Intranasal Administration of Exosome Packaged Catalase-SKL, a Novel Antioxidant Delivery System Across the Blood-Brain Barrier in mice: Biodistribution and Safety

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Anatomy and Cell Biology
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Abstract

A core pathological feature of AD is the over-production of reactive oxygen species (ROS), which mediate oxidative stress in the brain. Peroxisomes play a crucial role in mitigating ROS accumulation due to the presence of catalase, an antioxidant enzyme found within the organelle. As the brain ages, progressive dysfunction of peroxisomes and decreased localization of catalase into the organelle contributes to elevated ROS. A suitable therapy to address the increased accumulation of ROS in AD has yet to be identified. Our team has engineered a recombinant derivative of the antioxidant enzyme catalase (CAT-SKL) that specifically targets peroxisomes, thereby providing powerful organelle-based antioxidant and anti-inflammatory effects. In the present study, we aimed to (i) establish the packaging CAT-SKL into macrophage-derived exosomes — endogenous membrane vesicles for the transport of proteins between cells — to effectively target our therapeutic to cells undergoing oxidative stress while minimizing degradation in the bloodstream, (ii) determine the safety and biodistribution in vivo, and (iii) assess the protective effects of prophylactic intranasal administration of exosomes loaded with CAT-SKL (exoCAT-SKL). CAT-SKL was packaged into macrophage-derived exosomes via sonication, and loaded exosomes were purified using size exclusion chromatography or ultracentrifugation. Male and female, control, and 3xTG AD transgenic mice were intranasally administered exoCAT-SKL, and tissues of interest were investigated. The diffuse labelling of CAT-SKL in these mice's brains supports the intranasal administration of exosomes as an effective delivery method to bypass issues surrounding bioavailability, antigenicity, and the blood-brain barrier. Histopathological assessments of the brain and off-target tissue demonstrated no toxicity. Further behavioural and immunohistochemical analysis is required to determine whether exoCAT-SKL can prevent and ameliorate pathological and behavioural outcomes in 3xTG AD mice.

Keywords: Alzheimer’s disease, Oxidative Stress, Drug delivery and toxicology, Exosomes, Therapeutic antioxidants, Catalase
Lay summary

This project investigated the use of nanosized vesicles called exosomes to carry and deliver CAT-SKL, an antioxidant molecule, into a preclinical transgenic mouse model of Alzheimer’s disease (AD). Human patients with AD demonstrate progressive dysfunction of peroxisome-mediated lipid oxidation leading to excessive accumulation of reactive oxygen species (ROS). CAT-SKL specifically targets these peroxisomes and provides antioxidant, anti-inflammatory and anti-ageing effects. Intranasal administration of CAT-SKL loaded exosomes can penetrate the brain, potentially preventing and ameliorating pathological, biochemical, and behavioural outcomes. This project also provided further validation for the use of nanoparticles to deliver therapeutics to the brain.
Statement of co-authorship

The work presented as Aim 1 in this study was presented at the 2019 Society for Neuroscience Annual Meeting with the title Evaluating the Neuroprotective Potential of Exosome Delivered Catalase-SKL in a Pre-clinical Model of Alzheimer's disease and was coauthored by Dr. Sarah Hayes, Dr. Patti Kiser, Sureka Selvakumaran, Dr. Brian Allman, Dr. Paul Walton and Dr. Shawn Whitehead. In addition, a manuscript of the work presented as Aim 1 in this study titled Intranasal Administration of Exosome Packaged Catalase-SKL, a Novel Antioxidant Delivery System Across the Blood-Brain Barrier: Biodistribution and Safety is being prepared with coauthors Dr. Sarah Hayes, Sureka Selvakumaran, Dr. Elena Batrakova, Dr. Brian Allman, Dr. Paul Walton, Dr. Shawn Whitehead & Dr. Patti Kiser. Experiments of Aim 1 of this study was aided by Dr. Sarah Hayes. Histological analysis of off-target tissue (Table 2) was conducted by Dr. Patti Kiser and Sureka Selvakumaran.
Acknowledgements

I want to thank my supervisors Dr. Shawn Whitehead and Dr. Paul Walton, as well as the Department of Anatomy and Cell Biology, for the opportunity to pursue my master's degree. Dr. Whitehead and Dr. Walton have continually guided and supported my research journey. They have both inspired and motivated me to continue pursuing science and research. I am honoured to have worked in Dr. Whitehead's lab since I was in high school and incredibly appreciative of all of the wisdom he has shared over the years.

I want to express my appreciation and gratitude to my supervisory committee members Dr. Brian Allman and Dr. Patti Kiser. Thank you for your patience and for sharing your expertise throughout my master's degree. A special thank you to Dr. Lin Zhao, Dr. Lynn Wang and Dr. Sarah Hayes, for their invaluable technical expertise to overcome many crucial elements of this project.

Thank you to all of the Vulnerable Brain Lab members for their help throughout my master's degree. Thank you to Kyra Keer and Romit Bhusari, who have spent many hours helping me with experiments and tissue processing.

Lastly, I'd like to express my gratitude to my friends and family for always being there for me.
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<td>Beta-amyloid</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>ApoE4</td>
<td>Apolipoprotein E4 allele</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
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<tr>
<td>sAPP-α</td>
<td>α-secretase cleaved amyloid precursor protein</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain-barrier</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>CAT-SKL</td>
<td>Catalase-serine-lysine-leucine</td>
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<td>ExoCAT-SKL</td>
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<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>KANL</td>
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<tr>
<td>MWM</td>
<td>Morris water maze</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEX5p</td>
<td>Peroxisomal targeting signal type 1 receptor</td>
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<td>Parkinson’s disease</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
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Section 1: INTRODUCTION
1.1 Alzheimer’s disease

Alzheimer's disease (AD) is a neurodegenerative disorder that causes a progressive decline in a person's cognitive and behavioural abilities (Neugroschl and Wang, 2011). AD has a devastating impact not only on the diagnosed individual but also on caregivers and healthcare systems (Koca, Taşkapilioğlu and Bakar, 2017). In Canada, over half a million people are living with AD, and the number of AD cases is expected to double in the next 15 years (Alzheimer's Disease International, 2019). Globally, in 2006, an estimated 26.6 million people were living with AD, and over 106 million cases are projected by 2050 (Brookmeyer et al., 2007). Symptoms of AD begin with confusion, amnesia, and progress to severe cognitive impairments such as memory and language deficits, and executive dysfunction ( Förstl and Kurz, 1999; Humbert et al., 2010). Eventually, the deterioration and loss of critical bodily functions such as the ability to swallow cause severe complications and lead to death (Zanetti, Solerte and Cantoni, 2009). This progression is variable as the typical life expectancy following diagnosis is between 3 and 9 years. Currently, Canada is ill-equipped to manage the growing costs and lacks the infrastructure to manage this public health crisis (Chambers, Bancej and Mc Dowell, 2016). Understanding the etiology and pathology of AD are essential first steps in combating the AD epidemic and reducing the burden to healthcare resources.

AD can be categorized into early-onset and late-onset AD. Two universal hallmark pathologies of the AD brain are extracellular β-amyloid (Aβ) plaque deposition and intracellular aggregates of tau protein forming neurofibrillary tangles. As AD progresses, these two pathologies are strongly linked to neuroinflammation, the loss of neurons and synapses, as well as the enlargement of ventricles due to whole brain atrophy (Perl, 2010). Several genetic mutations have been identified as the cause of the early-onset autosomal dominant form of AD. These mutations
are found in the amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2) (Piaceri, Nacmias and Sorbi, 2013). In particular, APP, PS1, and PS2 mutations affect the processing of APP at γ-secretase or β-secretase cleavage sites. These mutations result in an increased production of Aβ peptides leading to an increased accumulation of amyloid plaques (Bhadbhide and Cheng, 2012; Kelleher and Shen, 2017). The majority of AD cases are not categorized as early-onset. It is estimated that up to 70% of cases are of the late-onset category (Piaceri, Nacmias and Sorbi, 2013). The most prevalent genetic risk factor for developing late-onset AD is the apolipoprotein E (ApoE) 4 allele (Vanitallie, 2013; Safieh, Korczyn and Michaelson, 2019). ApoE plays an essential role in cholesterol transport and regulating lipid metabolism in the brain (Huang and Mahley, 2014). Possession of the ApoE 4 allele results in reduced Aβ clearance by the glymphatic system and astrocytes, one of the brain's glial cell types. As such, individuals homozygous for the ApoE 4 allele have up to 12 times the risk of developing AD (Michaelson, 2014; Fernandez et al., 2019). The etiology of AD remains convoluted due to the complexity of the disease as it involves a combination of protein misfolding and aggregation, neuroinflammation, and oxidative stress, amongst other environmental factors (Jiang et al., 2013).

Although genetics convey additional risks for affected individuals, the most significant known risk factor for AD is age (Guerreiro and Bras, 2015). The majority of AD patients are older than 65, with a higher incidence among older populations. Suggested practices by the National Institute of Health to prevent AD or delay the age of onset include: managing comorbidities such as diabetes and high blood pressure, increasing physical activity, maintaining a healthy balanced diet, and staying cognitively stimulated (National Institute of Health, 2018). These nonpharmacologic measures are also crucial in the management of symptoms as a cure for AD does not yet exist (Dharmarajan and Gunturu, 2009). Available treatments that target Aβ
production, aggregation, or clearance have not led to curative clinical outcomes (Yiannopoulou and Papageorgiou, 2013; Mehta et al., 2017). The most common drug option, acetylcholinesterase inhibitors, have been shown to slow the progression of the disease but often lead to off-target toxicity such as hepatotoxicity and adverse gastrointestinal symptoms (Ali et al., 2015). Other drugs such as N-Methyl-D-aspartate inhibitors are better tolerated however are typically prescribed for advanced stage AD with modest benefits in patients (Olivares et al., 2013). Developing potential medications that are both safe and effective requires further knowledge of how AD disrupts the brain.

1.2 Anatomical brain areas associated with AD

As AD progresses, different areas of the brain are affected, highlighting the connections between anatomical and cognitive changes (Raskin et al., 2015). In earlier stages of the disease, cells of the hippocampus begin to degenerate, causing deficits in memory and learning. Significant reductions of neurons are seen in the CA1 and less so in CA3 hippocampal areas of patients with AD (Padurariu et al., 2012), which may partially explain the loss of memory seen in AD patients. The hippocampus is an especially vulnerable and essential brain area strongly tied to the cognitive impairments accompanied with AD (Halliday, 2017).

In the moderate AD stage, cerebral cortical atrophy occurs, causing severe behavioural deficits and the inability to perform simple tasks (Bakkour et al., 2013). Post-mortem examination of AD brains reveals a reduction in total brain size and weight, with widened sulci indicating the loss of cortical interneurons and neurotransmitter pathways (Whitwell, 2010). In human and rodent models of AD, a loss of cholinergic neurons is observed in the basal forebrain, while noradrenergic degeneration is observed in the locus coeruleus (Mufson et al., 2008). While this phenomenon is
observed in the moderate stage for humans, it is not replicated in rodent models of AD until later stages of the disease. Instead, preclinical moderate stage AD mouse models feature neuroinflammation marked by microgliosis, astrocytosis, and deposits of amyloid plaques (Saito and Saito, 2018).

Findings in rodents show that early-stage neuroinflammation and white matter etiologies contribute to AD progression in addition to grey matter etiologies. In particular, white matter inflammation and demyelination have been reported in the corpus callosum and the internal capsule preceding Aβ plaques, perhaps driving Aβ production (Raj et al., 2017). Developing drugs to target the hippocampus and white matter inflammation early on in AD progression may prevent or reduce cognitive decline.

