The Development of a Brain Penetrating Erythropoietin for the Treatment of Alzheimer's Disease

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The Development of a Brain Penetrating Erythropoietin for the Treatment of Alzheimer's Disease

By

Joshua Yang

A Dissertation submitted to the Faculty of Keck Graduate Institute of Applied Life Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Applied Life Sciences

Claremont, California

2022
We, the undersigned, certify that we have read this dissertation of Joshua Yang and approve it as adequate in scope and quality for the degree of Doctor of Philosophy.
Abstract of the Dissertation

The Development of a Brain Penetrating Erythropoietin for the Treatment of Alzheimer's Disease

By

Joshua Yang

Keck Graduate Institute of Applied Life Sciences: 2022

The development of a neurotherapeutic for the treatment of Alzheimer’s Disease (AD) is challenging due to limited endpoint efficacy. Erythropoietin (EPO), a hematopoietic neurotrophin, is a potential therapeutic for AD but has limited blood-brain barrier (BBB) permeability. A chimeric fusion protein of EPO bound to the transferrin receptor monoclonal antibody (cTfRMAb) can act as a molecular trojan horse for brain drug delivery, shuttling EPO into the brain via the transvascular route. However, cTfRMAbs have Fc-effector function adverse effects, and removal of the Fc N-linked glycosylation site by substituting Asn with Gly (cTfRMAb-N292G-EPO) reduces this Fc effector function. Hence, developing this therapeutic for the treatment of AD is the central goal of the present dissertation.

In previous studies, the cTfRMAb-N292G-EPO protein displayed a higher-than-expected clearance and lower plasma concentrations compared to the non-mutant protein. The first objective was to determine the dosage by which the clearance
mechanisms are saturated to get plasma exposure comparable to the non-mutant protein while maintaining a favorable safety profile through intraperitoneal and subcutaneous parenteral routes of administration.

Previous studies also showed that the BBB-penetrating cTfRMAb-EPO reduces amyloid-beta (Aβ) in transgenic mice, but the neuroprotective effects on tau pathology are not known. The second objective was to investigate the effects of chronic dosing of the cTfRMAb-N292G-EPO fusion protein in a PS19 mouse model of tauopathy.

Radiolabeled molecules are the gold standard for measuring brain protein concentration. However, radioactive particles may be potentially hazardous for investigators. The last objective was to develop an alternative method to radiolabeled molecules for measuring cTfRMAb concentration. The method developed in this dissertation is the Amicon Concentration Method, which concentrates brain homogenate samples.

Our results demonstrated that although cTfRMAb-N292G-EPO is efficacious in Tauopathic mice, high doses of cTfRMAb-N292G-EPO are needed to equate to comparable plasma exposure to the nonmutant protein. This increase in dose leads to hematologic adverse effects similar to those associated with high-dose recombinant EPO treatment. The data obtained from the groundwork for future studies using the non-mutant cTfRMAb-EPO at low doses in multiple AD mouse models that combine different hallmarks of AD pathology
I dedicate my dissertation to my loving parents, Dr. Joseph Yang and Dr. Lily Ma whose support and words of encouragement helped me to persevere and thrive in the Ph.D. program. I will always appreciate all that they have done for me and for being there for me throughout the entire doctorate program.
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ABBREVIATIONS
Alzheimer’s disease (AD)
Amyloid-beta (Aβ)
Area Under the Plasma Concentration-Time Curve (AUC)
Asparagine (Asn)
Blood-brain barrier (BBB)
Bovine Serum Albumin (BSA)
Central nervous system (CNS)
Chinese Hamster Ovary (CHO)
Complete Blood Count (CBC)
Chimeric Monoclonal Antibody Targeting the Mouse Transferrin Receptor (cTfRMAb)
Chimeric Transferrin Receptor Antibody-Erythropoietin Fusion Protein Therapeutic (cTfRMAb-EPO)
Mutant Chimeric Transferrin Receptor Antibody-Erythropoietin Fusion Protein Therapeutic (cTfRMAb-N292G-EPO)
Dentate Gyrus (DG)
Dissociation Constant (KD)
Enzyme-linked immunosorbent assay (ELISA)
Erythropoietin (EPO)
Erythropoietin Receptor (EPOR)
Fc Gamma Receptor (FcyR)
Glycine (Gly)
Hematocrit (HCT)
Hematoxylin & Eosin (H&E)
Hemoglobin (HGB)
Injection Dose (ID)
Intraperitoneal (IP)
Intravenous (IV)
Open-Field Behavioral Test (OFT)
Optical Density (OD)
Paraformaldehyde (PFA)
Percentage of injected dose per volume of plasma (%ID/mL)
Pharmacokinetics (PK)
Phosphate buffered saline (PBS)
Prnp-MAPT*P301S PS19Vle (PS19)
Phospho-tau (pTau)
Maximum Binding ($B_{\text{max}}$)
Maximum Plasma Concentration ($C_{\text{max}}$)
Mean corpuscular volume (MCV)
Mean Residence Time (MRT)
Molecular Trojan Horse (MTH)
Monoclonal antibody (Mab)
Neonatal Fc Receptor (FcRn)
Neurofibrillary tangles (NFTs)
Receptor-mediated transcytosis (RMT)
Red blood cells (RBCs)
Reverse transcription-polymerase chain reaction (RT-PCR)
Size exclusion chromatography (SEC)
Standard Error of the Mean (SEM)

Subcutaneous (SQ)

Tissue Protein Extraction Reagent (T-PER)

Transferrin receptor-1 (TfR1)

Tris-Buffered Saline (TBS)

Trist- Buffered Saline containing 1% bovine serum albumin (TBSB)

Tris-Buffered Saline containing 0.05% Tween 20 (TBST)

Triton X-100 (TX100)

Volume of Distribution at Steady State (V\textsubscript{SS})

White blood cell (WBC)

Wildtype (WT)
Alzheimer’s Disease:

Alzheimer’s disease (AD) is a chronic neurodegenerative disease that is characterized by age-related, progressive cognitive dysfunction, psychiatric symptoms, and behavioral disturbances [4]. AD is the most common form of dementia and may contribute to up to 80% of dementia cases [5]. The prevalence of AD dementia today is about 5.8 million Americans age 65 and older with a death toll of 122,019 in 2018, which is the latest year where data are available [6]. AD is the 6th leading cause of death in the US and totals an estimated $305 billion in long-term care and hospice services for people age 65 and older [6]. The symptoms of AD can range from mild memory loss to functional decline and behavioral disturbances. Gradual memory loss is the first symptom in the vast majority of AD cases [4]. The functional decline and behavioral disturbances may include apathy, problems with language, disorientation, mood swings, motivation loss, physical aggression, and self-care mismanagement [7].

