SQSTM1/p62 Expression Effects on Mitochondria and Protection from Neurodegeneration: A Proposed Role from Mitochondrial Dynamics to Learning and Memory

by

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Abstract

Sequestosome 1(SQSTM1/p62) is a multi-domain scaffolding protein involved in multiple cellular processes from endocytosis to protein degradation by both the ubiquitin proteasome system and autophagy. In its role as a degradation pathway regulator, p62 has been localized to the mitochondria where is participates in mitophagy. However, the aforementioned roles of p62 all have in common stress applied to the cell to drive multiple survival pathways. The question of a physiological role for p62 at the mitochondria has not been addressed. By using innovative cell culture techniques to isolated hippocampal neurons from mouse brain, I have shown that p62 participates in physiological mitochondrial function. I identify for the first time that p62 localization plays a role in regulating mitochondrial morphology, genome integrity and mitochondrial import of a key transcription factor as well as, present evidence that these responses to the presence of p62 extend beyond the protein's immediate influence on membrane potential. Changes in mitochondrial function have been associated with Affective spectrum and anxiety disorders. Thus, proteins that affect mitochondrial energetics and function could prove to be attractive targets for drug treatment for these psychiatric behavioral disorders. I report on the generation of a transgenic mouse overexpressing SQSTM1/p62 or a single point mutant (P392L) in the UBA domain of SQSTM1/p62. Mitochondrial energy output and functionality is improved in the overexpressing mouse compared to Wild Type. These elevated levels of mitochondrial functionality correlate directly with discernible improvements in mouse behaviors related to affective spectrum and anxiety disorders. We also describe how overexpression of SQSTM1/p62

improves spatial learning and long term memory formation in these transgenic mice. These results suggest that SQSTM1/p62 provides an attractive target for therapeutic agents potentially suitable for the treatment of anxiety and affective spectrum disorders.

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Finally, I dedicate this work to my mentor, my friend, Dr. Marie Wooten. You left us too soon, …….there are no words to express my feelings and any I have tried to put down on paper seem to cheapen the depth of emotions. The only passage I can provide is from your favorite aria by Bocelli…..these words mean I will never forget.

"When you are far away,
I dream of the horizon
And words fail,
And Yes….
I know that you are with me"

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Table of Contents

Abstract ii

Acknowledgements iv

Introduction 1

Chapter 1. Isolation and Culture of Hippocampal Neurons from Prenatal Mice 8

1.1 Short Abstract 9

1.2 Long Abstract 10

1.3 Protocol Text 11

1.4 Representative Results 16

1.5 Discussion 18

1.6 Acknowledgements 22

1.7 Disclosure 22

1.8 References 24

Chapter 2. Role for Sequestosome 1/p62 in Mitochondrial Dynamics, Import and Genome Integrity 36

2.1 Highlights 37

2.2 Abstract 38

2.3 Introduction 39

2.4 Materials and Methods 41

2.5 Results and Discussion 46

2.6 Conclusion 54

2.7 Acknowledgements 54

2.8 References....55

Chapter 3. Behavioral Effects of SQSTM1/p62 Overexpression in Mice: Support for a Mitochondrial Role in Depression and Anxiety....69

3.1 Abstract....70

3.2 Introduction....71

3.3 Methods....74

3.4 Results....81

3.5 Discussion....86

3.6 Acknowledgements....91

3.7 References....92

Summary and Conclusions....104

List of Tables

Table 1.1 Table of specific reagents and equipment.....23

Table 3.1 Table of behavioral test statistics.....99

List of Figures

Figure 1.A Graphic overview of entire harvest and dissociation procedure 26

Figure 1.1 Dissection of the prenatal mouse brain 27

Figure 1.2 Location of the hippocampus in the prenatal mouse brain 28

Figure 1.3 Dissociation of hippocampal tissue in trypsin solution 29

Figure 1.4 Pasteur pipet tips used in trituration of hippocampal tissue 30

Figure 1.5 Hippocampal neurons isolated using this procedure and plate in NB media ..31

Figure 1.6 Hippocampal neurons contaminated with glial cells grown for 7 days and Stained with the organelle marker MitoTracker Red CM-H2XRos 32

Figure 1.7 Hippocampal neurons grown for 7 days and stained with the organelle marker MitoTracker Red CM-H2XRos 33

Figure 1.8 Hippocampal neurons grown for 7 days, fixed with 4% paraformaldehyde/ PBS and immuno-stained with monoclonal anti-tubulin β antibody 34

Figure 1.9 Hippocampal neuronal cultures were grown for 5 days and transfected With GFP-LC3β DNA construct using Lipofectamine 2000 35

Figure 2.1 Mitochondrial localization of p62 in MEF cells 65

Figure 2.2 p62 regulates mitochondrial morphology autonomous of cell type.....66

Figure 2.3 Abnormal mitochondrial metabolism and increased oxidative stress in $p62^{-/-}$ cells.....67

Figure 2.4 p62 protects mitochondrial genome integrity 68

Figure 3.1 Demonstration that EFGP tagged proteins were effectively expressed in mouse brain.....100

Figure 3.2 p62 overexpression increases the functionality of mitochondria in the hippocampus 101

Figure 3.3 p62 overexpression influences affective disorder behavior patterns 102

Figure 3.4 p62 overexpression affects spatial learning and memory tasks 103

List of Abbreviations

SQSTM1/p62	Sequestosome 1/p62
HBSS	Hank's Balanced Salt Solution
AraC	Arabinoside
FUdR	5-fluoro-2'-deoxyuridine
GFP-LC3	Green Fluorescent Protein – LC3
PBS	Phosphate Buffered Saline
TFAM	Mammalian Mitochondrial Transcription Factor A
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
UPS	Ubiquitin Proteasome System
UBA	Ubiquitin Associated Domain
PB1	Phox and Bem1 Binding Domain
ROS	Reactive Oxygen Species
PINK1	PTEN Induced Putative Kinase 1
HDAC6	Histone Deacetylase 6
mtDNA	Mitochondrial DNA
rRNA	Ribosomal RNA
tRNA	Transfer RNA
$\Delta\psi m$	Membrane Potential
AD	Alzheimer's Disease

MEF	Mouse Embryonic Fibroblast
DMEM	Dulbecco’s Modified Eagle Medium
HRP	Horse Radish Peroxidase
ECL	Enhanced Chemiluminescent
BSA	Bovine Serum Albumin
TBS	Tris Buffered Saline
PFA	Paraformaldehyde
FITC	Fluorescein Isothiocyanate
ER	Endoplasmic Reticulum
OXPHOS	Oxidative Phosphorylation
LTP	Long Term Potentiation
AMPA	AMPA type Glutamate Receptor
EGFP	Enhanced Green Fluorescent Protein
AMPK	5’-AMP-activated Kinase

Introduction

Sequestosome 1/p62 , also known as A170 in mouse or ZIP in rat, has been identified as a ubiquitin binding protein (Vadlamudi, et al. 1996) and is localized to aggresomes of various neurodegenerative diseases (Zatloukal, et al. 2002). p62 play critical roles in neurodegeneration, cancer and obesity through regulation of cell signaling, protein degradation and transcription factor activation (Moscat and Diaz-Meco, 2009; Rodriques, et al. 2006; Wooten, et al. 2006). p62 contains multiple protein interaction domains endowing the protein with scaffolding capabilities (Moscat, et al. 2007) and is shown to be important in two degradation pathways, the Ubiquitin Proteasome System (UPS) and autophagy (Bjørkøy, et al. 2006; Wooten, et al. 2006). In the UPS, p62's C-terminal UBA domain interacts with polyubiquitin tagged proteins shuttling them to the proteasome for degradation (Seibenhener, et al. 2004). In autophagy, the N-terminal PB1 domain of p62 polymerizes allowing the interaction of p62's LIR domain with the autophagic marker protein LC3 leading to mediation of the autophagic process (Bjørkøy, et al. 2005; Pankiv, et al. 2007). p62 itself can be degraded by either pathway (Seibenhener, et al. 2004; Ichimura, et al. 2008) however, autophagic defects causing accumulation of p62 containing aggregates in response to stress lead to increases in reactive oxygen species (ROS), DNA damage, tumorigenesis and ultimately cell death (Komatsu, et al. 2007; Matthew, et al. 2009).

Mitochondrial function is directly related to mitochondrial structure (Campello and Scorrano, 2010). Mitochondrial fragmentation correlates with bioenergetic defects in the electron transport chain leading to elevated oxidative stress yielding increased mtDNA mutations (Chen, et al. 2005; Frank, 2006; Guillery, et al. 2008). Defects in mitochondrial dynamics causing dysfunction in the mitochondria have been linked to multiple neurodegenerative diseases (Chen and Chan, 2009). Mitochondrial fusion is required for mtDNA stability and been shown to protect against neurodegeneration (Chen, et al. 2007). When fusion is disrupted, loss of membrane potential and decreased cellular respiration has been reported (Chen, et al. 2005). Efficient mitochondrial function and turnover depends on continuous structural remodeling through fusion and fission (Knott, et al. 2008). Fission produces daughter mitochondria which can become normal functioning mitochondria or become depolarized and be eliminated by mitophagy (a process similar to autophagy but specific for the removal of damaged mitochondria). The interplay between fusion, fission and autophagy are believed to work as a bioenergetic quality control mechanism for addressing damaged mitochondria (Twig, et al. 2008).

Growing lines of evidence correlate p62 with clustering of damaged mitochondria. p62 has been shown to be recruited to depolarized mitochondria in PINK1/parkin expressing cells, both proteins linked to the pathology of Parkinson's disease (Okatsu, et al. 2010). Depolarized mitochondria change morphology from normal ribbon-like structures to a more fragmented form and migrate to the perinuclear region. This process is reported to require recruitment of p62 and its adaptor protein HDAC6 leading to mitophagy (Lee, et al. 2010; Okatsu, et al. 2010). Although there are some conflicting reports in the literature concerning the exact role of p62 in the mitophagic process, it is clear that p62 is indispensable in the polymerization and

transportation of damaged mitochondria to aggregates leading to mitophagy (Geisler, et al. 2010; Narendra, et al. 2010). It should also be noted that this diverse suite of p62 functions is always described under stress or pathological conditions. What role p62 plays in the physiological mitochondria remains unclear.

Previous work in our laboratory has revealed that p62 protects cells from oxidative damage caused by free radicals thus promoting cell survival while loss of p62 results in increased oxidative damage of nuclear DNA (Du, et al. 2009a; Du, et al. 2009b). mtDNA has been shown to be more prone to oxidative damage than nuclear DNA. Lack of histone protection of the mitochondrial genome, reduced repair mechanisms in the mitochondria as well as the close localization to the major production site of ROS (Electron Transport Chain) are directly responsible for this increase in mtDNA damage. Damage to mtDNA directly results in apoptotic cell death, decreased mitochondrial membrane potential ($\Delta\psi m$) and is also a hallmark of neurodegenerative diseases (Acevedo-Torres, et al. 2009; Hauptmann, et al. 2009). Our lab has extensively characterized a mouse knock-out model for the p62 gene as a mediator of ubiquitin dependent trafficking of interacting proteins (Babu, et al. 2008). We have shown $p62^{-/-}$ mice possess an Alzheimer's disease phenotype, biochemically exhibiting increased levels of polyubiquitinated tau protein, reduced neurotrophic factors and manifesting behaviors associated with sporadic AD (Babu, et al. 2008). Evidence to date, collected in our lab as well as others, places p62 at an intersection of trafficking proteins and organelles for degradation as a scaffolding protein and regulation of cellular dynamics and function as a regulatory protein.

Evidence is also accumulating that mitochondrial function is related to the pathophysiology and treatment of behavioral disorders (Einat, et al. 2005). In general, anxiety disorders are the most prevalent psychiatric disorders diagnosed and have been related back to

altered energy metabolism, mitochondrial transport and oxidative stress. ATP production through the mitochondrial electron transport chain is necessary for the survival of neurons, and protein signaling cascades have been shown to mediate synapse changes, as well as other long term changes in neuronal structure (Calkins, et al. 2011). Altered levels of proteins specifically involved in neurotransmission, energy metabolism and oxidative stress have been found in lab mice diagnosed as "high anxiety" by elevated-plus maze analysis (Filiou, et al. 2011). Deletion of Bcl-2, a major modulator of mitochondria function, has been related to increased anxiolytic activity in a mouse behavior model (Einat, et al. 2005). Furthermore, monoamines, such as serotonin, have for years been included in causative hypotheses related to depression. Substantial decreases in serotonin levels in brain regions of a genetic mouse model of bipolar disorder were correlated with decreased mtDNA levels and multiple mtDNA deletions (Kasahara, et al. 2006). Changes in mitochondrial functionality resulting in inflammation have also been offered as an explanation for major depression disorders (Gardner, et al. 2011). Collectively, such studies have revealed a strong correlation between proteins specific to mitochondrial function and behavior patterns associated with affective spectrum disorders.

I propose to examine the relationship between p62 and mitochondrial dynamics using $p62^{-/-}$ tissue and cells as the model system. I also propose to investigate what effects overexpression of p62 has in a directed murine model on mitochondrial function and gross behaviors affected by neurodegeneration. By combining information obtained from both the absence and overexpression of p62 in the brain of different mouse models, I believe I can begin to elucidate what physiological role p62 could play in normal function of the mitochondria and give insight into unraveling its association with neurodegenerative diseases.

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Chapter 1.

Isolation and Culture of Hippocampal Neurons from Prenatal Mice

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Short Abstract:

We provide a protocol for the culture of highly purified hippocampal neurons from prenatal mouse brains without the use of a feeder glial cell layer.

Long Abstract:

Primary cultures of rat and murine hippocampal neurons are widely used to reveal cellular mechanisms in neurobiology. By isolating and growing individual neurons, researchers are able to analyze properties related to cellular trafficking, cellular structure and individual protein localization using a variety of biochemical techniques. Results from such experiments are critical for testing theories addressing the neural basis of memory and learning. However, unambiguous results from these forms of experiments are predicated on the ability to grow neuronal cultures with minimum contamination by other brain cell types. In this protocol, we use specific media designed for neuron growth and careful dissection of embryonic hippocampal tissue to optimize growth of healthy neurons while minimizing contaminating cell types (i.e. astrocytes). Embryonic mouse hippocampal tissue can be more difficult to isolate than similar rodent tissue due to the size of the sample for dissection. We show detailed dissection techniques of hippocampus from embryonic day 19 (E19) mouse pups. Once hippocampal tissue is isolated, gentle dissociation of neuronal cells is achieved with a dilute concentration of trypsin and mechanical disruption designed to separate cells from connective tissue while providing minimum damage to individual cells. A detailed description of how to prepare pipets to be used in the disruption is included. Optimal plating densities are provided for immuno-fluorescence protocols to maximize successful cell culture. The protocol provides a fast (approximately 2 hour) and efficient technique for the culture of neuronal cells from mouse hippocampal tissue.

Protocol Text:

1) **Setup prior to harvest**

1.1) To generate prenatal pups for neuron harvest, schedule breeding between adult mice 19 days prior to the day of neuron isolation. *(C57BL/6 mice ages 2-8 months were used in matings for the purposes of developing this protocol).* Successful mating can be confirmed by detection of a vaginal plug in the female, palpitation or visual confirmation of pregnancy.

1.2) The day prior to neuron isolation:

1.2A) For immunofluorescence applications, coat glass coverslips in a 24 well plate with a light coating of 3:1 Collagen 1, Rat Tail:poly-D-Lysine solution.

1.2B) For cell culture applications, coat appropriate size tissue culture plastic ware with a light coating of 3:1 Collagen 1, Rat Tail:poly-D-Lysine solution.

1.3) Rest the plates uncovered in a tissue culture hood under a UV light overnight.

1.4) Wash the plates with sterile Hank's Balance Salt Solution (HBSS) prior to use. Coated plates can be filled with HBSS and stored up to one week at 4^{o}C in the dark.

2) **Tissue Harvest**

2.1) Sterility is always a factor when growing primary cell cultures and as such, the greatest caution should be exercised to ensure the most sterile environment possible. With careful attention to sterile technique, initial dissection and harvest of neural tissue for this protocol can

be completed outside of a laminar flow hood with minimal risk of contamination. After initial harvest, all subsequent steps should be conducted under maximum sterile conditions within a hood rated for cell culture.

2.2) Euthanize a pregnant mouse at approximately 19 days post-fertilization by decapitation. Use of anesthesia to euthanize the pregnant female is not recommended as anesthesia is known to cause brain cell death (Stratmann, et al., 2010). Using dissecting scissors and forceps, create an opening in the mid-ventral side of the mouse to completely reveal body cavity.

2.3) Prenatal pups will be located towards the posterior of the mouse's body cavity and should be easily visible in the uterus. With autoclaved sterile forceps, open the uterus and remove pups. Decapitate pups with fresh sterile scissors and place removed head on sterile gauze under a dissecting microscope. Sterile, autoclaved instruments can be flame cleaned using alcohol and an open flame prior to and during use.

2.4) Using sterile scissors, open cranium of pup from back of the neck to the nose. This procedure can normally be completed by inserting one tip of the scissors into the vertebral foramen and then proceeding anteriorly. Carefully remove the entire brain with forceps. Place the brain on sterile gauze. Using a sterile scalpel, remove the cerebellum and incise down the midline of the brain to separate it into two hemispheres. (Figure 1.)

2.5) Grasp a small section of meninges surrounding the hippocampus with sterile forceps and pull it gently away. Although it is not strictly necessary to remove the meninges before isolating the hippocampus, the presence of the meninges can make the dissection of the hippocampus more difficult due to the toughness of the membrane. In either case, the hippocampus will be more clearly visible after the meninges have been removed. The hippocampus is a curved

structure that starts in the distal part of the hemisphere and bends ventrally (Figure 2.). As the inner, concave, side (caudal) is facing a ventricle, it is already free. Therefore, to isolate the hippocampus, one needs to cut along the convex outer side. After the dissection, gently lift each hippocampi with sterile tissue forceps and transfer into a small tissue culture dish with warmed (37^{o}C) HBSS under a cell culture hood. Brain tissue can be combined from multiple pups

3) **Tissue Dissociation**

3.1) Using a sterile scalpel, gently mince brain tissue in 3 ml of sterile HBSS in a 100mm Tissue Culture dish.

3.2) Transfer the minced tissue and HBSS to a 15 ml conical tube. Add 1.5 ml of HBSS and 0.5 ml of 0.25% Trypsin solution to a total volume of 5 ml.

3.3) Cap and gently invert the tube 4-5 times to mix. Try to avoid producing bubbles as DNA released from the digested tissue will adhere to the bubbles and cause the minced tissue to float instead of settling to the bottom of the tube (Figure 3.).

3.4) Incubate hippocampal tissue at 37^{o}C for 15 minutes, inverting tube as above every 5 minutes.

3.5) Allow the tissue to settle to the bottom of the tube.

3.6) Carefully remove excess solution using a sterile pipet, leaving tissue undisturbed at the bottom of the tube.

3.7) Wash tissue pellet with 5 ml of HBSS at 37^{o}C for 5 minutes. Repeat a total of 3 times. Allow tissue to completely settle to the bottom of the tube each time before proceeding to the next wash step.

3.8) Remove the final wash from the tissue pellet and replace with 2 ml of fresh HBSS.