1.3 Beta-amyloid, amyloid cascade hypothesis and neuroinflammation

The amyloid cascade hypothesis has been the most prominent AD research hypothesis since the early 1990s (Hardy and Allsop, 1991; Selkoe, 1991; Hardy and Higgins, 1992; Ricciarelli and Fedele, 2017). The hypothesis posits that excessive Aβ causes AD and triggers a cascade of downstream symptoms and pathologies such as cognitive decline, progressive neuronal injury, neuroinflammatory responses, and oxidative injury (Barage and Sonawane, 2015). Aβ is derived from APP, which is ubiquitously expressed but predominantly in the central nervous system (CNS) (Puig and Combs, 2013). APP is a highly conserved transmembrane protein; however, the exact function of APP is not fully understood. It is accepted that it plays a role in cell growth, cell survival, and synapse physiology (Bhadbhade and Cheng, 2012). The production of Aβ is a normal cellular process and begins with sequential cleavage of APP by two intramembranous endoproteases, β-secretase and γ-secretase. β-secretase cleaves APP into a protein that is 99 amino
acids in length, and γ-secretase subsequently cleaves the protein at the γ-cleavage site to 38 - 42 amino acids (Chow et al., 2010; Macleod et al., 2015). A third endoprotease, α-secretase, can also cleave APP; however, the resulting peptide (sAPP- α) does not lead to Aβ formation (Zhang et al., 2011). Ninety-percent of the cleaved APP is 40 amino acids in length (Aβ40), and the less abundant cleaved APP is 42 amino acids in length (Aβ42) (Qiu et al., 2015). The two isoforms of Aβ differ by two amino acids, isoleucine and alanine, with Aβ42 more highly related to AD pathogenesis compared to Aβ40. Aβ42 demonstrates increased aggregation and fibril formation, which contribute to amyloid plaques formation (Cline et al., 2018). Aβ42 has also been reported to have higher cellular toxicity, and clinical experiments suggest mutations causing AD result in an increase in the Aβ42 to Aβ40 ratio (Lehmann et al., 2018). Both isoforms of Aβ can be found in the cerebrospinal fluid (CSF) with 50-70% in the form of Aβ40 and roughly 10% as Aβ42 in healthy adults. Aβ37, Aβ38, Aβ39, and Aβ43 isoforms make up the remaining Aβ found in the CSF (Qiu et al., 2015). An increased ratio of CSF Aβ42 to Aβ40 in combination with CSF concentrations of tau species has been used as a diagnostic tool of AD (Hansson et al., 2019).

Several mechanisms exist to catabolize Aβ42 for degradation or transport out of the CNS. Enzymes such as neprilysin and insulin degrading enzyme play essential roles in the extracellular degradation of Aβ while transport proteins called low-density lipoprotein receptor-related protein I are responsible for transcellular transport of Aβ across the blood-brain-barrier (BBB) (Deane et al., 2009; Grimm et al., 2013). The amyloid cascade hypothesis proposes that neurodegeneration arises when Aβ is overproduced, and the clearance and degradation are reduced, causing the buildup of Aβ (Barage and Sonawane, 2015).

The hydrophobic nature of Aβ42 enables the formation of β sheet protein structures, and other structures such as fibrils and oligomers (Baldassarre et al., 2017). Aβ oligomers are reported
to be more toxic than Aβ fibrils and cause oxidative damage, cell membrane disruption, and induce neural cell apoptosis pathways (Verma, Vats and Taneja, 2015; Sengupta, Nilson and Kayed, 2016). In addition, Aβ triggers the activation of inflammatory responses marked by reactive microglia activation and reactive astrocytosis (Hensley, 2010). Microglia and astrocytes respond to insults caused by Aβ by transitioning from a ramified form to an active phagocytic form and migrating to the site of injury. The number of activated microglia and astrocytes has been shown to increase in relation to the amount of Aβ plaques (Bolmont et al., 2008). Scavenger receptors on microglia and its phagocytic nature allow for modest Aβ engulfment, but the activated microglia phenotype is accompanied by the release of pro-inflammatory cytokines and ROS (Doens and Fernández, 2014). Aβ deposits are difficult to remove, and chronic microglial activation as seen in AD leads to a 'frustrated phagocytosis' phenotype, causing an extended release of inflammatory molecules such as interleukin-1β, interleukin-6, and tumour necrosis factor-α (Majerova et al., 2014; Wang et al., 2015). This phenomenon causes a toxic environment for neurons and also leads to neurotoxic injuries such as demyelination, synaptic dysfunction, and ultimately neuron death (Lull and Block, 2010). Therefore, exploring neuroinflammation is a promising strategy that may lead to novel approaches and ameliorate the burden of AD.

1.4 Reactive oxygen species and oxidative stress in Alzheimer’s disease

Oxidation and reduction (redox) reactions in living organisms are utilized in fundamental processes and are necessary for overall health. An unavoidable consequence of redox reactions is the production of ROS (Zuo et al., 2015). ROS is an umbrella term that includes active oxygen-containing compounds such as hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻), hydroxyl radical (OH⁻), amongst other radicals. ROS is primarily produced in the mitochondria through the
electron transport chain and the oxidative phosphorylation pathway (Ray, Huang and Tsuji, 2012). In particular, H$_2$O$_2$ is continuously produced by the mitochondria and accounts for 40% of the H$_2$O$_2$ in the cytosol (Boveris and Cadenas, 2001). Compared to other ROS, H$_2$O$_2$ is a stable molecule that can diffuse easily between cells and react with free metals (Groeger, Quiney and Cotter, 2009). The Fenton reaction occurs when H$_2$O$_2$ reacts with iron or copper to produce the damaging hydroxyl radical (Winterbourn, 1995).

$$\text{Fe}^{2+} + \text{H}_2\text{O} \rightarrow \text{Fe}^{3+} + [\text{H}_2\text{O}_2^-] \rightarrow \text{OH} + \text{OH}^-$$

ROS are signalling messengers that play a role in normal cellular function (Dröge, 2002), but become problematic when they are chronically present. ROS modulate cell cycle pathways and maintain the balance between cell proliferation and apoptosis at normal levels (Finkel, 2011). ROS also play an important role in the immune system as cells utilize ROS to undergo the phagocytosis process and fight against foreign bodies (Dupré-Crochet, Erard and Nüße, 2013). Neutrophils, monocytes, macrophages and microglia are significant cellular sources of ROS. These cells utilize cellular enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to secrete ROS and serve as both a protective and inflammatory mechanism (Haslund-Vinding \textit{et al.}, 2017). Toll-like receptor-4 on the surface of microglia is activated by the binding of foreign bodies to NADPH oxidase triggering downstream ROS production to breakdown pathogens (Fiebich \textit{et al.}, 2018). However, ROS can be extremely harmful if produced in excess of normal physiologic levels (Zuo \textit{et al.}, 2015). Downstream effects due to the overproduction of ROS include lipid peroxidation, nucleic acid damage, protein oxidation, and enzyme inhibition, all leading to cellular dysfunction and cell death (Mylonas and Kouretas, 1999;
Lipid peroxidation occurs when ROS targets polyunsaturated fatty acids of membrane phospholipids in the brain (Sultana, Perluigi and Butterfield, 2013). These reactions lead to the production of highly reactive aldehydes such as 4-hydroxy-2-nonenal and malondialdehyde. Reactive aldehydes are strong electrophiles and react with amino acids and nucleic acids, leading to the inhibition of DNA, RNA, cell membrane function, and protein synthesis (Matveychuk et al., 2011).

Antioxidants are endogenous molecules that function to counteract free radicals and neutralize oxidants by breaking them down into less reactive molecules (Pham-Huy, He and Pham-Huy, 2008). However, excessive ROS levels can overwhelm the body’s antioxidant mechanisms, resulting in oxidative stress. Increased oxidative stress is linked to ageing and disease (Liguori et al., 2018). The over-production of ROS can be attributed to several mechanisms such as mitochondrial electron-transport chain dysfunction, overwhelmed antioxidant mechanisms, microglia activation pathways, amyloid deposition, and metal abnormalities (Valko et al., 2007; Su et al., 2008). These primary sources of ROS imbalances have been implicated in both the pathogenesis of AD as well as an outcome of the disease (Wang et al., 2014; Manoharan et al., 2016). Aβ42 induces oxidative stress in the brain by binding with transition metal ions such as iron and copper generating H₂O₂ and other hydroxyl radicals (Huang et al., 1999). Markers of increased lipid peroxidation have also been correlated with amyloid plaques deposition (Praticò et al., 2001). More research is required to characterize oxidative sources and the cellular consequences of excessive ROS. This is of particular importance since the regulation of ROS through cellular antioxidant systems is crucial for maintaining cell viability and ultimately organ function in patients with AD.
1.5 Peroxisomes and cellular mechanisms of ROS mitigation

Mammalian peroxisomes are essential organelles that perform oxidation reactions involved in the breakdown of amino and fatty acids to produce metabolic energy (Cooper, 2000). Peroxisomes are a significant source of oxidative metabolism comprising up to 20% of total cellular oxygen consumption and oxidative stress (Wanders, Waterham and Ferdinandusse, 2016). Within the nervous system, peroxisomes are found in all neural cell types such as neurons, microglia, astrocytes, and oligodendrocytes. Peroxisomes of the nervous system are involved in a variety of catabolic and anabolic reactions, including the biosynthesis of lipids, critical for the formation of myelin and membrane processes of oligodendrocytes (Baes and Aubourg, 2009). A harmful consequence of these oxidation reactions is the production of H$_2$O$_2$. The primary source of H$_2$O$_2$ produced in peroxisomes occurs through the β-oxidation of fatty acids (Bonekamp et al., 2009). This catabolic process oxidizes fatty acids into acetyl-CoA subunits; however, the high-energy electrons that are generated are not coupled to a respiratory chain destined for ATP synthesis like those seen in the mitochondria. Instead, these electrons are coupled to oxygen reduction reactions to form reactive H$_2$O$_2$ in turn upsetting the cellular redox balance, and damaging DNA, proteins and lipids (Mylonas and Kouretas, 1999).

Peroxisomes contain many antioxidant enzymes that combat the effect of ROS, including superoxide dismutase, peroxiredoxin, epoxide hydrolase, and catalase (Fransen et al., 2012). Catalase is a 62 kDa enzyme that plays a critical protective role because it catalyzes H$_2$O$_2$ back into water and oxygen (Schrader and Fahimi, 2006).

\[
\text{Catalase} \\
2 \text{H}_2\text{O}_2 \quad \rightarrow \quad 2 \text{H}_2\text{O} + \text{O}_2
\]
Like all other peroxisomal matrix proteins, endogenous catalase is transcribed in the cytosol and targeted to peroxisomes through the use of 4 amino acid residues at its carboxy terminus, lysine-alanine-asparagine-leucine (-KANL), making up a peroxisome-targeting signal sequence (PTS1) (Sheik et al., 1998). The peroxisomal targeting signal type 1 receptor (PEX5p) recognizes the targeting sequence on catalase leading to internalization of the enzyme for H$_2$O$_2$ homeostasis (Terlecky, Koepke and Walton, 2006). These protective antioxidant systems can be overwhelmed by ageing, cellular disturbances and peroxisomal dysfunction.

In cell culture models of ageing, the serial passaging of cells demonstrated decreased catalase import into peroxisomes resulting in increased oxidative stress and cell senescence (Koepke et al., 2007). Some diseases, such as the rare congenital Zellweger syndrome, are marked by PEX5p mutations leading to deficient catalase import and excessive H$_2$O$_2$ buildup (Berger et al., 2016). Peroxisomal dysfunction contributes to the pathogenesis of a variety of other diseases, including cancer, diabetes, and AD (Cipolla and Lodhi, 2017). It is clear that peroxisomes and catalase enzyme function as mediators of H$_2$O$_2$ metabolism. Additional research is required to understand how this peroxiosomal pathway can potentially contribute to AD prevention. The effectiveness of drugs that specifically target peroxisomes and H$_2$O$_2$ accumulation remains to be determined.

### 1.6 Targeted antioxidant CAT-SKL

One of the major limitation of endogenous catalase is that the residues that make up its PTS1 functionally deteriorate as cells age (Koepke et al., 2007). Due to this deterioration, strategies have been previously developed to improve the shuttling of catalase to peroxisomes. The engineered catalase (CAT-SKL, United States patents 7601366 and 8663630) used in this study
replaces the -KANL terminus with a serine-lysine-leucine (-SKL) sequence to increase peroxisomal targeting, thereby allowing for higher enzymatic metabolism of H$_2$O$_2$ (Koepke et al., 2007; Giordano et al., 2015; Nell et al., 2017). The -SKL sequence is a more efficient peroxisome targeting signal for importing proteins compared to the -KANL sequence and even functions when PEX5p receptors are mutated (Koepke et al., 2007). CAT-SKL is effective in mitigating oxidative stress in several in vitro and in vivo studies. Subcutaneous injections of CAT-SKL improved subnormal intraretinal uptake of manganese and reduced oxidative liver damage in diabetic mice (Giordano et al., 2015). CAT-SKL also protected against Aβ induced neurotoxicity and oxidative stress in cultured primary cortical/hippocampal neurons (Giordano et al., 2014; Nell et al., 2017). These pre-clinical studies suggest that CAT-SKL holds potential as a new therapeutic approach to address the increased accumulation of ROS in disorders such as Parkinson’s and AD (Valko et al., 2007; Price, Terlecky and Kessel, 2009; Cipolla and Lodhi, 2017; Nell et al., 2017). However, the use of therapeutic antioxidants such as CAT-SKL has been hindered by physiological barriers such as degradation of the antioxidant before reaching its cellular target (Moosmann and Behl, 2002). In addition, exogenous CAT-SKL alone has not been shown to penetrate the BBB. Identifying a delivery system to effectively cargo CAT-SKL to the brain is essential to investigate its protective effects in neurodegenerative diseases.

