striatum.
- Demonstration of early and extensive induction of the cell stress marker heme oxygenase 1, following rotenone insult in rat striatum.

7. REPORTABLE OUTCOMES (May 2002-April 2003)

Manuscripts

Browne SE and Beal MF. The Energetics of Huntington's Disease. Neurochem. Res. 2003 In press.

Browne SE, Beal MF. Toxin-induced mitochondrial dysfunction. Int Rev Neurobiol. 2002; 53:243-79.

Andreassen OA, Dedeoglu A, Stanojevic V, Hughes DB, Browne SE, Leech CA, Ferrante RJ, Habener JF, Beal MF, Thomas MK. Huntington's Disease of the Endocrine Pancreas: Insulin

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Wu AS, Aguirre N, Calingasan NY, Browne SE, Crow JP, Kiaei M, Beal MF. Iron porphyrin treatment extends survival in a transgenic animal model of amyotrophic lateral sclerosis. *J. Neurochem.* 2003; 85:142-150.

Browne SE, Yang L, Berger SE, DiMauro J-PP, Fuller SW, Beal MF. Metabolic changes in the G93A mouse model of familial amyotrophic lateral sclerosis precede pathologic changes. *Neuron* (2003) *Submitted.*

Abstracts

Browne SE, DiMauro J-PP, Albrecht RJ, Burr HN. The evolution of metabolic defects in a knock-in mutant mouse model of huntingtons disease. Soc. Neurosci. Abs. 2003; 29: In press

Burr H, DiMauro J-PP, Gregorio J, Browne SE. [^{14}C]-2-Deoxyglucose in vivo autoradiographic studies reveal alterations in cerebral metabolism precede pathologic changes in two mutant mouse models of Huntingtin's disease. Brain 03 & Brain/PET 03, J.CBF Metab. (2003) In press.

Gregorio J, DiMauro J-P P, Narr S, Fuller SW, Browne SE. Cerebral metabolism defects in HD: Glucose utilization abnormalities in multiple HD mouse models. Soc. Neurosci. Abs. (2002) 28: 195.10

Wu AS, Aguirre N, Calingasan NY, Browne SE, Crow JP, Kiaei M, Beal MF. Iron porphyrin treatment extends survival in a transgenic animal model of amyotrophic lateral sclerosis. Soc. Neurosci. Abs. (2002) 28: 789.1

Gregorio J, Burr H, Klivenyi P, Gardian G, von Borstel RW, Saydoff JA, Beal MF, Browne SE. Manipulating oxidative damage and energy metabolism: Novel neuroprotectants in HD mouse models. HDF: Changes, Advances and Good news $(CAG)_n$. (2002) 18.

Browne SE. Disruptions of cellular energy metabolism in HD: evidence for treatment effects? Frontiers in Neurodegeneration – Huntington's disease. (2002).

Invited Presentations

May 7th 2002	Biochemistry Department Seminar Series University of Maryland, Baltimore, MD, USA. *"Animal Models of Neurodegenerative Disorders: Insights into Pathogenesis"*
July 20th –21st 2002	Hereditary Disease Foundation, Mary Jennifer Selznick Workshop, Cardiff, WALES, UK. *"Behavioral Assessment in Mouse Models of Huntington's Disease."*
October 16th-18th 2002	Young ALS Investigator's Workshop Lafayette, PA, USA. *"Cerebral Energy Metabolism in G93A Mice."*
April 15th 2003	American Society for Pharmacology and Experimental Therapeutics, Symposium: Animal Models of Neuropsychiatric Diseases. San Diego, CA, USA. *"Modeling Huntington's Disease in the Mouse: Mechanistic and Therapeutic Insights"*
June 5th 2003	University of Edinburgh, SCOTLAND, UK, Department of Neuroscience Seminar Series. *"The Cerebral Energetics of Huntington's Disease".*

8. CONCLUSIONS

The overall goals of this proposal were to gain insight into the roles of defects in CNS energy metabolism and oxidative stress in mechanisms of neuronal death and dysfunction in neurodegenerative disorders. Outcomes may impact therapeutic strategies for treatment of both degenerative disorders and neurotoxin exposure. Previous studies in human and animal models have implicated the involvement of mis-metabolism and oxidative damage in the pathogenesis of several neurodegenerative diseases including Parkinson's disease (PD). This project concentrated largely on using *in vivo* techniques in whole animal models of degenerative disorders, to gain insight into disease mechanisms at all stages of pathogenesis.