4) **Neuron Trituration**

4.1) Before beginning trituration steps, you will need to prepare fire polished Pasteur pipets. Using a Bunson burner, hold a sterile 9 inch Pasteur pipet tip (Figure 4a.) in the flame until diameter of the pipet opening is approximately 0.5mm in size and edges of the pipet opening have been rounded slightly (Figure 4b.). Allow pipet to completely cool before beginning the trituration process.

4.2) Using a normal sterile 9 inch Pasteur pipet, gently triturate the tissue a total of 7 times. Larger tissue pieces are normal at this point and should be allowed to settle to the bottom of the tube prior to moving to the next step.

4.3) Transfer the supernatant to a fresh sterile 50 ml conical tube.

4.4) To the remaining tissue, add 2 ml of sterile HBSS and triturate a total of 5 times using the fire polished Pasteur pipet.

4.5) Allow all remaining larger tissue pieces to settle to the bottom of the tube and combine supernatant with the previous supernatant for a total of 4 ml dissociated neuronal cells.

5) **Cell Plating**

5.1) Count the dissociated cells using a hemocytometer.

5.2) As a general rule, once cell numbers have been determined, subtract 20% from that final number to account for any cell death that may occur after plating.

5.2) Cells can be plated using the recommendations below:

For coverslips in a 24 well plate – 6 x 10^4 cells/well in 0.5 ml

For 60 mm Tissue Culture plates – 4 x 10^5 cells/plate in 3 ml

For 100 mm Tissue Culture plates – 6 x 10^6 cells/plate in 6 ml

5.3) Mix appropriate cell numbers with indicated volume of Neurobasal Plating Media (Neurobasal Media containing B27 Supplement [1ml/50ml], 0.5 mM Glutamine Solution, 25 μM Glutamate (Mr 147.13 g/mol), Penicillin (10,000units/ml)/Streptomycin (10,000μg/ml) [250μl/50ml], 1mM HEPES (Mr 238.3 g/mol), 10% Heat Inactivated Donor Horse Serum) and add cells to plates. Swirl plates gently to distribute cells evenly. HI-Donor Horse Serum is added to the Plating Media to enrich the cells during the first 24 hours of growth. The cells are subsequently weaned from the serum and returned to a Serum-Free environment by serial reduction of the serum at each media replacement. It should also be noted that while glutamate at higher concentrations is toxic to neuronal cell cultures, at the lower concentrations added here, it will inhibit the attachment of non-neuronal cells (Price and Brewer, Protocols for Neural Cell Culture, 2001). However, it should only be added to the plating media for the first 24 hours in culture and subsequently left out of any Feeding Media to prevent neurotoxicity of the cells.

5.4) Place neurons in a 37^{o}C, 5% CO2 incubator overnight.

5.5) Remove half the volume of media from the cells and replace with same volume of Neurobasal Feeding Media (Neurobasal Media containing B27 Supplement [1ml/50ml], 0.5 mM Glutamine Solution, Penicillin (10,000units/ml)/Streptomycin (10,000µg/ml) [250µl/50ml], 1mM HEPES (Mr 238.3 g/mol) .

5.6) Neurons should be fed every 4 days by removing half of the old media and replacing it with the same volume of fresh Neurobasal Feeding media. Neuronal processes should begin to be visible on Day 1 (Figure 5a.) and become prevalent by Day 10 (Figure 5b.).

Representative Results:

The ability to grow and culture primary neuronal cells has become an indispensible part of neuroscience. Primary cultures allow the researcher to analyze specific cellular pathways, chemical modification and treatment, target localization and growth patterns in a controlled environment. Many of these procedures utilize sophisticated methodology to visualize specific changes in cell responses. In this case, hippocampal neurons are used to study specific neuronal pathways that would prove difficult, if not impossible to analyze in the intact brain. Preparation of near homogeneous populations of neurons from specific areas of the brain is critical for studying brain function. Molecular effects in individual neurons can be instrumental in delineating higher order pathways such as memory or learning. As this protocol yields relatively pure cultures of hippocampal neurons, without the need of a feeder layer of glial cells, these neurons are easily utilized for immunofluorescence studies. However, as with all primary culture from organs containing multiple cell types, some contamination by less desired cells can occur.

In isolation of neuronal cells, contamination by glial cells can be a common problem. Glial cells can be easily detected upon microscopic visualization of the culture as their morphology differs significantly from the target neurons (Figure 6). The impact of glial cell contamination will depend on the planned use of the cultures. If cells are being used for immuno-fluorescence examination, glial contamination can be nothing more than an inconvenience when trying to photograph individual neurons. However, if the neuronal cultures are to be used for biochemical analysis, any significant contamination by glial cells could cause major changes in the results. Ways to address glial cell contamination are outlined further in the Discussion.

Once neurons have been successfully isolated and grown in culture, one typical application is to examine cellular processes immuno-fluorescence techniques. As illustrated in Figure 7, organelles, such as the mitochondria, can be stained using vital dyes added to the culture media prior to fixation. Endogenous cellular proteins can be visualized from fixed cells using standard immuno-fluorescence techniques (Figure 8). Once neuronal cells are fixed, specific antibodies for proteins of interest can be introduced to the cell and these proteins can be visualized using a fluorescence microscope. Cultured neurons also provide the researcher with the means to examine individual protein effects on neuronal functions. Using a variety of techniques including DNA transfections, electroporation or viral transduction, proteins can be overexpressed in neuronal cells (Figure 9). How neural cells respond to the effects of over-expressed proteins can have direct inferences on how the brain may respond and offers the possibility of identifying cellular targets for drug treatments. The details of these types of experiments go beyond the scope of this paper but they do illustrate that cultures prepared by this technique are suitable for a wide array of down-stream applications. However, the overall simplicity of this protocol, as well

as, the short time period required to prepare these neuronal cultures make this an ideal method for use in today's neuroscience laboratory.

Discussion:

Hippocampal cultures have been used in molecular biology for more than 20 years. While in principle, neuronal cultures can be made from any part of the brain, hippocampal cultures have proven to be the most popular due to the relatively simple architecture of the nerve cell population in the hippocampus (Kaech and Banker, Nature Protocols, 2006). Hippocampal cultures are typically made from late-stage embryonic tissue. This tissue is easier to dissociate and contains fewer glial cells than does mature brain tissue (Banker and Cowen, Brain Res, 1977). Isolation of hippocampal neurons from embryonic tissue also decreases shearing damage to axons and dendrites due to fewer adhesion contacts (Brewer, J of Neurosci Methods, 1997). While hippocampal cultures are most often generated from rats due to the relatively easier isolation of the hippocampus, mice can also be used with the same protocols if appropriate care is taken during tissue isolation. Once neurons are cultured, the ability to use advanced molecular techniques to analyze subcellular localization and trafficking can be employed. This can be especially advantageous when analyzing embryonic lethal transgenic mice as it provides the ability to study protein interactions that would result in the death of the embryo.

The hippocampus has been implicated in both spatial and contextual learning (Burwell, et al, J of Neurosci, 2004) and memory (Gluck, et al, Neural Networks, 2005). Growth of primary cultures from the hippocampus can allow a correlation between subcellular biological events and their effects on the brain's ability to learn and remember.

As with all neural cells, neurons grown from hippocampal cultures require critical growth factors, hormones and amino acids. In the brain, these factors are provided by glial cells. This symbiotic relationship can also be carried into a culture environment by growing a "feeder" layer of glial cells along with the cultured neurons. However, glial cells will also produce cytotoxic factors during their lifespan (Wallace and Johnson, J of Neurosci, 1989) which can be toxic to cultured neurons. To circumvent this, neurons have been grown in serum-free media such as Neurobasal medium supplemented with B27. The B27 supplement is optimized for survival of hippocampal neurons but will support growth of other neuronal cultures as well (Brewer, et al, J Neurosci Res, 1993). L-glutamine is an essential amino acid for energy production and protein synthesis in cell culture. However, glutamine can be labile over time, degrading into ammonia and carboxylic acid byproducts once added to culture media. Glutamax, a cell culture supplement from Invitrogen, can be used as a direct substitute for L-glutamine if desired. Glutamax is more stable in media but slightly more expensive. Growth of neurons in serum-free media allows the study of effects of growth factors and hormones on neuronal growth and differentiation.

We submit a protocol for the rapid isolation of hippocampal neurons from mouse prenatal embryos using Neurobasal media and the B27 supplement for growth of neurons in a serum free environment without the use of feeder cells. As with all cultured primary cell protocols, it is advantageous to minimize the growth of non-desired cell types (i.e. glial cells). This is most readily accomplished by careful dissection of the hippocampus from surrounding regions of the brain. Prenatal cells also contain a comparatively small number of glial cells aiding in this goal. However, glial cell contamination can still occur. It is possible to reduce glial cell contamination by treatment with cytosine arabinoside (AraC) at early time points in the culture (Mao and

Wang, Brain Res Dev Brain Res, 2001, Mao and Wang, Brain Res Mol Brain Res, 2001). AraC can be used as an anti-mitotic agent to reduce the population of non-neuronal cells capable of DNA synthesis. However, to avoid possible toxic effects of treatment on neurons, it should be used at its lowest effective does (5μM) and not added after 3-4 days of culture (Wallace and Johnson, J Neurosci, 1989). Another option would be the use of 5-fluoro-2'-deoxyuridine (FUdR) treatment to decrease the proportion of fibroblastic-reactive microglial cells (Oorschot, Exp Brain Res, 1989).

Once harvested, hippocampal tissue can be treated with a dilute trypsin solution to dissociate/disaggregate adherent cells. However, prolonged exposure to higher concentrations of trypsin can be detrimental to cell subculture so time in trypsin solution is most often limited to between 3-5 minutes. The diluted concentration of trypsin used in this protocol does allow longer times of enzyme incubation to increase individual cellular disassociation but care should be taken to strictly adhere to the time points provided. Papain can be used as an alternative enzyme and has been proven to be more effective and less destructive with certain tissues such as retinal neurons (Shen J, et al, Jap J of Physiology, 1995).

Cell dissociation is followed by trituration of the tissue. This has proven to be the most important step in consistent neuronal culture and is highlighted by two important points. First, two sterile Pasteur pipets are used during this process. The first is used to grossly disrupt tissue/cellular association. The opening size of this first pipet should be between 1.0-1.5 mm. Careful selection of pipets directly from the vendor package can usually result in appropriate pipets for this first trituration. The second Pasteur pipet used is "fire-polished" over a Bunsen burner to reduce the size of the opening to approximately 0.5 mm in diameter. This also results in "polishing" the glass ring of the opening to a smoother surface reducing mechanical damage to cells as they pass

through. Secondly, the speed/force of trituration through the pipet is of major importance. As the cells dissociate from the whole tissue, they are of course more fragile. Thus, "rough" treatment as this point would be detrimental to survival of the neuronal cells. Trypsinized tissue should be passaged through the pipet at a consistent but firm flow rate. A common mistake by the researcher is the idea of having to completely disassociate the tissue until there is no discernible structure remaining. In most cases, this will result in massive neuronal death as the cells will be too damaged by the repeated mechanical manipulation. The indicated number of times to pass the tissue through the pipet will result in sufficient numbers of neurons without resulting in gross damage to the population.

Once the cells have been dissociated and pooled, a Trypan Blue stain of an aliquot of cells will provide a ratio of live to dead cells. Typically, 75-80% of the cells should survive the harvest process and can be used for plating. Trypan Blue staining can also be used to obtain an accurate cell count when done on a hemocytometer. In practice, once a total cell count is achieved, add 20% to the total and use that cell number for plating to account for the approximate amount of cell death. Numbers provided in the above protocol are a good starting point to achieve good densities of neurons. If neuron density is too low at plating, cell growth will likely not be sufficient as plating density is an important variable in propagation of the cell population. Cells should be fed every four days with B27/Neurobasal Feed media. Neuronal growth under these conditions can easily be carried out to 10-14 days in culture and has been used to propagate neurons out to 30 days in culture when seeded at 80 cells/mm^2 (Brewer, J Neurosci Research, 1995).

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Disclosure:

We have no conflict of interest and we have nothing to disclose.

Table 1. Specific reagents and equipment:

Name of Reagent	Vendor	Catalog Number
Rat Tail Collagen 1	BD Biosciences	354236
Poly-D-lysine Solution	Chemicon	A-003-E
Hanks Balanced Salt Solution	Invitrogen	14175-095
Trypsin Solution (1X) 0.25%, liquid	Invitrogen	15050-065
NeuroBasal Medium (1X) liquid	Invitrogen	21103-049
B27 Supplement (50X) liquid	Invitrogen	17504-044
L-Glutamine 200mM (100X) liquid	Invitrogen	25030-149
Penicillin(10,000 units/ml / Streptomycin(10,000μg/ml	Invitrogen	15140-148
HI-Donor Horse Serum	Atlanta Biologicals	S12150H

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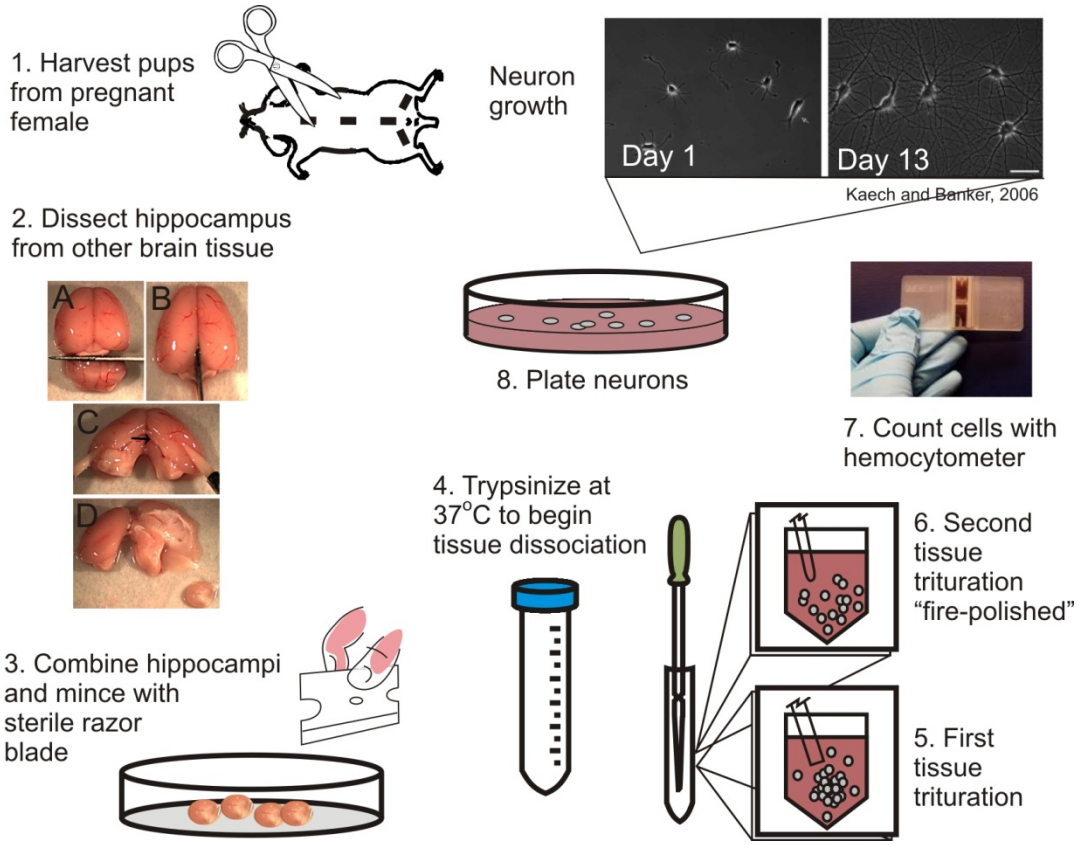

Figure A. Graphic overview of entire harvest and dissociation procedure.

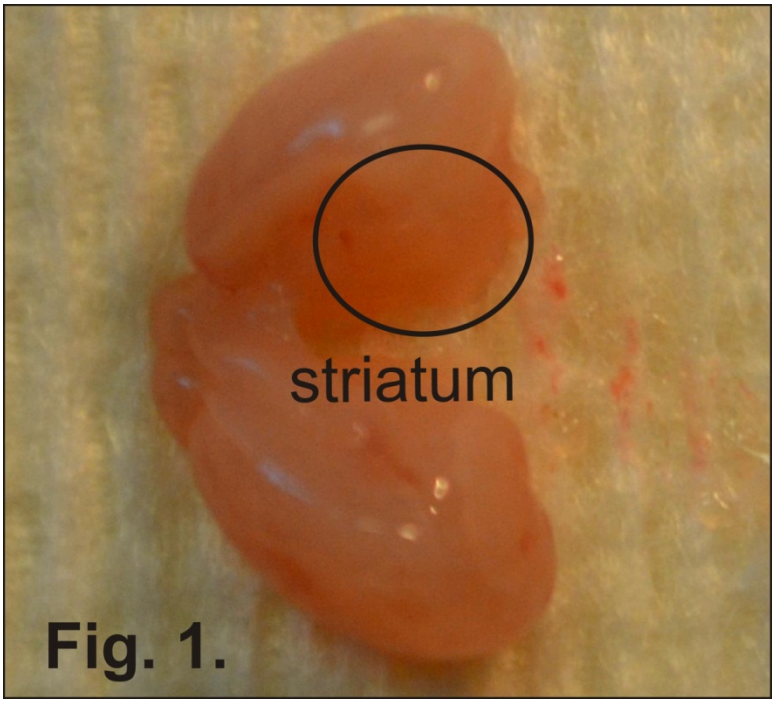

Figure 1. Dissection of the prenatal mouse brain. The first incision is down the midline of the brain separating it into two hemispheres**.**

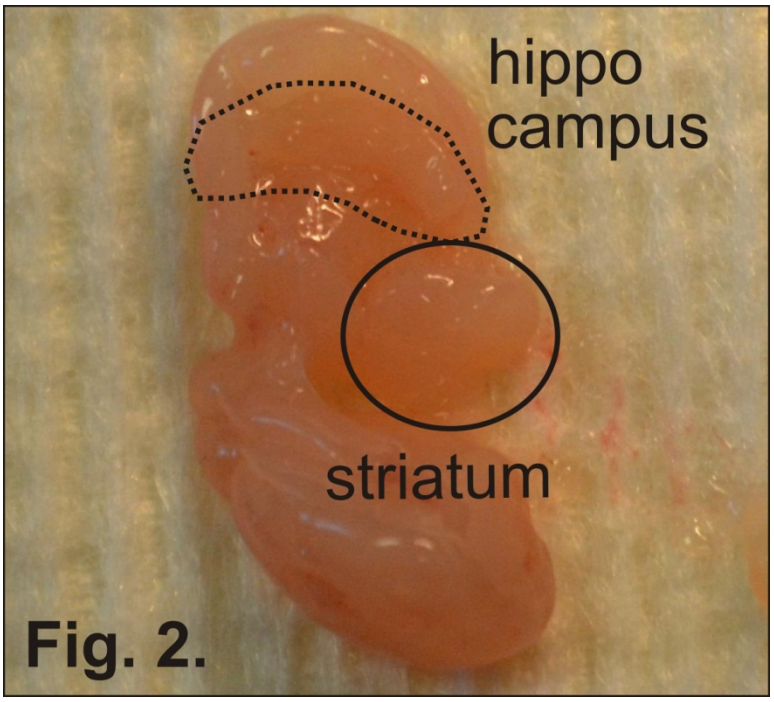

Figure 2. Location of the hippocampus in the prenatal mouse brain. The striatum is moved aside to visualize the hippocampus and is noted by the curved “kidney bean” type structure in the distal region of each hemisphere.