1.7 Blood-brain barrier and the intranasal delivery pathway – function and potential

The BBB is a unique microvasculature system that supports the CNS allowing for strict regulation of transport of molecules between the blood and the brain. The restrictive nature of the BBB is due to its anatomical architecture. A monolayer of polarized endothelial cells makes up the walls of the blood vessels (Serlin et al., 2015). These cells are securely sealed to each other by
a network of proteins, including claudins and occludins, to form tight junctions (Luissint et al., 2012). Supporting connective tissue cells called pericytes on the abluminal (brain facing) surface wrap the endothelial cells and play a role in endothelial proliferation and migration (Liu et al., 2012). Astrocytes extend their cellular processes to sheath the entire endothelial cell border to regulate the BBB permeability and blood flow. This barrier acts to protect the brain from foreign bodies and other circulating pathogens (Daneman and Prat, 2015). The breakdown of the BBB causes disease pathologies and is observed in severe neurological diseases such as Parkinson’s disease, amyotrophic lateral sclerosis and AD (Sweeney, Sagare and Zlokovic, 2018). Although the BBB serves a crucial function in maintaining the brain's protected environment, its selective nature can become a limiting factor when designing potential therapeutics. Therefore the BBB is a major barrier that must be addressed in order to evaluate the efficacy of candidate therapies (Ballabh, Braun and Nedergaard, 2004).

One approach to penetrate the BBB in treating neurodegenerative diseases is to implement the intranasal delivery of therapeutics (Erdő et al., 2018). Intranasal delivery utilizes both intracellular and extracellular pathways to the brain. The extracellular route directly connects from the nasal cavity to the brain and begins with crossing the nasal epithelium. The nasal epithelium is composed of diverse cells connected by tight junctions more permissible than that of the BBB (Yang et al., 2017). Lipophilic molecules can freely pass through these tight junctions, while hydrophilic molecules diffuse through the tight junctions based on their size. Once the drug has passed through the nasal epithelium, drugs are transported through the subarachnoid space of the brain and directly into the CSF. The intracellular route employs the olfactory and trigeminal nerves. It begins with endocytosis of the drug by olfactory sensory cells and subsequent axonal transport to their synaptic clefts in the olfactory bulb. The drug is further exocytosed, and
surrounding neurons repeat the neuronal translocation to other brain regions. Extracellular transport of drugs is much faster than intracellular transport and is the main pathway used in intranasal administration (Crowe et al., 2018a). While the intranasal route offers direct and rapid access of lipophilic molecules to the brain, issues regarding the stability of drug delivery remain. Targeting exogenous CAT-SKL through the nasal epithelium is impeded by protein degradation and the enzymes' hydrophilic outer surface (Domínguez, Sosa-Peinado and Hansberg, 2010; Pharmaceu Sci, Shah and Shao, 2017). The use of a lipophilic nanodelivery system that can protect CAT-SKL from degradation would allow for both efficient and targeted delivery of CAT-SKL to the brain (Li et al., 2017).

1.8 Exosomes and their potential as therapy delivery tools

Recent research into extracellular vesicles has garnered much attention and suggests a more significant physiological role than previously recognized. Almost all eukaryotic cells produce extracellular vesicles as a method of cell-to-cell communication (Tetta et al., 2013). Extracellular vesicles carry biological cargo, including proteins, and nucleic acids, which are often taken up by surrounding cells or transported through the circulatory system to distant cells (Margolis and Sadovsky, 2019). Proteins and nucleic acids released from exosomes are able to carry out various functions such as modulating cellular function and gene expression (Yáñez-Mó et al., 2015; Iraci et al., 2016; Jiang and Gao, 2017).

Exosomes are unique, nanosized extracellular vesicles, ranging from 30 nm to 150 nm in size (Théry et al., 2006). Exosomes originate from endosomes, which are internal invaginations (endocytosis) of the cell plasma membrane (Zhang et al., 2019). Endosomes undergo a maturation process from early through to late endosomes via interactions with the Golgi complex, where they
become increasingly acidic. During the late endosome stage, intraluminal vesicles form, and the late endosome migrates towards the plasma membrane (Scott, Vacca and Gruenberg, 2014). These intraluminal vesicles are contained within the late endosome until fusion occurs between the late endosome membrane and the plasma membrane, where they are subsequently released as exosomes into the extracellular space. Alternatively, exosomes can be transported for degradation in lysosomes before reaching the plasma membrane (Zhang et al., 2019). Exosome membranes have similar bilipid composition to that of the plasma membrane with additional lipids added from the Golgi complex. The enriched lipid composition of exosomes increases their stability during transport and allows for passage through membranes such as the BBB (Li et al., 2017). Once an exosome reaches its target cell, it can be internalized by several potential mechanisms, including receptor-dependant binding, direct fusion, and phagocytosis (McKelvey et al., 2015).

The innate ability of exosomes to permeate biological barriers with low immunogenicity and toxicity while acting as agents of cellular communication offers a potential strategy to deliver biologics of interest into cells (Barile and Vassalli, 2017; Jiang and Gao, 2017). Several methods have been successfully implemented to load exogenous cargo into exosomes allowing for the targeted cellular delivery (Zhuang et al., 2011; Tetta et al., 2013; Qu et al., 2018; Pomatto et al., 2019; Zhang et al., 2019). Low loading capacity has been demonstrated from the passive incubation of exosomes with the selected cargo. Hydrophobic drugs can diffuse through the lipid membrane of exosomes along the concentration gradient (Zhuang et al., 2011; Haney et al., 2015). Other passive techniques include treating cells with drugs and collecting their secreted exosomes loaded with the drug (Pascucci et al., 2014). Mechanical methods have also been implemented to load exosomes actively. Haney et al. demonstrated that the permeabilization of macrophage-derived exosomes with saponin and sonication techniques provide high loading efficiency (Haney
et al., 2015). In addition, techniques such as electroporation, freeze-thaw cycles, membrane permeabilizers have been used to load exosomes with small drug molecules and miRNA (Luan et al., 2017; Pomatto et al., 2019).

The biodistribution of systemically administered exogenous exosomes is similar to other therapeutics such that accumulation primarily occurs in the liver, kidney, and spleen. Exosomes have low immunogenicity and are efficiently excreted in the bile, renal system, and circulating phagocytic cells (Lai et al., 2014). In vivo neuroimaging studies using nanoparticle tracking technology have indicated that intranasal administration of exosomes efficiently penetrates the BBB and demonstrates superior brain accumulation compared to intravenous administration (Betzer et al., 2017). Zhuang et al. showed that intranasal administration of exosomes loaded with curcumin was up-taken by microglia while Haney et al. demonstrated that intranasal administration of exosomes loaded with catalase can effectively be delivered to neurons of mice as a neuroprotective method against Parkinson’s disease (Zhuang et al., 2011; Haney et al., 2015). Using macrophage-derived homologous exosomes of the same species as a delivery tool allows for biologics to overcome barriers such as biodistribution, antigenicity, and the BBB. These physiological hurdles have historically limited the therapeutic potential of proteinaceous reagents in neurodegenerative diseases such as AD.

1.9 3xTG AD mouse model

There are numerous animal models to choose from to study AD, each with a distinct set of AD pathophysiology. Most commonly used are transgenic rodents that foster characteristic AD-related genetics. These genes are either disease-causing or serve as increased risk factors and include APP, tau, PS1, and ApoE (Hall and Roberson, 2012). Although any single model cannot
completely recapitulate AD pathology, mouse models conserve many of the mammalian genetic and physiological characteristics observed in humans. Mice are a cost-effective reductionist system that enables experimental manipulation in preclinical testing to develop new drugs (Perlman, 2016). The model selected for this thesis was the 3xTG-AD mouse line, which combines three mutant transgenes. The first is the human APP gene carrying the Swedish mutation. The APP Swedish double mutations cause two amino acid substitutions (lysine to asparagine and methionine to leucine) adjacent to the β-secretase site of APP. These mutations have been reported to increase total Aβ levels (Haass et al., 1995). Mice carrying the APP Swedish mutations such as the 3xTG-AD mouse line accumulate high levels of both soluble and insoluble Aβ and develop amyloid pathology (Giménez-Llort et al., 2013). The second mutation involves the M146V mutation of presenilin, a gene that encodes proteins required for the catalytic subunit of γ-secretase. Two hypotheses surround how presenilin mutations lead to neurodegeneration and cognitive impairments in AD. The amyloid hypothesis proposes that presenilin mutations increase the production of Aβ42 or the Aβ42 to Aβ40 ratio, thus leading to increased amyloid plaque deposition. The presenilin hypothesis proposes that presenilin mutations cause the loss of presenilin function and reduces essential functions in learning, memory and neuronal survival (Elder et al., 2010; Kelleher and Shen, 2017) The third and final mutation characterizing this mouse model is the P301L mutation of human tau. (Sterniczuk et al., 2010). Tau mutations cause the changes in tau protein splicing, phosphorylation state, and interactions with tubulin leading to prominent neurofibrillary tangles found in AD patients (Goedert, 2005). Tau mutations themselves do not cause AD, and their role in AD pathology is unclear. Current research shows that reducing tau pathology has been linked to improving cognitive impairment in learning and memory (Iqbal et al., 2010). Overall, these findings suggest that combining tau mutations with APP and PS1
mutations provide an aggressive mouse phenotype with a combination of AD pathologies that strongly mimic what is seen in human AD patients (Hall and Roberson, 2012).

The AD-like pathology of 3xTG-AD mice has been well described in previous studies. These mice express human APP within the pyramidal cell layer of the hippocampus as early as two months of age (Belfiore et al., 2019). The three transgenes cause 3xTG-AD mice to first develop extracellular Aβ deposition and tau pathology at six months of age (Oddo et al., 2003; Belfiore et al., 2019). Neuroinflammation marked by microglia activation in the hippocampus is also evident in these mice as early as six months and increases with age (Belfiore et al., 2019). Behaviour deficits have also been reported in 3xTG-AD mice at 6.5 months of age, as indicated by spatial and memory deficits in the Barnes maze (Stover et al., 2015). Morris water maze testing has shown differences in learning and memory in 6-month-old 3xTG mice compared to control B6129FS mice (Stimmell et al., 2019). These findings highlight the utility of the 3xTG mouse model due to the presentation of pathological and cognitive features reminiscent of human AD.

1.10 Rationale

Excess production of ROS, causing oxidative stress in the brain, is a core pathological feature of AD (Valko et al., 2007). The use of non-specific antioxidants treatments has led to inconsistent results due to physiological barriers common in human subjects and preclinical models of AD. These barriers include the degradation of therapeutics in the blood and the inability of candidate agents to cross the BBB to penetrate the brain (Moosmann and Behl, 2002). Current research has indicated that the dysfunction of peroxisomes, a cellular organelle, contributes to this increase in harmful ROS (Cipolla and Lodhi, 2017). Peroxisomes play a crucial role in ROS homeostasis balancing the production of H₂O₂ from β-oxidation of fatty
acids with antioxidant functions of the enzyme catalase, which is active within the organelle (Bonekamp et al., 2009). As the brain ages, progressive dysfunction of peroxisomes and decreased localization of catalase into the organelle contributes to elevated ROS contributing to the oxidative stress response in AD (Cheignon et al., 2018). This phenomenon has also been observed in preclinical rodent models of AD. In particular, the 3xTG AD mouse used in the present study has elevated levels of PMP70, a peroxisomal population marker, as early as 3 months of age reflecting the need for more efficient fatty acid β-oxidation (Fanelli et al., 2013).

Increased markers of lipid peroxidation and oxidative stress are also observed in 3xTG AD mice prior to traditional neuropathological hallmarks indicating the necessity for antioxidant therapy at the early stages of AD (Resende et al., 2008). The engineered CAT-SKL used in our lab can target this early AD pathology by effectively targeting peroxisomes and providing organelle-based antioxidant, anti-inflammatory effects (Koepke et al., 2007; Giordano et al., 2014; Nell et al., 2017).

A major obstacle with using CAT-SKL as a therapeutic is finding a method to efficiently and safely deliver the enzyme to the brain while minimizing off-target effects. We proposed that packaging CAT-SKL into exosomes could overcome this obstacle (Iraci et al., 2016). The use of exosomes is a promising delivery method as it can cross the BBB, thereby delivering therapeutics to cells of the CNS (Betzer et al., 2017; Li et al., 2017). Previous work by Haney et al. also demonstrated that exosomes loaded with endogenous catalase decreased neuroinflammation and increase neuronal survival in a mouse model of Parkinson’s disease (PD) (Haney et al., 2015). Early stages of PD and AD exhibit similar pathologies such as microglia activation, excessive secretion of ROS, and lower levels of antioxidant enzymes (Xie et al., 2014). Therefore, exosome-mediated delivery of CAT-SKL is an intuitive strategy to target ROS in 3xTG AD mice.
1.11 Hypothesis and aims

*Overarching Thesis Hypothesis:*

Intranasal administration of exoCAT-SKL will penetrate the BBB and provide non-toxic, targeted antioxidant protection in mice.