The underlying cause of AD is not fully understood [4]. Risk factors linked to AD may include age, environmental factors, genetics, and predisposition to head injuries, depression, and hypertension [4]. The hallmark pathology of AD is characterized by amyloid plaques, neurofibrillary tangles (NFTs), and chronic inflammation.

Amyloid Plaques:
Amyloid plaques are composed primarily of amyloid-beta (Aβ). Aβ is comprised of 36-43 amino acids, derived from the amyloid precursor protein (APP) [8]. APP is an integral membrane protein found in many tissues in the central nervous system (CNS) including the brain and spinal cord. APP is a complex molecule, and its primary function is not completely understood. However, APP has been hypothesized to be involved in synaptic formation regulation [9] and neural activity regulation [10]. The regulation of neural functions by APP fragments including Aβ includes cell excitability, synaptic transmission, and long-term potentiation [10]. The APP gene, located on chromosome 21, consists of 18 exons [11]. The APP gene is ancient and highly conserved, but recent mutations may potentially have resulted in unrelated amyloid-forming sequences [12]. Genetic studies identified mutations in the APP, presenilin 1 (PSEN1), and presenilin 2 (PSEN2) genes that lead to the accumulation of Aβ [13]. Critical APP mutations may increase the likelihood and familial susceptibility to AD [14, 15].

APP undergoes cleaving and post-translational modifications to create Aβ peptide fragments. Through the amyloidogenic pathway, APP is initially cleaved by β-secretase (BACE1), releasing the large N-terminal ectodomain (sAPPβ) into the extracellular space and leaving behind the 99 amino acid C-terminal domain on the membrane [16]. The large C-terminal domain is subsequently cleaved by γ-secretase, releasing 36-43 fragments of amino acid peptides called Aβ [16]. The Aβ peptides that contain 42 residues (Aβ 1–42) are more neurotoxic because the extra amino acids increase the probability of misfolding, leading to subsequent aggregation [17]. The misfolding and aggregation of Aβ have been hypothesized to lead to the progression of AD.
**Aβ Mechanisms of Action for the Progression of AD:**

The amyloid cascade hypothesis has been the prevailing and most influential hypothesis for the progression of AD since its discovery in 1984. This hypothesis postulates that the deposition of Aβ peptides in the brain parenchyma progresses AD, and this concept has inspired and guided research in the AD field for the last twenty years [18]. There are four main postulated mechanisms by which the accumulation of Aβ leads to the onset of AD. These mechanisms are amyloid accumulation, neurotransmitter dysfunction & synaptic loss, oxidative stress, and neuroinflammation.

Amyloid accumulation leads to neurotoxicity and neuronal death. In the amyloid cascade hypothesis, the initial deposition of Aβ leads to the formation of extracellular senile plaques and tau-associated NFTs [19]. The accumulation of senile plaque aggregates disrupts normal cell functions, leading to neuronal death. Aβ plaques also induce abnormal hyperphosphorylation of tau, generating NFT and greater neurotoxicity in the brain [20]. Tau is a microtubule-associated protein (MAP) with a primary role in maintaining microtubule stability in axons and stimulating tubulin assembly into microtubules in the brain [21, 22]. The tau protein primarily constitutes the abnormal fibrous assembly known as the paired helical filaments (PHFs) of NFTs [23-25]. Under normal, maintenance conditions, tau is phosphorylated, which reduces its affinity for tubulin and destabilizes the axon, and is targeted for removal via neuronal secretion or lysosomal degradation [26]. Removal of damaged proteins by proteolysis and the synthesis of new proteins are important for the preservation of brain homeostasis [27]. However, the continual exposure of neurons to aggregated Aβ results in the
hyperphosphorylation of tau and NFT formation, which builds up in the neurons and results in neuronal death [28]. In AD, the development of NFTs occurs in 3 stages: 1. Pre-neurofibrillary tangle (pre-NFTs), 2. Intra-neuronal neurofibrillary tangle, and 3. Extra-neuronal neurofibrillary tangle [29]. At least 19 tau amino acids are phosphorylated with pre-NFTs phosphorylated at serine (S) 199, S202, and S409, with intra-neuronal NFTs, phosphorylated at S396 and threonine (T) 231, and extra-neuronal NFTs phosphorylated at S396 [29, 30]. Phosphorylation at these positions can lead to a conformational change of the original tau molecule. The abnormally hyperphosphorylated tau can self-aggregate into tangles of PHFs and neutralize inhibitory signals, leading to damage to the neuron [31]. This damage can lead to neurotransmitter dysfunction and synaptic loss.

Aβ and NFT disrupt normal synaptic transmission to neighboring regions throughout the brain [32]. The accumulation of Aβ causes synaptic degeneration [33]. Synaptic homeostasis is maintained when oligomerized Aβ and complement proteins tag synapses for removal by microglial cells [26]. In normal conditions, synaptic tagging for removal is a short-lived signal that phosphorylates tau [26]. However, the continual exposure of neurons to Aβ not only leads to hyperphosphorylation of tau but also tau-dependent Aβ toxicity. In vivo models in JNPL3 (P301L) transgenic mice, which have overexpression of human tau with P301L mutation and cause non-Alzheimer tauopathy, demonstrate this link between Aβ and the accumulation of tangles [34]. Injection of synthetic Aβ into P301L mouse brains produces a 5-fold elevation of tangles near the site of injection [35]. Crossing P301L transgenic mice with the Swedish (K670N/M671L) double mutant transgenic mice with overexpression of human APP results in a hybrid
mouse with similar plaque formation as the parental APP$_{\text{Swe}}$ strain and an accelerated
tangle formation compared to the parental tau$_{P301L}$ strain, modelling familial early-onset
of AD [36]. The various soluble, hydrophobic (diffusible) Aβ oligomers can bind to
different components of the neuronal and non-neuronal cellular membrane, triggering
complex patterns of synaptic dysfunction and vast network disorganization [37]. The
synaptic dysfunction and network disorganization are caused by improper synaptic
communication due to an imbalance of neurotransmitter levels, causing downstream
synaptic loss and cognitive decline.