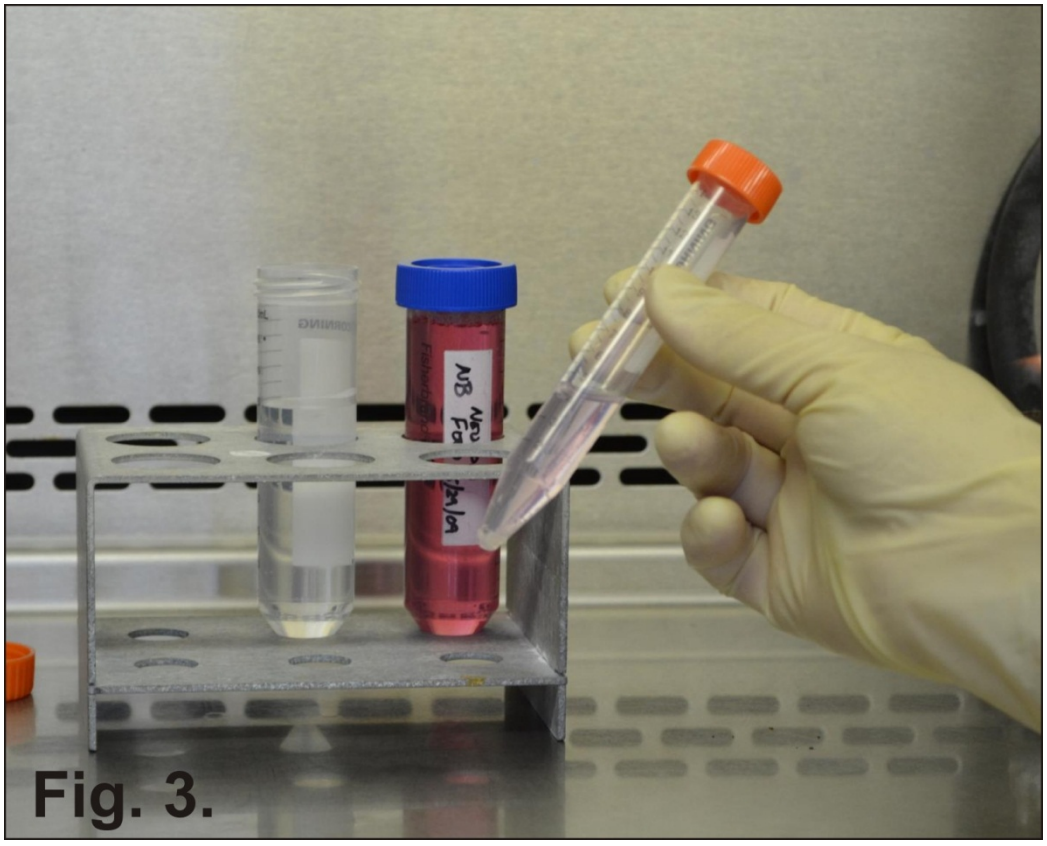

Figure 3. Dissociation of hippocampal tissue in trypsin solution.

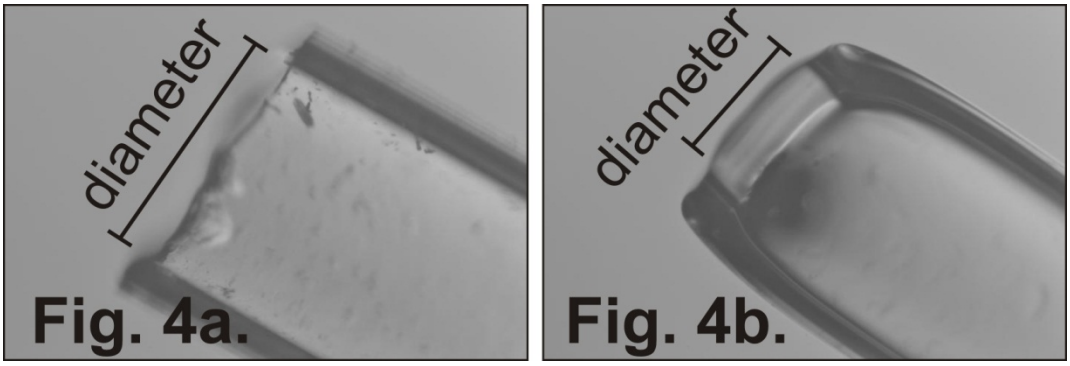

Figure 4. Pasteur pipet tips used in trituration of hippocampal tissue. (a) Normal Pasteur pipet tip. (b) Fire-polished Pasteur pipet tip. Take note of the rounded edges and the approximate 50% decrease in pipet opening size.

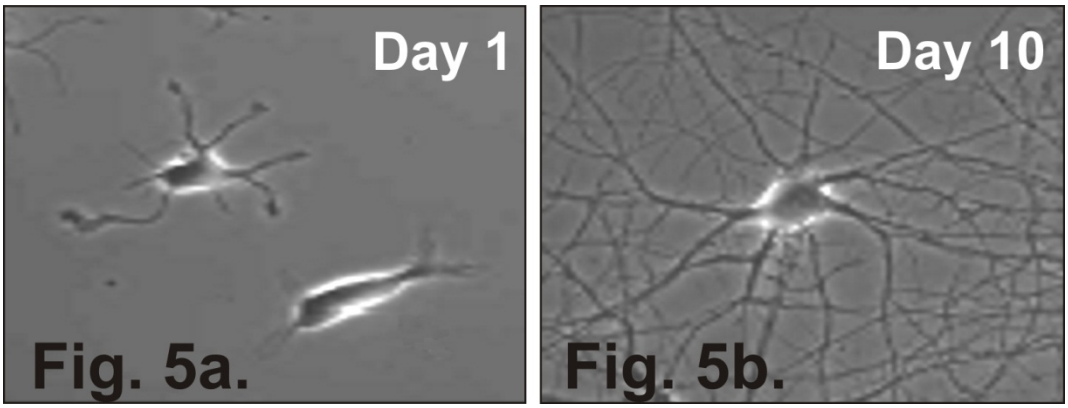

Figure 5. Hippocampal neurons isolated using this procedure and plated in NB Media. (a) Cell growth 1 day post-plating. Neuronal processes begin to be visible during Day 1. (b) Cell growth 10 day post-plating, neurites are branched and overlapping.

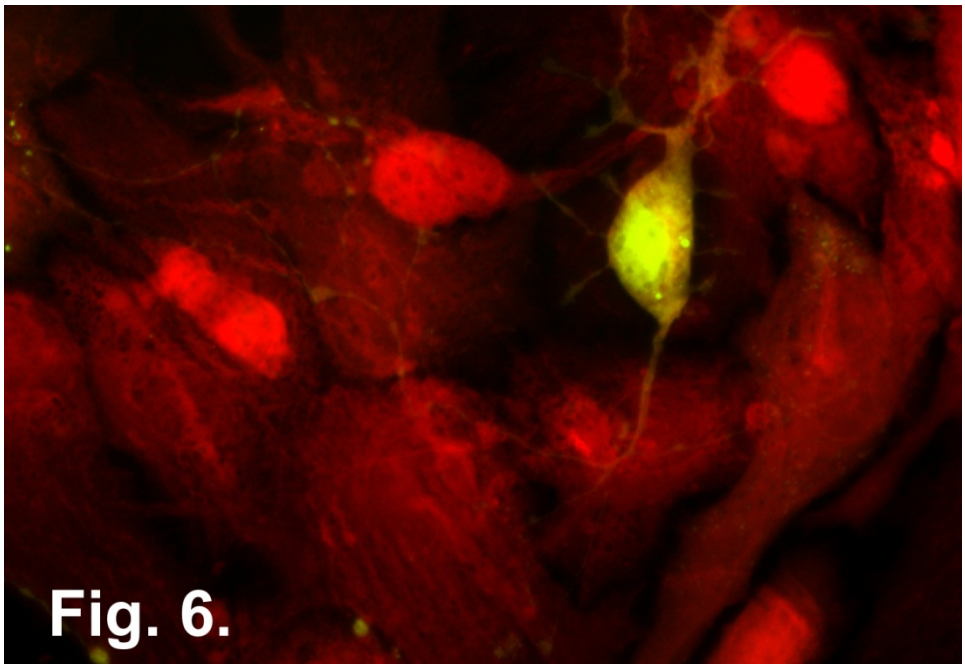

Figure 6. Hippocampal neurons contaminated with glial cells grown for 7 days and stained with the organelle marker MitoTracker Red CM-H2XRos (Invitrogen #M7515) and transfected with GFP-LC3 using Lipofectamine 2000 (Invitrogen #11668019). Mitochondria are visible in all cells however only a single neuron was successfully transfected with the fluorescent construct. Contamination with glial cells makes analysis of GFP-LC3 expression in the neuronal processes difficult to visualize.

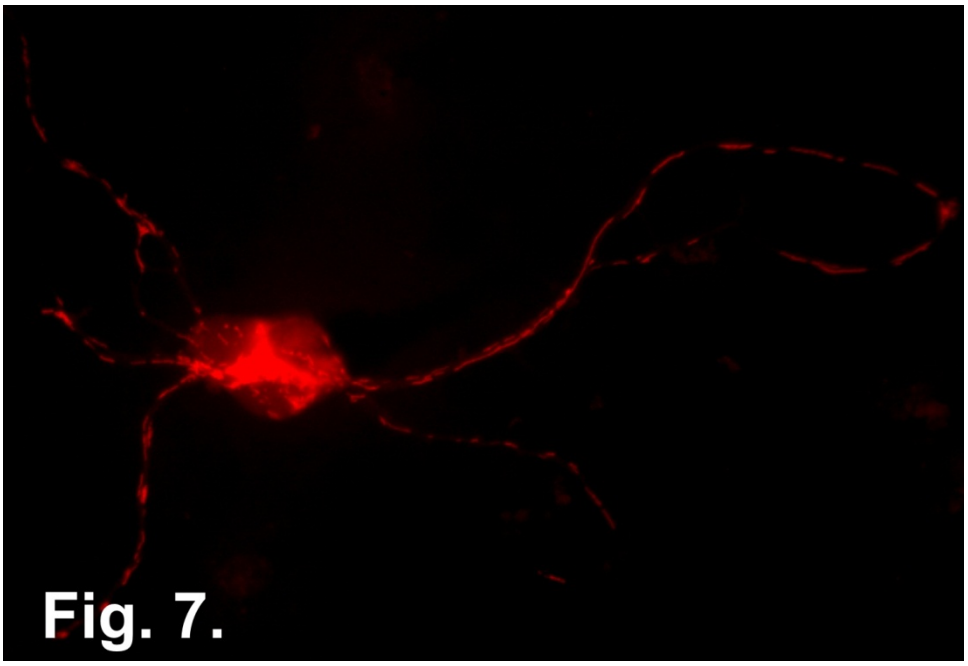

Figure 7. Hippocampal neurons grown for 7 days and stained with the organelle marker MitoTracker Red CM-H_2 XRos (Invitrogen #M7513). This vital dye is used to stain active mitochondria in tissue culture cells. The cells were fixed in 4% paraformaldehyde/PBS and visualized by fluorescent microscopy. The dye itself is non-fluorescent until oxidized in the mitochondria. Active mitochondria can be seen throughout the neuronal processes.

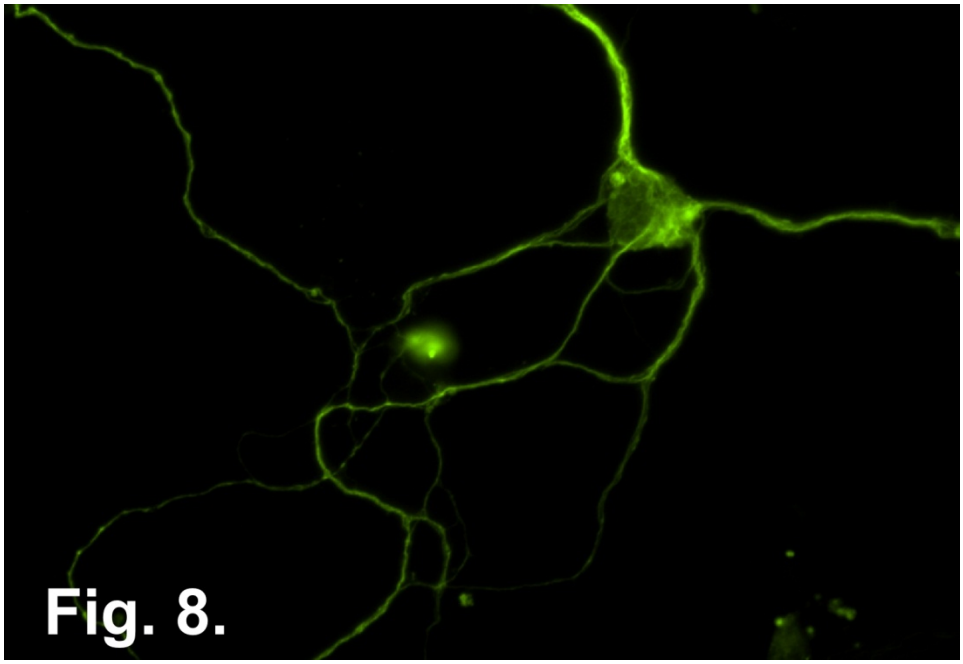

Figure 8. Hippocampal neurons grown for 7 days, fixed with 4% paraformaldehyde/PBS and immuno-stained with monoclonal anti-tubulin β antibody (Sigma #T0198). Following primary antibody, Oregon Green labeled goat-anti-mouse secondary antibody (Invitrogen #O11033) was added and fluorescence visualized by microscopy.

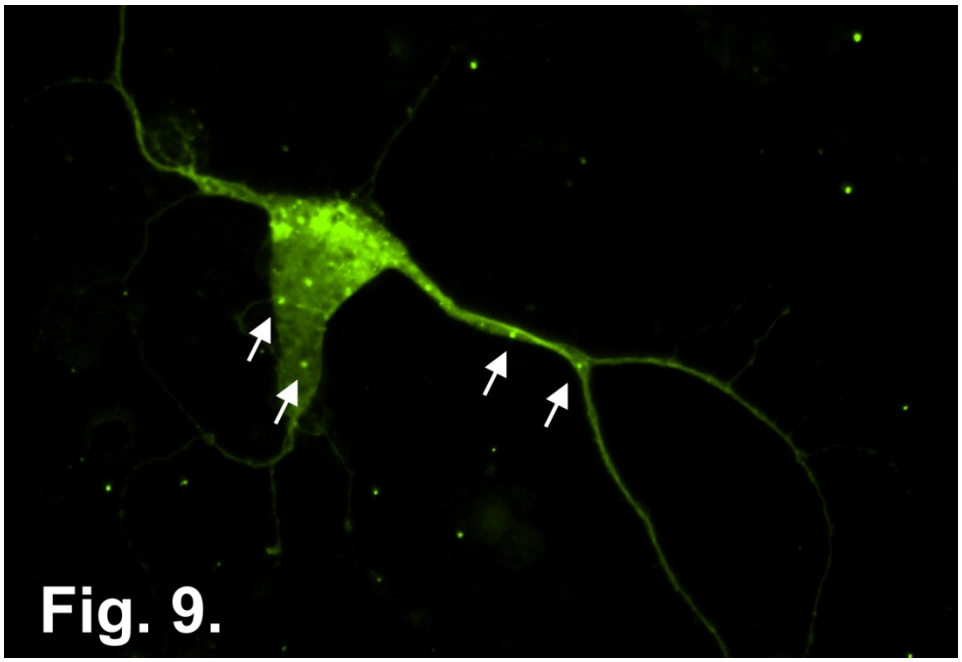

Figure 9. Hippocampal neuronal cultures were grown for 5 days and transfected with GFP-LC3β DNA construct using Lipofectamine 2000 (Invitrogen #11668019). At Day 7, cells were fixed using 4% paraformaldehyde/PBS and aggresomes with GFP tagged LC3β incorporated into their outer membrane were visualized using fluorescent microscopy. Aggresomes are located throughout the cell body and neurites and are denoted with arrows.

Chapter 2.

A Role for Sequestosome1/p62 in Mitochondrial Dynamics, Import and Genome Integrity

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Keywords:

mitochondria integrity; oxidative stress; p62; TFAM; mitochondrial import

Highlights

p62 is localized to the mitochondria where it plays a physiological role.

p62 affects both mitochondrial morphology and function.

p62 is required to maintain mitochondrial genome stability.

p62 regulates the import of TFAM to mitochondria.

mtDNA biogenesis is affected by p62 beyond regulation provided by $\Delta\psi m$.

Abstract

As a signaling scaffold, p62/SQSTM1 plays important roles in cell signaling and degradation of misfolded proteins. While localization of p62 to mitochondria has been reported, a description of its function once there, remains unclear. Here, we report p62 is localized to mitochondria in non-stressed situations and demonstrate that deficiency in p62 exacerbates defects in mitochondrial membrane potential and energetics leading to mitochondrial dysfunction. We report on the relationship between mitochondrial protein import and p62. In a p62 null background, mitochondrial import of the mitochondrial transcription factor TFAM is disrupted. When p62 is returned, mitochondrial function is restored to more normal levels. We identify for the first time that p62 localization plays a role in regulating mitochondrial morphology, genome integrity and mitochondrial import of a key transcription factor. We present evidence that these responses to the presence of p62 extend beyond the protein's immediate influence on membrane potential.

1. Introduction

Sequestosome 1/p62 was identified as a ubiquitin binding protein [1], and has been localized to aggresomes of various neurodegenerative diseases [2]. p62 plays critical roles in neurodegeneration, cancer, and obesity through regulation of cell signaling, protein degradation and NF-κB activation [3-5]. p62 contains several interaction motifs that endow the protein with scaffolding abilities [3] and is important in both the UPS and autophagy degradation pathways. In the UPS, p62 interacts with polyubiquitinated proteins through its C-terminal UBA domain allowing shuttling to the proteasome for degradation [6]. Mediation of autophagy occurs by p62 polymerizing through an N-terminal PB1 domain and interaction with the autophagic marker protein LC3 [7,8]. p62 itself is degraded by autophagy with autophagic defects causing accumulation of p62 in response to stress stimuli [9]. Inability to clear p62-containing aggregates increases ROS levels, DNA damage, tumorigenesis and cell death in the absence of autophagy [10,11].

Growing lines of evidence correlate p62 with clustering of damaged mitochondria. p62 is recruited to depolarized mitochondria in PINK1/parkin-expressing cells, two proteins linked to the pathology of Parkinson's disease (PD) [12]. Efficient mitochondrial function and turnover depends on continuous structural remodeling through fusion and fission [13]. Daughter mitochondria produced by fission can either maintain intact membrane potential or become depolarized. Depolarized mitochondria may restore their membrane potential and return to normal fusion/fission equilibrium or remain as non-fusing mitochondria to be eliminated by autophagy. It has been proposed that fission acts as an autophagic checkpoint [14]. Depolarized mitochondria change their morphology to a more fragmented form and show perinuclear clustering ("mito-aggresomes"). Completion of the mitophagy pathway appears to be dependent

on the recruitment of both p62 and HDAC6 [12,15]. Although there are conflicting reports as to p62's role in mitophagy, it is known to be indispensable in the polymerization and transportation of mitochondria to aggregates [16,17]. This diverse suite of p62 functions is known to take place under stress or pathological conditions. However, whether p62 is localized to mitochondria and what its function might be under physiological conditions remains unclear.

