Final Report

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Date: April 12, 2021
Title of Project: Molecular analyses of pre-synaptic compartments in diseased human AD brains.

Summary: (approximately 1,000 words).
Neuronal synapses are the specialized connections between neurons and/or with organs in the body. In the brain it is the function of such connections that allow us to think, learn, remember, and respond to our environment. The loss of synapses in the brain is a common feature in many neurodegenerative conditions, such as Alzheimer's Disease (**AD**).

The loss of synaptic function which triggers synaptic loss can be brought about by either a lack of energy that drives synaptic communication between neurons, and/or a lack of the protein synthesis needed for neuro-chemical metabolism and -transmitter release at synapses. Energy production for synaptic communication is driven by mitochondria that are enriched within nerve terminal endings, whereas protein synthesis can originate close to the neuron's cell body for transport to the distal nerve terminal *or occur locally within the nerve terminal ending*. The protein machinery (translation) needed to make proteins from RNA is also an energy driven process. Hence it makes sense that the pre-synaptic compartment of synapses contains both mitochondria and the translation machinery to allow synapses to regulate the synthesis of neuro-chemicals and the associated synaptic proteins needed for synaptic function.

With support from the Brain Foundation we examined ultrastructure, proteome profiles, respiration, and calcium influx in response to membrane depolarization, of isolated nerve terminal endings (termed **synaptosomes, Figure 1A**) from cryopreserved human autopsy material stored in the Queensland Brian Bank (**QBB**). This included control and vulnerable regions from diseased brains (**AD** and Motor Neuron Disease [**MND**]). The following is a summary of our research progress.

We have completed our ultrastructural analyses of synaptosomes from frozen autopsy brains and found that the size and synaptic contents were the same as seen in synaptosomes prepared from non-frozen and frozen mouse brains: namely, they were 500 nm in diameter, and contained mitochondria and synaptic vesicles (**Figure 1B** to **1E**). There was no difference between synaptosomes prepared from non-frozen and frozen mouse brains. These results demonstrate that synaptosomes from frozen human brain contain the intracellular elements needed for respiration (energy production) and neuro-transmitter release from synaptic vesicles.

Based on these observations, we then conducted a series of biochemical and functional assessments of re-thawed human synaptosomes isolated from control and vulnerable regions of AD and MND frozen autopsy brains. First, we conducted proteomic profiling and confirmed the presence of proteins needed for the release of neuro-transmitters from nerve terminals (**Figure 2**). We also observed low levels of proteins that are enriched in post-synaptic regions, which is not surprising because isolated synaptosomes have post-synaptic membranes tethered to them *via* the adhesion proteins neuroligin and neurexin. Interestingly, we did observe an increase in the expression of stress response proteins in synaptosomes prepared from vulnerable regions of AD brains (**Figure 3**). This preliminary observation is currently being pursued.

Next, we examined the mitochondrial respiratory capacity of human synaptosomes isolated from re-thawed control and vulnerable regions of AD and MND frozen autopsy brains. To achieve this goal, we first compared the oxygen consumption rates (**OCR**) of mitochondria from synaptosomes prepared from frozen brain tissue (mouse and human) to synaptosomes prepared from non-frozen (acutely dissected) mouse brain tissue. We demonstrated that synaptosomes prepared from frozen/re-thawed brain tissue (mouse and human) displayed mitochondrial respiration. Further, we were able to show that basal respiration rates could be transiently increased by depolarization induced by the addition of veratridine – a compound that prevents Na^+ channel inactivation to increase ATP hydrolysis (**Figure 4**). These

observations, to our knowledge, are the first to demonstrate that synaptosomes prepared from frozen-thawed autopsy brains can respire, and suggest that they have the capacity to provide energy (ATP) to drive nerve terminal function. Given that human synaptosomes can respire, we then wanted to compare the mitochondrial respiratory capacity of synaptosomes prepared from diseased (vulnerable) and non-diseased (relatively spared) regions from AD and MND brains. For AD brains the oxygen consumption rates (**OCR**) of synaptosomes from vulnerable and protected regions were not significantly different (**Figure 5A**). For MND brains we also observed no significant difference in the oxygen consumption rates of synaptosomes from control and vulnerable regions (**Figure 5B**).