Aβ1-42 disrupts the synaptophysin and vesicle-associated membrane protein 2
(VAMP2) complex at the presynaptic terminals, elevating the number of primed vesicles
and exocytosis [38]. Synaptophysin and VAMP2 protein complex are important for
docking and fusion of synaptic vesicles in the presynaptic membrane. Synaptic
transmission and vesicular exocytosis are dependent on the formation of the soluble N-
ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex [39]. Aβ
oligomers interfere with vesicular exocytosis by binding to syntaxin 1a, preventing the
formation of the SNARE complex [39].

Aβ also causes vesicular depletion by inducing a rapid increase in intracellular
calcium and miniature currents, leading to a surge in vesicular neurotransmitter release
such as acetylcholine, serotonin, and histamine [40]. Because more immature
presynaptic vesicles are released with fewer neurotransmitters, the neurons are unable
to reach the depolarization threshold needed for action potential propagation, leading to
excitotoxicity. This progression of AD can affect other cellular processes such as energy
production, cellular structure, mitochondrial function, and cell cycle progression.
Aβ and tau aggregates can lead to mitochondrial dysfunction and adversely impact energy production. While many studies found that elevated levels of soluble, fibrillar oligomeric Aβ correlate with oxidative stress in neuronal cells, the exact mechanism is still debated [41]. The primary function of mitochondria is to produce energy through cellular respiration and the electron transport chain (ETC). However, this process creates reactive oxygen species (ROS) that lead to oxidative stress and damage cellular structure and biomolecules. Oxidative stress is strongly linked to the development of neuronal death and dysfunction, and these problems are further compounded due to age-related reduction in efficiency of the ETC [27]. Redox-active metals bound to Aβ can catalyze the production of ROS and can contribute to oxidative damage to surrounding molecules [42]. Oxidative stress induces further Aβ accumulation and tau phosphorylation, leading to a self-perpetuating cycle. LDL receptor-related protein 1 (LRP1) is a multifunctional protein that can bind to Aβ oligomers and whose primary function is to control the efflux of Aβ from the brain to the blood via the blood-brain barrier (BBB) [43]. However, Aβ oxidizes LRP1, disrupting the clearance of Aβ and elevating the accumulation of Aβ in the brain [42]. Aβ can also interact with receptor for advanced glycation end products (RAGE), a multiligand receptor that initiate inflammatory responses. RAGE mediates the continuous entry of circulating Aβ into the CNS but does not eliminate brain-derived Aβ [44]. This interaction results in a positive influx of Aβ across the BBB and increases the expression of proinflammatory cytokines [45].

The accumulation of Aβ in the brain can lead to neuroinflammation. In the CNS, microglia are specialized macrophages involved in synaptic remodeling and tissue
surveillance, maintaining CNS health. Microglia roam the environment, scanning for and phagocytizing damaged, apoptotic cells, pathogens, or foreign materials such as cellular debris and Aβ plaques [46, 47]. However, pathologic stimuli, including Aβ plaque accumulation, can result in sustained microglial activation and the release of proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α), affecting the surrounding brain tissues [48]. Microglial cells have Fcγ receptors, which can be activated by Immunoglobulin G (IgG) [49]. Activated microglial cells are involved in Aβ plaque clearance. However, sustained microglial activation can perturb this clearance process through the secretion of proinflammatory cytokines, which worsens the condition through tissue damage. Proinflammatory cytokines such as IL-18 increase levels of BACE1 and APP expression, resulting in higher levels of Aβ [50]. IL-18 also has been shown to elevate the expression of glycogen synthase kinase-3β (GSK-3β) and cyclin-dependent kinase 5, which are involved in the hyperphosphorylation of tau [51]. This positive feedback loop of Aβ accumulation, microglial activation, and hyperphosphorylation of tau will ultimately lead to irreversible neuronal damage in the CNS.

**Current AD Treatments:**

Before the FDA approval of aducanumab for the treatment of AD, there was no approved therapeutic for the treatment of AD since 2003. More than 200 investigational programs and disease-modifying treatments have failed or were abandoned in the last decade due to late initiation of treatments during AD progression, inappropriate drug dosages, inappropriate treatment targets, or insufficient understanding of AD
pathophysiology [52]. The approved treatments for AD are symptomatic and do not target the underlying cause of the disease. Two types of drugs are used to treat mild to moderate cognitive symptoms associated with AD: Cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists. Typically, these symptomatic treatments are taken in conjunction to improve cognition.

Cholinesterase inhibitors work by blocking the enzyme cholinesterase from breaking down acetylcholine [53]. Acetylcholine is an important neurotransmitter associated with memory, and by inhibiting its breakdown, more acetylcholine can exert its effect at the post-synaptic terminal in neurons [53].

NMDA receptor antagonists also act on the excitation and firing potential in the synapse of neurons. Overactivation of NMDA receptors can cause an overabundance of calcium (Ca\(^{2+}\)) ions, leading to synaptic dysfunction [54]. Glutamate is the primary excitatory neurotransmitter in the brain and is important for the rapid excitation of the synapses [55]. However, continual stimulation of glutamatergic receptors can result in excitotoxicity by the excess release of calcium [56]. NMDA receptor antagonists bind to NMDA receptors, blocking glutamate binding [57]. This blockage prevents the synapse from depolarizing the cell and relieves the normal Mg\(^{2+}\) block of NMDA receptor-coupled channels, disallowing the release of Ca\(^{2+}\) from entering the neuron [57].