The structural state of mitochondria is directly related to the organelle's functional status [18]. Mitochondrial fragmentation correlates with bioenergetic defects and elevated oxidative stress leading to increased mtDNA mutations [19-21]. Meanwhile, mitochondrial fusion is required for mtDNA stability in skeletal muscle and protects against neurodegeneration in the cerebellum [22]. Disruption of fusion leads to loss of membrane potential and decreased cellular respiration [19]. Defects in mitochondrial dynamics causing dysfunction have been linked to multiple neurodegenerative diseases [23].

The mitochondrial genome encodes rRNAs, tRNAs and proteins important for cell respiration and ATP generation [24,25]. mtDNA is more prone to oxidative damage than nuclear DNA due to mitochondria being the major source of ROS, a lack of histone protection in mtDNA and reduced mitochondrial DNA repair ability. mtDNA damage results in decreased membrane potential ($\Delta\psi m$), increased apoptotic cell death [26], and is a hallmark of neurodegenerative diseases [27,28]. Depletion of mtDNA can result in mitochondrial change, such as fragmentation and reduction in number of cristae [29]. Thus, maintenance of the mitochondrial genome is crucial for cell survival.

Our previous studies revealed that p62 protected cells from oxidative damage and promoted cell survival while defects in p62 resulted in oxidative damage to nuclear DNA in

association with various neurodegenerative diseases [30,31]. In the current study, the relationship between p62 and mitochondrial dynamics was investigated using $p62^{-/-}$ tissues and cells. $p62^{-/-}$ mice possess an AD-like phenotype [32] and exhibit mitochondrial morphology and mtDNA damage associated with neurodegenerative diseases. Our goal was to elucidate the relationships between p62, mtDNA stability and biosynthesis. We also wished to examine how p62 might affect mitochondria morphology and function. We show for the first time p62 plays a role in maintaining functional mitochondria energetics and is integral for increased mtDNA stability.

2. Materials and Methods

2.1 Reagents and antibodies.

All chemicals in this project were obtained from Sigma (St. Louis, MO). Lipofectamine 2000 transfection reagent and ATP synthase antibody were from Life Technologies (Carlsbad, CA). p62 antibody was from ABCAM (Cambridge, MA). All other antibodies were from Santa Cruz (Santa Cruz, CA).

2.2 Cell culture and transfection.

WT and $p62^{-/-}$ Mouse Embryonic Fibroblast (MEF) cells were cultured in DMEM media with 10% fetal bovine serum and pen/strep in a 37°C incubator in 5% CO2. Cells were transfected using Lipofectamine 2000 transfection reagent in OPTIMEM media as directed in reagent insert for a total of 48 hours prior to harvest.

2.3 Western blot and analysis.

The cell lysate or isolated mitochondria was subject to SDS-PAGE in polyacrylamide gels. Samples were Western blotted with primary antibody from sources described above and HRP-tagged secondary antibody from GE Healthcare Life Sciences (Pittsburgh, PA) and processed with ECL detection reagent. Following exposure of the labeled membrane to Hyperfilm-ECL detection film, the Un-Scan-it Gel and Graph Digitizing software (Silk Scientific, Orem, UT) was used to scan and quantify the signal from the Western blot, and data were analyzed statistically (Win-SAS, Microsoft, Seattle, WA).

2.4 Mitochondria isolation.

Following trypsinization, MEF cells were collected by centrifugation and mitochondria isolated essentially as described by Wieckowski, et al. 2009 [33]. Briefly, washed cells were homogenized on ice with a Teflon pestle followed by centrifugation twice at 600xg for 5 min. The Post Nuclear Pellet (PNP) was collected from the pellets and the supernatant was centrifuged at 7000xg for 10 min. The cytosolic fraction (Cyto) was obtained from the supernatant while the crude mitochondria pellet (C. Mito) was collected in the pellet. The pellet was washed with MRB Buffer (250mM Mannitol, 5 mM HEPES, pH 7.4 and 0.5 mM EDTA) before being layered over a Percol gradient. The gradient was centrifuged at 95,000xg for 30 min with a dense band containing purified mitochondria localized at the bottom of the tube. This band was collected and washed with MRB buffer before being suspended in a small volume of MRB Buffer containing protease inhibitors. Protein concentration was determined by Bradford

assay (Bio-Rad Laboratories, Hercules, CA) and subjected to SDS-PAGE and Western blot or used in further experiments as mitochondrial lysates.

2.5 Immuno-Electron Microscopy

Immuno-TEM was performed essentially as described by Liu, et al. 2004 [34]. Briefly, mitochondria were isolated as by Percol gradient centrifugation, collected in homogenization buffer and fixed by addition of 4% formaldehyde, 0.4% glutaraldehyde, 4mM $CaCl_2$ in 0.1M cacodylate buffer, pH 7.3. Mitochondrial pellets were collected and washed with 0.1M cacodylate/2mM $CaCl_2$ prior to embedment in LR White (EMS). Sections (~80nm) were collected to 300 mesh nickel grids and etched with saturated sodium periodate (Sigma) prior to blocking with 4% Acetylated BSA in Tris buffered saline. Grids were incubated with SQSTM1/p62 antibody (1:100) in 1% Ac-BSA/TBS overnight at 4^oC followed by goat-anti-rabbit conjugated with 20nm colloidal gold (EMS) for 1 hour at room temperature. Sections were postfixed in 2% glutaraldehyde, rinsed in distilled water and contrasted with 2% uranyl acetate and lead citrate.

2.6 Immunocytochemistry.

For immunocytochemical analysis, MEF cells or other cells as indicated were grown on coverslips in 24-well plates in DMEM, and where indicated, transfected with myc- p62 plasmid. Cells were stained with MitoTracker Red (Life Technologies, Grand Island, NY) prior to fixation with warm 4% PFA in PBS and permeabilized with 0.1% TX-100 for 10 min. The cells were subsequently blocked in 3% nonfat dry milk in PBS and incubated with primary antibody overnight at 4°C, washed and incubated with secondary antibody coupled to FITC (Invitrogen, Carlsbad, CA) in blocking buffer for 2 h. After washing in PBS, the coverslips were mounted on

slides using Vectashield Hardset Mounting media (Vector Laboratories, Burlingame, CA), and analyzed using a Zeiss Axiovert fluorescent microscope with Zeiss PLAN-Apochromatic 63X 1.4 Oil DIC lens. Images were collected and magnified with NIS-Elements AR software (Nikon, Melville, NY). Representative images are shown for all fluorescent applications from an approximate pool of 100 cells analyzed. Mitochondrial morphologies were quantitated in a blinded study as described in figure legends

2.7 mtDNA copy number and oxidative damage of mtDNA.

Genomic DNA was isolated from brain tissue using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Following elution with ddH_2O, purified DNA was stored at -80°C. Relative Quantitative real time PCR was employed to determine mtDNA copy number. mtDNA was amplified with mtDNA primer: MDF: 5'-CCTATCACCCTTGCCATCAT-3'and MDR: 5'-GAGGCTGTTGCTTGTGTGAC-3'. Nuclear DNA was amplified by nuclear DNA primer: NDF: 5'- ACATCTGTTGCTCCGGCTCTCATT-3' and NDR: 5'-GCAAGCTCAAAGGGCAAGGCTAAA-3'. RT-QPCR was conducted using an ABI 7500 Real Time PCR system (Applied Biosystems, Carlsbad, CA) using Power SYBR Green PCR Master Mix. Relative mtDNA levels were calculated by the ΔΔCt method as described (Acevedo-Torres et al, DNA Repair 8:126). Larger 10 Kb fragments were amplified by GeneAmp XL PCR kit (Applied Biosystems, Carlsbad, CA) using the primer set: mt5733F: 5'-CCAGTCCATGCAGGAGCATC-3' and mt15733R: 5'- CGAGAAGAGGGGCATTGGTG-3'. A small 91bp fragment was amplified by primer set: Mt13597F: 5'-CCCAGCTACTACCATCATTCAAGT-3' and Mt13688R: 5'-GATGGTTTGGGAGATTGGTTGATGT-3'. Aliquots of the 10 Kb PCR products were resolved on 0.8% agarose gels, while 91 bp PCR products were resolved on a 2% agarose gel. Density of

bands were scanned and quantified. Relative amplifications of 10 Kb fragments were normalized to the 91 bp small fragments. The average lesion frequency per 10kb (λ) was calculated as λ=ln AD/AO.

2.8 ATP Assay.

ATP production was measured using the ATP Determination Kit (Life Technologies, Grand Island, NY). Briefly, MEF cells were detached from tissue culture plates by trypsin treatment and collected by brief centrifugation followed by PBS wash. Cell pellets were resuspended in isolation buffer (5mM HEPES, pH 7.2; 225mM Mannitol, 75mM Sucrose, 1mM EGTA, and protease inhibitors) followed by 5 times syringe using a 23 gauge needle. Samples were centrifuged at 1,500xg for 5 min prior to supernatants being used for ATP assay following the manufacturer's directions and measured with a luminometer. Each sample was measured in triplicate.

2.9 ROS Assay.

ROS levels were measured using the fluorogenic dye H_2DCFDA (Life Technologies, Grand Island, NY). MEF cells were grown in 24 well tissue culture plates. Growth media was removed from attached cells and replaced with fresh DMEM media prior to treatment directly into the media with 10μM H_2DCFDA. Fluorescence was measured immediately post stain addition (T=0 min) and again at T=30 min. H_2DCFDA interacts with ROS produced in living cells and released into the culture media showing an increase in fluorescence when excited at 485nm and measured emission at 528nm.

2.10 Membrane potential assay.

Briefly, cells were grown in DMEM media and harvested following trypsinization. Cells were washed and resuspended in media containing 5μM JC-1 followed by incubation at 37^{o}C for 30 minutes. Cells were pelleted and washed with PBS, resuspended in PBS and counted with an Accuri C6 Flow Cytometer. JC-1 fluorescence was measure in FL1 (530nm) and FL2 (585nm) channels with 50,000 events captured.

2.11 Statistical analyses.

Data were expressed as the mean ± SEM of different group. Possible differences between group means and statistical significance between WT and p62$^{-/-}$ MEF cells or mice were analyzed using one way ANOVAs (SAS v9.2, SAS Institute Inc.). For significant differences, alpha was set at 0.05.

3. Results and Discussion

3.1 p62 localizes to mitochondria under physiological conditions.

Mitochondrial dysfunction is a major characteristic of neurodegenerative disease, cancer and obesity [35,36]. p62 is known to be mitochondrially associated under stress conditions [12], including oxidative stress [37]. Correlatively, our research has shown that absence of p62 results in higher oxidative damage in mouse cells and tissues [31]. We reasoned that these two

observations were interrelated, and thus sought to examine if p62 plays an undiscovered role in mitochondrial function and oxidative metabolism. Using embryonic fibroblasts, we first investigated whether p62 localized to mitochondria under physiological conditions in our model system. Mitochondrial fractions were isolated from WT and $p62^{-/-}$ MEF cells and subjected to Western blot analysis. Mitochondrial and organelle marker proteins were used to determine the purity of mitochondrial fractions (Fig. 1 A). Mitochondrially associated proteins were detected predominantly in mitochondrial fractions while cellular markers were effectively limited to pre-fractionated lysate or cytoplasmic fractions. One notable exception was the ER protein calnexin found in crude mitochondrial fractions of both WT and $p62^{-/-}$ samples. This is likely due to the intimate association of mitochondria with ER [38,39]. However, calnexin was absent from further purified mitochondrial fractions. Endogenous p62 distribution was also examined and a pool of endogenous protein was found to be localized to purified mitochondria of WT MEF cells under physiological conditions while the absence of p62 from $p62^{-/-}$ cells was also confirmed (Fig. 1 A).

Mitochondrial localization of p62 was visualized by immunofluorescence and immuno-TEM. WT MEF cells were treated with MitoTracker Red prior to fixation and immunostaining for endogenous p62. Using FITC-labeled secondary antibody, unstimulated pools of p62 were found associated with mitochondria (Fig. 1 B). Colocalization was quantitatively estimated using the NIS Elements software (Nikon). A Mander's Overlap Coefficient value (40) of 0.749 was obtained suggesting colocalization of p62 with the mitochondria. Further, mitochondria isolated from WT MEFs followed by immuno-TEM with p62 antibody (Fig. 1 C) showed labeling not only localized to the Outer Mitochondrial Membrane (OMM) but was also observed associated with the Inner Mitochondrial Membrane (IMM). These data indicate that p62 could be found

inside, as well as on the surface of mitochondria. These results are consistent with recent reports that portions of mitochondrially localized p62 are protected from proteinase K digestion [37]. It is possible p62 localization spans all mitochondrial compartments and plays a similar role as the ATPase ATAD3A which specifically functions to regulate mitochondrial dynamics [41].

3.2 Localization of p62 to the mitochondria affects mitochondrial morphology.

Since p62 localized to mitochondria and mitochondrial shape orchestrates mitochondrial function [18], we asked if p62 localization could affect mitochondrial morphology. Using a variety of cell types, plus or minus p62, we examined mitochondrial structure by MitoTracker Red staining. We found the morphology of mitochondria, irrespective of cell type, became fragmented in the absence of p62 (Fig. 2 A). Mitochondrial structure was individually quantitated showing an increase in fragmented morphology in the absence of p62 while normal "tubular-like" mitochondrial structure was seen in WT cells consistently across cell types.

We next reasoned that if removal of p62 results in mitochondrial fragmentation, restoring p62 to $p62^{-/-}$ cells could rescue mitochondrial morphology. $p62^{-/-}$ MEFs were transfected with myc-tagged p62, mitochondria stained with MitoTracker Red and immuno-stained for tagged p62. Only cells showing immunofluorescence for FITC-myc , indicating successful introduction of exogenous p62, were used in the analysis. When myc-p62 was introduced, mitochondrial morphology did revert to a ribbon-like tubulo-reticular network indicative of normal mitochondria. (Fig. 2 B). These results suggest that morphological changes in mitochondria may be directly correlated with mitochondrial localization of p62. Fragmentation is directly associated with mitochondrial depolarization and energetics defects [42]. Since p62 localizes to mitochondria, restores normal mitochondrial morphology and possibly spans the mitochondrial

membranes [37], we reasoned p62 could be in a position to affect mitochondrial depolarization and energetics.

3.3 p62 is required for normal mitochondrial function.

Growing lines of evidence show a positive correlation between mitochondria fragmentation and loss of membrane potential, decreased mitochondrial energetics, and mtDNA damage response [19,24,26,29]. Based on initial observations of atypical mitochondrial morphology in $p62^{-/-}$ cells, we anticipated elevated mitochondrial dysfunction and genomic instability. In order to evaluate if $p62^{-/-}$ mitochondrial morphology correlated with mitochondrial energetics, we examined $\Delta\psi m$ in WT and $p62^{-/-}$ cells by staining with JC-1. JC-1 selectively enters potentiometric mitochondria and reversibly changes color from red to green as $\Delta\psi m$ decreases. $p62^{-/-}$ cells showed an increase in green fluorescence when examined by flow cytometry (Fig. 3 A) indicating a significant portion of the mitochondria displayed decreased membrane potential when compared to WT cells. Defects in $\Delta\psi m$ have been shown to negatively impact mitochondrial dynamics and energy production [43], therefore, we sought to examine if p62 might play a role in maintaining functional mitochondrial energetics.

To address p62 effects on energy production, we measured the capacity of mitochondria to synthesize ATP in MEF cells plus or minus p62. Significant impairment in ATP production was observed in $p62^{-/-}$ cells cultured in DMEM media with glucose as the major energy substrate suggesting p62 is required for normal cellular energetics through glycolysis (Fig. 3 B). When cells were cultured in glucose free media containing galactose as the energy source to force mitochondrial respiration, ATP levels were likewise depressed in the absence of p62. We did not observe an increase in ATP production when ATP synthase was inhibited with oligomycin

seemingly indicating the lack of p62 does not cause reversal of ATP synthase [44, 45]. Moreover, the decrease in ATP production was abrogated when p62 was introduced into a null background showing ATP production can be rescued by p62 restoration. Glucose tolerance tests in aged $p62^{-/-}$ mice have also shown them to be defective in glucose uptake and insulin tolerance [4] indicative of glycolytic defects. Interestingly, overexpression of p62 not only recovered ATP production to WT levels, but also appeared to increase overall ATP levels above control (Fig. 4 B). While decreased $\Delta\psi m$ of $p62^{-/-}$ MEFs no doubt affects ATP levels in mitochondria, restoration of ATP above WT levels seems to be a clear indication that presence of p62 is integral to mitochondrial energetics in a manner that extends beyond simply impacting membrane potential.

Changes in $\Delta\psi m$ are initiating events during activation of NADPH oxidase and ROS production [46]. We reasoned that decreased $\Delta\psi m$ of $p62^{-/-}$ MEFs along with deregulation of OXPHOS would result in an increase in ROS production [47]. Indeed, analysis of ROS from culture media of WT and $p62^{-/-}$ MEFs showed increased levels in $p62^{-/-}$ MEFs which was again abrogated with the addition of p62 (Fig. 3 C). mtDNA damage is a hallmark response to ROS-induced oxidative stress [48]. p62 has been shown to protect nuclear DNA from oxidative damage [31]. We therefore hypothesized that p62's association with mitochondria could also confer protection to mtDNA. To evaluate this possibility, we examined damage to mtDNA in the hippocampus of WT and $p62^{-/-}$ mice. We observed an increase in *in vivo* oxidative damage from mtDNA fragments from $p62^{-/-}$ mice (Fig. 3 D). Similar results have been seen in p62's role in the Keap1-Nrf2-Nqo1 pathway and mitochondrial aging [49]. Consequently, the observed correlation between elevated ROS levels and increased mtDNA damage in the absence of p62 as well as the fact that p62 can abrogate the effects of mtDNA damage in a null background was

consistent with our hypothesis that p62 plays a general role in mediating oxidative stress. Furthermore, ROS acts as a key modulator of mtDNA copy number [50] while oxidative stress promotes mitochondria fission in neurons [13]. Overall, our results indicate a deficiency in p62 could be an underlying cause of increased oxidative stress related damage to mtDNA.

3.4 p62 is integral to maintaining mitochondrial stability and mtDNA biogenesis

Normal mitochondrial dynamics maintain mtDNA copy number while fragmentation results in mtDNA mutations and loss of mtDNA [13,22]. In addition to mtDNA damage, total quantity of mtDNA is reported to regulate energy metabolism [24]. We investigated if loss of p62 affected the physical volume of the mitochondrial genome pool in $p62^{-/-}$ mice. We examined total DNA copy number in mitochondria from brain of age matched WT and $p62^{-/-}$ mice (Fig. 4 A). mtDNA copy number accumulated with age, peaking at 12-months in both genotypes. . Increased levels of mtDNA have been observed in both human and mouse brain tissue previously [51; 52] possibly due to a p62 dependent role in mitophagy [16]. However, $p62^{-/-}$ consistently measured below WT levels after 3 months of age. In order to investigate if mtDNA variation was tissue specific, we also examined mtDNA copy number in mouse liver and adipose tissues at 9 months (Fig. 4 B). Decreases in mtDNA levels were seen in both tissues indicating loss of mtDNA copy number in $p62^{-/-}$ mice could be universal. mtDNA copy number of $p62^{-/-}$ mice decreased compared to age-matched WT mice which correlates with the appearance of early AD phenotypes [32]. This result is consistent with investigations that propose mtDNA decrease occurs as an early event preceding loss of mitochondria function [27,53,54].

