Resource Sharing Plan for Mitochondrial Dysfunction in Neurodegeneration of Aging

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The investigators on the program project have two mechanisms for resource sharing. Dr. Anatoly Starkov maintains a web site that has "everything you want to know about mitochondria" including multiple protocols. The address is http://oxphos.org.

The other resource-sharing plan is focused on this Program Project. The Burke Rehabilitation Center, which includes the Burke Rehabilitation Hospital and the Burke Medical Research Institute, has long had a public domain internet web site (http://www.burke.org). Thus, a tab on that site has been established under "Research/Faculty/Gary Gibson" (Resource Sharing Plan for Mitochondrial Dysfunction In Neurodegeneration Of Aging) that includes downloadable information on tools and publications related to the program project.

All finalized data published in this program project are published in publically available journals or in publically available abstracts from scientific meetings. The list of publications is available at the end of this section.

Preliminary data are discussed in group meetings. They will not be posted on a public site because they are preliminary.

The following tools and reagents have either been developed as part of this program project or their use optimized. The part we are responsible for will be made available upon request.

1. Tools related to Project 1

1.1. TGase assay using dot blots

1.2. Specific inhibitors of TGase. ZDON, Boc-DON, cystamine

1.2.1 Chemical inhibitors. ZDON

1.2.2 Molecular inhibitors (RNAi)

1.3. Methods for measuring gene activation

1.3.1. Methods for measuring activation of PGC-1-α and cytochrome c

1.3.2 cytochrome c promoter reporter construct fused to luciferase.

1.3.3 cDNA for wild-type transglutaminase

1.3.4 cDNA for mutant transglutaminase that lacks the ability to transamidate

1.3.5. A 66 base, inactive fragment lacking the CRE and NRF-1 sites.

1.4 HDAC inhibitors sodium butyrate, trichostatin,

1.5 Chromatin immunoprecipitation assays

1.6 Transglutaminase 2 antibody

1.7 Enzyme activity measures

1.7.1 Citrate synthase

1.7.2 ND6 levels

1.8 Live/dead assays (Calcein AM/ethidium homodimer)

1.9 Transglutaminase 2 constructs for the human wt protein and mutants (TG2-C277A; TG2-R580A; TG2-R580A Y516F);

1.10 Chromatin precipitation assays.

1.11. Cell models of Huntington Disease.

1.11.1 Immortalized striatal cells generated from the HdhQ111 knockin mice (STHdhQ111) which bear full length htt with an expanded polyQ tract of 111 CAG repeats.

1.11.2. Mouse embryonic fibroblasts

1.11.3 SH-SY5Y cells that express expanded polyglutamine repeats

1.11.4. Human myoblasts

1.12. Drosophila models of Huntington Disease. Expresses an exon1 fragment of mhtt that contains a 93 glutamine repeat

1.13 Chromatin precipitation assays.

1.14 5 shRNA from Open Biosystem directed against mouse transglutaminase 1 (TG1). One of the sequences recognizes also the rat isoform. We are currently testing their efficiency in reducing TG1 mRNA levels and protein levels. The shRNAs are inserted in a lentiviral backbone (pLKO.1), so we used the most promising shRNA to produce a lentivirus able to knock down TG1 in mouse cells.

1.15. Plasmid overexpressing human TG1 (vector: pSPORT6).

2. Tools related to Project 2 and the mitochondrial core. (Also see oxphos.org)

Techniques available for studying purified mitochondria (Dr. Anatoly Starkov)

2.1. Preparation of non-synaptic mitochondria from small amounts of tissue. Percol purified mitochondria from various brain structures.

2.2 Measurements on isolated mitochondria.

2.2.1. Bioenergetics

2.2.1.1. Respiration rates

2.2.1.2 Resting, phosphorylated and uncoupled respiration to determine whether there are any abnormalities in the respiratory chain, substrate transporters, and ADP phosphorylating system.