*Aim 1:*

Demonstrate that intranasal administration of exoCAT-SKL can penetrate the BBB, assess the brain biodistribution, and determine potential toxicity in young healthy mice.

*Aim 2:*

Assess the protective effects of prophylactic intranasal administration of exoCAT-SKL using pathological, biochemical, and behavioural outcomes in control B6129FS and 3xTG AD mice.
Section 2: METHODS
2.1 Isolation of naïve exosomes and biotinylated catalase-SKL (bCAT-SKL)

Naïve exosomes from Raw 264.7 macrophages were collected and isolated using gradient centrifugation and were provided by our collaborators Dr. Batrakova (UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA). The preparation and purification of bCAT-SKL and CAT-SKL was provided by the Shilton laboratory (Schulich School of Medicine and Dentistry, Western University, London, ON, CA). The methods used to isolate exosomes are previously described by Haney et al. (Haney et al., 2015). The genetic reengineering of the catalase enzyme and purification of bCAT-SKL or CAT-SKL is as previously described (Koepke et al., 2007).

2.2 ExoCAT-SKL preparation

A sonication technique was implemented to incorporate biotinylated CAT-SKL (bCAT-SKL) into exosomes. Naïve exosomes isolated from Raw 264.7 macrophages were diluted in phosphate-buffered saline (PBS) to a concentration of 0.15 mg/mL exosome protein. A bCAT-SKL solution of 0.5 mg/mL was prepared. 250 μL of exosomes solution was combined with 125 μL of bCAT-SKL to achieve a final concentration of 0.1 mg/mL of total protein. This bCAT-SKL and exosome mixture was then sonicated for six cycles of 4 seconds pulse and 2 seconds pause (output power 9 watts – level 3), chilled on ice for 2 minutes, and sonicated again for six cycles of 4 seconds pulse and 2 seconds pause (Sonic Dismembrator Model 100, Fisher Scientific). The sonicated mixture was then chilled on ice for 5 minutes, followed by a 37 °C water bath for 10 minutes, and 30 minutes at room temperature. The bCAT-SKL loaded exosomes (exoCAT-SKL) were subsequently purified from unbound bCAT-SKL in solution by gel-filtration/size exclusion chromatography with Sepharose CL-6B column (Sigma-Aldrich) or the Optima MAX-XP.
Benchtop Ultracentrifuge at 50 k RPM for 70 minutes using an MLA130 rotor at 4 °C. (Beckman Coulter, California, USA). A total of 24 fractions of 1 mL each were collected from the gel-filtration chromatography column. For exoCAT-SKL purified using ultracentrifuge methods, the supernatant from the ultracentrifuge solution was replaced with 0.1 M phosphate buffer solution (PBS) and spun down again at 50 k RPM for 70 minutes at 4 °C. The remaining exoCAT-SKL pellet was resuspended in 0.2 M sucrose solution. ExoCAT-SKL purified by ultracentrifuge was stored at -80 °C for up to 5 weeks and underwent one freeze-thaw cycle before use. Gel-filtration and western blot techniques, as described above, were implemented to confirm successful ultracentrifuge purification and activity following one freeze-thaw cycle. A flowchart depicting the methods of exosome loading of catalase-SKL and purification of exoCAT-SKL is displayed in figure 1.
Figure 1. Flowchart depicting methods of exosome loading of CAT-SKL and purification. 250 uL of exosome stock (0.15 mg/ml) is sonicated with 125 uL of 0.5mg/mL CAT-SKL. Two methods of purification of loaded exoCat-SKL from free CAT-SKL are implemented: 1) by gel filtration and 2) by ultracentrifugation. Loading of exosomes is verified via BCA and H$_2$O$_2$ decomposition assays, and Western Blots (exosome markers: CD9, TSG101, HSP70). Dotted arrows represent one-time validation techniques by gel-filtration and western blots of collected supernatant and re-dissolved exoCAT-SKL from ultracentrifugation. Confirmed loading and purification of exoCat-SKL precede intranasal injection.
2.3 ExoCAT-SKL verification

Following exoCAT-SKL loading and purification, western blot techniques were implemented to identify fractions containing exoCAT-SKL or CAT-SKL as well as verify ultracentrifuge methods. Protein concentrations of each fraction were determined using bicinchoninic acid assays (Thermo Fisher Scientific, Massachusetts, USA). Exosome membrane proteins, TSG101 and CD9, were detected using primary rabbit anti-TSG101 (1:1000; Abcam, Massachusetts, USA) or rabbit anti-CD9 (1:500; Abcam, Massachusetts, USA) antibodies respectively. Supplemental exosome protein markers, HSP70, were detected using anti-HSP70 (1:1000; Abcam, Massachusetts, USA). CAT-SKL protein bands were detected using rabbit anti-catalase (1:1000; Abcam, Massachusetts, USA) antibodies. Membranes were incubated using secondary horseradish peroxidase-conjugated goat anti-rabbit antibodies, and protein bands were detected using chemiluminescence substrate and visualized on a ChemiDoc Imaging System (BioRad, Philadelphia, USA).

Catalase activity of prepared exoCAT-SKL was determined by hydrogen peroxide (H$_2$O$_2$) decomposition assays (Storrie and Madden, 1990; Koepke et al., 2008; Walton and Pizzitelli, 2012). Protein concentrations of each fraction were determined using bicinchoninic acid assays (Thermo Fisher Scientific, Massachusetts, USA). Samples of each fraction collected from the gel-filtration column were added to a reaction mixture of 0.02 M imidazole buffer (pH 7.0), 1 mg/mL bovine serum albumin (BSA), 0.2% Triton-X, and 0.06% hydrogen peroxide and incubated at room temperature for 10 minutes. The reaction was completed using a titanium oxysulfate stop solution (TiOSO$_4$ in 2N H$_2$SO$_4$), which reacts with the remaining hydrogen peroxide not decomposed by the CAT-SKL to produce a yellow peroxotitanium complex. Optical density (OD)
of each sample was measured at 405 nm, and catalase activity was calculated based on the
difference in OD per minute per mg total protein.

2.4 Animal Handling and Care

All animal handling and experimental procedures were approved by Western University
Animal Care Committee (AUP 2018-068) and carried out in accordance with the guidelines of the
Canadian Council on Animal Care and National Institute of Health Guides for the Care and Use
of Laboratory Animals. For Aim 1, 29 wildtype (C57BL6) mice (17 males, 12 females) between
the ages of eight and 16 weeks were involved in the study. For Aim 2, a total of 19 animals, nine
3xTG-AD mice and ten control (B6129SF2/J), were used in the study. All mice were housed under
standard conditions (12:12 light/dark cycle, at 22-24°C) and maintained on a standard diet provided ad libitum.

2.5 Aim 1 – Timeline

The timeline for aim 1 of the study is shown in figure 2.
Figure 2. **Aim 1 timeline.** Biotinylated CAT-SKL was loaded into exosomes via six cycles of sonication for 4 seconds and 2 seconds in-between. The loaded exosomes (exoCAT-SKL) were subsequently purified from unbound CAT-SKL using either gel filtration or ultracentrifugation. Loading of exosomes was verified via BCA and H₂O₂ decomposition assays and Western Blots (exosome markers: CD9, TSG101, HSP70). Male and female 8-week old C56BL/6 wildtype mice received intranasal administrations of empty exosomes, low dose exoCAT-SKL (30 μL), or high dose exoCAT-SKL (60 μL), once per week for four consecutive weeks. Mice were subsequently sacrificed and processed for tissue collection. Pathological and histological assessments were performed on the brain as well as off-target tissue.

2.6 **Aim 1 – Treatment groups and dosing regimen**

Animal numbers for each experimental group were as follows: male empty exosomes, n=7; male low exoCAT-SKL, n=5; male high exoCAT-SKL, n=5; female empty exosomes, n=2; female low exoCAT-SKL, n=5; female high exoCAT-SKL, n=5. Mice assigned to high exoCAT-SKL groups were subjected to 3 intranasal administrations of 10 μL per nostril of prepared exoCAT-SKL while mice assigned to low exoCAT-SKL groups were subjected to 3 sequential intranasal administration of 5 μL per nostril. All intranasal administrations were performed by holding mice in a supine position and held there for 2 minutes following each administration. Mice assigned to the empty exosome group were subjected to 3 intranasal administration of 5 μL of exosomes per nostril. All mice received intranasal administration of empty exosomes, low or high dose exoCAT-SKL once per week for four consecutive weeks.
The dosing regimen to test the safety of intranasal exoCAT-SKL injection in this study is based upon previous work by Nell et al. in which rats were treated with intraperitoneal CAT-SKL injections once per week for four sequential weeks at 1 mg/kg (Nell et al., 2017). A similar injection paradigm was explored by Giordano et al. in which C57Bl/6 mice were subcutaneously injected once a week with CAT-SKL for 3 to 4 months at 1 mg/kg (Giordano et al., 2015). In this study, multiple doses, in addition to empty exosomes, were used to determine any potential dose-dependent responses.

2.7 Aim 2 – Pilot study timeline

The timeline for aim 2 of the study is shown in figure 3.

![Figure 3. Aim 2 pilot study overview](image)

**Figure 3. Aim 2 pilot study overview.** CAT-SKL was loaded into exosomes via six cycles of sonication for 4 seconds and 2 seconds in-between. Loaded exosomes were subsequently purified from unbound CAT-SKL using ultracentrifugation. Loading of exosomes was verified via BCA and H2O2 decomposition assays and Western Blots (exosome marker: CD9). Batches of CAT-SKL loaded exosomes (exoCAT-SKL) were stored at -80°C for up to 5 weeks for subsequent injections. Male and female 8-week old B6129SF2/J control mice, 3xTG-AD mice, received intranasal administrations of empty exosomes, or 60 uL of exoCAT-SKL once per week for 24 consecutive weeks. Immediately following the last injection, the Morris water maze task was implemented to evaluate mouse behaviour, learning deficits, memory and cognitive impairments. Mice were sacrificed and perfused following behavioural testing; the brains and off-target tissue were collected and processed. Pathological and histological assessments were performed on all tissue.

2.8 Aim 2 – Treatment groups and numbers

The treatment groups and numbers for aim 2 of the study is shown in figure 4.
Figure 4. **Aim 2 cohort 1 treatment groups and numbers.** A total of 10 B6129SF2/J control mice and nine 3xTG-AD mice make up cohort 1 of Aim 2. Of the control mice, listed following are the treatment groups: males receiving empty exosomes, females receiving empty exosomes, males receiving high dose exoCAT-SKL, females receiving high dose exoCAT-SKL. Of the 3xTG mice, listed following are the treatment groups: males receiving empty exosomes, females receiving empty exosomes, males receiving high dose exoCAT-SKL, females receiving high dose exoCAT-SKL.

2.9 Euthanasia and Tissue Collection

Following the final injection (week 4), mice were euthanized using a CO\textsubscript{2} chamber and weighed. Transcardial perfusion using 0.01 M PBS (pH 7.35), followed by 4% paraformaldehyde (pH 7.35), was performed for all mice. Brains were subsequently extracted, weighed, and submerged in paraformaldehyde for 24 hours and then transferred to a 30% sucrose solution until brains were saturated and fully submerged. Brains were sectioned coronally on a cryostat at 30 \textmu m thickness approximately from bregma 3.75 mm to bregma -7.50 mm, sorted into series and stored in cryoprotectant at -20°C until used for histological and immunofluorescent techniques. All other organs were harvested, weighed, trimmed, and stored within cassettes and embedded in paraffin wax. As per the Society of Toxicologic Pathology, a list of all organs collected for histopathologic
examinations is listed in Table 1 (Bregman et al., 2003).

**Table 1: List of tissues included for histological examination**

<table>
<thead>
<tr>
<th>Organ Systems</th>
<th>Organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secretory Glands</td>
<td>Adrenal, Harderian, Kidney, Mammary, Parathyroid, Pituitary, Pancreas, Salivary, Thymus, Thyroid</td>
</tr>
<tr>
<td>Muscular Skeletal</td>
<td>Femur with articular cartilage, Bone marrow, Skeletal muscle (thigh), Skin</td>
</tr>
<tr>
<td>Reproductive</td>
<td>Epididymis, Seminal Vesicle, Testes, Ovary, Prostate, Uterus, Vagina</td>
</tr>
<tr>
<td>Central and Peripheral Nervous</td>
<td>Brain, Eye with optic nerve, Peripheral nerve (sciatic), Spinal cord (cervical, midthoracic, lumbar)</td>
</tr>
<tr>
<td>Gastrointestinal Tract</td>
<td>Cecum, Colon, Duodenum, Esophagus, Gall Bladder, Ileum, Jejunum, Liver, Stomach, and Urinary Bladder</td>
</tr>
</tbody>
</table>

**2.10 Histology**

Organs and brain sections were mounted onto slides and stained with 0.1% Mayer’s hematoxylin solution and 0.5% eosin Y solution, dehydrated, and coverslipped at the Robarts Research Institute Molecular Pathology Core Facility. Free-floating brain sections were pre-washed in 0.01 M PBS, mounted on glass slides, and dried overnight.