Loss of copy number was further seen in $p62^{-/-}$ MEF cells where mtDNA levels decreased compared with WT cells (Fig. 4 C). Of note, when p62 was introduced to $p62^{-/-}$ MEF

cells, mtDNA levels returned to a point consistent with WT whereas overexpression of p62 in WT cells resulted in a 3-fold increase in mtDNA. This is significant in that restoration of p62 not only rescued the damaged mitochondrial phenotype of $p62^{-/-}$ MEFS, but overexpression also increased the levels of mtDNA in normal cells. mtDNA levels are correlated with functional energetics [24] and our data demonstrate that the presence of p62 is required for normal mitochondrial energy production (Fig. 3 B). Collectively, our results lead to the unequivocal conclusion that p62 plays a central role in mitochondrial biogenesis and mitochondrial energetics. The full magnitude of this observed p62/mitochondria association remains largely unexplored and presents a fertile area for subsequent research.

One aspect of the p62/mitochondria relationship we investigated was related to the observation of a physical association between p62 and mitochondrial membranes. p62 has been shown to be a component of the nuclear pore complex, directly implicating it in protein import [55]. Recently, parkin was reported to protect the mitochondrial genome by association with mitochondrial transcription factor A (TFAM) prior to its import into mitochondria [56,57]. TFAM promotes mtDNA replication/transcription [58], protects mtDNA from damage [59,60] and affects repair of oxidatively damaged mtDNA [59,61]. Moreover, replication of mtDNA is dependent on TFAM import and activated transcription [62]. As levels of mtDNA were restored in $p62^{-/-}$ cells and increased in WT cells overexpressing p62, we reasoned that p62 might play a role in TFAM/mitochondria association and import. Such a relationship might represent one avenue by which p62 contributes to stabilized energetics and protection of the mitochondrial genome. We isolated mitochondria from WT and $p62^{-/-}$ MEF cells and analyzed levels of TFAM protein (Fig. 4 D). WT cells showed significantly more mature TFAM localized into the mitochondria than did $p62^{-/-}$ MEFs while mitochondrial protein TOM40 remained relatively

unchanged. . Interestingly, this correlates well with the levels of mtDNA observed in Fig. 4C indicating TFAM transcriptional activity is not affected. When myc-p62 constructs were expressed to increase p62 levels, a noticeable increase in TFAM protein was detected in WT mitochondria while restoration of p62 in $p62^{-/-}$ MEFs increased TFAM in the mitochondria to levels approaching WT basal expression. Post-translational modification (maturing) of TFAM occurs following import into mitochondria [63], thus increases in mature TFAM protein in transfected $p62^{-/-}$ MEFs provide clear evidence that mitochondrial import was restored.

A direct correlation between TFAM and mtDNA copy number has been seen in multiple systems [58,59,64]. Moreover, decreased mtDNA levels and respiratory chain function were found in $TFAM^{-/-}$ adipose cells [65]. We demonstrated a decrease in mitochondrial TFAM protein levels along with associated energetic defects and decreased mtDNA copy number in $p62^{-/-}$ MEFs. Expression of p62 in a null background system effectively restores mitochondrial energetics, in many instances approaching WT levels. Our data also indicated that while restoring p62 expression resulted in normal levels of mitochondrial import, overexpression of p62 increased TFAM import into mitochondria resulting in higher levels of total mtDNA. This indicates p62 is integral to maintaining normal mitochondrial function and biogenesis. The exact *in vivo* import mechanism is yet unknown but the evidence indicates it is multifactorial. Lack of p62 obviously results in decreased $\Delta\psi m$ (Fig 3 A). Variation in membrane potential is well known to directly affect import dynamics [66] and therefore no doubt accounts for some proportion of the observed p62/TFAM import relationship. But importantly, based on increased levels of TFAM in mitochondria as well as the increase in total mtDNA from p62 overexpressed cells, p62 plays an important, *independent* role in the functionality of normal mitochondrial dynamics which extends beyond solely altering $\Delta\psi m$.

5. Conclusion

In conclusion, our study not only confirmed that p62 localizes to the mitochondria under non-stressed, physiological conditions but also further defines a critical role for p62 in the normal functioning of mitochondria. We show that p62 is integral to normal mitochondrial dynamics and, by regulation of the mitochondrial transcription factor TFAM, maintains mitochondrial genome stability. These functions appear to be the results of direct interactions of p62 with mitochondria that extend beyond generalized physiological responses.

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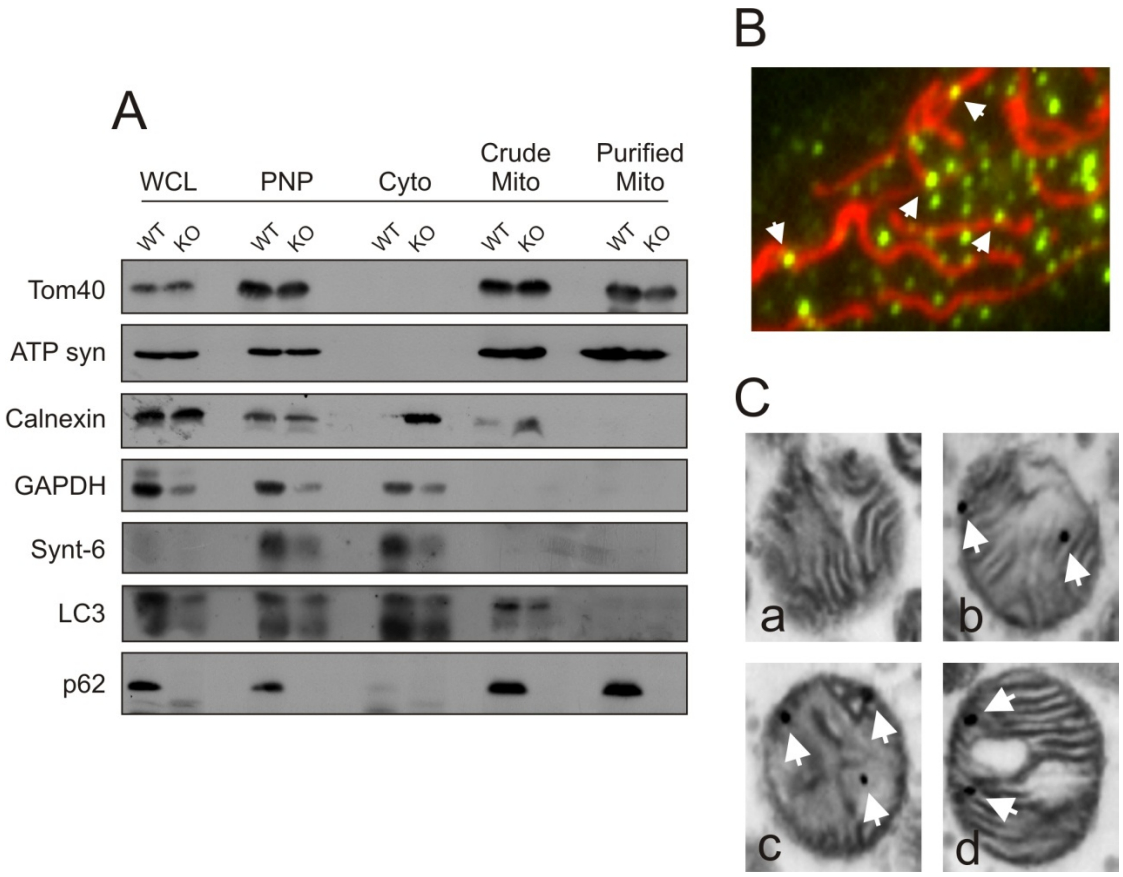

Fig. 1. Mitochondrial localization of p62 in MEF cells. (A) Mitochondrial fraction was isolated from WT and $p62^{-/-}$ MEF cells by Percoll gradient and centrifugation. Whole cell lysate (WCL), post-nuclear supernatant (PNS), cytoplasm fraction (Cyto) or mitochondrial fraction (Mito) was subjected to 10% SDS-PAGE and immunoblotted with antibodies to p62 or protein markers. TOM40 was used as outer membrane (OMM) marker and ATP synthase as inner membrane (IMM) marker for mitochondria. The cellular compartment markers are calnexin (endoplasmic reticulum), GAPDH (cytoplasm), syntaxin 6 (trans-Golgi network) and LC3-II (autophagosome). (B) FITC-labeled p62 (arrows) associates with MitoTracker Red stained mitochondria in WT MEF cells. (C) Purified mitochondria from WT MEF cells were prepared for TEM and stained with colloidal gold labeled p62 antibody. **a** – negative control containing only colloidal gold secondary antibody; **b-d** - granules of p62 are localized to the OMM as well as with internal cristae.

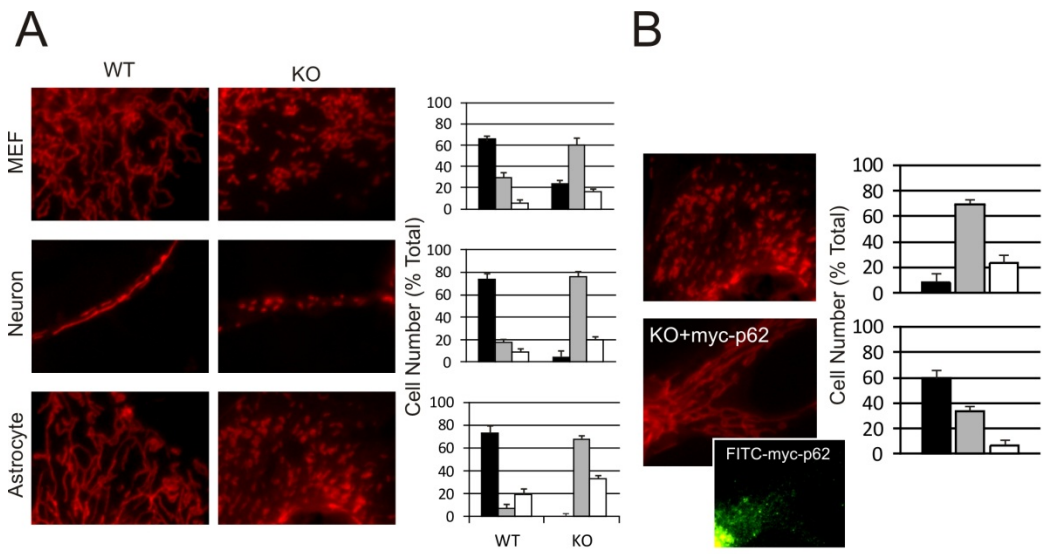

Fig. 2. p62 regulates mitochondrial morphology autonomous of cell type. (A) $p62^{-/-}$ cell types were compared to WT for mitochondrial morphology and visualized by MitoTracker Red staining. Morphology was classified as Tubular (black bar), Fragmented (grey bar) or Intermediate (white bar) and cells counted in each class. Mitochondria from a minimum of 100 cells were classified for each cell type. (B) $p62^{-/-}$ MEF cells were transfected with myc-p62 to analyze reversion of mitochondrial phenotype. For transfected cells, only those exhibiting expression of FITC-myc-p62 were used in the analysis. Inset shows FITC-myc-p62 immunofluorescence for represented cell. Mitochondria were classified and counted as in (A). Data for all graphs are mean +/- s.e.m., $P< 0.05$.

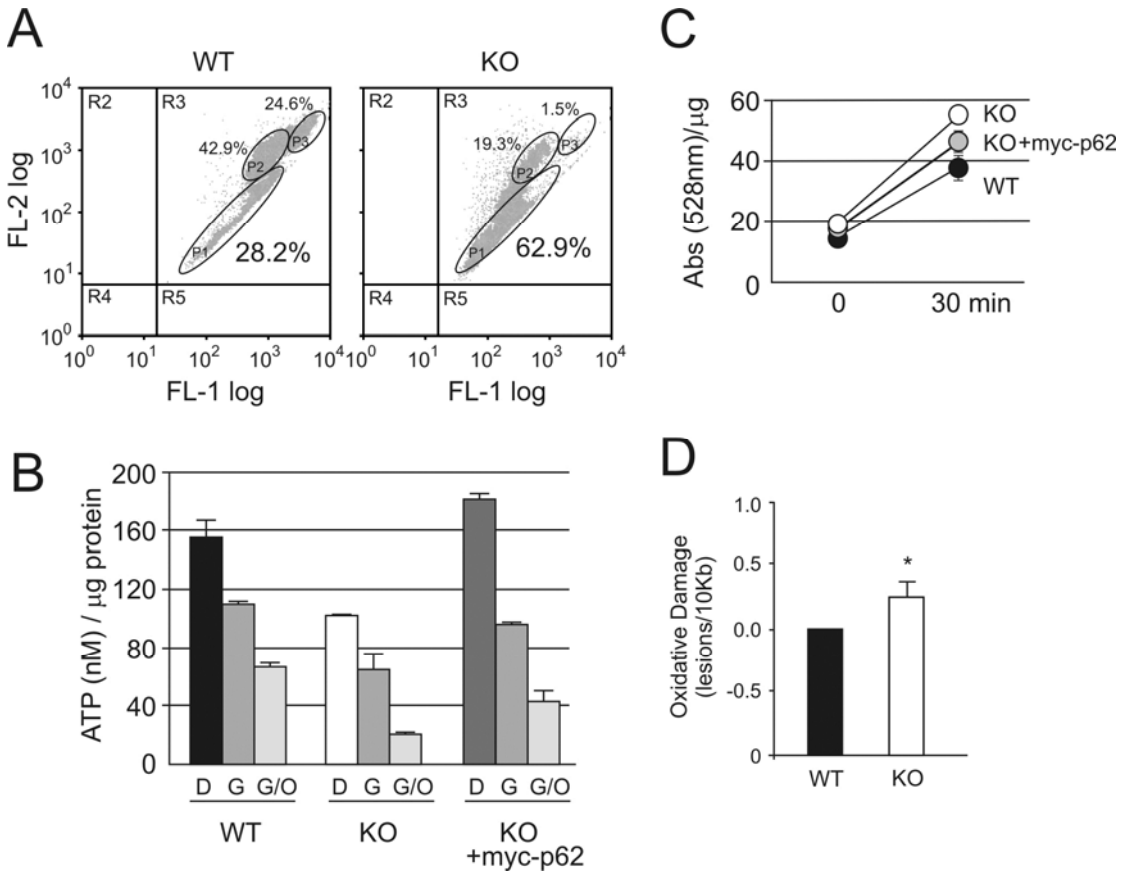

Fig. 3. Abnormal mitochondrial metabolism and increased oxidative stress in p62$^{-/-}$ cells. (A) Membrane potential was analyzed using JC-1 staining in WT or p62$^{-/-}$ MEF cells. Cells were counted by flow cytometry. (B) Mitochondrial ATP production in WT, p62$^{-/-}$ MEF cells minus/plus exogenous myc-p62. Cells were grown in DMEM (D), Galacotose media (G) or Galactose media plus oligomycin (G/O). (C) WT or p62$^{-/-}$ MEF cells minus/plus exogenous myc-p62 were analyzed for ROS production by detection with H_2DCFDA at 485nm at times indicated. (D) Comparison of the oxidative damage of mitochondrial DNA fragments in WT and p62$^{-/-}$ mice. Data for all graphs are mean +/- s.e.m., $P < 0.05$.

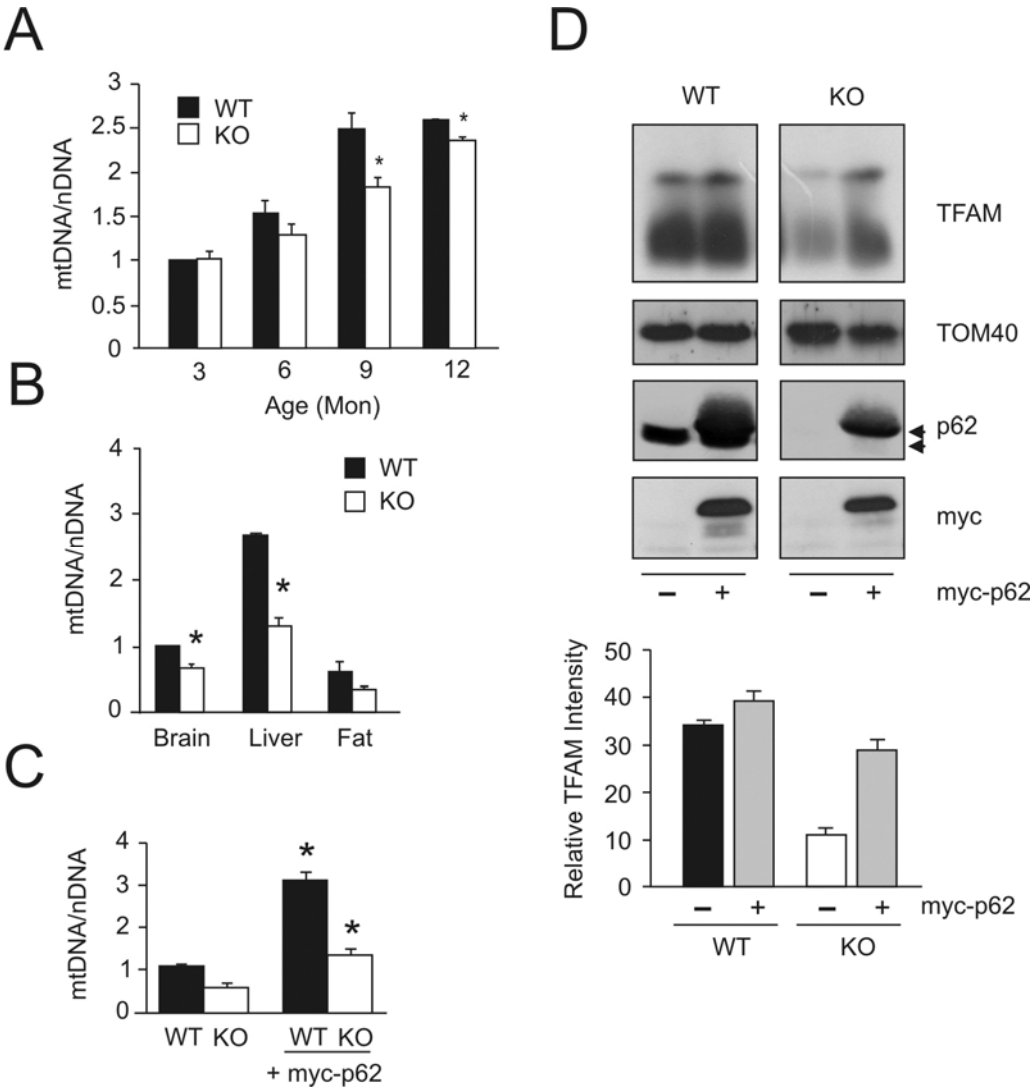

Fig. 4. p62 protects mitochondrial genome integrity. (A) Quantitative analysis of mtDNA copy in WT and p62$^{-/-}$ mouse brain was analyzed across ages. (B) mtDNA copy number in tissue from WT and p62$^{-/-}$ mice. (C) mtDNA copy number quantitated in WT and p62$^{-/-}$ MEF cells with overexpression of myc-p62. (D) Mitochondrial TFAM import was analyzed by Western blot. Mitochondria were isolated from WT and p62$^{-/-}$ MEFs transfected with myc-p62 by Percol gradient centrifugation and levels of TFAM in the mitochondria analyzed. Resulting blots were analyzed by densitometry and graphed for relative TFAM intensity. Blots shown are representative of 4 different experiments. Data for all graphs are mean +/- s.e.m., $p < 0.05$.