2.2.1.3 Spare respiratory capacity of mitochondria, their phosphorylation efficiency at the level of ATPase and substrate phosphorylation,

2.2.1.4. Mitochondrial Membrane potential under the conditions described above.

2.2.2 ROS. ROS emission by isolated mitochondria and determine the contribution of various sites known to generate ROS such as respiratory chain Complex I, Complex III and dihydorlipoyl dehydrogenase

2.2.3. ROS scavenging capacity

2.2.4. Calcium handling. Methods to measure the Permeability Transition Pore Ca2+threshold and the maximum Ca2+capacity of isolated mitochondria and to evaluate the activity of Ca2+release pathways in mitochondria

2.2.5. Enzyme profile of mitochondria several key TCA, respiratory chain, and ROS defense system enzymes. Specifically, the maximum activities of malate, succinate, isocitrate, alpha-ketoglutarate dehydrogenases, citrate synthase, respiratory chain complexes I, III, and IV, malic enzyme, glutathione reductase, and glutathione peroxidase can all be assessed.

2.3.1 Unique cells.

2.3.1.1.1. gC1QR knockdown cells with varied expression of the protein.
2.3.1.1.2. CBARA1 knockdown cells with varied expression of the protein.

2.4 Recombinant gC1qR protein (mature form lacking mitochondria targeting sequence)

2.5 Validated QPCR primers (SyberGreen; mouse brain and liver) for PPAR-regulated proteins involved in peroxisome and mitochondria biogenesis and the major antioxidant proteins:

2.5.1. GenBank Accession NM_017366; NCBI Protein Accession NP_059062
Mus musculus acyl-Coenzyme A dehydrogenase, very long chain (Acadvl), nuclear gene encoding mitochondrial protein, mRNA; amplicon Size 150
Forward Primer CTACTGTGCTTCAGGGACAAC
Reverse Primer CAAAGGACTTCGATTCTGCCC

2.5.2. GenBank Accession NM_007382; NCBI Protein Accession NP_031408
Mus musculus acyl-Coenzyme A dehydrogenase, medium chain (Acadm), nuclear gene encoding mitochondrial protein, mRNA; amplicon Size 110
Forward Primer AGGGTTTAGTTTTGAGTTGACGG
Reverse Primer CCCCGCTTTTGTCATATTCCG

2.5.3. GenBank Accession NM_013495 NCBI Protein Accession NP_038523
Mus musculus carnitine palmitoyltransferase 1a, liver (Cpt1a), nuclear gene encoding mitochondrial protein, mRNA; amplicon Size 100
Forward Primer CTCCGCCTGAGCCATGAAG
Reverse Primer CACCAGTGATGATGCCATTCT

2.5.4. Mus musculus Cox1 (from PMID: 19131594)
Forward Primer TCGCAATTCCTACCGGTGTC
Reverse Primer CGTGTAGGGTTGCAAGTCAGC

2.5.5. GenBank Accession NM_013671 NCBI Protein Accession NP_038699
Mus musculus superoxide dismutase 2, mitochondrial (Sod2), nuclear gene encoding mitochondrial protein, mRNA; amplicon Size 113
Forward Primer CAGACCTGCCTTACGACTATGG
Reverse Primer CTCGGTGGCGTTGAGATTGTT

2.5.6. GenBank Accession NM_009804 NCBI Protein Accession NP_033934
Mus musculus catalase (Cat), mRNA; amplicon Size 181
Forward Primer AGCGACCAGATGAAGCAGTG
Reverse Primer TCCGCTCTCTGTCAAAGTGTG

2.5.7. GenBank Accession NM_011144 NCBI Protein Accession NP_035274
Mus musculus peroxisome proliferator activated receptor alpha (Ppara), transcript variant 1; amplicon Size 153
Forward Primer AGAGCCCCATCTGTCCTCTC
Reverse Primer ACTGGTAGTCTGCAAAACCAAA

2.5.8. GenBank Accession NM_001127330 NCBI Protein Accession NP_001120802
Mus musculus peroxisome proliferator activated receptor gamma (Pparg), transcript variant 1, mRNA; amplicon Size 139
Forward Primer TTTTCCGAAGAACCATCCGATT

Reverse Primer ATGGCATTGTGAGACATCCCC

2.5.9. GenBank Accession NM_001164230 NCBI Protein Accession NP_001157702
Mus musculus nuclear respiratory factor 1 (Nrf1), transcript variant 5, mRNA; amplicon Size 90
Forward Primer AGCACGGAGTGACCCAAAC
Reverse Primer TGTACGTGGCTACATGGACCT

2.5.10. GenBank Accession NM_009360 NCBI Protein Accession NP_033386
Mus musculus transcription factor A, mitochondrial (Tfam), nuclear gene encoding mitochondrial protein, mRNA; amplicon Size 122
Forward Primer ATTCCGAAGTGTTTTTCCAGCA
Reverse Primer TCTGAAAGTTTTGCATCTGGGT