**2.11 Immunofluorescence**

Immunofluorescence was performed on free-floating sections to visualize bCAT-SKL in the brain. Following PBS washes, sections were blocked in 1% BSA diluted in 0.01 M PBS/0.1% Triton X and subsequently incubated in Streptavidin Texas Red Conjugate (1:250, Abcam, Massachusetts, USA) for 2 hours diluted in antibody buffer. Sections were mounted on glass slides,
and DAPI fluorescent mounting medium (Abcam, Massachusetts, USA) was applied, and slides were subsequently coverslipped.

2.12 Immunohistochemistry

Immunohistochemistry was performed on free-floating sections to visualize microglia in the brain. Following PBS washes, sections were blocked in 1% horse serum diluted in 0.01 M PBS/0.1% Triton X and subsequently incubated overnight at 4°C in primary rabbit polyclonal antibody against the ionized calcium-binding adaptor molecule-1 (anti-Iba-1; 1:1000; Wako Chemicals USA Inc., Richmond, VA, USA). Sections were subsequently incubated with biotinylated anti-rabbit secondary antibody (1:500, Vector Laboratories, Inc. Burlingame, CA, USA) followed by a 1-hour incubation with avidin-biotin complex (ABC kit, Vector Laboratories, Inc. Burlingame, CA, USA) reagent. Sections were developed using 0.05% 3, 3’ diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis MO, USA). Stained sections were then mounted on glass slides, air-dried, dehydrated in increasing concentrations of ethanol and xylene, and coverslipped with DePex mounting media (DePex, BDH Chemicals, Poole, UK).

2.13 Microscopy

Immunofluorescent, immunohistochemical, and histological imaging of organs and brain sections were taken using Nikon Eclipse Ni-E upright microscope with a Nikon DS Fi2 black and white camera head or colour camera. Images were taken using the NIS-Elements Imaging Software Version 4.30.02 (Nikon Instruments Inc., Melville, NY, USA) with consistent settings. Brain regions of interest included the prefrontal cortex, striatum, dorsal and ventral hippocampus, and the cerebellum.
2.14 Immunohistochemistry Quantification

Entire brain sections stained for IBA1 positive cells were imaged at 2X to quantify changes in general microglia populations. Sections of bregma 1.7 mm to bregma 1.8 mm were screened for positive IBA1 signal to determine regions of interest. All analysis and quantification were carried out using 64-bit ImageJ software (Version 1.48u4, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Areas of interest included the hippocampus and the corpus callosum and were manually outlined using the polygon selection tool. Selected areas were cropped and converted into grayscale 8-bit images and underwent universal thresholding. The percent area of positive IBA1 signal (percent of the total area) was measured for each region and expressed as a weighted mean.

2.15 Behaviour: Morris Water Maze

To assess brain areas associated with AD, cognitive testing using preclinical animal models has proven useful. Hippocampal dependent spatial learning and memory can be assessed in rodents using the Morris water maze (MWM) task, first developed by Richard Morris in the early 1980s (Morris, 1984). Tools such as the Morris water allows for the investigation of behavioural deficits caused by vulnerable brain areas as well as the impact of novel drugs. The MWM protocol published by Frame et al. 2019 was used in this study and explained as followed (Frame et al., 2019).

*MWM equipment and layout:*

The objective of the task is for each mouse to learn the location of an escape platform in a circular polyurethane water tank, 122 cm in diameter, 81 cm height (San Diego Instruments,
catalogue number: 7000-0723, USA) using distal spatial cues. An escape platform made of clear acrylic, 10 cm diameter, 56 cm height (San Diego Instruments, catalogue number: 7500-0310, USA) was hidden in the pool using an opaque water mixture of non-toxic white tempera paint (0.34 g/L). The pool was filled within 20 cm to the top such that the escape platform was submerged ~1 cm below the surface. The water temperature was maintained at 24 ± 1°C throughout all testing days. Different spatial cues (distinct in colour and shape; blue triangle, yellow cross, red heart, green circle, grey diamond) were placed around the tank on the north, east, south and west walls of the testing area with transparent tape 1-2 m away from the pool. A camera was placed on the ceiling directly above the tank, allowing for complete capture of the entire tank in the same plane. Using ANY-Maze software (ANY-maze, Stoelting CO, USA), a virtual map of the MWM tank was drawn. The pool was virtually divided into four equal quadrants using horizontal and vertical lines that cut through the center point. Quadrants were labelled North-east, North-west, South-west, and South-east. The submerged exit platform area was also virtually marked using the ANY-maze software. All mice completed an 8-day protocol – 3 days of habituation, four days of learning, and a probe trial for 24 h long-term memory on the final day.

**Habituation period:**

All mice underwent three days of habituation. Mice were acclimatized to the holding area for 30 minutes on day 1 and returned to the normal housing room. On day 2, each mouse was placed on the exit platform positioned in the center of the maze. Immediately after placing the mouse on the platform, the mouse was given 15 uninterrupted seconds to acclimate to the exit platform and extra-maze visual cues. On day 3, mice were acclimatized to the training procedure. The exit platform remained in the center of the maze in the testing area. The trial began when a
mouse was placed in the water on the perimeter of the maze at a randomly chosen cardinal position (south) facing the wall of the pool. Each mouse was given 90 seconds to find the exit platform. Once the mouse found the platform, the mouse was again given 15 seconds before removing it from the testing area.

Learning period:

Over four days, four training sessions were implemented to induce spatial learning following the acclimation period. The exit platform was moved from the center of the pool to the center of the Northwest quadrant for the duration of all learning trials. The objective of these trials was to have the mice recognize the extra-maze visual cues to help establish the spatial location of the hidden exit platform. On the first day, each mouse performed 4 trials with a 10-minute inter-trial time interval starting at the same cardinal position (south) used during the habituation session and facing the pool wall. Mice were given 90 seconds to search and locate the exit platform. An additional 15 seconds was given to the mouse to sit on the platform and absorb the surroundings. If the mouse had not found the platform in the allocated time, the mouse was gently guided to the platform and ensured 15 seconds on the platform before being removed from the pool. For each subsequent trial, mice were released in the cardinal direction that was one quarter turn clockwise (east, north, then west) from the previous trial. The initial starting position for each subsequent day was one quarter turn clockwise from the starting position of the previous day. Using the ANY-maze software, each mouse's latency and distance to the exit platform, and mean swim speed was recorded.
**Probe trial:**

A probe trial (exit platform was removed from the pool) was conducted to assess 24-hour long-term memory following the last learning trial. The probe trial began with each mouse being placed at the same cardinal direction (north) used during the habituation period. Mice were allowed 60 seconds to explore the maze. As recorded using ANY-maze software, the amount of time spent, and the distance travelled in the target quadrant (Northwest - where the exit platform had been during the learning period) is taken as an index of the mouse's learning and 24-hour memory capacity. Mean swim speed and latency to where the exit platform had previously been was also analyzed.

**Flag Trial:**

Immediately following the probe trial, a flag trial was implemented to test the visuospatial navigation of each mouse. This test was used to control for potential differences in the MWM task between mice attributed to motivation or visual acuity. Extra-maze visual cues were removed from the testing area, and the exit platform was returned to the pool and placed in the center of the southeast quadrant diametrically opposite the original position (northwest) of the learning period. A flag was attached to the platform above the surface of the water, marking its location. Each mouse was allowed to acclimate to the new set of conditions and placed on the platform for 30 seconds. Immediately after, each mouse was given a second trial and placed back in the pool at the same cardinal position (south) as the habituation period facing the wall. Due to the removal of extra-maze visual cues, the platform's location can only be discovered due to the flag's presence. The latency and distance travelled to the platform, as well as mean swim speed was recorded using ANY-maze software.
2.16 Data and Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0. Collected data were analyzed by either a three-way or two-way analysis of variance (ANOVA), followed by a Fisher’s LSD test where applicable. All data are expressed as the mean ± the standard error of the mean (SEM) with statistical significance considered at a p-value < 0.05. Statistical significance is indicated using symbols * and ‡.
Section 3: RESULTS
3.1 Aim 1 Results:

3.1.1 bCAT-SKL loaded exosomes can be purified using gel filtration and maintain enzymatic activity.

Short burst sonication was implemented to load bCAT-SKL into naïve exosomes derived from Raw 264.7 macrophages by disrupting the exosome membrane allowing for bCAT-SKL incorporation. Sonication was used due to its high loading efficacy compared to other protein incorporation techniques such as saponin permeabilization, freeze-thaw cycles, or extrusion (Haney et al., 2015). Unbound bCAT-SKL was removed using gel filtration chromatography with a Sepharose 6BCL column as described in the methods. Protein concentrations of collected fractions were measured using a BCA assay to confirm the separation of packaged and unpackaged bCAT-SKL (Figure 5A). Results of the representative assay demonstrated that fractions 6 and 7 contained relatively higher protein concentrations. The first peak (fractions 6 and 7) corresponds to both exosome proteins as well as packaged bCAT-SKL. The second protein peak (fractions 9-14) corresponded to the unbound bCAT-SKL. This double peak pattern was mirrored in bCAT-SKL enzymatic activity, as indicated in a representative H$_2$O$_2$ decomposition assay of collected fractions (Figure 5B). Early fractions 6 and 7 correspond to the catalase activity of exosome packaged bCAT-SKL, while later fractions 9-14 showcase the catalase activity of unbound bCAT-SKL. Western blot techniques were used to confirm the presence of both exosome proteins and bCAT-SKL in early fractions and bCAT-SKL alone in later fractions (Figure 5C). Supplemental exosome markers CD9 and HSP70 were also utilized to confirm the presence of loaded exosomes (Figure 2D). The separation function of gel-filtration chromatography is dependent on molecular size. Larger molecules are filtered through the column at a faster rate than smaller molecules.
bCAT-SKL (a smaller molecule than exosomes) can only be collected into earlier fractions if incorporated into exosomes. The combination of all three techniques demonstrated the successful loading and purification of exoCAT-SKL without disrupting its enzymatic activity.

**Figure 5. Loaded exoCAT-SKL can be purified using gel-filtration chromatography.**

A) Representative BCA assay of collected fractions following gel filtration. The protein peak corresponding to exosome packaged bCAT-SKL occurred in early fractions (6-7; red) while the unbound catalase protein peak occurred in later fractions (10-14; blue).

B) Representative H$_2$O$_2$ decomposition assay of collected fractions following gel filtration. The H$_2$O$_2$ decomposition activity peak corresponding to exosome packaged bCAT-SKL occurred in early fractions (6-7; red) while the unbound catalase activity peak occurred in late fractions (9-16; blue). Fractions 9-16 displayed catalase activity outside the linear range of the H$_2$O$_2$ decomposition assay.

C) Representative western blot confirmed the presence of catalase and exosomes in fraction 6. Unbound catalase was found in later fractions 11-14.

D) Supplemental exosome markers CD9 (24 kDa) and HSP70 (70 kDa) were used to confirm the presence of loaded exosomes in gel filtration fractions.
3.1.2 Ultracentrifuge purification of ExoCAT-SKL and stability of enzymatic activity following freeze-thaw cycle

Ultracentrifuge purification was used as an alternative method to produce exoCAT-SKL. Stronger centrifugal forces were required to pellet CAT-SKL compared to exoCAT-SKL due to their difference in molecular weight. An MLA13-rotor spun at 50 k RPM, as used in this study, translated to 100,000 x g force and had been shown to pellet exosomes (Szatanek et al., 2015). Western blot techniques were implemented to confirm the successful purification of exoCAT-SKL using ultracentrifuge methods (Figure 6A). Exosome membrane protein, CD9, and CAT-SKL proteins were found within the resuspended pellet, and only CAT-SKL protein was found in the first supernatant. Trace levels of CAT-SKL protein were found in the second supernatant, confirming the successful removal of unbound CAT-SKL. The catalytic activity of purified exoCAT-SKL before and after one freeze-thaw cycle was confirmed using H$_2$O$_2$ decomposition assays (Figure 6C). Thawed exoCAT-SKL was also filtered through a gel-filtration column, and western blot assays of collected fractions confirmed the presence of CAT-SKL within exosomes (Figure 6D). Results of the western blot and H$_2$O$_2$ decomposition assays confirm successful exoCAT-SKL ultracentrifuge purification and retention of enzymatic activity following one freeze-thaw cycle.
Figure 6. Loaded exoCAT-SKL can be purified using ultracentrifuge techniques, and thawed exoCAT-SKL retains enzymatic activity. A) Representative western blot confirmed the presence of both CAT-SKL and exosomes in the resuspended exoCAT-SKL pellet (labelled EXO CAT). Only CAT-SKL was found in the first supernatant and trace levels in the second supernatant. B) Representative H_{2}O_{2} decomposition assay showcased strong exoCAT-SKL enzymatic activity before freezing. A 1:50 dilution of the resuspended exoCAT-SKL equated to 9 ng/µL CAT-SKL. C) Representative western blot confirmed the presence of CAT-SKL and exosomes in early fractions (fractions 5-7) collected from gel-filtration assay following one freeze-thaw cycle. D) Representative H_{2}O_{2} decomposition assay showcased thawed exoCAT-SKL retained strong enzymatic activity.
3.1.3 Intranasal administration of exoCAT-SKL demonstrates the ability to penetrate the BBB in vivo.