Chapter 3.

Behavioral Effects of SQSTM1/p62 Overexpression in Mice: Support for a Mitochondrial Role in Depression and Anxiety.

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Mitochondria; SQSTM1/p62; Animal models; Affective spectrum disorders; Depression; Anxiety

Abstract

Affective spectrum and anxiety disorders have come to be recognized as the most prevalently diagnosed psychiatric disorders. Among a suite of potential causes, changes in mitochondrial energy metabolism and function have been associated with such disorders. Thus, proteins that specifically change mitochondrial functionality could be identified as molecular targets for drugs related to treatment for affective spectrum disorders. Here, we report generation of transgenic mice overexpressing the scaffolding and mitophagy related protein Sequestosome1 (SQSTM1/p62) or a single point mutant (P392L) in the UBA domain of SQSTM1/p62. We show that overexpression of SQSTM1/p62 increases mitochondrial energy output and improves transcription factor import into the mitochondrial matrix. These elevated levels of mitochondrial functionality correlate directly with discernible improvements in mouse behaviors related to affective spectrum and anxiety disorders. We also describe how overexpression of SQSTM1/p62 improves spatial learning and long term memory formation in these transgenic mice. These results suggest that SQSTM1/p62 provides an attractive target for therapeutic agents potentially suitable for the treatment of anxiety and affective spectrum disorders.

1. Introduction

While the molecular mechanisms behind neurodegeneration are often ambiguous, dysfunction of mitochondrial dynamics has emerged in recent years as a hallmark feature of neurodegenerative disease. Dysfunction of mitochondria leads to impaired energy metabolism, reduced ATP production, and disruption of mitochondrial calcium homeostasis and increased production of oxygen radicals [5]. Cell types with the highest energy demands, such as muscle or neurons, appear to be the most susceptible to mitochondrial dysfunction [77].

Evidence is accumulating that mitochondrial function is related to the pathophysiology and treatment of behavioral disorders [18]. In general, anxiety disorders are the most prevalent psychiatric disorders diagnosed and have been related back to altered energy metabolism, mitochondrial transport and oxidative stress. ATP production through the mitochondrial electron transport chain is necessary for the survival of neurons, and protein signaling cascades have been shown to mediate synapse changes, as well as other long term changes in neuronal structure [7]. Altered levels of proteins specifically involved in neurotransmission, energy metabolism and oxidative stress have been found in lab mice diagnosed as “high anxiety” by elevated-plus maze analysis [19]. Deletion of Bcl-2, a major modulator of mitochondria function, has been related to increased anxiolytic activity in a mouse behavior model [18]. Furthermore, monoamines, such as serotonin, have for years been included in causative hypotheses related to depression. Substantial decreases in serotonin levels in brain regions of a genetic mouse model of bipolar disorder were correlated with decreased mtDNA levels and multiple mtDNA deletions [42]. Changes in mitochondrial functionality resulting in inflammation have also been offered as an explanation for major depression disorders [20]. Collectively, such studies have revealed a strong correlation

between proteins specific to mitochondrial function and behavior patterns associated with affective spectrum disorders.

The ubiquitous cytoplasmic protein Sequestosome1 (SQSTM1/p62) was originally cloned as a phosphotyrosine-independent ligand of the $p56^{lck}$ Src homology (SH2) domain [62] and identified as a ubiquitin binding protein [76] via its UBA domain [10]. Mutations in the C-terminal UBA domain of p62, particularly those affecting ubiquitin binding, have been associated with Paget's Disease of Bone [9, 48]. The most common of these mutations, P392L, impairs ubiquitin binding however, the transcription factor NF-κB is constitutively activated when ubiquitin binding is absent with this mutation [65]. p62 is also implicated in multiple cellular activities including antioxidant metabolism [34], autophagy/mitophagy [23], and the ubiquitin proteasome system [69], as well as serving as a scaffold for aPKC/TRAF6 mediated activation of NF-κB [17]. Interestingly, loss of NF-κB activity in $p62^{-/-}$ brain contributes to increased generation of Reactive Oxidative Species (ROS) in neurons and glial cells [17] while NF-κB presence in brain cells is required for long term potentiation (LTP), neurogenesis and memory formation [2, 14]. Moreover, p62 mediates the phosphorylation of the AMPA receptor subunit GluR1 by atypical PKC further implicating that it plays a role in synaptic transmission and neuronal plasticity [35].

Growing lines of evidence support a strong correlation between p62 expression and mitochondrial function. p62 is recruited to mitochondria in PINK1/Parkin expressing cells [61], as well as being required for the completion of the mitophagy pathway [49, 61]. Recently, we have shown that p62 has an endogenous association with mitochondria where it spans the mitochondrial membrane and affects import of the mitochondrial transcription factor TFAM [70]. Loss of TFAM import into the mitochondria matrix results in decreased mitochondrial

DNA (mtDNA) ultimately leading to mitochondrial dysfunction. Overexpression of p62 in Mouse Embryonic Fibroblast (MEF) cells results in increased levels of mtDNA. Further, restored expression of p62 rescues TFAM import into the mitochondrial matrix resulting in restoration of mitochondrial functionality [70].

Previously, our laboratory analyzed the behavior of mice in which the p62 gene had been deactivated ($p62^{-/-}$ mice). These mice were tested for their ability to complete spatio-temporal tasks as well as their susceptibility to overall affective spectrum disorders [64]. Absence of p62 from neural tissue in these mice resulted in hippocampal dependent cognitive decline and increased anxiolytic behavior. These mice also displayed a significant degree of depression when compared to wild-type (WT), a phenotype indicative of mitochondrial defects [42], while maintaining normal motor neuron function [64]. Loss of synapses and tau-pathology, as well as other signs of neurodegeneration, are also characteristic of the loss of p62 suggestive of traits related to Alzheimer's disease [45,72]. Thus, p62 appears to be a prime candidate for a protein that changes the functionality of mitochondria and also affects changes in behavior patterns in a mouse model.

Because removal of p62 has proven to be deleterious to mitochondria function, as well as to mouse behavior, we reasoned that the converse, namely increasing levels of p62 in neural tissue, might theoretically improve mitochondrial functionality and as a direct response, abrogate atypical behaviors related to mitochondrial dysfunction. This hypothesis was the focus of the research described in this paper. Our goal was to examine the involvement of mitochondrial function in affective spectrum disorders, in learning and memory, and to explore the role that p62 plays in this context. To investigate these relationships, we designed a study to explore directed p62 ***overexpression*** in the hippocampus of a C57BL/6 mouse and the effects p62 has on

mitochondrial function. Central to our question was the corresponding examination of behavioral outcomes, specifically those related to affective spectrum disorders, learning and memory. We show that by increasing the levels of p62 in the hippocampus of OEp62 mice, mitochondrial function is improved and gross behavioral patterns, especially those related to anxiety and depression, are changed. By using p62:P392L overexpressing mice, we provide evidence that p62's ability to bind ubiquitin through its C-terminal UBA domain plays a role in controlling p62's ability to affect both mitochondrial function and behavior in overexpressing mice.

2. Methods

2.1 Animals

Mice overexpressing p62 (OEp62) or a mutated form of the protein (p62:P392L) in the hippocampus were generated by our laboratory. C57BL/6 mice were used as the surrogate strain for the development of both OEp62 and P392L overexpressing mice. The Thy-1 vector has been widely used to obtain brain-specific expression of exogenous proteins and tends to display restricted expression to the hippocampus [25]. We generated two constructs employing the Thy 1.2 vector and insertion of an EGFP-tagged p62 cDNA (either full length p62 or mutant P392L p62 construct) by insertion into the Xho1 site of the pTSC21k Thy 1.2 expression vector. The EGFP-p62 construct encompasses the EGFP translation initiation codon, signal peptide and p62 translation initiation codon, signal peptide, termination codon and poly-A tail. The full length clone was sequenced to ensure fidelity during PCR and forward orientation. The construct encodes a 97Kd fusion protein under the influence of the Thy 1.2 promoter. Founder mice were generated at the Auburn University College of Veterinary Medicine Transgenics Facility using C57BL/6 as the donor strain and germ line transmission analyzed by EGFP-p62 expression using

sequence specific primers. Donor mice were supplied by the Transgenics Facility. Once founder mice were identified, they were crossed back into the WT C57BL/6 strain and offspring characterized for EGFP-p62 expression. Mice positive for construct expression were inter-crossed to establish a strain of mice overexpressing EGFP-p62 or EGFP-p62:P392L in neural tissues, specifically the hippocampus. Mice were caged in standard barrier cages on a ventilated rack in an animal room with constant temperature (~22+/-1 ^{o}C) on a 12 hour light/dark cycle with ad lib food and water. All experiments, unless otherwise indicated, were performed with age matched (6 month) mice in the early phase of the light cycle under standard room fluorescence. There was no significant difference in maze performance between sexes of mice, thus experiments were performed using age-matched subjects only. All procedures were submitted to and approved by IACUC and were conducted following NIH guidelines. Mice used in each behavioral testing paradigm were naive and not used for other tests.

2.2 Neuronal Cell Culture

Hippocampal neurons and astrocytes were cultured as described previously [71]. Briefly, the hippocampus was dissected from day 19 embryonic mice. Hippocampi were combined in NB Media (Life Technologies, Carlsbad, CA) supplemented with 10% FBS and neuronal cells triturated. A cell count was performed and cells were plated to poly-lysine:collagen coated plates and grown for 7 days in culture at 37^{o}C and 5% CO_2. On day 7, cells were stained with 50nM MitoTracker Red (Life Technologies) for 30 minutes followed by fixation in 4% paraformaldehyde/PBS. Images were generated using a Nikon A1/T1 confocal microscope and Nikon Elements software.

2.3 Western blot and analysis.

Cell lysates or isolated mitochondria were subjected to SDS-PAGE in polyacrylamide gels. Samples were Western blotted with primary antibody (phospho-AMPK/AMPK – Cell Signaling Technology, Danvers, MA; TFAM, p62 – Abcam, Cambridge, MA; $p62^{Hum}$ – BD Biosciences, San Jose, CA; β-actin – Sigma-Aldrich, St. Louis, MO) and HRP-tagged secondary antibody from GE Healthcare Life Sciences (Pittsburgh, PA) and processed with ECL detection reagent (GE Healthcare). Following exposure of the labeled membrane to Hyperfilm-ECL detection film, the Un-Scan-it Gel and Graph Digitizing software (Silk Scientific, Orem, UT) was used to scan and quantify the signal from the Western blot, and data were analyzed statistically (Win-SAS, Microsoft, Seattle, WA).

2.4 Mitochondria isolation.

Following trypsinization, MEF cells were collected by centrifugation and mitochondria isolated essentially as described by Wieckowski, et al. 2009 [78]. Briefly, washed cells were homogenized on ice with a Teflon pestle followed by centrifugation twice at 600x*g* for 5 min. The post nuclear pellet was collected from the initial spin and further centrifuged at 7000x*g* for 10 min. The cytosolic fraction was obtained from the supernatant while the crude mitochondria pellet (C. Mito) was collected in the pellet. The pellet was washed with MRB Buffer (250mM Mannitol, 5mM HEPES, pH 7.4 and 0.5mM EDTA) before being layered over a Percol gradient. The gradient was centrifuged at 95,000xg for 30 min with a dense band containing purified mitochondria localized at the bottom of the tube. This band was collected and washed with MRB buffer before being suspended in a small volume of MRB Buffer containing protease inhibitors. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA)

and subjected to SDS-PAGE and Western blot or used in further experiments as mitochondrial lysates.

2.5 ATP Assay.

ATP production was measured using the ATP Determination Kit (Life Technologies, Carlsbad, CA). Briefly, MEF cells were detached from tissue culture plates by trypsin treatment and collected by brief centrifugation followed by PBS wash. Cell pellets were resuspended in isolation buffer (5mM HEPES, pH 7.2; 225mM Mannitol, 75mM Sucrose, 1mM EGTA, and protease inhibitors) followed by 5 times syringe using a 23 gauge needle. Samples were centrifuged at 1,500x*g* for 5 min prior to supernatants being used for ATP assay following the manufacturer's directions and measured with a luminometer. Each sample was measured in triplicate. Results are representative of three separate experiments.

2.6 mtDNA copy number determination.

Genomic DNA was isolated from brain tissue using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Following elution with ddH_2O, purified DNA was stored at -80°C. Relative Quantitative real time PCR was employed to determine mtDNA copy number. mtDNA was amplified with mtDNA primer: MDF: 5'-CCTATCACCCTTGCCATCAT-3'and MDR: 5'-GAGGCTGTTGCTTGTGTGAC-3'. Nuclear DNA was amplified by nuclear DNA primer: NDF: 5'- ACATCTGTTGCTCCGGCTCTCATT-3' and NDR: 5'-GCAAGCTCAAAGGGCAAGGCTAAA-3'. RT-QPCR was conducted using an ABI 7500 Real Time PCR system (Applied Biosystems, Carlsbad, CA) using Power SYBR Green PCR Master Mix. Relative mtDNA levels were calculated by the ΔΔCt method as described [1].

2.7 Behavioral Equipment and Procedures

2.7.1 Open Field Maze

Measurement of locomotor activity and anxiety-related behavior was performed simultaneously using a multiple unit Open Field Maze consisting of four activity chambers (San Diego Instruments, CA). Each chamber measured 50cm x 50cm and a single subject animal was placed in the center of each at the beginning of a 10 minute test period. Testing was done under low lighting conditions (~30 lux) from incandescent lamps in set place in the testing room. Mice were recorded on videotape and analysis was performed using the SMART Video Tracking software (PanLab, Harvard Apparatus). The field for each maze was divided into sixteen 12.5cm x 12.5cm squares for analysis. The outer 12 squares were considered the Outer Zone while the inner 4 squares (25x25 cm) constituted the Inner Zone for analysis. Time spent in the outer area (closest to the walls) was measured as anxiety-related behavior and compared to time spent in the central area of the chamber. Total ambulatory distance was also recorded for each mouse to ensure there were no motor defects between genotypes.

2.7.2 Elevated Plus Maze

An Elevated Plus Maze (Colbourne Instruments, Allentown, PA) was used to measure anxiety-like behavior. Briefly, the maze consisted of 2 arms (50 x 10cm) open to the environment and 2 arms enclosed by darkened plastic walls (50cm x 10cm x 30cm). The arms are connected by a 10cm x 10cm central platform and elevated 50cm off the floor. Naive mice were tested in low light conditions (~30 lux) from incandescent lamps set in place in the testing room by placing the mouse into the center of the maze facing an open arm and recording

explorative behavior for a period of 5 minutes using the SMART software. The number of open and closed arm entries, time spent in both types of arms, total distance traveled in each arm as well as total distance overall were analyzed.

2.7.3 Forced Swim Test

Depression-like behavior was analyzed using methods similar to the Porsalt Forced Swim Test [58]. Mice were placed in the middle of a 1 liter Pyrex beaker filled with 12cm of water and activity was videotaped for 6 minutes. Water was warmed to approximately 28^{o}C and water depth prevented the subject from touching the bottom of the beaker. Water was completely changed between individual subjects. Activities were defined as either 1) *immobility* – floating either without movement or only small movements to stay above water; 2) *swimming* – active movement traveling within quadrants of the beaker; and finally 3) *climbing* – actively trying to escape, usually by attempting to climb the walls of the beaker. Behaviors were counted every 5 seconds during the test and totals were averaged within each genotype.

2.7.4 Barnes Maze

Spatial learning and memory was tested using a Barnes Maze [4, 27]. Testing occurred in a darkened room with a single bright incandescent light (~2000 lux) shining down on the surface of the maze platform. A metronome producing 85 decibels was used to further stimulate the escape response. A video tracking camera was suspended above the maze while extra-maze cues were positioned at specific places around the maze and maintained throughout the testing process. An escape hole was designated with an escape box installed beneath the surface of the maze allowing the subject to escape exposure to the adverse stimuli with a different escape hole

designated for each mouse. Mice were adapted to the maze during 4 separate 3 minute trials on the first day of testing with a 15 minute intertrial interval. The mice were placed in a hinged box, released at the center of the maze and allowed to explore until the escape hole was located and the mouse entered the escape box. Upon entering the box, the metronome was turned off and the box was closed allowing the subject to remain hidden for 2 minutes prior to return to the home cage. Prior to the next subject, the maze was cleaned with a 2% ethanol solution to remove any remaining olfactory clues. The spatial acquisition phase was conducted over the next 5 days as described for day 1 and latency time was measured until the subject located and entered the escape box to determine how fast the mouse learned the location of the escape hole. Four trials per day were performed for each mouse with a 15 minute intertrial interval.

Short term memory was accessed with a probe trial on day 5, approximately 2 hours after completion of the acquisition phase. The mouse was placed in the maze as described above with light and metronome as adverse stimuli, however, the escape box was removed from the maze. Discrete zones were designated for each hole in the maze using SMART Video Tracking software (PanLab, Harvard Apparatus) and distance traveled throughout the maze was tracked for a total of 90 seconds. Time spent in the target zone (location of the prior escape hole) was indicative of how well the mouse remembered the location of the escape hole over a relatively short time. This same protocol was repeated on Day 12 for analysis of the subject's long term ability to remember the location of the escape hole.

2.8 Statistical Methods

The overall results for all data were expressed as the mean ± S.E.M. Statistical analyses (*t*-tests) were performed using Excel 2010 (Microsoft, Redmond WA) and SAS 9.2 (SAS

Institute, Cary NC). Corrections for confounded multiple *t*-tests were made using the step-up Bonferroni adjustment (*Hoch* option; SAS procedure MULTTEST). The step-up modification to the Bonferroni has been shown to provide additional power for controlling the *post hoc* family-wise error rate [32]. Individual *t*-tests were used throughout because the study was designed to evaluate specific comparisons between control (WT) and individual treatment (OEp62 or p62:P392L). Global comparisons among all three genotypes were not of general interest. Hard boundaries for statistical significance (i.e. $p<0.05$) were not used [33, 44]. *t*-values (degrees of freedom), raw *p*-values and adjusted *p*-values are reported for each comparison along with our interpretation for the reader's consideration. The number of subjects (N) used in behavior testing varied between a minimum of 5 to a maximum of 28. Specific numbers of replicates are described below in the Results for each experiment and in Table 1.

3. Results

3.1 Generation of OEp62 and p62:P392L overexpression mice.

Mice overexpressing p62 specifically in neural tissue were generated as described in Materials and Methods. Human EGFP-p62 cDNA, either native or containing the P392L substitution, was inserted in the Thy 1.2 expression vector and founder mice generated. These founders were backcrossed into the C57BL/6 background and colonies of each genotype established. The Thy 1.2 expression vector directed p62 overexpression specifically to neural tissue (Fig. 1A). Human specific antibody recognized only the EGFP-tagged p62 expressed as a 97Kd fusion protein in various sub-regions of the brain. p62 expression was increased mainly in the hippocampal region of the mouse brain, however, expression was also modest in the brain stem region as well as seen to a lesser extent in the striatum and cortex. Because expression was

highest in the hippocampal region, slices of 6 month adult brains were analyzed under fluorescent microscopy to verify EGFP-62 expression in this region of the brain (Fig. 1B). The expressed fusion protein was identifiable in the hippocampus of slice cultures when excited at 488nm.