2.5.11. GenBank Accession NM_015729 NCBI Protein Accession NP_056544
Mus musculus acyl-Coenzyme A oxidase 1, palmitoyl (Acox1), mRNA; amplicon Size 283
Forward Primer TAACTTCCTCACTCGAAGCCA
Reverse Primer AGTTCCATGACCCATCTCTGTC

2.5.12. GenBank Accession NM_011434 NCBI Protein Accession NP_035564
Coding DNA Length 465
Gene Description Mus musculus superoxide dismutase 1, soluble (Sod1), mRNA; amplicon Size 139
Forward Primer AACCAGTTGTGTTGTCAGGAC
Reverse Primer CCACCATGTTTCTTAGAGTGAGG

2.5.13. NCBI GeneID 26379 GenBank Accession NM_007953 NCBI Protein Accession NP_031979
Mus musculus estrogen related receptor, alpha (Esrra), mRNA; amplicon Size 168
Forward Primer CTCAGCTCTCTACCCAAACGC
Reverse Primer CCGCTTGGTGATCTCACACTC

2.5.14. GenBank Accession NM_010295 NCBI Protein Accession NP_034425 Coding DNA Length 1914
Mus musculus glutamate-cysteine ligase, catalytic subunit (Gclc), mRNA; amplicon Size 125
Forward Primer GGGGTGACGAGGTGGAGTA
Reverse Primer GTTGGGGTTTGTCCTCTCCC

2.5.15. GenBank Accession NM_019913 NCBI Protein Accession NP_064297 Coding DNA Length 501
Gene Description Mus musculus thioredoxin 2 (Txn2), nuclear gene encoding mitochondrial protein,; amplicon Size 127
Forward Primer TGGGCTTCCCTCACCTCTAAG
Reverse Primer CCTGGACGTTAAAGGTCGTCA

2.5.16. GenBank Accession NM_007452 NCBI Protein Accession NP_031478 Coding DNA Length 774
Mus musculus peroxiredoxin 3 (Prdx3), nuclear gene encoding mitochondrial protein, mRNA; amplicon Size 100

Forward Primer GGTTGCTCGTCATGCAAGTG
Reverse Primer CCACAGTATGTCTGTCAAACAGG

2.5.17. GenBank Accession NM_012021 NCBI Protein Accession NP_036151 Coding DNA Length 633
Mus musculus peroxiredoxin 5 (Prdx5), nuclear gene encoding mitochondrial protein, mRNA;
Forward Primer GGCTGTTCTAAGACCCACCTG
Reverse Primer GGAGCCGAACCTTGCCTTC

2.5.18. GenBank Accession NM_013711 NCBI Protein Accession NP_038739 Coding DNA Length 1584 Gene Description Mus musculus thioredoxin reductase 2 (Txnrd2), nuclear gene encoding mitochondrial protein, mRNA; amplicon Size 86
Forward Primer GATCCGGTGGCCTAGCTTG
Reverse Primer TCGGGGAGAAGGTTCCACAT

2.5.19. GenBank Accession NM_025794 NCBI Protein Accession NP_080070
Mus musculus electron transferring flavoprotein, dehydrogenase (Etfdh), mRNA; amplicon Size 140
Forward Primer GTGCGACTAACCAAGCTGTC
Reverse Primer GGATGAACAGTGTAGTGAGTGG

2.5.20. GenBank Accession NM_008160 NCBI Protein Accession NP_032186 Coding DNA Length 606
Gene Description Mus musculus glutathione peroxidase 1 (Gpx1), mRNA; amplicon Size 105
Forward Primer AGTCCACCGTGTATGCCTTCT
Reverse Primer GAGACGCGACATTCTCAATGA

2.5.21. GenBank Accession NM_007393 NCBI Protein Accession NP_031419
Mus musculus actin, beta (Actb), mRNA; amplicon Size 154
Forward Primer GGCTGTATTCCCCTCCATCG
Reverse Primer CCAGTTGGTAACAATGCCATGT

3. Development of new tools and reagents related to project 3,

3.1 Transgenic Mice.

3.1.1. DLST+/-mice. Targeted disruption of the mitochondrial dihydrolipoamide succinyltransferase (the E2k subunit of KGDHC) provides a specific interruption of KGDHC activities. DLST+/-heterozygous mice had lower message and protein levels for E2k, leading to reduced brain KGDHC activity. A partial reduction in KGDHC activity did not result in any significant changes in body weight, ratio of sex and ratio of genotype. Methods for genotyping these mice were also standardized.