Immunofluorescent and confocal imaging techniques were implemented to visualize bCAT-SKL deposition in WT mice brains. Mice were intranasally administered empty exosomes, low, or high doses of exoCAT-SKL (Figure 7). bCAT-SKL was immunofluorescently labelled to identify its biodistribution 1 hour following the final administration. The imaging revealed diffuse bCAT-SKL positive signal throughout the brain (bregma 0.5 to bregma -2.75) including areas of interest such as the prefrontal cortex, corpus callosum, and the hippocampus. Higher magnification of brain sections showed bCAT-SKL localization in the perinuclear region (Figure 7 - yellow arrow) and the vessel walls (Figure 7 - white arrow). Images of the control brain injected with empty exosomes demonstrated a complete absence of bCAT-SKL fluorescent signal (Figure 7 - Control 40X). Fluorescent imaging of bCAT-SKL demonstrated that intranasal administration of exoCAT-SKL is an effective delivery mechanism to penetrate the BBB and target the CNS of mice.
Figure 7. Intranasal administration of exoCAT-SKL penetrated BBB in vivo. Pervasive biodistribution of biotinylated CAT-SKL (red) following low and high dose intranasal exoCAT-SKL injection was observed throughout the brain. 40X magnification and 40X-Digital Enhancement (DE; 40X area resize) of the prefrontal cortex, corpus callosum and hippocampus are showcased. The white arrow in the figure showcases the vasculature deposition of bCAT-SKL. Yellow arrow showcases cellular deposition of bCAT-SKL.
3.1.4 Short-term in vivo exoCAT-SKL administration does not exhibit toxicity in the brain

Histopathological analysis of H&E stained brain tissue determined whether degenerative or toxic changes occurred in mice following four weeks of exoCAT-SKL treatment compared to control mice (Figure 8). Serial sections throughout the brain (bregma 2.8 mm to bregma -6.6 mm), including anatomical structures encompassing predicted exoCAT-SKL absorption pathways such as the piriform cortex, were analyzed to account for widespread bCAT-SKL biodistribution. Representative images of the prefrontal cortex, corpus callosum, hippocampus, and cerebellum are showcased. Neuronal injury within the grey matter was not observed under microscopic examination in any of the treatment groups evidenced by the absence of eosinophilia of the cytoplasm, shrinkage or disappearance of the nucleolus, disintegration of the cell, amongst other characteristics of injury. Neuronal atrophy or neuronal necrosis was not observed in any treatment group. Reactive inflammation was not observed, and a normal distribution of neural cells was observed in all treatment groups. No vasculature abnormalities nor lesions were identified throughout the tissue and white matter tracks did not contain degenerative changes (i.e. vacuolation, spheroids). No evidence of toxicity was identified throughout the brain for all treatments and doses.
Figure 8. ExoCat-SKL does not cause toxicity in the brain in vivo. The prefrontal cortex (bregma 2.8 mm), corpus callosum (bregma 0.5 mm), hippocampus (bregma -1.8 mm), and cerebellum (bregma -6.6 mm) sections were selected to represent the entirety of the brain. All sections were examined for features of toxicity, including but not limited to spongiosis, neuron degeneration, inflammation, and vasculature abnormalities. Representative 10X magnification of boxed areas is showcased. All features of toxicity are negative in all areas of mice treated at different doses of exoCAT-SKL.
3.1.5 Short term in vivo intranasal exoCAT-SKL administration does not exhibit off-target toxicity.

In addition to assessing the toxicity of exoCAT-SKL in the brain, off-target toxicity was assessed to validate the utility of this therapeutic approach. The Society for Toxicologic Pathology's (STP) best practices guideline for toxicologic histopathology was utilized to ensure high-quality histologic assessments. No significant differences were observed in relative organ weight of the brain, liver, spleen, nor in the thymus, heart, kidney, adrenal glands, testis and ovaries compared between treatment groups. All features of toxicity were negative in off-target tissue (Table 2).

### Table 2: Features of toxicity of tissues included for histological examination

<table>
<thead>
<tr>
<th>Organ Systems</th>
<th>Organs</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secretory Glands</td>
<td>Adrenal, Harderian, Kidney, Mammary, Parathyroid, Pituitary, Pancreas, Salivary, Thymus, Thyroid</td>
<td>Negative</td>
</tr>
<tr>
<td>Muscular Skeletal</td>
<td>Femur with articular cartilage, Bone marrow, Skeletal muscle (thigh), Skin</td>
<td>Negative</td>
</tr>
<tr>
<td>Reproductive</td>
<td>Epididymis, Seminal Vesicle, Testes, Ovary, Prostate, Uterus, Vagina</td>
<td>Negative</td>
</tr>
<tr>
<td>Central and Peripheral Nervous</td>
<td>Eye with optic nerve, Peripheral nerve (sciatic), Spinal cord (cervical, midthoracic, lumbar)</td>
<td>Negative</td>
</tr>
<tr>
<td>Digestive</td>
<td>Cecum, Colon, Duodenum, Esophagus, Gall Bladder, Ileum, Jejunum, Liver, Stomach, and Urinary Bladder</td>
<td>Negative</td>
</tr>
</tbody>
</table>
3.2 Aim 2 pilot study results

3.2.1 Behaviour testing: Morris water maze

The Morris water maze task was implemented to examine if 24 weeks of intranasal exoCAT-SKL injections improved memory or learning deficits in control and 3xTG AD mice. All mice underwent four days of learning, followed by a probe trial on the 5th day to assess 24-hour long-term memory. The amount of time spent, and the distance travelled in the target quadrant (area of the maze where the exit platform had been during the learning period) during the probe trial was taken as an index of each mouse’s learning and 24-hour memory capacity.

Latency to platform and swim speed during the learning period

Following the last exoCAT-SKL injection, all mice were subjected to a 4-day training regimen with four trials each day for a total of 16 spatial learning trials. No significant interactions between time, genotype, and treatment were observed for both latency to platform by trial and by day. A significant time-dependent effect was observed for the latency to platform by trial ($F_{15,240} = 4.154$, $P < 0.001$) (Figure 9A), and for the latency to platform by day ($F_{3,60} = 5.816$, $P < 0.01$) (Figure 9B). No significant interactions between variables were observed in measured swim speeds. Significant genotype dependent differences in swim speed were observed between groups (Figure 9C). The control empty exosome group swam significantly slower than the 3xTG AD empty group across the days.
Figure 9. Morris water maze learning trials following 24 weeks of intranasal injections of either empty exosomes or high dose exoCAT-SKL. A) The mean latency to the exit platform for each treatment group per trial is plotted. Vertical dotted lines indicate the first trial of the following day (i.e. trial 5 is 24 hours following trial 4). Mice that did not reach the platform during the trial were recorded as 90 seconds (time indicating the trial endpoint). No significant interactions between time, genotype, and treatment were observed for the latency to platform by trial. A significant time-dependent effect was observed ($F_{15,240} = 4.154$, $P < 0.001$). B) The mean latency to the exit platform area of each treatment group per day is plotted. No significant interactions between time, genotype, and treatment were observed for the latency to platform by day. A significant time-dependent effect was observed ($F_{3,60} = 5.816$, $P < 0.01$). C) The mean swim speeds of each treatment group were compared per day during the learning trials. No significant interactions between variables were observed in measured swim speeds. Significant genotype dependent differences in swim speed were observed between groups The Control Empty group swam slower than both 3xTG groups on day 1, day 2, and day 3. Significant differences were observed between Control High Dose ExoCAT-SKL group compared to both 3xTG groups on day 3 and day 4. Values are presented as mean ± SEM (Three - way ANOVA).
Distance to the exit platform during the learning period

The distance to the exit platform during the learning period accounts was analyzed to account for variability in swim speeds between treatment groups. No significant interactions between time, genotype, and treatment were observed for both distance to platform by trial and by day. Significant time-dependent ($F_{15,240} = 3.340, P < 0.0001$), genotype-dependent ($F_{1,240} = 23.81, P < 0.0001$), and treatment-dependent sources of variation ($F_{1,240} = 5.503, P < 0.05$) were observed in the distance to platform by trial (Figure 10A). In addition, significant time-dependent ($F_{3,60} = 5.639, P < 0.01$), genotype-dependent ($F_{1,60} = 19.85, P < 0.0001$), and treatment-dependent sources of variation ($F_{1,60} = 4.589, P < 0.05$) were observed in the distance to platform by day. The same pattern was seen in the latency to the exit platform during the learning period.
Figure 10. Morris water maze learning trials following 24 weeks of intranasal injections of either empty exosomes or high dose exoCAT-SKL. A) The mean distance to the exit platform area of each group per trial is plotted. The distance to the platform reflects path efficiency. Vertical dotted lines indicate the first trial of the following day (i.e. trial 5 is 24 hours following trial 4). Mice that did not reach the platform during the trial were recorded with the total distance swam reflecting the trial endpoint. No significant interactions between time, genotype, and treatment were observed for the distance to platform by trial. Significant time-dependent ($F_{15,240} = 3.340$, $P < 0.0001$), genotype-dependent ($F_{1,240} = 23.81$, $P < 0.0001$), and treatment-dependent sources of variation ($F_{1,240} = 5.503$, $P < 0.05$) were observed in the distance to platform by trial. The distance to the platform was significantly shorter over the course of the trials for all treatment groups except for 3xTG AD mice treated with high dose exoCAT-SKL. B) The mean distance to the platform of each group per day is plotted. No significant interactions between time, genotype, and treatment were observed for the distance to platform by day. Significant time-dependent ($F_{3,60} = 5.639$, $P < 0.01$), genotype-dependent ($F_{1,60} = 19.85$, $P < 0.0001$), and treatment-dependent sources of variation ($F_{1,60} = 4.589$, $P < 0.05$) were observed. Values are presented as mean ± SEM (Three-way ANOVA).
**Probe trial: 24-hour long-term memory retention does not demonstrate significant differences**

Twenty-four hours following the last learning trial, mice were subjected to a probe trial. Several measurements were recorded as an index of each mouse's memory capacity: the percentage of time spent in the target quadrant (Figure 12A), the number of removed platform area entries (Figure 12B), latency to removed platform area (Figure 12C) and distance to removed platform area (Figure 12D). No significant differences were observed in any of the interactions and measurements between all treatment groups.
Figure 11. Indications of short-term memory during the probe trial. A) The time spent in the target quadrant is expressed as a percent of the total time of the probe trial. The horizontal dotted line represents the theoretical amount of time spent in the target quadrant due to chance. No significant differences amongst groups were observed. B) The number of removed platform area entries per group are indicated. No significant differences were observed. C) The mean latency to the removed platform area of each group is plotted. Mice that did not reach the area during the trial were recorded as 60 seconds (time indicating the trial endpoint). No significant differences were observed. D) The mean distance to the removed platform area of each group is plotted. Mice that did not reach the area during the trial were recorded with their total swim distance during the entire trial. No significant differences were observed. Values are presented as mean ± SEM (Two-way ANOVA).
Flag Trial:

On the final day immediately following the probe trial, a single flag trial was conducted as a control procedure to examine if any differences observed in the MWM task was attributed to extraneous factors such as visual impairment and lack of motivation. All mice were acclimated to a flag placed upon the platform indicating its position in the MWM. The latency to the flagged platform was recorded (Figure 12). No significant differences between genotypes, treatments, and interactions were observed demonstrating that all mice had similar motivation and abilities to find the exit platform.

![Figure 12. Latency to the platform during flag trial.](image)

The mean latency to the platform of each group is plotted. All mice reached the platform within the allocated trial time. No significant differences were observed between groups. Values are presented as mean ± SEM (Two – way ANOVA).
3.2.2 Anatomical changes

Mice were weighed once a week on the day of the exoCAT-SKL intranasal administration for 24 weeks. The change in body weight was calculated as the mouse's weight on the day of sacrifice (Figure 13A) subtracted by the weight of the mouse on the first intranasal injection. No significant sources of variation were observed in the interactions between genotype and treatment for all measurements. No significant body weight changes were identified between all treatment groups (Figure 13C). Post-mortem brain weights were also recorded on the day of sacrifice (Figure 13B). The relative brain to body weight was calculated as the ratio of the brain weight to the mouse. No significant differences were observed in normalized brain weights amongst all treatment groups (Figure 13D).
Figure 13. Weight gain, brain weight, and relative brain weight. A) The mean weight of each treatment group at time of sacrifice. B) Means of raw brain weight at the time of sacrifice of each treatment group is plotted. C) The mean change in body weight over 24 weeks of injections was tracked for all treatment groups. No significant differences were observed. D) The relative brain to body weight is expressed as a percentage of total body weight. No significant differences were observed between all treatment groups. Values are presented as mean ± SEM (Two-way ANOVA).
3.2.3 Long-term exoCAT-SKL administration does not exhibit toxicity in the brain in vivo.