3.2 p62 overexpression increases the functionality of mitochondria in the hippocampus.

To examine the effects of elevated p62 levels on mitochondrial functionality, we first looked at mitochondrial morphology in hippocampal neurons. Neurons were isolated from day 19 embryonic pups and grown for 7 days in culture. Mitochondria were stained with MitoTracker Red (Life Technologies, Carlsbad, CA) and visualized using confocal microscopy (Fig 2A). Overexpression of p62 showed an increase in "tubulo-reticular" structure in OEp62 mice when compared to WT however, mitochondria from neurons expressing the P392L mutant appeared to be very similar to WT morphology.

As mitochondrial morphology drives mitochondrial function, we next examined aspects of ATP production in hippocampal tissue from overexpressing mice. AMPK acts as a metabolic switch for ATP production in the mitochondria [60] with increases in the ratio of AMP:ATP affecting the activation state of AMPK [74]. As a cellular energy response element, low levels of ATP positively activate AMPK which then results in downstream regulation of signaling pathways and cyclic replenishing of cellular ATP levels. Over expression of p62 caused a decrease in phosphorylated AMPK compared to WT (Fig 2B). These results suggest that cellular ATP levels in hippocampal tissue could be higher due to increased p62 levels enhancing mitochondrial homeostasis. We therefore examined ATP levels in hippocampal tissues of overexpressing mice and compared them to WT (Fig 2C). OEp62 mice showed increased levels

of ATP correlating with the decreased expression of activated AMPK. Interestingly, mutation of P392L in the ubiquitin binding domain (p62:P392L), yielded ATP levels more consistent with the lower levels seen in WT suggesting a pathway by which p62 could regulate mitochondrial ATP production potentially based on its ability to bind to ubiquitin or poly-ubiquitinated substrates.

The mitochondrial transcription factor A (TFAM) has been reported to protect the mitochondrial genome once imported into mitochondria [30]. TFAM promotes mtDNA replication/transcription [24] and aids in the repair of oxidatively damaged DNA [39, 81]. As we observed an increase in mitochondrial functionality as measured by ATP production, we next examined import of this important transcription factor. Mitochondria were isolated from the hippocampal region of the mouse brain and levels of TFAM associated specifically with the mitochondria measured by Western blot (Fig. 2D). TFAM expression localized to the mitochondria was increased in both OEp62 and p62:P392L mice hippocampi when compared to WT. As post translational modification (maturing) of TFAM occurs following import into mitochondria [47], increases in mature TFAM protein indicate an increase in mitochondrial import capability specific to p62 expression levels. mtDNA copy number is often used as a direct correlation of mitochondrial TFAM levels [24, 38, 39] and as a measure of functional mitochondrial energetics [66]. Thus, we measured total mitochondrial DNA present in the hippocampus of our overexpressing mice. Levels of mtDNA were essentially consistent with WT (Fig. 2E).

3.3 p62 overexpression changes behavior patterns related to affective spectrum disorders.

Recent studies have suggested that Bcl-2 protein imbedded in the inner mitochondrial membrane is a major modulator of mitochondrial function [54] and that overexpression of the Bcl-2 gene reduces anxiety-like behaviors in a mouse model [67]. Mitochondrial function has been related to anxiety disorders caused by the deletion of Bcl-2 [18]. Genetic inactivation of p62 in mice leads to increases in anxiogenic behavior [64] accompanied by mitochondrial dysfunction [70]. Because we demonstrate here that increased expression of p62 results in increased mitochondrial functionality, we sought to examine behaviors in p62 overexpressing mice related to affective spectrum disorders. We hypothesized that increased mitochondrial function could in fact provide relief in anxiety related behaviors. Results and statistical analysis from behavior testing are summarized in Table 1.

3.3.1 Mice overexpressing p62 show no change in locomotor activity in the Open Field Maze.

OEp62 (N=12) and p62:P392L (N=28) mice were observed in an Open Field Maze for any difference gene expression levels would have on general locomotor activity (Fig. 3A). Distances traveled in the maze during 10 minute trials were essentially the same for overexpressing mice when compared to WT (N=24). However, p62 overexpressing mice spent significantly more time exploring the center of the open field (Fig. 3B), a measure reflecting decreased thigmotaxis and anxiety related behavior [73].

3.3.2 Mice overexpressing p62 have variable differences in measured anxiety in the Elevated Plus Maze.

An Elevated Plus Maze was used to measure anxiogenic behavior in overexpressing mice. OEp62 mice (N=12) showed a *decrease* in anxiety-related behavior by spending more time on the open arms of the Elevated Plus Maze than comparable WT mice (N=20). Conversely, p62:P392L mice (N=6) showed a significant *increase* in anxiogenic behavior (Fig 3C) raising the possibility of ubiquitin binding by p62 playing a role in both mitochondria functionality (Fig. 2) and behavioral pattern modification.

3.3.3 Mice overexpressing p62 show different behavior patterns in the Forced Swim Test.

A modified Forced Swim Test was used to access depression in overexpressing mice compared to WT [11]. Immobility time of OEp62 (N=11) and p62:P392L (N=6) mice did not measurably differ from that of WT (N=15) (Fig. 3D). However, other behavioral traits did show variation during the testing period. Specifically, there were significant differences in swimming and climbing behaviors between WT and overexpressing mice (Fig. 3E). WT mice exhibited climbing behavior greater that what was seen in the overexpressing genotypes while conversely, overexpressing mice spent much more time in swimming behavior when compared to WT.

3.4 p62 overexpression effects on spatial learning and memory tasks in mice.

Spatial learning in p62 overexpressing mice was measured using a Barnes Maze. While all three genotypes exhibited equal search times for the hidden escape box in Day 3 of the acquisition, both OEp62 and p62:P392L mice continued to show slight improvement in latency to escape compared to WT during Days 4 and 5 of the acquisition phase. At the end of the 5 day

acquisition phase for the maze, both genotypes of p62 overexpressing mice (OEp62, N=5; p62:P392L, N=9) performed better in latency measurements than did WT mice (N=8; Fig.4A).

To measure effects of p62 overexpression on memory, both short and long term probe trials were undertaken using the Barnes Maze after the acquisition phase. Short term memory was assessed two hours after the final acquisition measurements were taken and showed no significant differences in short term memory between genotypes (Fig. 4B). Long term memory measurements were undertaken six days after the last acquisition measure. At this time, OEp62 mice showed significantly greater ability to remember escape box locations compared to both WT and p62:P392L (Fig. 4B).

4. Discussion

Mitochondrial dysfunction has recently been associated with the pathophysiology of affective spectrum and anxiety disorders [20]. Patients exhibiting major depression display significantly decreased mitochondrial function [21] while treatment with anxiolytic as well as anti-depressant drugs has been shown to improve mitochondrial respiration thus increasing anti-oxidant activity [75]. Mitochondrial dysfunction can be caused by numerous deficiencies related to the electron transport chain and thus to energy production. We have previously shown that shutdown of the SQSTM1/p62 gene can result in decreases in mitochondrial energy production [70], as well as increased oxidative damage to mitochondrial DNA [15, 16]. Other key modulators of mitochondrial function include regulation of intracellular Ca^{+2} levels by Bcl-2 [59] and abrogation of electron transfer along the respiration chain by coenzyme Q10 (CoQ10) deficiency [51]. Overexpression of either of these proteins results in improved mitochondrial

function [59, 68], an observation we have also seen with p62 overexpression in mouse embryonic fibroblasts [70].

Neurodegeneration refers to progressive death and loss of neurons in the brain. The most common form of cell death in neurodegeneration is through apoptotic pathways related to mitochondria [20]. Specifically, increases in reactive oxygen species (ROS), changes in mitochondrial fusion and fission rates, Ca^{+2} homeostasis, and mitochondrial membrane permeability all lead to mitochondrial disease and dysfunction [70]. In this study, by increasing the levels of p62 expressed in brain tissue, we have shown improvement in mitochondrial functionality. Morphologically, hippocampal mitochondria showed increased fusion resulting in increases in ATP production and mitochondrial import of the major mitochondrial transcription factor A (TFAM). TFAM is known to have a role in replication of mitochondrial DNA, and also in its maintenance [40]. Furthermore, overexpression of TFAM results in decreased ROS production as well as increased mitochondrial ATP production and function [29]. With increased TFAM presence in hippocampal mitochondria of OEp62 mice, an increase in mtDNA could also be expected. However, p62 also exhibits a scaffolding function for the delivery of mitochondria in the cell for disposal by mitophagy [23, 31]. Increased p62 levels would not only increase the amount of mtDNA present, but would also improve mitophagy thus playing a two stage role in regulating mtDNA levels. Increased TFAM presence in mitochondria also ameliorates age dependent impairment of cognitive functions in the brain [29]. It should also be noted that while increases in p62 resulted in increased mitochondrial functionality, the role of ubiquitin binding also appears to play a major role in ATP production. Given p62's transmembrane presence in the mitochondria [50], it is possible that the C-terminal UBA domain of p62 is required for electron transport chain functionality on the inner mitochondrial membrane. Regardless, one can surmise

that increases in mitochondrial functionality in the mouse brain appear to ameliorate neurodegeneration resulting in an associated change in learning, memory and long term potentiation.

In the present study, we observe an overall improvement in the affective spectrum disorder behavior patterns in mice exhibiting increased mitochondria functionality. This study also suggests a role for functional mitochondria in the learning and long term memory patterns of these mice. p62 overexpressing mice displayed no gross physiological changes when compared to age matched WT nor was any inhibition of locomotor ability observed between genotypes. Thus, measurements related to distance and latency in the maze could be used to compare behaviors of the mice. Two separate methods for anxiety measurement were used in this study. Movement of mice in the open field away from the walls is an indication of increased exploratory behavior and a decrease in thigmotaxis in mice [73]. The Elevated Plus Maze is the standard measure of anxiety-like behavior in rodents [8, 46]. The anxiety measures used here were of the exploratory nature and related to a mouse's desire to examine new environments. A reduction in anxiety-like behavior was observed in the overexpressing mice when compared to WT. Interestingly, mice lacking ubiquitin binding ability of p62 displayed a decrease in anxiogenic behavior when tested on the Elevated Plus Maze. Decreased anxiety has been previously seen in mice overexpressing the ubiquitin ligase KF-1 resulting in ubiquitination and degradation of target proteins in dopaminergic neurons [28]. Conversely, increased anxiety-like behavior has been recorded where decreases in ubiquitin ligase activity of Parkin [37] and RNF41 [43] have been observed. p62 has a scaffolding function of binding to K63-ubiquitinated proteins and targeting those proteins to the proteasome for degradation [69]. The inability of p62 to associate with K63-tagged cargoes would result in an increase in aggregated proteins in the

cell. How this lack scaffolding function affects anxiety-like behaviors requires further study but should not be overlooked.

A relationship between depression and mitochondrial dysfunction has been examined in a number of studies. Mutations in mitochondrial proteins have been found in the pre-frontal and frontal cortices of post-mortem samples obtained from patients diagnosed with major depression [41] while levels of electron transport chain activity were found to be decreased below control levels in a major depressive disorder study [3]. While p62 overexpressing mice showed no difference from WT in immobility time in the Forced Swim Test, which measures despair/depression, when specific behaviors were distinguished in the test, a notable difference was seen between overexpressing mice and their WT counterparts. WT mice exhibited specific climbing or thrashing behavior characterized by upward directed movements with the front paws on the side of the swim chamber. Studies have revealed that catecholaminergic agents decrease immobility in the swim chamber with a corresponding increase in climbing behavior [52]. Conversely, p62 overexpressing mice exhibited significantly increased swimming behavior over controls, a behavior associated with increases in 5-HT-related compounds [11]. Several 5-HT receptors have been suggested to play a role in mediating antidepressant behavior [53]. Furthermore, antidepressant treatment not only mediates the effects of 5-HT-related compounds on behavior [11, 12], but also affects electron transport and ATPase activity in the mitochondria [13].

Serotonergic pathways are known to play a major role in despair and depression. Multiple 5-HT receptors have been implicated in mechanisms underlying learning and memory [79]. However, the role of 5-HT receptors in learning and memory is complex. Some studies have reported improvement in cognition and memory formation [56] while others have reported

limited involvement of chronic 5-HT depletion in spatial learning and memory [63]. We found in this study, that p62 overexpression improved spatial learning in the Barnes Maze while also increased the ability of the mice to retain long term memory. Activation of 5-HT1A by its agonist 8-OH-DPAT has been shown to significantly enhance learning acquisition and memory formation as well [26]. Cognitive impairments are coincident with loss of long term potentiation in $p62^{-/-}$ mice [64]. Furthermore, we have previously demonstrated that p62 is an AMPA receptor interacting protein and this interaction, along with aPKC, results in delivery of the GluR1 subunit to neuronal surfaces in the hippocampus mediating synaptic plasticity and long term potentiation [36]. Our results are further suggestive of p62 involvement in the mediation of 5-HT cognitive functions in the hippocampus. This new evidence for a role for p62 in abrogating behavioral responses associated with depression-related behaviors may provide a new target for development of antidepressant drugs or drugs effective in other 5-HT mediated disorders.

In conclusion, our study demonstrated that SQSTM1/p62 plays a role in mitochondrial functionality and provides a link to affective spectrum disorders such as anxiety and depression, as well as affecting cognitive learning and memory. It is possible that changes in behavior patterns can be related to p62 function outside of the mitochondria as it is ubiquitously expressed and plays a myriad of different roles in the cell [6, 22, 80]. However, p62 clearly plays a crucial role in maintaining mitochondrial homeostasis [70] and as we show here, overexpression improves functionality of the mitochondria. Given the increasing scientific support for a relationship between mitochondria and affective disorders [21, 55, 57], p62 would seem to be a novel target for potential drug discovery to treat anxiety and affective disorders.

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Table 1.

Behavior Test Statistics

Test	I.D.	Mean +/- S.E.	N	Comparison	df	*t*	*p*	Adjusted *p*
Open Field Maze								
Locomotion	WT	615.16 +/- 34.88	24	WT v. OEp62	34	0.202	0.421	0.421
	OEp62	600.45 +/- 76.59	12	WT v. p62:P392L	50	1.462	0.075	0.225
	p62:P392L	555.79 +/- 22.83	28	OEp62 v. p62:P392L	38	0.738	0.232	0.421
Thigmotaxis								
Outer Zone (%)	WT	82.78 +/- 1.27	24	WT v. OEp62	34	10.005	5.75E-12	<0.0001
	OEp62	41.78 +/- 5.33	12	WT v. p62:P392L	50	6.673	9.76E-09	<0.0001
	p62:P392L	57.46 +/- 3.34	28	OEp62 v. p62:P392L	38	-2.59	0.007	0.007
Inner Zone (%)	WT	17.22 +/- 1.27	24	WT v. OEp62	34	-9.933	6.93E-12	<0.0001
	OEp62	58.22 +/- 5.35	12	WT v. p62:P392L	50	-6.673	9.76E-09	<0.0001
	p62:P392L	42.54 +/- 3.34	28	OEp62 v. p62:P392L	38	2.562	0.007	0.007
Elevated Plus Maze								
Anxiety	WT	0.52 +/- 0.046	20	WT v. OEp62	32	2.079	0.023	0.058
	OEp62	0.34 +/- 0.078	12	WT v. p62:P392L	26	0.87	0.196	0.196
	p62:P392L	0.61 +/- 0.111	6	OEp62 v. p62:P392L	20	-1.997	0.029	0.059
Forced Swim Test								
Immobility Time (%)	WT	26.79 +/- 3.72	17	WT v. OEp62	24	0.543	0.296	0.386
	OEp62	29.92 +/- 4.45	13	WT v. p62:P392L	19	0.293	0.386	0.386
	p62:P392L	25 +/- 2.03	8	OEp62 v. p62:P392L	15	0.782	0.223	0.386
Swimming	WT	17.11 +/- 2.73	8	WT v. OEp62	18	-3.101	0.003	0.007
	OEp62	35.09 +/- 2.25	12	WT v. p62:P392L	12	-3.529	0.002	0.006
	p62:P392L	31.17 +/- 1.51	6	OEp62 v. p62:P392L	16	0.345	0.367	0.367
Climbing	WT	36.11 +/- 5.96	8	WT v. OEp62	18	4.17	0.0003	0.0009
	OEp62	15.27 +/- 3.14	12	WT v. p62:P392L	12	2.464	0.015	0.029
	p62:P392L	22.83 +/- 1.42	6	OEp62 v. p62:P392L	16	-1.769	0.048	0.048
Barnes Maze								
Acquisition Day 5	WT	31.51 +/- 9.15	8	WT v. OEp62	11	1.458	0.086	0.173
	OEp62	13.71 +/- 4.18	5	WT v. p62:P392L	15	1.746	0.051	0.152
	p62:P392L	14.96 +/- 3.75	9	OEp62 v. p62:P392L	12	-0.209	0.419	0.419
Probe Trial 1	WT	36.6 +/- 4.85	8	WT v. OEp62	11	0.247	0.405	0.405
(Short Term)	OEp62	34.98 +/- 2.64	5	WT v. p62:P392L	15	-0.926	0.185	0.369
	p62:P392L	42.64 +/- 4.37	9	OEp62 v. p62:P392L	12	-1.219	0.123	0.369
Probe Trial 2	WT	22.86 +/- 1.98	7	WT v. OEp62	10	-3.676	0.002	0.006
(Long Term)	OEp62	41.46 +/- 5.54	5	WT v. p62:P392L	14	4.78	0.32	0.32
	p62:P392L	25.34 +/- 4.28	9	OEp62 v. p62:P392L	12	2.333	0.019	0.039

Adjusted *p* = Step-up Bonferroni Adjustment for Multiple Comparisons

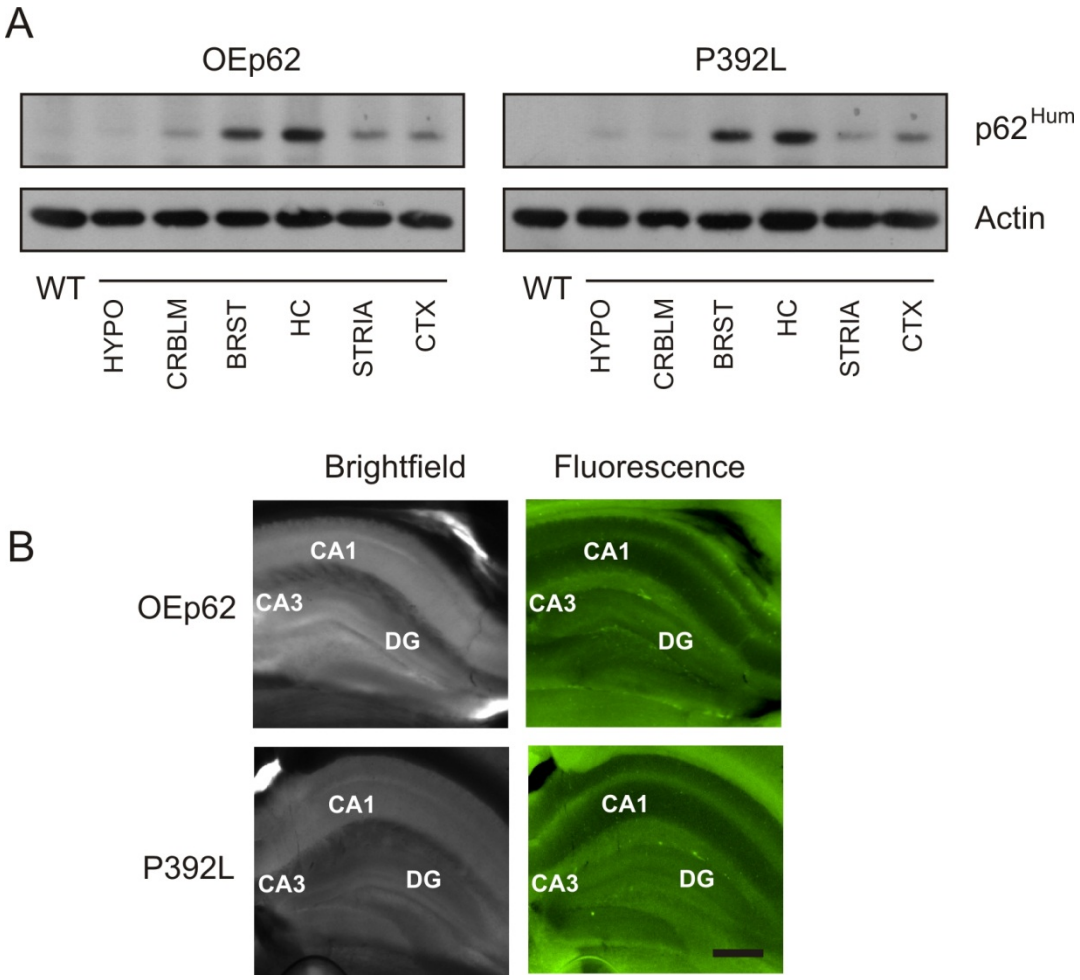

Fig. 1. Demonstration that EFGP tagged proteins were effectively expressed in mouse brain. A) Overexpressed EGFP-p62 protein was mainly localized to the hippocampus in transgenic mice. Mouse brain from either OEp62 or p62:P392L expressing mice were dissected into regions as indicated (HYPO=hypothalamus; CRBLM=cerebellum; BRST=brain stem; HC=hippocampus; STRIA=striatum; CTX=cortex) and Western blotted with $p62^{Hum}$ antibody to detect human specific overexpressing p62 protein. β-actin levels were used as control to indicate loading. B) Sagittal plane slices (200μm) were made through the hippocampal region of mouse brains from OEp62 and p62:P392L mice and examined under a fluorescent microscope at 488nm to show localization of EGFP tagged proteins in the hippocampal region. Bright field images are provided for structural reference. CA1, CA3 and DG regions in the hippocampus are labeled for orientation. Scale bar = 300μm.