The release of neuro-transmitters from nerve terminal endings is governed by the influx of Ca^{2+} ions. This influx is *via* voltage gated Ca^{2+} channels that open when the terminal membrane is depolarized, triggered by the arriving action potential. We therefore used a Ca^{2+}-sensitive fluorescent indicator that detects the rise in intracellular Ca^{2+} ions in response to membrane depolarization by KCl (**Figure 6A**). Synaptosomes prepared from human control and disease-vulnerable brain regions in neurodegenerative disease (AD and MND) showed no difference in Ca^{2+} ion levels after KCl membrane depolarization: that is, synaptosomes from both regions showed a rapid rise in intracellular Ca^{2+} in response to KCl (arrow in **Figure 6B**).

Collectively our respiratory and Ca^{2+} mobilization findings are not surprising if one considers that the remaining nerve terminals are not vulnerable, given their presence at disease end-stage. We are continuing with these experiments to increase our biological sample size for meaningful statistical comparisons and to consolidate our conclusions. The fact that we have shown that frozen-thawed human brain tissue can be used to study the functions of nerve-endings opens a new research door into the study of neurodegenerative diseases.

Hypothesis vs Findings

Utilizing human disease-affected autopsy brain tissue cryo-stored in the Queensland Brain Bank (**QBB**), we expected to see different profiles for synaptic protein content, respiration, and Ca^{2+} influx in synaptosomes isolated from disease-vulnerable regions compared with control regions. The fact that we did not see any significant differences in these parameters did challenge our original hypothesis that the remaining synapses at end-stage disease would show decreases in these pre-synaptic parameters. This may be because following the loss of vulnerable synapses in the early and mid-disease periods, the remaining synapses at end-stage disease remain functional. This opens a research opportunity to understand what allows the end-stage synaptic population to remain functional, and/or the possibility to look for disease-related fingerprints that may lead to subsequent synapse death.

Unanswered Questions

An interesting finding that we are keen to pursue with more biological samples is the up-regulation of stress-response proteins in synaptosomes from disease-vulnerable regions of AD brains. Increased stress response within the cytoplasmic compartments of cells – such as the nerve terminal – can lead to a halt in the translation of new proteins and/or an increase in oxidative free radicals, both of which can degrade cellular function and trigger synaptic loss.

What these research outcomes mean

We have demonstrated that frozen-thawed human and mouse brain tissue can be used to study the metabolic function of nerve terminals; that is, synaptosomes prepared from cryopreserved brain tissue satisfy the metabolic requirements for a pre-synaptic model. With mouse synaptosomes we can interrogate metabolic function prior to and during the loss of synaptic connections; findings can then be translated to human synaptosomes. Perhaps what is most exciting is our observations that remaining synapses at disease end-stage remain metabolically functional. Understanding the basis of this may have implications for how we may prevent or slow the rates synaptic loss in disease and old age.

Data Figures cited in the Summary.