In the first three years of this grant we made substantial progress in characterizing the nature of changes in cerebral energy metabolism seen in theR6/2, N171-82Q and *Hdh* mouse models of HD, both *in vivo* and *in vitro*. We have also shown that cerebral glucose metabolism is impaired in the G93A transgenic mouse model of FALS (overexpressing human mutant SOD1) at 60 days of age, and ATP generation is depressed as early as 30 days. Our observations suggest that energetic dysfunction may play an intrinsic role in the pathogenesis of the motor neuron disorder seen in both HD and ALS mouse models, since alterations precede symptomatic and pathological changes in these animals.

In the period of this grant report, we have provided further evidence of metabolic compromise in the R6/2 mouse model of HD (reduced ATP levels in symptomatic mice, and alterations in high energy phosphates around the time of the first symptoms). We have also begun to address the inter-relationship between energetic defects and oxidative damage in the context of another degenerative disorder, PD. Again using *in vivo* approaches, we have made progress towards optimizing experimental conditions for assessing the temporal association between inhibiting complex I activity at specific mitochondrial subunit sites, and producing free radicals and hence oxidative damage. While experiments still need to be fine-tuned, we have novel and tantalizing data showing the induction of oxidative damage to lipids, and the induction of the cell stress-responder heme oxygenase-1, at a time point shortly after complex I inhibition. These observations have not been reported before, and in future experiments will be explored further to determine *in vivo* how rapidly oxidative damage occurs.

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10. APPENDIX

C.V. Attached

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1989-1993 **Ph.D., Neuroscience**
Viva Feb. 1994 University of Glasgow, Scotland.
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Academic Posts:
1993-1996 **Post-Doctoral Research Fellow**, Neurology Research, Massachusetts General Hospital (MGH) and Harvard Medical School (HMS), Boston MA (P.I.: MF Beal MD).
1996-1999 **Instructor**, Neurology Research, MGH and HMS, Boston MA.
1997-1999 **Assistant**, Neurology Service, Harvard Medical School, Boston MA.
1999 - Present **Assistant Professor** of Neuroscience, Weill Medical College of Cornell University, New York NY.
2001 – Present **Assistant Professor**, Weill Graduate School of Medical Sciences of Cornell University; Program in Neuroscience.

Principal Investigator:
1998-2003 ***USAMRAA: DAMD 7-98-1-8620***
Neurotoxin Exposure Treatment Research Program:
"Bioenergetic defects and oxidative damage in transgenic mouse models of neurodegenerative disorders". $ 1,010,237

2000-2002 ***Huntington's Disease Society of America:***
"The role of energy metabolism in pathogenesis in transgenic mouse models of HD and another CAG repeat disorder". $ 85,000

1997-2003 ***Huntington's Disease Society of America:***
Coalition for the Cure:
"Metabolic defects and oxidative damage in a transgenic mouse model of Huntington's disease." *Co-PI.* $ 90,000pa

Investigator:
1999-2008 ***NIH: 1R01 NS39258 (Beal PI)***
'Bioenergetics in animal models of Huntington's Disease"
Investigator $203,134pa

Grants Pending:
2003 ***NIH: R01; (Reddy PI)***
"Mitochondrial energy metabolism and Alzheimer's disease"
Co-PI.

2003 ***NIH: 1P01 AG14930 (Gibson PI) – Comp. Renewal***
"Mitochondrial dysfunction in transgenic mice with neurodegeneration"
Co-Project Leader.

Post-Doctoral Fellowships Held:

1993-1995 ***Huntington's Disease Society of America:***
"Mitochondrial energy metabolism and oxidative damage in Huntington's disease." $ 60,000

1995 ***Sandoz Foundation for Gerontological Research:***
"Effect of impairment of mitochondrial energy metabolism and oxidative damage on cerebral glucose metabolism." $ 20,000

1996-1999 ***Amyotrophic Lateral Sclerosis Association:***
"An *in vivo* investigation of the cerebral metabolic consequences of motor neuron disease: Measurement of local cerebral glucose utilization in a transgenic mouse model of familial ALS." $120,000

1996-1999 ***Muscular Dystrophy Association:***
"Cerebral energy metabolism in a transgenic mousemodel of ALS." $180,000

Teaching:

2000-present Weill Graduate School of Medical Sciences of Cornell University,
Course Director, Lecturer: "The Neurobiology of Degenerative Diseases". Neuroscience Program,
Lecturer: "Molecular Neuropharmacology". Neuroscience and Pharmacology Programs.

2002-present **Course Director:** Prograom in Neuroscience Seminar series.