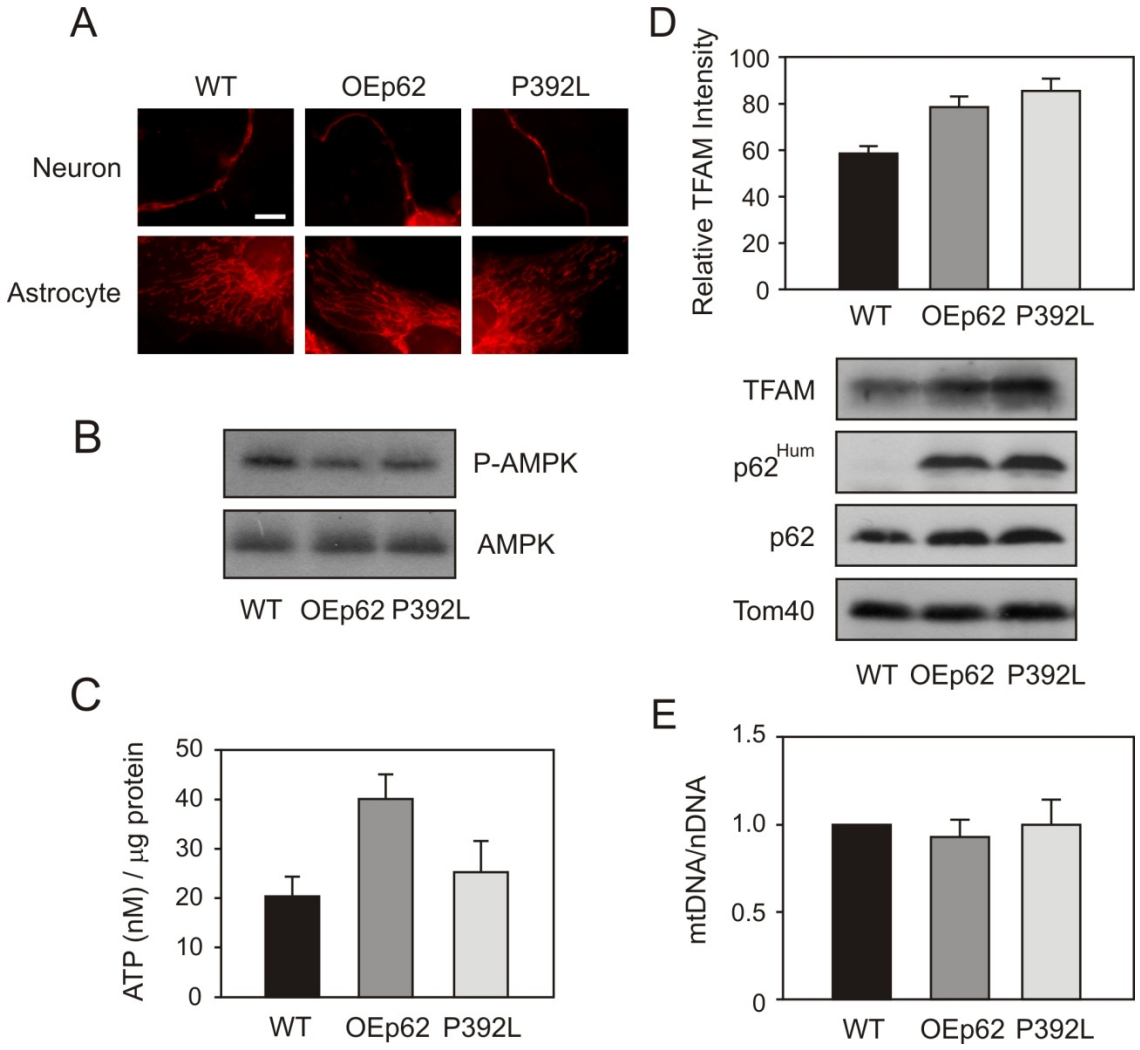

Fig. 2. p62 overexpression increases the functionality of mitochondria in the hippocampus. A) Mitochondrial morphology was altered in OEp62 mice compared to WT and p62:P392L. Primary neuronal cells were cultured from dissected embryonic Day 19 hippocampus. At culture day 7, mitochondria were visualized by staining with MitoTracker Red and examined using a confocal microscope. B) Activated AMPK levels were decreased in p62 overexpressing tissue. Hippocampal lysates from WT, OEp62 and p62:P392L mice were separated on SDS-PAGE and phospho-AMPK levels compared to total AMPK examined by Western blot. C) Overexpression of p62 results in increased ATP production. Total ATP levels in hippocampal lysates were measured by luciferase assay and compared to WT. D) Mitochondrial import was increased with p62 overexpression. Mitochondria were isolated from hippocampal lysates and import of TFAM examined by SDS-PAGE and Western blot. Presence of overexpressed protein was confirmed with $p62^{Hum}$ antibody and compared to native protein. E) p62 overexpression did not show increased mtDNA levels. Total mitochondrial DNA copy number was quantitated by RT-PCR.

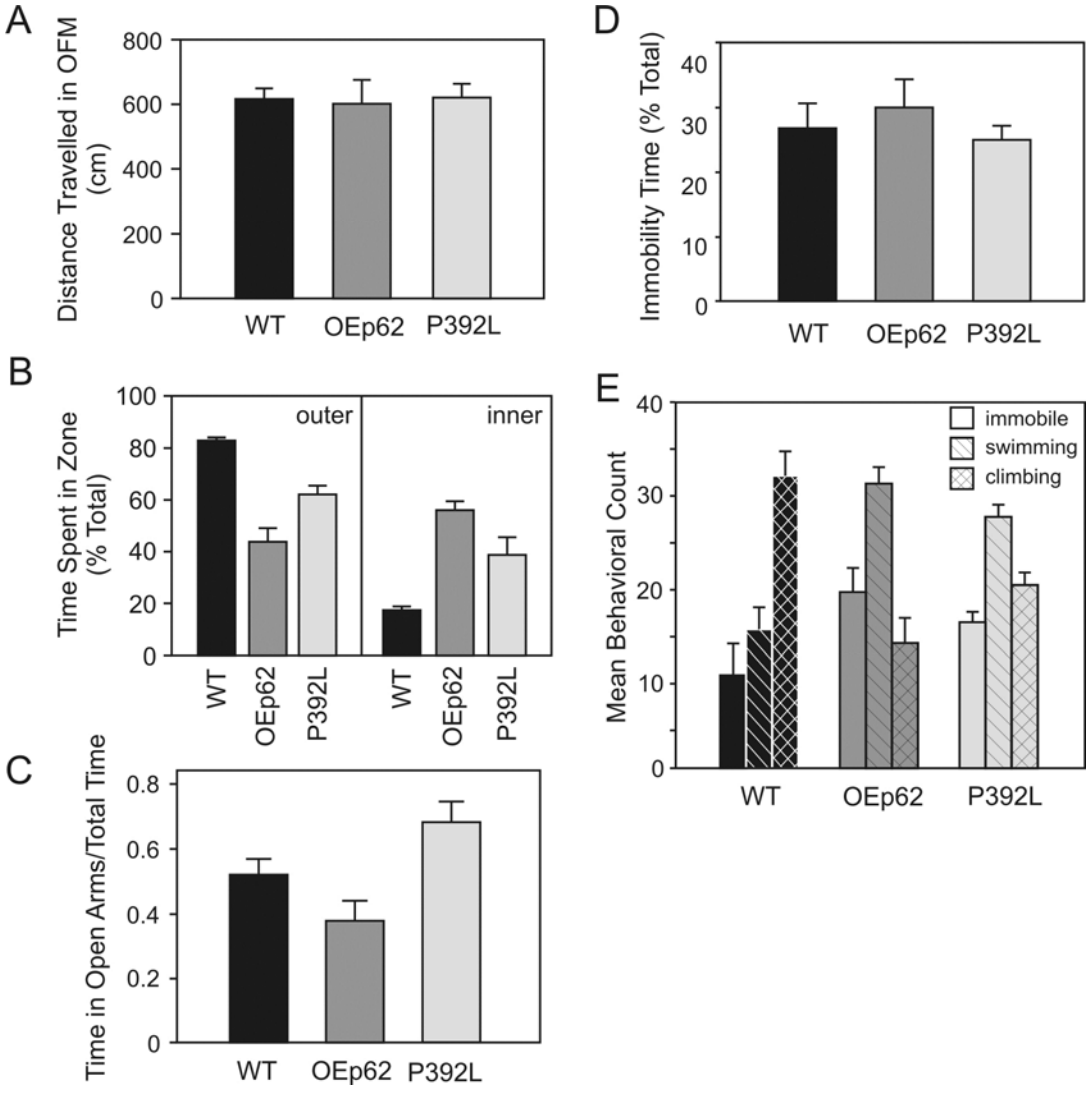

Fig. 3. p62 overexpression influences affective disorder behavior patterns. A) Behavior in the Open Field Maze: there was no significant difference in distance traveled in the maze between genotypes. B) Open field behavior analyzed by zone: p62 overexpression resulted in increased maze exploration toward to the center and away from the walls by genotypes. C) Anxiety measure in the Elevated Plus Maze: overexpression of p62 substantially reduced the time spent in open arms of the maze, however, expression of P392L protein increased time spent on open arms. D) Depression measure in the Forced Swim Test: no difference in behavior was indicated when using immobility time as a measure. E) Mean behavior exhibited during the Forced Swim Test: overexpression of p62 changed the overall behavior patterns resulting in increased swimming activity and decrease climbing compared to WT. Refer to Table 1 for analysis of statistics.

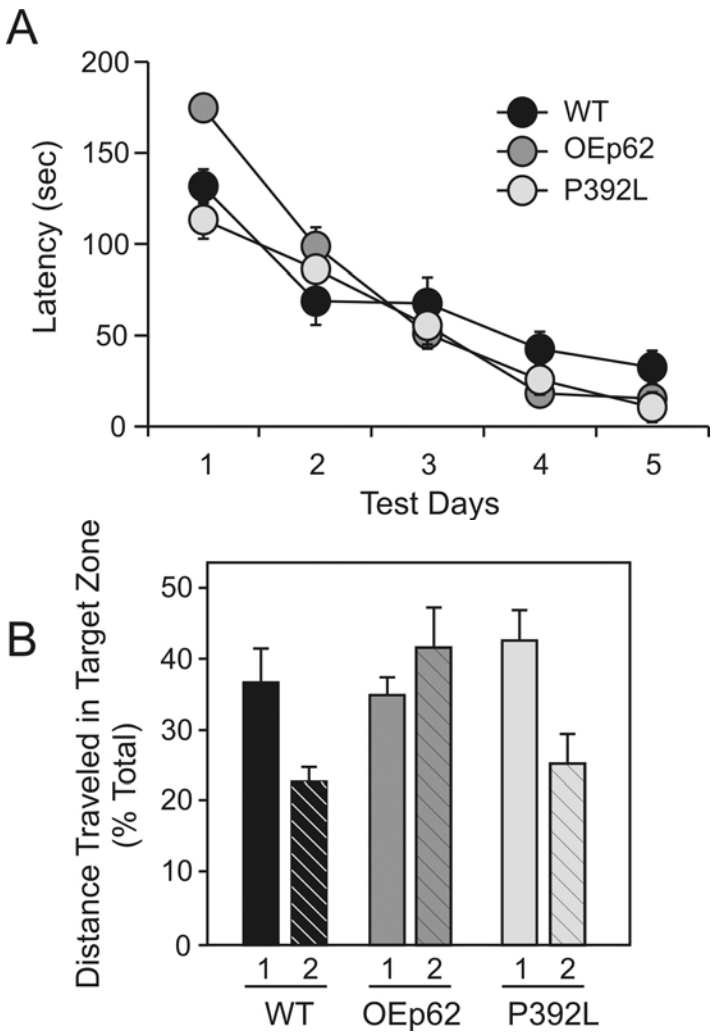

Fig. 4. p62 overexpression affects spatial learning and memory tasks. A) Latency times to complete the Barnes Maze: overexpression of p62 improved latency times compared to WT on last day of Acquisition period. B) Probe Trials in the Barnes Maze (1=Probe Trial 2 hours post Day 5 acquisition; 2=Probe Trial 6 days post Day 5 acquisition): no significant differences in short term memory measure during Probe Trial 1 were observed between genotypes, however overexpression of p62 improved long term memory in Probe Trial 2 compared to WT and p62:P392L. Refer to Table 1 for analysis of statistics.

Summary and Conclusions.

Mitochondria have been intimately linked with neurodegeneration and neurodegenerative diseases. Specifically, dysfunction of the electron transport chain in neuronal cells leads to increased ROS production which results in increased damage to DNA and misfolding of proteins. A cells inability to handle these damaged products is directly proportional to the level of neurodegeneration observed in the affected organism. SQSTM1/p62 has previously been linked with neurodegeneration and autophagic processes for clearance of aggregated and misfolded proteins. A mouse model lacking p62 has shown increased neurodegeneration and tau protein insolubility leading to an AD phenotype as well as, exhibiting premature aging related to increased levels of oxidative damage and mitochondrial dysfunction. p62 has also been identified as an integral component to the mitophagy pathway for clearance of damaged mitochondria. Such dysfunctional mitochondria exhibit impaired energy metabolism, but also are being recognized as a significant pathophysiology of behavioral disorders, specifically those related to anxiety. Thus, p62 could be an intriguing target for potential drug discovery to treat not only neurodegenerative diseases, but also anxiety and affective disorders.

The ability to grow and culture primary neuronal cells is an indispensable part of neuroscience. Primary cultures allow analysis of specific cellular pathways, target localization and organelle movement which would be impossible to decipher in intact brain tissue. To study the effects p62 has on signaling cascades, mitochondrial movement and morphology, it was paramount to first establish a working culture protocol for the growth of hippocampal neurons.

Besides exhibiting a relatively simple cellular architecture, the hippocampus is implicated in learning, memory and affective spectrum disorders. While hippocampal cultures are most typically generated from rats, the models used here were mouse models which are generally more difficult to isolate hippocampal neurons from due to the difference in size of the hippocampus in embryonic pups. Thus, a culture protocol and growth of relatively pure primary cultures of hippocampal neurons from embryonic mouse brain was established and used throughout.

As previously mentioned, p62 plays an important role in the degradation of misfolded proteins and dysfunctional mitochondria. However, these results have been described in systems under induced cellular stress. By using a p62 knock out mouse model, I was able to examine what role p62 plays at the mitochondria under non-induced conditions. p62 was localized to the mitochondria where a role in mitochondrial regulation and import was defined for the first time under non-stress induced conditions. I was able to show that p62 is integral for maintaining normal mitochondrial dynamics and, by regulating the import of the transcription factor TFAM, maintains mitochondrial genome stability. This importantly identifies p62 as not only being important for the trafficking and clearance of dysfunctional mitochondria, but also a required protein for normal mitochondrial function.

Changes in mitochondrial energy metabolism and function are among a list of potential causes for affective spectrum and anxiety disorders. Growing lines of evidence support a strong positive correlation between p62 expression and normal mitochondrial function. Loss of p62 specifically leads to mitochondrial dysfunction and, as has been shown previously, decreased hippocampal dependent cognitive ability and increase anxiolytic behavior. As overexpression of p62 in exogenous cell culture leads to improved mitochondrial dynamics, I sought to examine

the effect increasing levels of p62 in neural tissue would have on mitochondrial functionality and behaviors associated with mitochondrial dysfunction.

The laboratory has previously generated two mouse models with brain specific expression of fluorescently tagged p62 protein. One strain overexpresses the full length p62 protein while the other expresses a UBA domain p62 variant with the inability to bind ubiquitin chains. Using these two mouse models, I have shown that p62 overexpression increases the functionality of mitochondria in neural tissues as evidenced by the increase in ATP production and transcription factor import. Importantly, especially as relates to ATP production, the ability of p62 to bind ubiquitin plays a major role in mitochondria functionality.

Affective spectrum disorder behavior patterns are linked to dysfunctional mitochondria. As p62 can improve mitochondrial dynamics, I examined behavioral patterns of both mouse models for affective spectrum and anxiety related behaviors and compared them to WT mice. Overexpression of p62 showed an overall improvement in anxiety and depression behaviors in mice. This study also suggested a role for improved mitochondrial function in learning and long term memory formation, as well as again implicating p62's ability to interact with ubiquitin possibly playing a role in behavioral patterns.

Taken as a whole, I have shown for the first time that p62 is required for normal mitochondrial function as well as identifying it as an attractive novel target for potential drug discovery to treat anxiety and affective spectrum disorders. The ability of p62 to interact with ubiquitin chains or ubiquitinated substrates appears to play an important role in mitochondria functionality and associate behaviors but requires further study, not only at the molecular level,

but also in relation to how behavior patterns are regulated and how memory is learned and formed.