FIGURE 1

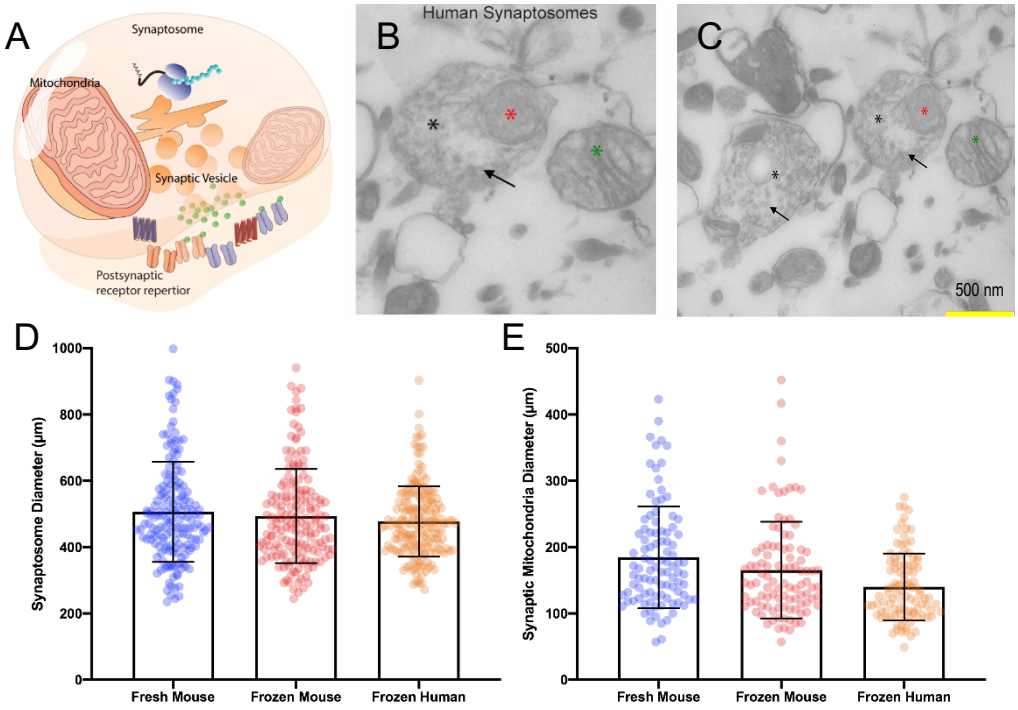


***Figure 1**: Synaptosomes isolated from frozen autopsy human brains contain the subcellular elements needed for nerve-terminal function. **A,** schematic representation of the subcellular elements of the nerve terminal – including mitochondria, RNA machinery, synaptic vesicles, and voltage gated Ca^{2+} channels (purple bars). **B** and **C,** electron micrographs of a mouse synaptosome (B) and a human synaptosome (C). The human synaptosome had been prepared from human cryopreserved autopsy material. Red asterisks represent synaptic mitochondria; green asterisk represents free mitochondrial contamination. Scale bar = 500 nm. **D** and **E**, bar graphs represent the mean sizes of fresh mouse, frozen mouse, and frozen human autopsy sample synaptosome (D) and mitochondria (E) diameter (μm^2).*

FIGURE 2

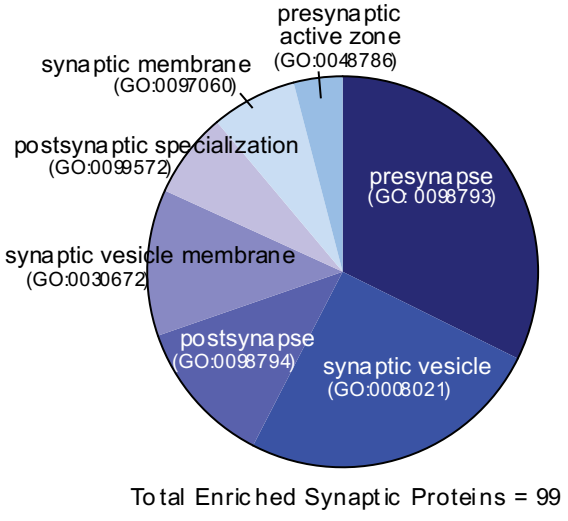


***Figure 2.** Proteomic profiling of synaptosomes prepared from slow-frozen, rapid-thawed (0.32 M sucrose) cortex. Information identified from a database comparison with a low false detection rate ($p > 0.05$) was established in Protein Pilot and clustered based on biological components using PANTHER software. Groups were scored and the figure was created using GraphPad Prism 8.*