2000-2001 **Lecturer:** "Brain and Mind", Neuroanatomy, Weill Medical College.

Students:

2001-2004 Ph.D. Rotation Supervisor: Adriana Galvan , Carl Wonders, Tina Higgins.

20003 B. Med (Australia) Supervisor: Debbie Fried.

Percent Effort: Research: 70%, Teaching:15%, Administration:15%

Prizes/Awards:

1989 Wellcome Trust Ph.D. Studentship

1997 *HDSA / Astra Merck Scholarship*
WFN HD Research Meeting, Sydney, Australia.

Professional Affiliations:

British Neuroscience Association UK (1991-present)
Society for Neuroscience (1994-present)
International Society of Cerebral Blood Flow and Metabolism (1997-)
American Society for Neurochemistry (2002-present)

Journal Reviewer:

J. Neuroscience
J. Cerebral Blood Flow & Metabolism
Eur. J. Neuroscience
Stroke
J. Neurochemistry
Exp. Neurology
Brain Research

Techniques:

In Vivo (rats and mice):

[^{14}C]-2-deoxyglucose autoradiographic measurement of local cerebral metabolic rates for glucose, in conscious rats and mice.
[^{14}C]-methylglucose autoradiography.
[^{125}I]-MK-801 autoradiography.
Stereotactic intracerebral injections: excitotoxin and mitochondrial toxin lesions of discrete brain regions.
Mechanical visual pathway lesions by orbital enucleation.
Vascular cannulation. Systemic drug administration.

In Vitro: (human and rodent CNS tissue):

Quantitative ligand binding autoradiography in brain sections and homogenate preparations.
Histological lesion analysis.
Densitometric measurement of ischemic and excitotoxic lesion volume.
Mitochondrial isolation from tissue.
Spectrophotometric metabolic enzyme activity assays.
Mitochondrial and nuclear DNA extraction.
HPLC detection of oxidative damage products.
Immunocytochemistry
Affymatrix gene microarry.

REFERENCES

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PUBLICATIONS

1. **BROWNE SE** and Beal MF. The Energetics of Huntington's Disease. *Neurochem. Res* (2003) *In press.*

2. Klivenyi P, Ferrante RJ, Gardian G, **BROWNE SE**, Chabrier P-E, Beal MF. Neuroprotective effects of BN82451 in a transgenic mouse model of Huntington's disease. *J. Neurochem. (2003) In press.*

3. Yang LC, Sugama S, Lorenzl S, **BROWNE SE**, Gregorio J, Chirichigno J, Joh TH, Beal MF, Albers DS. Minocycline exacerbates MPTP toxicity in an *in vivo* model of Parkinson's disease. *J. Neurosci. Res* (2003) *In press.*

4. Wu AS, Aguirre N, Calingasan NY, **BROWNE SE**, Crow JP, Kiaei M, Beal MF. Iron porphyrin treatment extends survival in a transgenic animal model of amyotrophic lateral sclerosis. *J. Neurochem.* (2003) 85:142-150.

5. **BROWNE SE,** Beal MF. Toxin-induced mitochondrial dysfunction. *Int Rev Neurobiol.* (2002) 53: 243-79.

6. Andreassen OA, Dedeoglu A, Stanojevic V, Hughes DB, **BROWNE SE**, Leech CA, Ferrante RJ, Habener JF, Beal MF, Thomas MK. Huntington's Disease of the Endocrine Pancreas: Insulin Deficiency and Diabetes Mellitus due to Impaired Insulin Gene Expression. *Neurobiol Dis* (2002) 11:410-424.

7. **BROWNE SE**, Lin L, Mattson A, Georgievska B, Isacson O. Cognitive deficits correlate with sustained cerebral hypometabolism after selective degeneration of the basal forebrain cholinergic system in rats. *Expt. Neurol.* (2001) 170: 36-47.

8. Jeitner TM, Bogdanov MB, Mattson WR, Daikhin Y, Yudkoff M, Folk JE, Steinman L, **BROWNE SE,** Beal MF, Blass JP, Cooper AJL. N(epsilon)-(gamma-L-glutamyl)-L-lysine (GGEL) is increased in cerebrospinal fluid of patients with Huntington's disease. *J Neurochem.* (2001) 79:1109-1112.

9. Andreassen OA, Dedeoglu A, Ferrante RJ, Jenkins BG, Ferrante KL, Thomas M, **BROWNE SE**, Friedlich A, Hersch SM, Borchelt DR, Ross CA, Beal MF. Creatine increases survival and delays motor symptoms in a transgenic animal model of Huntington's disease. *Neurobiol. Ageing.* (2001)) 8: 479-491.