In June 2021, the Food and Drug Administration (FDA) approved aducanumab (Aduhelm) developed by Biogen Inc. under the FDA’s accelerated approval pathway for the treatment of mild cognitive impairment or dementia in AD patients, which was a controversial decision. Aducanumab is the first drug approved in the US to treat one of the hallmarks of AD by targeting Aβ plaques in the brain through intravenous infusion.
Aducanumab is a human, immunoglobulin gamma 1 (IgG1) monoclonal antibody directed against aggregated soluble and insoluble forms of Aβ and cerebral amyloid plaques [58]. In 22-month-old female Tg2576 transgenic mice, aducanumab was shown to cross the BBB, bind to parenchymal Aβ, and ultimately reduce both soluble and insoluble Aβ in a dose-dependent manner [59]. Similarly, a Phase 1b study with 165 randomized patients found that treatment with aducanumab also reduced brain Aβ plaques in a dose- and time-dependent manner as measured by florbetapir PET imaging [59]. Despite the preclinical and Phase 1b clinical data, there are some controversies associated with the FDA approval of aducanumab.

Initially, in November 2020, Biogen’s application to the FDA’s Peripheral and CNS Drugs Advisory Committee for aducanumab approval was declined based on utilizing amyloid plaque reduction as a surrogate endpoint and the efficacy of the drug in clinical trials was inconclusive [60]. The 2 large phase III clinical trials were stopped prematurely by Biogen with one trial trending positive and the other displaying no clinical benefits from aducanumab [61]. As a result, the efficacy of aducanumab cannot be concluded by clinical trials with differing outcomes [61]. Participants also exhibited some side effects including headaches, nausea, dizziness, and brain swelling, leading to amyloid-related imaging abnormalities (ARIA) [62]. ARIA represents a spectrum of image abnormalities taken by magnetic resonance imaging (MRI) including vasogenic edema (ARIA-E) and microhemorrhages (ARIA-H) [63]. 26% of participants in the clinical trials receiving a 10 mg/kg dose of aducanumab and experienced ARIA-E, displayed side effects. Additionally, the apparent reduction in amyloid accumulation by aducanumab administration may be due to continued and advanced cerebral cell death
and not improvement of the disease [64]. Due to the limitations and controversies surrounding aducanumab, other neurotherapeutics may be needed for better clinical outcomes for the treatment of AD.

**Erythropoietin:**

The hormone and hematopoietic growth factor erythropoietin (EPO) is a polar, acidic glycosylated cytokine, composed of 4 alpha-helical bundles and weighs about 30.4 kDa [65]. Recombinant human EPO (rHuEPO) is currently approved by the FDA for the treatment of chronic kidney disease, cancer, or anemia [66]. During times of hypoxia, EPO is secreted by the kidneys to stimulate red blood cell (RBC) production in the bone marrow and act as an anti-apoptotic agent for erythrocytic progenitors [65]. EPO maintains RBC mass by promoting the survival, proliferation, and differentiation of erythrocytic progenitors.

EPO binds to EPO receptors (EPOR), leading to the activation of the cytoplasmic Janus kinase 2 (JAK2) signaling cascade, triggering downstream mitogenesis [67]. The binding of EPO to EPOR causes a conformational change to the receptor, leading to phosphorylation of pre-associated Jak2 kinases [68, 69]. EPOR is a highly conserved type I transmembrane protein that belongs to the cytokine superfamily [70]. Human astrocytes produce EPO, and EPOR expression was found in human neurons, astrocytes, and microglial cells grown in culture [71]. The expression of EPOR is upregulated in the temporal cortex and hippocampal astrocytes in patients with AD and mild cognitive impairment [72]. The EPORs found in the cerebral cortex and
hippocampus suggest a role in preventing neuronal death and stimulating neuronal plasticity [73]. The expression of EPOR in select regions of the brain allows for the utilization of EPO as a potential neurotherapeutic.

Although the mechanism of action of EPO on synaptic transmission and synaptic plasticity is not fully understood, EPO attenuates synaptic transmission and induces synaptic plasticity in the hippocampus of rats and mice [74]. Acute EPO administration to rats improved the magnitude of long-term potentiation and protect it from depotentiation [75]. Increased exposure to EPO facilitates long-term potentiation in the hippocampus, improving neuronal function, learning, and memory [76].

EPO is a promising candidate for the treatment of AD because it targets a spectrum of processes involved in AD pathology and progression including reducing Aβ load, limiting microglial activation, and rescuing synaptic loss [77]. EPO has also been shown to improve neurological outcomes in experimental AD by improving cognitive functions such as associative learning memory, recognition memory, and spatial long-term memory in aged Tg2576 and APPSwe mice [78-80]. Overall, EPO has evolved as a potential therapeutic agent for CNS disorders because of its robust neuroprotective effects.

Blood-Brain Barrier (BBB):

There are some challenges in utilizing EPO as a brain therapeutic. The main challenge is the low BBB penetrability of EPO. The BBB acts as a selective, semi-permeable barrier that separates the extracellular fluid of the brain from the blood. The
BBB is formed from brain microvascular endothelial cells forming tight junctions (TJs) along the capillary walls with astrocytic end-feet and pericytes embedding the basement membrane [81]. The primary function of the BBB is to shield and protect the brain from circulating pathogens and toxins, while selectively allowing certain vital molecules and nutrients into the brain. Small lipophilic molecules enter through the BBB via passive diffusion, while other essential small molecules such as glucose and amino acids require specialized transport proteins [82]. The molecular weight threshold for passive diffusion across the BBB is <400 Da (80 Å²), and the polarity of molecules cannot exceed 7 hydrogen bonds with solvent water [83]. Large proteins, including insulin, can enter the brain via active transport mechanisms including receptor-mediated transport [84]. Overall, the BBB is impermeable to about 98% of small molecules and 100% of biologic drugs [83, 85].

Due to its size and polarity, EPO cannot readily cross the BBB into the brain parenchyma unless leakiness caused by BBB disruption is present. Only about 0.05-0.1% of systemically administered EPO crosses the BBB [86]. As a result, a more invasive route of administration or higher doses would be needed to achieve a therapeutic effect [87]. To ameliorate this issue, a molecular Trojan horse can be used to shuttle the therapeutic into the brain through a non-invasive route of administration.