FIGURE 3

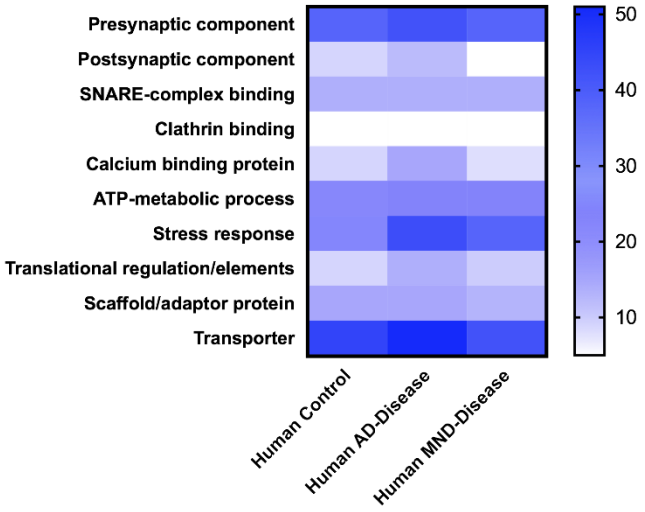


***Figure 3**. Upregulation of stress response proteins in synaptosomes prepared from the vulnerable regions of AD brains. The shade/intensity of blue indicates increasing protein levels. Proportion of relative abundance protein groups identified post 95% cut off for peptide sequence detection coverage.*

FIGURE 4

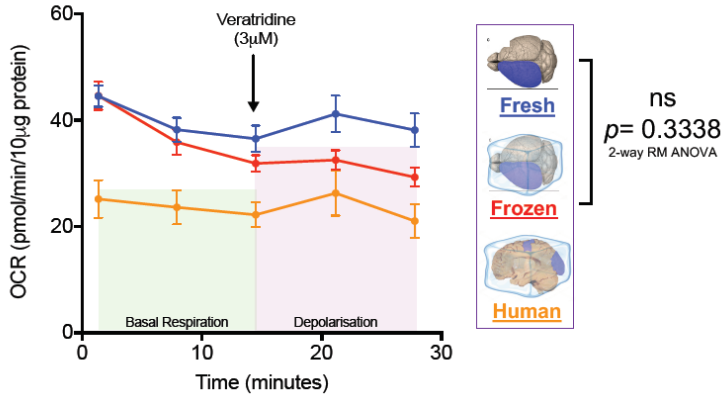


***Figure 4.** Respiration rates of frozen and fresh (non-frozen) prepared synaptosome show similar Oxygen consumption rates (**OCR**) over 30 mins. Shown are the means of OCRs for mouse synaptosome prepared from fresh (blue), and frozen thawed (red) brains. The OCR for synaptosomes prepared from frozen-thawed autopsy brains are shown in orange. As reported from the literature, mouse brains are metabolically more active compared to humans, hence the expected higher OCRs for mouse.*