10. Albers DS, Augood SJ, Park LCH, **BROWNE SE**, Martin DM, Adamson J, Hutton M, Standaert DG, Vonsattel JPG, Gibson GE, Beal MF. Frontal lobe dysfunction in PSP: Evidence for oxidative stress and mitochondrial impairment. *J. Neurochem.* (2000). 74: 878-881.

11. **BROWNE SE**, Ferrante RJ, Beal MF. Oxidative stress in Huntington's disease. *Brain Pathol.* (1999) 9: 147-163.

12. **BROWNE SE**, Ayata C, Huang PL, Moskowitz MA, Beal MF. Lack of either endothelial or neuronal nitric oxide synthase isoforms does not differentially affect basal cerebral glucose metabolism in knockout mice. *J. Cereb Blood Flow Metab.* (1999) 19:144-148.

13. **BROWNE SE**. Neurodegenerative disease. *IDrugs* (1999) 2: 4-6.

14. Polidori MC, Mecocci P, **BROWNE SE**, Senin U, Beal MF. Oxidative damage to mitochondrial DNA in Huntington's disease parietal cortex. *Neurosci Lett* (1999) 272:53-6

15. Simon DK, Pulst SM, Sutton JP, **BROWNE SE**, Beal MF, Johns DR. Familial multisystem degeneration with parkinsonism associated with the 11778 mitochondrial DNA mutation. *Neurology* 1999; 53:1787-93.

16. **BROWNE SE**, Bowling AC, Baik MJ, Gurney M,Brown RH Jr., Beal MF. Metabolic dysfunction in familial, but not sporadic, amyotrophic lateral sclerosis. *J. Neurochem.* (1998) 71: 281-287.

17. **BROWNE SE**, Muir J, Robbins TW, Page KJ, Everitt BJ, McCulloch J. The cerebral metabolic effects of manipulating glutamatergic systems within the basal forebrain in conscious rats. *Eur. J. Neurosci.* (1998) 10: 649-663.

18. Matthews RT, Yang L, **BROWNE SE**, Baik MJ, Beal MF. Coenzyme Q_{10} administration increases brain mitochondrial concentrations and exerts neuroprotective effects. *PNAS* (1998) 95: 8892-8897.

19. **BROWNE SE**, Bowling AC, MacGarvey U, Baik MJ, Berger SC, Muqit MMK, Bird ED, Beal MF. Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. *Ann. Neurol.* (1997) 41: 646-653.

20. Ferrante RJ, **BROWNE SE**, Shinobu LA, Bowling AC, Baik MJ, MacGarvey U, Kowall NW, Brown RH Jr, Beal MF. Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *J. Neurochem.* (1997) 69: 2064-2074.

21. Beal MF, Ferrante RJ, **BROWNE SE**, Matthews RT, Kowall NW, Brown RH Jr. Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. *Ann. Neurol.* (1997) 42: 644-654.

22. Matthews RT, Ferrante RJ, Jenkins BG, **BROWNE SE**, Goetz K, Berger S, Chen IY, Beal MF. Iodoacetate produces striatal excitotoxic lesions. *J. Neurochem.* (1997) 69: 285-289.

23. Schulz JB, Matthews RT, Muqit MMK, **BROWNE SE**, Beal MF. Inhibition of neuronal nitric oxide synthase by 7-nitroindazole protects against MPTP-induced neurotoxicity in mice. *J. Neurochem.* (1995) 64: 936-939.

24. Macrae IM and **BROWNE SE**. Brain structures involved in the hypotensive effects of rilmenidine: evaluation by [^{14}C]2-deoxyglucose autoradiography. *J. Cardiovasc. Pharmacol.* (1995) 26 Suppl 2:S55-58.

25. **BROWNE SE** and Beal MF. Oxidative damage and mitochondrial dysfunction in neurodegen-erative diseases. *Biochem. Soc. Trans.* (1994) 22: 1002-1006.

26. **BROWNE SE** and Macrae IM. Differential patterns of local cerebral glucose utilisation associated with rilmenidine- or B-HT 933-induced hypotension. *Brain Res.* (1994) 666: 216-222.

27. **BROWNE SE** and McCulloch J. AMPA receptor antagonists and local cerebral glucose utilization in the rat. *Brain Res.* (1994) 641: 10-20.

Gregorio J, Burr H, Klivenyi P, Gardian G, von Borstel RW, Saydoff JA, Beal MF, Browne SE. Manipulating oxidative damage and energy metabolism: Novel neuroprotectants in HD mouse models. *HDF: Changes, Advances and Good news $(CAG)_n$.* (2002) 18.