**Molecular Trojan Horse:**

The molecular Trojan horse technology utilizes an antibody, a peptide or a peptidomimetic against an endogenous receptor to shuttle a therapeutic through the
BBB [88]. Under normal conditions, EPO cannot readily enter the brain parenchyma unless the BBB is disrupted [89]. In the molecular Trojan horse technology, genetic engineering of recombinant fusion proteins is created when a therapeutic biologic is fused with the molecular Trojan horse, a second peptide or monoclonal antibody that binds to a specific receptor on the BBB, allowing the therapeutic biologic to enter into the brain via receptor-mediated transcytosis [88]. These recombinant bifunctional fusion proteins can potentially be utilized as neurotherapeutics, bypassing the BBB on one hand and exerting desired pharmacological effects on the other.

The molecular Trojan horse of interest used herein is a chimeric monoclonal antibody against the mouse transferrin receptor (cTfRMAb) [90]. Although fusion proteins with a monoclonal antibody have been engineered against the human insulin receptor (HIR), the HIRMAbs do not bind to rodent insulin receptors, making drug delivery across the BBB impossible for in vivo mouse models [90].

The cTfRMAb is fused to EPO and the fusion protein is designated as cTfRMAb-EPO. The cTfRMAb-EPO fusion protein was produced in stably transfected Chinese hamster ovary (CHO) cells and purified by Protein G affinity chromatography [91]. This protein was formulated at a concentration of 0.7 mg/mL in 98 mM glycine, 148 mM NaCl, 28 mM Tris, 0.01% Polysorbate 80, pH=5.5, sterile filtered and stored at -80°C until use [91]. The cTfRMAb domain consists of a mouse Fc region and a rat Fab Variable region (8D3) [91]. This chimeric monoclonal antibody, derived from the rat, 8D3 monoclonal antibody consists of approximately 85% mouse amino acid sequence [92]. The dissociation constant (KD) for the TfRMAb is 2.6 ± 0.3 nM, and the effective dose (ED50) of EPO is 0.33 ± 0.04 nM [91].
The cTfRMAb-EPO fusion protein has two binding domains: a mouse transferrin receptor 1 (TfR1) binding domain and an EPOR binding domain. TfR1 is a 90 kDa transmembrane homodimer carrier protein that binds to two transferrin molecules [93]. The primary function of transferrin is to bind to and import iron into cells. Under normal, physiologic conditions, TfR1 is saturated by transferrin [94], and transferrin can therefore not be used as a vector to ferry drugs into the brain. TfR1 is one of two isoforms of transferrin receptor proteins. TfR2 is found primarily in the liver, while TfR1 is highly expressed on the brain capillary endothelium and kidneys [95]. Transferrin and cTfRMAb bind to different epitopes of TfR1, preventing interference with the physiological iron import into the cell [92]. The cTfRMAb-EPO fusion protein enters the brain through the transvascular route. The cTfRMAb domain of the fusion protein binds to the TfR1 of the brain endothelial cells lining the BBB in a bivalent format with high affinity [91]. This binding leads to endocytosis into the brain endothelial cells and to receptor-mediated transcytosis into the brain parenchyma [96]. The dense network of the cerebral vasculature allows widespread drug delivery throughout the brain through the transvascular route using this approach [85].

Receptor Binding Affinity Considerations:

The binding affinity of a drug is important in determining its pharmacokinetic (PK) profile and therapeutic dose. High-affinity TfRMAbs may be trapped in the brain vasculature due to their inability to dissociate from their target receptors [97]. As a result, low-affinity TfRMAbs were engineered. However, to achieve a therapeutic effect, low-affinity TfRMAbs require higher injection doses, triggering effector function
associated toxicity [98]. The typical injection doses for low-affinity TfRMAbs are between 20-50 mg/kg whereas the doses of high-affinity TfRMAbs range between 1-5 mg/kg [98]. Injecting higher doses will inevitably lead to more off-target-based toxicologic effects. Acute clinical signs associated with TfRMAb effector function include lethargy, spastic movements, reddish-brown urine, and a significant reduction in reticulocytes [98].

cTfRMAb-TNFR PK & Brain Uptake Study:

As an invasive route of administration, intravenous (IV) injections are not preferred because the study is geared towards long-term, chronic treatment of AD, a chronic neurodegenerative disease often managed in non-clinical settings. Subcutaneous (SQ) injections were ultimately chosen because of their pharmacokinetic profile with the cTfRMAb fusion protein. A PK and brain uptake study utilizing a fusion protein of cTfRMAb fused to the extracellular domain of the type-II TNF receptor (TNFR) that was radiolabeled with $[^3]$H-N-succinimidyl propionate was conducted in adult male C57BL/6J mice weighing between 25-27g [99]. Following the mice sacrifice, the brain and other major organs were harvested, weighed, and processed for determination of $^3$H radioactivity with Opti-Fluor O using a Tri-Carb 2100TR liquid scintillation counter [99]. The brain uptake (3-10 mg/kg doses) for this cTfRMAb-TNFR fusion protein following intraperitoneal (IP) and SQ injections were similar, but the plasma area under the curve (AUC) was 10 times higher following IP administration compared to the SQ group [99]. Because both the IP & SQ injections have similar brain uptake, SQ injection
is preferred because of a lower plasma AUC, leading to lower adverse effector function effects.

**Effects of cTfRMAb-EPO on Aβ Plaques:**


Previous work from our lab demonstrated that at 3 mg/kg injected IP doses, the cTfRMAb-EPO fusion protein significantly reduces the size and the amount of Aβ plaques in both the cerebral cortex and hippocampal regions when 5.5-month-old male APPswe/PSEN1dE9 (APP/PS1) transgenic mice were treated for 8-weeks [77]. However, after 8 weeks of injections with 3 days of injections per week, the study also found that the hematocrit, measured at week 10, was significantly lower for the mice injected with the fusion protein compared to the controls (data not shown in the paper), which was consistent with previous findings of lower reticulocytes with TfRMAbs [98]. As a hematopoietic cytokine, EPO was expected to increase reticulocyte count and maintain hematocrit [100]. However, the reduction in hematocrit and reticulocytes were attributed in part due to the TfRMAb Fc-effector function, resulting from the binding of TfRMAbs to reticulocyte TfR and leading to antibody-dependent cell-mediated cytotoxicity (ADCC) [98, 101]. As an immune mechanism, ADCC occurs when Fc effector cells, such as natural killer cells, recognize and eliminate target cells exhibiting specific antigens on its surface [101, 102].
Based on the previous study, reducing the dose of the cTfRMAb-EPO fusion protein may potentially reduce or eliminate the cTfRMAb-associated side effects. The preliminary goal of my Ph.D. research was to show the therapeutic potential of the BBB-penetrating EPO with limited effector function adverse effects.