Histopathological analysis of H&E stained brain tissue determined whether degenerative or toxic changes occurred in mice that underwent 24 weeks of intranasal exoCAT-SKL treatment. Mimicking Aim 1, serial sections throughout the brain, were analyzed to account for widespread bCAT-SKL biodistribution. Representative images of bregma 2.5 mm to bregma -6.2 mm are shown (Figure 14).

Neuronal injury within the piriform cortex, cerebral cortex, anterior and posterior corpus callosum, dorsal and ventral hippocampus, and the cerebellum was not observed under microscopic examination in any of the treatment groups. Features of toxicity examined included the absence of eosinophilia of the cytoplasm, shrinkage or disappearance of the nucleolus, disintegration of the cell, amongst other characteristics of injury. In addition, neuronal atrophy or neuronal necrosis was not observed in any treatment group. Reactive inflammation could not be accurately reported using H&E analysis; however, a normal distribution of neural cells was observed in all treatment groups. No vasculature abnormalities nor lesions were identified throughout the tissue and white matter tracks did not contain degenerative changes (i.e. vacuolation, spheroids). No evidence of toxicity was identified throughout the brain for both genotypes and treatments.
<table>
<thead>
<tr>
<th>Bregma Level</th>
<th>Control Empty Exosomes</th>
<th>Control High Dose exoCAT-SKL</th>
<th>3X TG Empty Exosomes</th>
<th>3X TG High Dose exoCAT-SKL</th>
</tr>
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<tbody>
<tr>
<td>2.5 mm</td>
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<tr>
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<tr>
<td>-6.2 mm</td>
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</tbody>
</table>

100 µm
Figure 14. Chronic exoCAT-SKL injections do not cause toxicity in the mouse brain *in vivo*. Representative sections of bregma 2.5 mm to bregma -6.2mm are selected to portray the entirety of the brain. All sections are stained with hematoxylin and eosin for features of toxicity, including but not limited to spongiosis, neuron degeneration, inflammation, and vasculature abnormalities. Representative 10X magnification of boxed areas of all treatment groups is showcased. All features of toxicity are negative in all areas for control and 3xTG AD mice treated with empty exosomes and high doses of exoCAT-SKL.
3.2.4 Neuroinflammation: Microglia reactivity

Immunohistochemical analysis of IBA1 stained brain tissue of mice intranasally injected with exoCAT-SKL for 24 weeks was performed to investigate microgliosis. Pathology in the corpus callosum and hippocampus were of particular interest due to their association with memory and cognitive function. Representative images of the of bregma 1.7 mm to bregma -1.8 mm are shown (Figure 15A). No significant differences were observed in the interactions between genotype and treatment. A significant genotype-dependent increase in Iba-1 positive microglia in the hippocampus (F_{1,32} = 34.57, \( P < 0.0001 \)) and the corpus callosum (F_{1,15} = 8.984, \( P < 0.01 \)) was observed. Both 3xTG AD groups had significantly more percent IBA1 microglia coverage in the hippocampus and corpus callosum than their control counterparts (Figure 15B,C).
Figure 15. Total microglia in the brain. A) Representative images of Iba-1 immunolabelled microglial cells throughout brain sections of bregma 1.7 mm to bregma 1.8 mm are showcased. 2X digitally enhanced magnification of boxed areas of the left column is shown for all treatment groups. 2x digitally enhanced insets of areas of interest included the piriform cortex, corpus callosum, medial septal nucleus, and hippocampus. B) Percent area of IBA1 positive microglia in the hippocampus is shown. No significant differences were observed in the interactions between genotype and treatment. A significant genotype effect was observed ($F_{1,32} = 34.57, P < 0.0001$). C) Percent area of IBA1 positive microglia in the corpus callosum is shown. No significant differences were observed in the interactions between genotype and treatment. A significant genotype dependent effect was observed ($F_{1,15} = 8.984, P < 0.01$). Values are presented as mean ± SEM (Two-way ANOVA).
Section 4: DISCUSSION
The results of this study provided the foundation for using macrophage-derived exosomes to deliver the recombinant antioxidant, CAT-SKL, to the brain. This approach could act as a potential therapeutic strategy for many neurodegenerative disorders by addressing two complementary issues: i) an important antioxidant cargo, and ii) a therapeutically promising delivery method.

The function of CAT-SKL in metabolizing H.O. into less toxic derivatives and its ability to target peroxisomes directly holds tremendous promise for managing cellular ROS. CAT-SKL was previously shown to protect neurons, reduce cell death and neuroinflammatory responses elicited in cell culture models of neurodegeneration (Giordano et al., 2014). In addition, studies have shown intraperitoneal injections of CAT–SKL reduced neuroinflammation and memory deficits in a rat model of AD (Nell et al., 2017). Although CAT-SKL had been demonstrated to be protective in numerous preclinical models, these previous studies were limited by biodistribution, systemic degradation, and, most importantly, the inability to penetrate the BBB.

4.1 Exosomes as therapeutic drug carriers

Results from the current study (Figure 1 and Figure 2) demonstrated that sonication techniques could be used to incorporate CAT-SKL into exosomes successfully. The hydrophilic core of exosomes is a suitable environment for water-soluble compounds such as CAT-SKL (Jiang and Gao, 2017). This approach allowed CAT-SKL to be protected from degradation in vivo while maintaining H.O. decomposition abilities (Figure 1 and Figure 2). Similar sonication techniques have been implemented to load exosomes successfully in other studies. Kim et al. reported that exosomes sonicated with chemotherapy medication, paclitaxel, did not disrupt the structure and content of exosome membranes and maintained stability in aqueous solution for over one month (Kim et al., 2016). In addition, Haney et al. also demonstrated mild sonication of exosomes in the
presence of endogenous catalase, provided effective loading capacity and improved its enzymatic stability against protease degradation (Haney et al., 2015).

Gel-filtration and ultracentrifuge techniques, as used in this study, demonstrated successful purification of exoCAT-SKL, providing promise for standardized production and purification of loaded exosomes (Figure 1 and Figure 2A). ExoCAT-SKL enzymatic activity was not disrupted following a freeze-thaw cycle as measured by H.O. decomposition assays (Figure 2D), allowing for long term storage in future studies. This work provides further evidence that exosomes could be a valuable tool as carriers of therapeutic proteins.

4.2 Safety of exoCAT-SKL within the brain

Although exoCAT-SKL holds tremendous potential as both an effective therapeutic and efficient drug transport technique, the safety of its use required investigation. The use of homologous exosomes in our study was derived from same-species monocytes, resulting in minimal immune responses (Ha, Yang and Nadithe, 2016). Thus, an emphasis was put on the toxicology profile of CAT-SKL in the brain and systemically in vivo. Accurate interpretation of mouse histology provided a fundamental step in determining the feasibility of exoCAT-SKL.

Drug delivery through the intranasal route has been demonstrated as an effective and non-invasive method for targeting drug uptake into the brain (Crowe et al., 2018b). This study demonstrated that the intranasal injection of exoCAT-SKL could successfully cross the BBB and deposit CAT-SKL into the brain (Figure 7). No apparent abnormalities nor features of toxicity were observed in the brains of mice treated for 4 weeks or 24 weeks of exoCAT-SKL injections in all treatment groups. This finding provided further evidence that CAT-SKL has non-toxic neuroprotective roles similarly described in other in vitro and in vivo studies (Giordano et al., 2015; Nell et al., 2017). Standard catalase without the recombinant c-terminus loaded within
exosomes have also been shown to be neuroprotective in preclinical models of Parkinson's disease (Haney et al., 2015). The protective capacity of standard catalase in neurodegenerative models highlights the increased potential of CAT-SKL in similar preclinical experiments.

4.3 Microgliosis

Research in AD has established that neuroinflammation contributes to the pathogenesis of AD in addition to other hallmarks such as Aβ and neurofibrillary tangles (Raj et al., 2017). It is well established that microglia contribute to the inflammatory response through their interactions with Aβ and their frustrated phenotype, causing the release of pro-inflammatory cytokines, chemokines and ROS (Xiang et al., 2006; Hemonnot et al., 2019). The IBA1 marker of microglia was used to measure microgliosis with increased microglia reflective of neuroinflammation and microglia activation (Imai et al., 1996; Lee et al., 2017). Results from this study demonstrated an increased microglia response in 34-week-old 3xTG AD mice compared to their control counterpart independent of exoCAT-SKL treatment. Consistent with these findings, previous work by Belfiore et al. found elevated levels of IBA1 labelled microglia in the medial, caudal, and rostral hippocampus of 3xTG AD mice at 6 months of age consistent with these findings (Belfiore et al., 2019). The current study found no significant differences in IBA1 labelled microglia in the corpus callosum or hippocampus between mice treated with exoCAT-SKL and mice treated with naive exosomes of either genotype (Figure 15). These findings contradict findings by Haney et al. which demonstrated that catalase reduced microgliosis in mice stereotaxically injected with 6-hydroxydopamine, a neurotoxic compound used to destroy dopaminergic neurons (Haney et al., 2015). In addition, Nell et al. 's findings demonstrated that intraperitoneal injections of CAT-SKL reduced microgliosis in the medial septal nucleus and thalamus of 6-month-old rats administered
with Aβ peptides (Nell et al., 2017). These contradictory results could be attributed to the concentration and frequency of exoCAT-SKL injections as well as the limited numbers used in this study. Nell et al. implemented intraperitoneal injections of CAT-SKL at 1 mg/kg once per week while Haney et al. implemented 10 intranasal injections every other day (Haney et al., 2015; Nell et al., 2017). The concentration of CAT-SKL that reached the brain was not quantifiable in this study, and preliminary results did not indicate CAT-SKL elicited a detectable effect on microgliosis.

### 4.4 Morris water maze

The Morris water maze task was implemented to discern any learning and memory differences induced by genotype differences or 24 weeks of exoCAT-SKL injections (Morris, 1984; Bromley-Brits, Deng and Song, 2011; Frame et al., 2019). Results from the MWM testing were preliminary, and discrepancies between our findings to that of others can be attributed to the lack of statistical power in Aim 2 of our study. All of the treatment groups demonstrated successful learning of the task by swimming to the platform significantly quicker and using less distance over the course of all trials except for the 3xTG High Dose exoCAT-SKL group (Figure 9, Figure 10). Genotype induced differences were not observed as the 3xTG Empty group performed similarly to Control Empty mice. This contradicts findings by Belfiore et al. that showcased 6-month-old 3xTG-AD mice developed learning deficits compared to age-matched controls (Belfiore et al., 2019). ExoCAT-SKL treatment showed beneficial effects exclusively in control mice as the Control High Dose exoCAT-SKL group performed better during the learning period in both latency and distance travelled compared to the Control Empty group (Figure 9, Figure 10). Similar exoCAT-SKL induced improvements were not demonstrated in 3xTG mice and could be attributed
to the aggressive phenotype of the 3xTG-AD mice at the age of testing (8.5 months) (Stimmell et al., no date; Oddo et al., 2003; Sterniczuk et al., 2010). Increased statistical power could also discern potential differences amongst treatment groups during the probe trial.

The flag trial acted as an important control to ensure that all the mice possessed the basic abilities to perform the tasks (Vorhees and Williams, 2006; Frame et al., 2019). These abilities include intact visual acuity, swimming away from the edge of the pool, and climbing onto the exit platform. The flag trial results did not identify any significant differences between the latency to the flagged platform among the treatment groups (Figure 12). This indicated that all of the mice had similar abilities to learn using visuospatial cues, swimming abilities, and motivation to find the platform. Therefore, any differences in learning and memory retention could be attributed to differences in genotype or treatment (Frame et al., 2019).