Kim .S-Y, Browne SE, Beal MF, Cooper AJ., Blass JP. Transglutaminases in Huntington Brain: Possible Relation to Selective Vulnerability. *American Soc. Neurochem.* (2002)

Browne SE. Disruptions of cellular energy metabolism in HD: evidence for treatment effects? *Frontiers in Neurodegeneration – Huntington's disease.* (2002).

INVITED SPEAKER

July 1994:	The Biochemical Society of Great Britain. Lancaster University, UK. *"Oxidative damage and mitochondrial dysfunction in neurodegenerative diseases."*
November 1995:	Centaur Pharmaceuticals Symposium on Spin Trapping Agents. San Jose, CA, USA. *"Spin trappingagents: Therapeutic potential inneurodegenerative disease."*
December 1995:	Wellesley College, Neurochemistry Seminar Program. Wellesley, MA, USA. *"Insights into functional activity in the brain using in vivo autoradiography techniques."*
May 1997:	HDSA "Coalition for the Cure" Symposium. Baltimore, MD, USA. *"Post-mortem studies and animal models of Huntington's disease."*
September 1997:	17th International World Federation on Neurology Research Group on Huntington's Disease. Sydney, Australia. *"Evidence of bioenergetic defects in Huntington's disease post-mortem brain and a transgenic mouse model of Huntington's disease."*
October 1997:	Institute of Neurology, Neurochemistry Symposium. University College London, UK. *"Roles of bioenergetic dysfunction and oxidative damage in the pathogenesis of familial and sporadic ALS."*
January 1998:	McLean Hospital, Neuroscience Seminar. Belmont, MA, USA. *"Cerebral metabolic defects and the pathogenesis of Huntington's disease."*
May 1998:	International Conference on Nitric Oxide: Peripheral and Central Actions. Antalya, Turkey. *"NO and basal ganglia degeneration."*
June 1998:	HDSA "Coalition for the Cure" Symposium. Atlanta, GA, USA. *"Animal models of Huntington's disease."*
October 2000:	HDSA "Coalition for the Cure" Symposium. San Diego, CA, USA.

	"Progress in studies into oxidative damage and creatine protection in animal models of HD."
November 2000:	Weill Medical College Neuroscience Program Symposium. New York, NY, USA. *"Huntington's Disease: Pathogenesis and Energy Metabolism."*
February 2001:	Huntington's Disease Society of America Public Meeting. Uniondale, NY, USA. *"Huntington's Disease and Energy Metabolism."*
June 16-17 2001	Hereditary Disease Foundation Workshop, Playa Del Rey CA, USA. *"Biomarkers for Huntington's disease".*
October 12th 2001	The 10th International Symposium on New Frontiers of Neurochemistry and Biophysics of Diagnosis and Treatment of Neurological Diseases. Florence, ITALY. *"The Role of Cerebral Energy Metaboism in the Pathogenesis of Huntington's Disease".*
November 28th 2001	Neuroscience Program Cornell University, Ithaca NY, USA. *"In Vivo Experimental Approaches in Models of Neurodegeneration".*
November 29th 2001	Neurobiology Department Seminar Series Cornell University, Ithaca NY, USA. *"Cerebral Energy Metaboism and the Pathogenesis of Huntington's Disease".*
February 1st 2002	Frontiers in Neurodegeneration – Huntington's Disease. Reisensburg, GERMANY. *"Disruptions of cellular energy metabolism in HD: evidence for treatment effects?"*
May 7th 2002	Biochemistry Department Seminar Series University of Maryland, Baltimore, MD, USA. *"Animal Models of Neurodegenerative Disorders: Insights into Pathogenesis"*
July 20-21 2002	Hereditary Disease Foundation, Mary Jennifer Selznick Workshop, Cardiff, WALES, UK. *"Behavioral Assessment in Mouse Models of Huntington's Disease."*
October 16-18 2002	Young ALS Investigator's Workshop Lafayette, PA, USA. *"Cerebral Energy Metabolism in G93A Mice."*
April 15 2003	American Society for Pharmacology and Experimental Therapeutics, Symposium: Animal Models of Neuropsychiatric Diseases. San Diego, CA, USA. *"Modeling Huntington's Disease in the Mouse: Mechanistic and Therapeutic Insights"*
June 5th 2003	University of Edinburgh, SCOTLAND, UK, Department of Neuroscience Seminar Series. *"The Cerebral Energetics of Huntington's Disease".*