Nine and a half-month-old male APPswe PSEN1dE9 (APP/PS1) transgenic mice were treated SQ with saline (n=11), cTfRMAb-EPO (3 mg/kg, n=7), or rhuEPO (0.6 mg/kg, n=9) 2 days per week for 6 weeks, compared to saline-treated wild-type mice (n=10). The TfRMAb-EPO’s initial dose is 3 mg/kg, but rhuEPO only constitutes 20% of that dose, equaling 0.6 mg/kg.

Blood was collected through the retro-orbital sinus at 4, 6, and 8 weeks following the initial injection, and shipped overnight at 4°C to Molecular Diagnostic Services, Inc. (San Diego, CA) for a complete blood count (CBC) differential. The parameters measured included hematocrit, red blood cell (RBC), reticulocyte, hemoglobin, and white blood cell (WBC) count.

Memory and exploration behavior were assessed at 6 weeks post-injection. Cardiac perfusion and mouse sacrifice were performed after 8 weeks of the start of the study. After the sacrifice, the brains of the mice were harvested to measure the amount of Aβ in the brain. The right cerebral hemispheres were immersion-fixed in 4% paraformaldehyde (PFA) and cryoprotected in sequential 10%, 20%, and 30%-sucrose solution at 4°C. The hemispheres were then frozen and mounted in Tissue-Tek OCT (Fisher Scientific, USA). The brain hemispheres were then sectioned into 20 μm sagittal sections with a cryostat (Micron Instruments, USA). Three sagittal sections per mouse, approximately 600 μm apart, containing both the cortex and the hippocampus were
stained using the Alexa Fluor 488-conjugated 6E10 MAb (Biolegend, USA) or the Thioflavin-S (Thio-S) staining (Sigma Aldrich, USA).

Thio-S staining was used to stain mature Aβ plaques, and the 6E10 MAb immunofluorescence was used to stain Aβ protein. For the Thio-S staining, tissue sections were mounted onto glass slides and washed sequentially with 70% and 80% ethanol for 1 minute, followed by incubation in 1% Thio-S solution in 80% ethanol for 15 minutes. This step was then followed by sequential washing with 70% and 80% ethanol for 1 minute each. For Aβ immunofluorescence, free-floating sections were washed in Phosphate Buffered Saline (PBS) for 5 minutes and treated with 70% formic acid for 10 minutes at room temperature. The brain sections were then blocked with 0.5% bovine serum albumin (BSA) in PBS containing 0.3% TritonX-100 for 1 hour at room temperature and incubated with 1 µg/mL of Alexa Fluor 488-conjugated 6E10 MAb overnight at 4°C. Both the 6E10 and Thio-S sections received a final wash with distilled water for 5 minutes and were coverslipped using Vectamount aqueous mounting media (Vector Laboratories, USA) and finally sealed with nail polish. The slides were stored at 4°C until confocal imaging.

Each brain section was imaged with two regions in the cortex and the entire hippocampus at 10X magnification with the Leica TCS SP5 confocal microscope (Leica Microsystems, USA). The images were analyzed with the NIH Image J software (version 1.52a). The parameters analyzed were the amyloid positive area divided by the total area analyzed expressed as a percentage, the number of amyloid positive stains per area of tissue analyzed, and the average plaque size.
The spatial memory of the mice was assessed using a modified Y-maze protocol. The protocol consists of a habituation training phase for visual encoding [103]. During this training phase, one arm of the Y-maze was blocked. The mouse was placed in the starting arm, facing the intersection between the open and close arm, and was allowed to explore the two open arms for 8 minutes before a resting phase [3]. The resting phase between the training phase and the test phase was 30 minutes. After the time delay of the resting phase, the testing phase began. During the testing phase, the mouse was placed back in the same position, facing the intersection between both open arms of the maze, at the starting arm with accessibility to all three arms for 8 minutes. This set of phases was conducted twice: once for the baseline at the start of the study before treatment and once for posttreatment at the end of 6 weeks of injections.

The camcorder recordings were used to identify the movements and preferences of the mouse for analysis. Ideally, a normal, non-diseased mouse would spend more time in the novel arm compared to an AD-affected mouse [104]. The preferences were recorded as a discrimination ratio for entries into each zone for both baseline and posttreatment. The discrimination ratio is the number of entries into the novel arm/(entries into novel arm + familiar arm). The percentage of entries into the novel arm was utilized as a measure of spatial reference memory [104]. These percentages of entry were used to compare the WT mice with the treatment groups.

All data are presented as the mean ± SEM, and all statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., USA). The Kruskal-Wallis with Dunn’s multiple comparisons test was performed for all non-normal data, comparing the mean of the APP/PS1 cTfRMAb-EPO and APP/PS1 rhuEPO mice.
section stains individually to the APP/PS1 saline mice section stains. CBC and WBC data were analyzed using Holm-Sidak’s multiple comparisons test, comparing each experimental group to the APP/PS1 saline control. The advantage of Holm-Sidak’s multiple comparisons test is that it can be used to analyze any set of P-values and has more power compared to the Bonferroni method. A one-sample t-test was performed for the Y-maze data, comparing the mean of each group to the hypothetical value of 50%. The behavior analysis was performed using the SMART Video Tracking Software (Panlab, Harvard Apparatus, USA). A two-tailed P value < 0.05 was considered statistically significant.

Spatial memory was assessed with the modified Y-maze. A discrimination ratio of 50% indicates that the mice’s decisions and movement were based on chance. The mice were 9.5 months old during the baseline testing and 11 months old during posttreatment testing. There were no significant differences in spatial memory of the baseline compared to the 0.50 discrimination ratio for all of the groups (Figure 1.1 A). However, for the posttreatment performance, the APP/PS1 saline and the rhuEPO mice both had a statistically significant decrease in the discrimination ratio, indicating that those mice were more likely to elect the familiar arm (Figure 1.1 B).