FIGURE 5

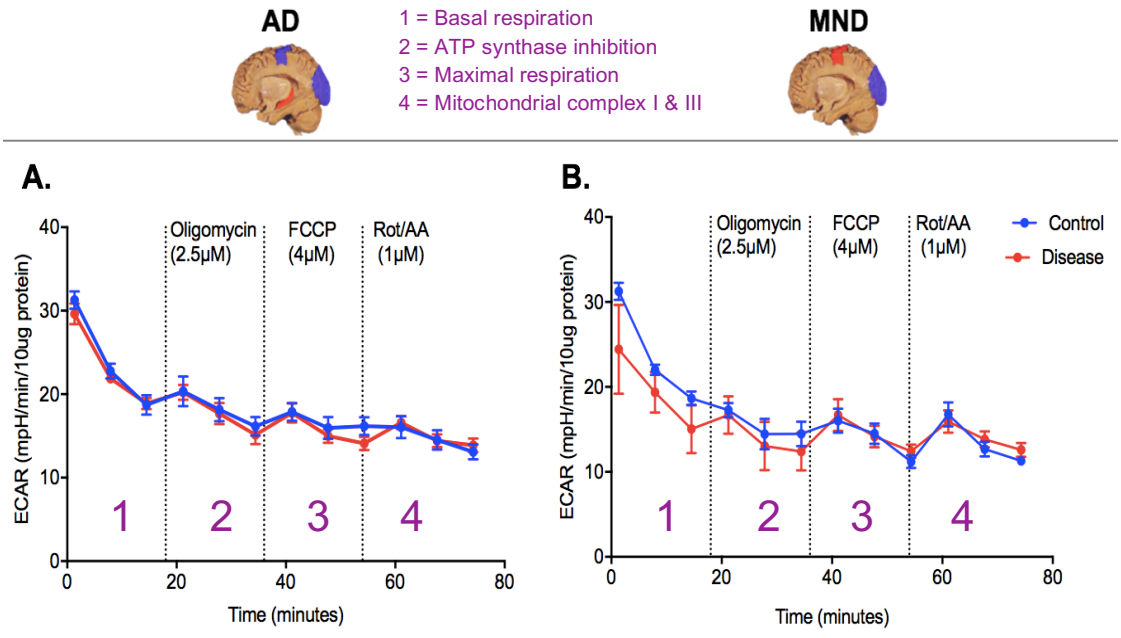


Figure 5: *Synaptosomes prepared from human control and disease-vulnerable brain regions in neurodegenerative disease (AD and MND) show no respiratory disparity. Basal OCR, oligomycin (2.5 µM) + FCCP (4 µM) + rotenone/antimycin-a (Rot/AA; 1 µM)-stimulated respiration was monitored in the Seahorse XFe96 analyser system (Agilent). Data points represent the mean ± SEM for normalized synaptosomal OCR (per 10 µg protein). Seven replicates (T = 7) were measured per sample. Three measurements were made per injection.* ***A****, In AD samples (n = 2), two brain regions were selected per individual; the mid-temporal lobe (hippocampus), representing disease, and a non-specific cortical region representing control (motor cortex and/or occipital cortex; Figure 4).* ***B****, the MND case (#5,* ***Table 1****) was selected for the primary motor cortex (disease) and the occipital lobe (control).*

FIGURE 6

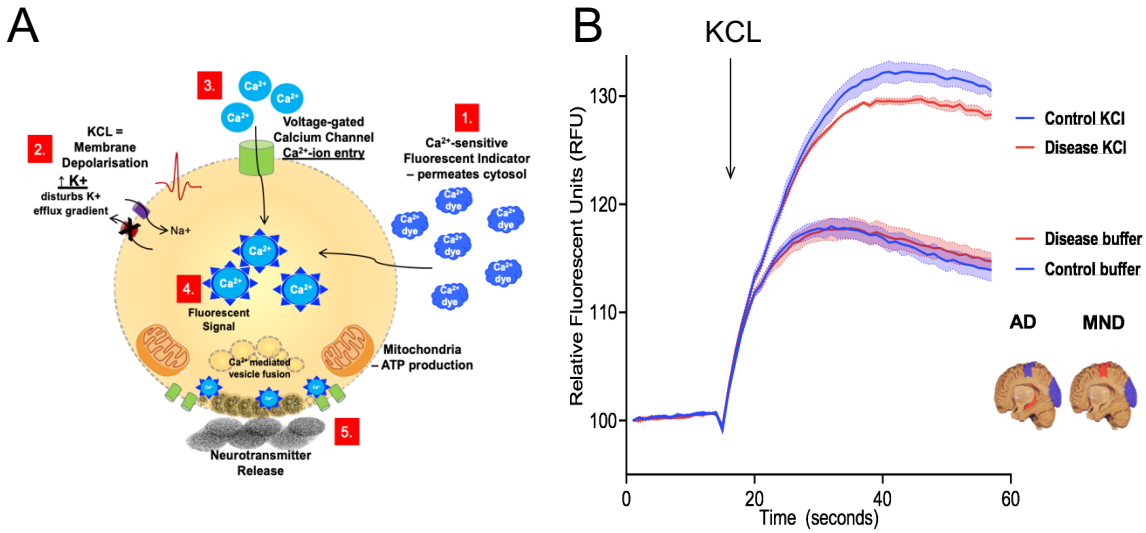
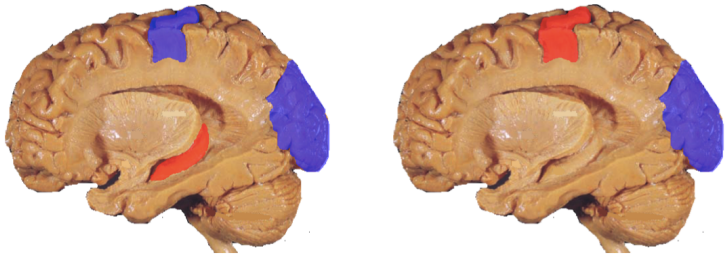