3.1.2. APP mutant mice and the methods for genotyping these mice.

3.1.3. Methods for crossing including genotyping of the APP/E2k crosses.

3.2 Cells.

3.2.1. HEK cells with diminished E2k by antisense strategy. Inhibitors block the action of the whole complex, so techniques were employed to test the consequences of different levels of diminished E2k-mRNA on the protein levels of the subunits, KGDHC activity and physiological response. Human embryonic kidney (HEK) Cells were stably transfected with an E2k sense or antisense expression vector. Sense control (E2k-mRNA-100) was compared to two clones in which the mRNA was reduced to 67% of the control (E2k-mRNA-67) or to 30% of the control (E2k-mRNA-30). The levels of the E2k protein in the clones paralleled the reduction in mRNA, and E3 protein levels were unaltered.

Growth rates of E2k-mRNA-67 and E2k-mRNA-30 were both reduced about 50% compared to the control. Unexpectedly, the clone with the greatest reduction in E2k protein (E2k-mRNA-30) had a 40% increase in E1k protein. The activity of the complex was only 52% of normal in E2k-mRNA-67 clone, but was near normal (90%) in the E2k-mRNA-30 clone.

3.2.2. Cultured neurons from embryos and adult mice from controls or DLST+/- mice. HEK cells are not neurons, so we tested the effects of diminished E2k in cultured neurons from DLST+/- mice. Cultures from embryos have the advantage that they are relatively pure and the techniques well characterized. However, enzymes of energy metabolism are low in embryonic brain and still developing. Thus, we established methods developed by G. Brewer for getting neurons from adults. His most recent version was published in Nature Protocols (Brewer and Torricelli, 2007). Although they are not as pure embryonic cultures they have tremendous advantage that they are from mature animals and AD is a disease of adult. Brewer has used this model to show striking changes in oxidative processes with aging. He has agreed to help us (see letter and cv).

3.2.3. SY5Y cells with inducible shRNA of E1k and E2k. Several SH-SY5Y cell lines stably expressing tetracycline repressor protein (tetR) were established. These cell lines (SH-SY5Y/tetR) showed that expression of exogenous gene can be tightly controlled by tetracycline (i.e. exogenous gene will only express in the presence of tetracycline).

Three siRNA expression vectors for both E1k and E2k as well as two negative siRNA control vectors were constructed. Sequences of the siRNAs were confirmed by automatic DNA sequencing. We are in the processing of generating siRNA inducible cell lines by stable transfection of the effective siRNA vector into the SH-SY5Y/tetR cells. The expression of siRNAs will be induced by addition of tetracycline. The effects of siRNAs on E1k and E2k expression will be tested by monitoring the levels of mRNA, protein and KGDHC activity.

3.3 Antibodies. Antibodies to all three subunits of KGDHC were developed and have become part of our usual experimental approach to study these problems. These are useful for Western blots as well as in vivo immunocytochemistry.

3.4 Inhibitors. Specific inhibitors are very complementary to the use of molecular biology to manipulate the proteins. The advantage is they act instantly; the disadvantage is they are not as specific as knocking down the message. We tested the ability of succinylphosphonates and several of its ethyl esters to inhibit brain KGDHC, other α-keto acid-dependent enzymes and KGDHC in intact cells. The esters allow the SP to enter the cells and then they are hydrolyzed. At a concentration of 0.01 mM, SP, its phosphonoethyl (PESP) and carboxyethyl (CESP) esters nearly completely inhibited isolated brain KGDHC. SP, PESP or CESP (0.01 mM) produced 70% inhibition of KGDHC in intact cells. The high specificity in targeting KGDHC, penetration into cells and minimal transformation by cellular enzymes indicate that SP and its esters are useful to study the effects of reduced KGDHC activity on neuronal and brain function. (Bunik et al., 2005)

3.5 Simple column purification of KGDHC. Purification of KGDHC is very critical step for post-translational modification (PTM) study by mass spectrometry. We established a simple one step method to obtain relatively pure KGDHC from Sigma KGDHC which contains numerous BSA and other contaminants. KGDHC (Sigma) (200 µl) was loaded onto a 2% ABT Agarose column (Agarose Bead Technologies, Tampa, FL) to remove contaminating bovine serum albumin. Five of the fractions with the highest KGDHC activity and least contaminants from four column purifications were pooled and used for used for further oxidants or modification experiments.