4.5 Off-Target exoCAT-SKL toxicology

Due to the inherent mechanisms of intranasal exoCAT-SKL delivery, off-target tissues were assessed to ensure the safety of repeated dosing. As with any intranasal drug, ExoCAT-SKL is likely to be aspirated and deposited into the lung, swallowed into the GI system, and distributed into the circulatory system (Erdő et al., 2018). Standard investigation of the relative organ weight of all tissues, including the brain, showed no significant differences in treatment groups and indicated no chemically induced changes to organs (data not shown). Histopathological assessment of the highlighted organs (as described in the methods) not only provides evidence that the intranasal administration of exoCAT-SKL is safe but also sheds light on the therapeutic potential of exoCAT-SKL for other organ disorders. Although exoCAT-SKL has a high likelihood of depositing into the lung (Imai et al., 2015), the histopathological assessment of lung tissue
indicated no perceived abnormalities across all treatments exemplifying the safety of exoCAT-SKL in terms of preserving the architecture and cellular makeup. In the liver, the absence of exoCAT-SKL toxicity supports other studies of subcutaneous injections of CAT-SKL being protective in preclinical models of diabetic retinopathy (Giordano et al., 2015). Assessments of the spleen across all treatments indicated that mice could tolerate exoCAT-SKL for all treatment dosages and durations. The absence of histopathological toxicity features in both the small and large intestines aligns with studies demonstrating protective effects of exogenous catalase in models of inflammatory bowel disease where exogenous catalase had been shown to decrease leukocytic ROS production, mucosal damage, and hyperemia (Ruh et al., 2000). Although the protective effects of exoCAT-SKL on off-target tissue were not investigated in the current study, the increased peroxisomal specificity of exoCAT-SKL may contribute to its safety. Compared to exogenous catalase, exoCAT-SKL limits the alterations of necessary redox biology residing in the cytoplasm or other organelles that maintain physiological functions (Valko et al., 2007; Finkel, 2011; Schieber and Chandel, 2014).

4.6 Limitations and future direction

Two of the biggest limitations of this study are the lack of statistical power for Aim 2 and the use of mouse models to model human AD. Although it is common practice to use transgenic mice for preclinical research, the genetic and physiological differences between mice and humans cause transformative and translational discrepancies in clinical studies (Ellenbroek and Youn, 2016). In addition, the statistical power of Aim 2 of this study was not adequate for evaluating the effects of exoCAT-SKL on behavioural and histological outcomes.
This study identified the distribution and safety of intranasal exoCAT-SKL administration; however, several questions remain regarding the efficacy and mechanisms of CAT-SKL. In particular, further investigation is required to identify the most effective dosing to prevent AD pathology in transgenic mice. Indicators of oxidative stress, such as the by-products of lipid peroxidation in brains of mice treated with and without exoCAT-SKL can be investigated using MALDI mass spectrometry (Leopold et al., 2018). In vitro experiments can also explore whether exoCAT-SKL is preferentially internalized within neural cell populations and the extent to which exoCAT-SKL can reduce ROS and oxidative stress in cell culture models. Future investigation can also assess the potential of exoCAT-SKL to ameliorate and even reverse AD outcomes in transgenic mice. The typical diagnosis of AD for most patients occurs following cognitive impairment and significant pathological load (Petersen, 2009). Giordano et al. have demonstrated that CAT-SKL can reverse the neurite loss caused by Aβ peptide administration in vitro (Giordano et al., 2014). Assessing the potential of exoCAT-SKL to reduce and reverse pre-existing pathological and behavioural correlates will increase its practicality.
Section 5: CONCLUSION
Developing potential therapeutic drugs for nervous system disorders such as AD must consider the disease's complex and debatable pathophysiology. This study has developed methods to test the safety, biodistribution, and therapeutic potential of a new drug and delivery mechanism, exoCAT-SKL. Results from the current investigation have provided further evidence that exosomes can act as carriers of recombinant proteins as well as other potential therapeutic proteins. The innate ability of exosomes to penetrate the BBB increases its potential in treating neurodegenerative diseases. Furthermore, the peroxisome targeting ability of CAT-SKL allows for Control of ROS and oxidative stress at the site of production.

CAT-SKL is a powerful antioxidant; however, its safety in the brain had never been investigated until now. A critical part of the risk assessment associated with exoCAT-SKL was assessing its toxicologic effects. Histopathology used in this study did not distinguish exoCAT-SKL induced abnormalities from normal histological variations. Analysis of brain tissue determined no features of toxicity such as necrosis, fibrosis, inflammation, and growth disturbances (i.e. neoplasia, hyperplasia). Off-target toxicity analysis also did not present risks associated with short-term and long-term intranasal administration of exoCAT-SKL.

AD is strongly tied to oxidative stress, lipid peroxidation, increased inflammatory mediators, and reduced antioxidant defences (Butterfield et al., 2001; Lull and Block, 2010; Cline et al., 2018). Although exoCAT-SKL did not demonstrate toxicity in mice, the efficacy of exoCAT-SKL in preventing AD pathology requires further analysis. Additional investigation is required to discern whether chronic exoCAT-SKL can reduce ROS and mediate neuroinflammation responses. Preliminary MWM maze results did not show behavioural
improvements in exoCAT-SKL treated 3xTD AD mice, but the promise remains in promoting learning and memory retention in wildtype mice.

This study addressed several barriers of using protein biologics in a therapeutic setting by answering questions surrounding bioavailability, toxicity, and penetrating the BBB. The use of exosomes to deliver compounds to cells in need allows for the re-evaluation of other proteinaceous reagents. In future clinical studies, using a patient's own monocytes as a source of homologous exosomes could also allow this delivery method to bypass any immune responses. Thus, in an era of personalized medicine, the use of exosomes will constitute a personalized delivery method that holds tremendous promise.
References


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10.1111/bpa.12368.


April 2020).


Stimmell, A. C. et al. (no date) ‘Impaired Spatial Reorientation in the 3xTg-AD Mouse Model of Alzheimer’s Disease’. doi: 10.1101/258616.


Appendix

AUP Number: 2018-068
PI Name: Whitehead, Shawn N
AUP Title: Exosome delivery of biologics to mice Approval Date: 07/01/2018
Official Notice of Animal Care Committee (ACC) Approval:
Your new Animal Use Protocol (AUP) 2018-068:1: entitled "Exosome delivery of biologics to mice" has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee University Council on Animal Care
Curriculum Vitae

QINGFAN LIU

HIGHLIGHTS OF QUALIFICATIONS

- Western University’s Faculty of Science National Scholarship recipient ($30,000)
  - Awarded for outstanding academic performance, achievement in extracurricular activities and commitment to community service
- 2019-2020 Canadian Institute of Health Research (CIHR) – Frederick Banting and Charles Best Canada Graduate Scholarship recipient ($17,500)
  - Awarded annually to 14 Master’s students at Western University who demonstrate high standards of achievement in undergraduate and early graduate studies
- 2019 Thales Student Innovation Championships 1st Place Winner ($20,000)
  - Awarded to the best innovative solution that solves a theoretical challenge using artificial intelligence across Canada

EDUCATION

Schulich School of Medicine and Dentistry  London, ON  2020-2024
  - Doctor of Medicine
Schulich School of Medicine and Dentistry  London, ON  2018-2020
  - Master of Science
    - Anatomy and Cell Biology
Western University  London, ON  2014-2018
  - Bachelor of Medical Science with Distinction
    - Honors Specialization in Interdisciplinary Medical Science, Minor in Mathematics
    - 2014-2018 Western University Dean’s Honor List

PUBLICATIONS

  *indicates both authors contributed equally.*

POSTER PRESENTATIONS


ORAL PRESENTATIONS


WORK EXPERIENCE AND COMMUNITY INVOLVEMENT

**Vulnerable Brain Laboratory Member**
Schulich School of Medicine and Dentistry London, ON 2012-Current

**Stroke, Alzheimer’s Disease, and Dementia Research Assistant**

- Performed perfusions, brain extractions, pathological and histological analysis on both rats and mice resulting in competency in animal experimentation and wet lab work
- Coordinated research projects investigating temporal and spatial characterization of brain inflammation following ischemic stroke resulting in presentation at 2016 Western Student Research Conference
- Analyzed rodent neuroanatomy using confocal and fluorescent microscopy and imaging software identifying pathological and histological abnormalities to produce figures for publications and poster presentations
- Investigated white matter inflammation and cognitive impairment in transgenic rats through Dean Undergraduate Research Opportunities Program (DUROP) resulting in co-first author publication in Journal of Neuroinflammation
- Combined 6 years of work experience in research leading to mentorship position and established foundation to pursue a master’s degree at Western University, Schulich School of Medicine and Dentistry

**Alzheimer’s Disease Stroke, Alzheimer’s Disease, and Dementia Research Mentor**
• Mentored three laboratory research volunteers in animal handling, microscopy, immunohistochemistry, amongst other laboratory techniques to develop their independence to take on individual projects
• Designated small research projects for laboratory volunteers and provided ongoing guidance until completion

Alzheimer’s Society Companion Program Volunteer
Alzheimer Society London and Middlesex  London, ON  2018-2020
• Provided weekly one-on-one visits for companion with mid-stage dementia promoting socialization and cognitive engagement, over time building a new friendship that improves the quality of life for the companion and their family

Western University Integrative Neuroscience Teaching Assistant
Western University  London, ON  2018-2019
• Employed as a teaching assistant for the course Integrative Neuroscience designed for 4th year medical science university students
• Facilitated student learning in the classroom, laboratory and online by guiding dissections and answering questions
• Provided technological support to professors and evaluated participation for in-class students improving grading objectivity and consistency

Western University Anatomy and Cell Biology Graduate Teaching Assistant Steward
Western University  London, ON  2018-2019
• Provided communication link amongst all the Graduate Teaching assistants and Post-Doctoral assistants in the department of Anatomy and Cell Biology at Western University
• Protected the rights of Graduate Teaching Assistants as bargained for in the Collective Agreement by the Public Service Alliance of Canada
• Organized TA Solidarity event for the department to discuss TA rights and resolve grievances

Western University Dragon Boat Team Dry-land Coach
Western University  London, ON  2016-2018
• Organized fitness regimes for over 120 team members up to four times a week to gradually build strength and endurance throughout the season
• Provided in depth instruction and classes on skills related to paddling, strength, and cardiovascular fitness to ensure members used proper form and trained safely
• Monitored the performance of each team member to provide individualized constructive feedback and supported team members in reaching personal goals
• Competed in regional, provincial, and national level regattas resulting in multiple Canadian Dragon Boat Championship podium finishes

Western University National Scholarship Mentor Link Program
Western University  London, ON  2016-2018
• Mentored national scholarship recipients year-round by connecting freshman students to Western University’s social environment and its academic resources to ensure smooth transitions into university life
• Provided one on one orientation of campus and student life for incoming first year students

Western University Dragon Boat Fundraising Executive
Western University  London, ON  2014-2016
• Coordinated sponsorships from corporate and local companies; fundraised over $1000 through creative and appropriate ideas for the team
• Designated tasks and provided training to fundraising representatives for effective communication with corporate sponsors

AWARDS AND DISTINCTIONS

Academic Achievements
• 2019-2020 Canadian Institute of Health Research (CIHR) – Frederick Banting and Charles Best Canada Graduate Scholarship recipient ($17,500)
  • Awarded annually to 14 master’s students at Western University who demonstrate high standards of achievement in undergraduate and early graduate studies
• 2019 Ontario Graduate Scholarship ($15,000) *Declined
• 2019 Schulich School of Medicine and Dentistry Dept. Anatomy and Cell Biology Travel Award
• 2014 Western University Faculty of Science National Scholarship Recipient ($30,000)
  • Awarded for outstanding academic performance, achievement in extracurricular activities and commitment to community service
• 2014-2018 Western University Dean’s Honor List
• 2013 Canadian Senior Mathematics Contest National Honor Roll
• 2013 Fermat Mathematics Contest Canadian Gold Medalist (Perfect Score)
• 2011 Pascal Mathematics Contest Canadian Gold Medalist (Perfect Score)

Extra-Curricular Achievements
• 2018 Thales Student Innovation Championship 1st Place Winner ($20,000)
  • Awarded to the best innovative solutions that solves a theoretical challenge using Artificial Intelligence across Canada
• 2018 Schulich School of Medicine and Dentistry Interdisciplinary Medical Science Speaker Series Guest Speaker
• 2017 Dean’s Undergraduate Research Opportunities Program Summer Award Recipient ($6,000)
  • Awarded to outstanding science undergraduate students to pursue medical research
• 2016 Western Student Research Conference Presentation
  • Project Title: Characterization of Microglia following Ischemic Stroke
• 2014 Schulich Leader Scholarship Nominee

Athletic Achievements
• 2019 Owl’s Head Quebec Super Spartan Race Finisher
• 2019 Subaru Triathlon Series Guelph Lake I – “Try a Tri” - Men’s 20-24 Gold Medalist
• 2018 Canadian Dragon Boat Championships University Mixed Division Bronze Medalists
• 2018 Canadian Dragon Boat Championships U24 Open Division Silver Medalists
• 2018 Toronto International Dragon Boat Festival University Division Finalist
• 2017 Canadian Dragon Boat Championships University A Division Finalists
• 2015 Canadian Dragon Boat Championships University Open Division Silver Medalists
• 2014 Alex Kelman SPECTRA (Sportsmanship, Participation, Excellence, Character, Teamwork, Respect and Achievement) Award Nominee
• 2014 Wil Rice and Bill Traut Athletic Award ($2500)