Figure 6*: Synaptosomes prepared from human control and disease-vulnerable brain regions in neurodegenerative disease (AD and MND) show no difference in Ca^{2+} mobilization.* ***A****, schematic diagram of the Ca^{2+} indicator assay. In brief****: (1)*** *Synaptosomes are pre-incubated with Calcium-4 indicator Dye. The dye permeates intracellularly and is then free to bind to Ca^{2+} ions within the synaptosome* ***(4).*** *Upon KCl exposure* ***(2),*** *synaptosomes are depolarized, which allows the rapid diffusion of Ca^{2+} ions into the synaptosome* ***(3).*** *The rise in Ca^{2+} is captured as increased relative fluorescent units (****RFU****).* ***B****, the rapid rise in Ca^{2+} after the addition of KCl – seen for synaptosomes prepared from both control and disease-vulnerable regions (AD + MND). In the AD samples, two brain regions were selected per individual: the mid-temporal lobe (hippocampus) and a non-specific cortical region that shows no abnormalities documented in macro/microscopic examination at autopsy. The same was conducted for MND sample (the diseased primary motor cortex and the control occipital lobe). Synaptosome samples were seeded (in triplicate [****T****]). Stimulation by KCl and non-stimulus buffer control was recorded after 10 baseline measurements. Lines here represent the mean response of control and disease regions ± SEM.*

TABLE 1. Control and diseased regions of AD and MND brains.

A. **AD Pathology** **B.** **MND Pathology**



Control	Disease	Control	Disease
Motor Cortex Occipital Cortex	Temporal Lobe (entorhinal cortex and hippocampus)	Occipital Cortex	Motor Cortex

#ID	Age	Sex	PMI (hours)	Years in Storage (Years)	Clinical Diagnosis and Autopsy Report	Regions of Interest
1	78	Male	< 24	< 15	AD (Braak III-IV)	Hippocampus
2	79	Male	< 24	< 15	AD (Braak III-IV)	Hippocampus
3	81	Male	24	15	AD (Braak III-IV)	Temporal Cortex
4	78	Male	47	17	AD (Braak III-IV)	Frontal Cortex
5	57	Female	< 48	< 15	MND	Motor Cortex
6	53	Male	44	12	MND	Motor Cortex
7	65	Male	59	12	MND	Motor Cortex
8	84.76	Male	84.76	18.93	MND	Motor Cortex
9	51.22	Male	51.22	12.07	MND	Motor Cortex
10	87.62	Male	87.62	11.53	Control	
11	77.84	Female	77.84	20.17	Control	*Absent from*
12	68.33	Female	68.33	17.55	Control	*disease-related*
13	76	Female	24	27	Pulmonary Embolus	*changes – Tissue*
14	67	Male	31	14	Melastatic Melanoma	*controls*
15	57	Female	9	21	Aortic stenosis	

***Footnote**: Regions and neurons that are vulnerable and spared from pathology in neurodegenerative diseases (AD and MND). **A**, in Alzheimer's pathology, the entorhinal cortex and hippocampus show early deficits and neuronal loss. These disease-vulnerable regions were selected for synaptosome isolation and compared with their respective regional controls. The occipital and motor cortices are relatively pathology-free until late in the disease (defined by Braak stage V-VI). **B**, in MND, upper motor neurons in the motor cortex are selectively vulnerable. The motor cortex was chosen to isolate synaptosomes with disease. The occipital cortex is free of pathology. All regions selected were confirmed in a database of histological morphology. The brain image annotated was sourced from color-atlas-of-anatomy-a-photog-study-of-the-humanbody-7th-ed-j-rohen-et-al-lippincott-2011*