3.6 Adeno- Viruses (AV) have been developed to knockdown E1k and to over express E1k. These work well in primary cultured neurons and in the N2a cell lines.

3.6.1 Overexpress E1: Ad-GFP-mOGDH

3.6.2. Knockdown E1: Ad-GFP-U6-OGDH-shRNA

3.7 Adeno-associated virus (AAV) have been developed to knockdown E1k or E2k in vivo.

3.7.1. E1: AAV2-GFP-U6-shOGDH
3.7.2. E2: AAV2-GFP-U6-DLSTshRNA

3.8 Gels

3.8.1. Blue native gels. Blue-Native gel has been shown to have the advantages of studying intact proteins or protein complexes with molecular weight up to 10 MDa and of preserving enzyme activity after electrophoresis (Schagger and von Jagow, 1991). We utilized both one two dimensional Blue-Native gel electrophoresis to achieve best

separation of KGDH complex from other contaminants and keep the complex intact and preserve the activity during electrophoresis.

3.8.2. SDS gels. Protein modifications have been determined by co-localizing proteins on SDS gels with antibodies to the primary antigen and antibodies to potential modifiers (eg nitration of glutathione).

3.9 Measurement of mitochondrial calcium by Rhod-2. Mitochondrial calcium and KGDHC are closely linked. Thus, methods were standardized to measure mitochondrial calcium by fluorescence microscopy and by cofocal use mitotracker green to select regions of interest (Kruman et al., 1998a; Kruman et al., 1998b). We see the appropriate decreases with FCCP+oligomycin andn appropriate increases with ionomycin.

3.10 In vivo therapies. Resveratrol treatment of plaques, brain glutathione, brain cysteine.

4. Technique and tools for Project 4.

4.1 Genotyping and breeding
- 4.1.1. APP-Tg19959 (two mutations in APP)
- 4.1.2. Tau-P301S mice (P301S mutation)
- 4.1.3. E2k +/- mice
- 4.1.4. Crosses of E2k and APP
- 4.1.5. Genetic models of Huntington Disease (R6/2, BAC-103)
- 4.1.6. MnSOD overexpressing mice

4.2 Immunocytochemistry, ELISA, and western blots
- 4.2.1 APP and amyloid (oligomers, monomers, plaque burden)
- 4.2.2. Tau protein and phosphorylation
- 4.2.3. Markers of oxidative stress (malondialdehyde, nitrotyrosine, carbonyls)
- 4.2.4. Markers for counting neurons (NeuN)
- 4.2.5. NOX1 immunoreactivity
- 4.2.6. Markers of inflammation (microglia and astrocytes)
- 4.2.7. Marker of medium spiny neurons in striatum (calbindin)
- 4.2.8. Markers of tau phosphorylation (AT8 and MC-1)

4.3 Stereological cavalieri method for assessing brain damage

4.4 Chemical mitochondrial toxins to induce neurodegenerative diseases
- 4.4.1. Malonate
- 4.4.2. 3-Nitroproprionic acid
- 4.4.3. MPTP

4.5 Behavioral tests
- 4.5.1. Morris Water Maze: spatial memory
- 4.5.2. Contextual fear conditioning: fear memory
- 4.5.3. Rotarod: motor coordination
- 4.5.4. Grid: muscular strength
- 4.5.5. Elevated plus maze: anxiety
- 4.5.6. Open field: locomotion

4.6 Activation of PGC-1alpha in vivo

4.7 Striatal injections of Adeno Associate Virus

4.8 Use of therapeutic agents
- 4.8.1. iNOS inhibitor N-iminoethyl-L-lysine
- 4.8.2. Resveratrol
- 4.8.3. Coenzyme Q10
- 4.8.4. Combination of CoQ10 with creatine
- 4.8.5. Bezafibrate
- 4.8.6. Lipoic acid and acetyl-L-carnitine

***Other techniques and conclusions using these techniques are detailed in publications that resulted from this Program Project on Mitochondria in Neurodegenerations of Aging*.**

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PROJECT 2

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