Four weeks after the initial injections, the red blood cell (RBC) count in the saline, cTfRMAb-EPO, and rhuEPO treated APP/PS1 mice were significantly higher by 9%, 10%, and 38%, respectively, compared to WT saline mice (Table 1.1). In the cTfRMAb-EPO mice, reticulocytes elevated by 108% compared to the WT saline mice at 4 weeks after the initial treatment. Some of the RBC indices were significantly altered by rhuEPO
treatment at 4 weeks. The rhuEPO mice had a significant increase in hemoglobin and hematocrit by 35% and 25%, respectively, compared to the WT saline mice.

Figure 1.1: Y Maze discrimination ratio results for (A) baseline and (B) posttreatment. There was a statistically significantly lower preference for the familiar arm in the discrimination ratio for both the SAL (n=10) and rhuEPO (n=8) APP/PS1 mice posttreatment. There was no significant change for the cTfRMAb-EPO (n=7) APP/PS1 or the wildtype saline (n=10) group [3]. Data are presented as mean ± SEM of 7-11 mice per group. A one-sample t-test was performed for the Y-maze data, comparing the mean of each group to the hypothetical value of 50%.

However, the rhuEPO mice reticulocyte count remained diminished by 84% and 56% at 6 and 8 weeks, respectively, compared with WT saline mice (Table 1.1). The levels of RBC, hemoglobin, and hematocrit in the rhuEPO mice were also significantly lower than the WT saline mice by 46%, 47%, and 50%, respectively, at 8-weeks following initial treatment (Table 1.1).
There were no significant reductions in the Thio-S positive Aβ plaque area in rhuEPO mice and cTfRMAb-EPO mice compared to the APP/PS1-saline mice in the cortical areas (Figure 1.2A & B) and hippocampal regions (Figures 1.2A & E). There were no significant reductions in the Thio-S number of positive Aβ-plaque stains in rhuEPO mice and cTfRMAb-EPO mice compared to the APP/PS1-saline mice in the cortical areas (Figure 1.2A & C) and hippocampal regions (Figure 1.2A & F). There were also no significant differences in the average Thio-S-plaque size between the groups in these regions (Figures 1.2A, D, &G). The lack of significance may be due to the low Aβ plaque load in the sensitivity of the staining. ThioS stains only the mature Aβ plaques, while the 6E10 antibody stains the Aβ peptides.
Table 1.1: Complete blood count and white blood cell differential at 4 weeks, 6 weeks, and 8 weeks after the start of the study in APP/PS1 and WT mice [3].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>4 weeks</th>
<th>6 weeks</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>APP/PS1</td>
<td>APP/PS1</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>cTfRMAb-EPO</td>
<td>rhu-EPO</td>
</tr>
<tr>
<td>RBC (10^3/μL)</td>
<td>8.9±0.1</td>
<td>9.7±0.2</td>
<td>9.8±0.2</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>13.6±0.2</td>
<td>14.3±0.5</td>
<td>14.4±0.3</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>40.0±0.8</td>
<td>41.5±1.3</td>
<td>43.0±1.6</td>
</tr>
<tr>
<td>WBC (10^3/μL)</td>
<td>4.7±0.6</td>
<td>6.9±1.5</td>
<td>8.0±0.2</td>
</tr>
<tr>
<td>Total Retic (%)</td>
<td>3.8±0.1</td>
<td>3.9±0.1</td>
<td>7.9±0.8</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 compared to WT-Saline  ††RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; WBC, White blood cell; Retic, reticulocytes
Figure 1.2: Representative images of Thio-S positive Aβ plaques in the brains of saline-, cTfRMAb-EPO- and rhuEPO-treated mice [3] (A). In the cortex, there was no significant reduction in the ThioS positive Aβ positive area (B) and number (C) in cTfRMAb-EPO- and rhu-EPO-treated mice compared to the saline mice. In the hippocampus, there was also no significant reduction in the ThioS positive Aβ positive area (E) and number (F) in rhuEPO-treated mice compared to the saline mice. There was no change in the average plaque size for both the cortex (D) and hippocampus (G). Data are presented as mean ± SEM of 7-11 mice per group. Kruskal-Wallis with Dunn's multiple comparisons test or one-way ANOVA with Holm-Sidak’s post-hoc test was used to compare to the APP/PS1-saline group. Scale bar = 100 μm.
There was a significant reduction in the 6E10-positive Aβ peptide area in rhuEPO mice (21% decrease; p<0.05) and cTfRMAb-EPO mice (32% decrease; p<0.05) compared to the APP/PS1-saline mice in the cortical areas (Figure 1.3A-B). There was also a significant reduction in the number of 6E10-positive Aβ-peptide stains in rhuEPO mice (20% decrease; p<0.05) and cTfRMAb-EPO mice (31% decrease; p<0.05) compared to the APP/PS1-saline mice in the cortical areas (Figures 1.3A & C). However, similar to the Thio-S, there were no significant differences in the average plaque size for both the rhuEPO and cTfRMAb-EPO mice compared to the APP/PS1-saline mice in the cortical areas (Figures 1.3A & D).

There was no significant reduction in the 6E10-positive Aβ peptide area in rhuEPO mice and cTfRMAb-EPO mice compared to the APP/PS1-saline mice in the hippocampal region (Figure 1.3A & E). Similarly, for the number of 6E10-positive Aβ-peptide stains in the hippocampal region, there was also no significant reduction in the rhuEPO mice and cTfRMAb-EPO mice compared to the APP/PS1-saline mice (Fig 1.3A & F). The lack of reduction in the hippocampal regions could be accounted for by the different mechanisms of deposition of Aβ plaques between the cortex and hippocampus [105]. Aβ deposition in the cortex precedes that in the hippocampus in the APP/PS1 mice [106]. There were also no significant differences in the average 6E10-positive stain size for both the rhuEPO and cTfRMAb-EPO mice compared to the APP/PS1-saline mice in the hippocampal areas (Figures 1.3A & G). Comparing the Thio-S and 6E10 staining results, both cTfRMAb-EPO and rhuEPO have a significant but modest effect on reducing Aβ load in the cortical regions.
Figure 1.3: Representative images of 6E10-positive Aβ peptide in the brains of saline-, cTfRMAb-EPO- and rhuEPO-treated mice [3] (A). In the cortex, there was significant reduction in the 6E10-positive Aβ positive area (B) and number (C) in cTfRMAb-EPO- and rhuEPO-treated mice compared to the saline mice. In the hippocampus, there was no significant reduction in the 6E10-positive Aβ positive area (E) and number (F) in both the cTfRMAb-EPO and rhuEPO-treated mice compared to the saline mice. There was no change in the average 6E10-positive stain size for both the cortex (D) and hippocampus (G). Data are presented as mean ± SEM of 7-11 mice per group. Kruskal-Wallis with Dunn's multiple comparisons test or one-way ANOVA with Holm-Sidak's post-hoc test was used to compare to the APP/PS1-saline group. *p<0.05, **p<0.01. Scale bar = 100 μm.
Rationale & Significance of the Research:

Due to the large number of failed neurotherapeutics and the controversies surrounding aducanumab, more research and efforts are needed to develop a neurotherapeutic for the treatment of AD. One potential drug candidate is the TfRMAb-EPO fusion protein. However, cTfRMAb-treated mice exhibit acute clinical signs associated with TfRMAb effector function including lethargy, spastic movements, and reddish-brown urine [98]. The TfRMAbs have been shown to induce hemolysis and a reduction in hematocrit and reticulocytes resulting from ADCC [98, 101]. Due to the adverse effects associated with the TfRMAb effector function, an alternative therapy may be more effective for the treatment of AD without the acute clinical signs associated with the TfRMAb effector function.

A novel, effectorless cTfRMAb-EPO fusion protein can be engineered to reduce or eliminate the effector function associated with cTfRMAbs while maintaining the therapeutic potential of the drug. This EPO therapeutic is the cTfRMAb-N292G-EPO fusion protein. The cTfRMAb-N292G-EPO fusion protein was produced by Genscript (Piscataway, NJ) in ExpiCHO cells that were grown in serum-free ExpiCHO Expression Medium (Gibco, Gaithersburg, MD) and formulated at a concentration of 0.78 mg/mL in 98 mM glycine, 148 mM NaCl, 28 mM Tris, 0.01% Polysorbate 80, pH=5.5, sterile filtered and stored at -80°C until use [1]. The cTfRMAb-N292G-EPO fusion protein has a single mutation in the Fc N-linked glycosylation site with a substitution of asparagine (Asn) with glycine (Gly) at position 292, which should make the molecule aglycosylated, and reduce the Fc effector function [1]. Typically, the Fc region of a monoclonal antibody binds to and interacts with Fc receptors, a protein found on the surface of
immune cells and platelets. This interaction leads to antibody-dependent cell-mediated cytotoxicity, where immune cells lyse cells bound by specific antibodies [107]. By mutating the Fc region of the antibody, the cTfRMAb-N292G-EPO should have reduced binding to Fc receptors and thus have reduced cellular cytotoxicity. This dissertation aspires to develop this mutant brain penetrating EPO for the treatment of AD.

Overall, this development process of utilizing the brain-penetrating EPO as a neurotherapeutic can also be applied to other drug candidates for the treatment of AD pathology. To fulfill the goals of the dissertation, the following specific goals were proposed:

a) To conduct a PK study on the cTfRMAb-N292G-EPO fusion protein through IP and SQ parenteral routes of administration and determine the best dose and route of administration. The plasma clearance and pharmacokinetics of therapeutic doses of N292G mutant TfRMAbs following different systemic routes of administration have been previously studied in our lab [3]. However, the mutant EPO fusion protein had a higher-than-expected clearance. The first aim of my research was to see if increasing the dosage could saturate these clearance mechanisms to get plasma concentrations comparable to the non-mutant while maintaining a favorable safety profile. (Chapter 2)

b) To study the effects of the cTfRMAb-N292G-EPO fusion protein on the PS19 mouse model of tauopathy. Our previous studies showed the efficacy of the cTfRMAb-EPO fusion protein on the APP/PS1 mouse model of amyloidosis. The second aim of my research was to see if chronic administration of the cTfRMAb-N292G-EPO fusion protein at the saturable dosage will be efficacious on phosphorylated tau, microgliosis, and hippocampal cellular health in in a mouse model of tauopathy. (Chapter 3)
c) To develop an alternative method to radiolabeled molecules for measuring the brain concentration of TffMAb-based fusion proteins. Brain homogenate samples were concentrated using the Amicon Concentration Method. (Chapter 4)
CHAPTER 2

ELIMINATING FC N-LINKED GLYCOSYLATION AND ITS IMPACT ON DOSING

CONSIDERATION FOR A TRANSFERRIN RECEPTOR ANTIBODY-ERYTHROPOIETIN FUSION PROTEIN IN MICE


Introduction:
Erythropoietin (EPO), a class-1 cytokine hematopoietic growth factor mainly produced in the kidneys, offers biological benefits beyond erythropoiesis. There is mounting evidence of the neuroprotective effects of EPO in neurodegenerative diseases [108, 109]. These neuroprotective effects include reducing inflammation, oxidative stress, and neuronal loss while elevating neurogenesis and angiogenesis [110]. Besides offering neuroprotection, studies in rodent models have shown that EPO can modulate the two major pathological hallmarks of Alzheimer’s disease (AD): extracellular amyloid-beta plaque accumulation and intracellular tau tangles, making it a potential treatment for AD [79, 111, 112]. However, there are some challenges associated with utilizing EPO as a brain therapeutic.

The blood-brain barrier (BBB) acts as a selective, semi-permeable barrier that separates the brain extracellular fluid from the blood [113]. The size and polarity of EPO prevent the macromolecule from passively diffusing across the BBB and reaching the brain parenchyma [86, 114, 115]. EPO penetration of the BBB in vivo is minimal, and the brain uptake of EPO is no greater than that of a substance confined to the plasma volume such as IgG in the monkey [90], or albumin in the mouse [86]. To ameliorate this issue, a molecular Trojan horse (MTH) can be used to shuttle EPO into the brain through a non-invasive transvascular route of administration [90, 91].

The MTH technology utilizes an antibody against an endogenous receptor-mediated transcytosis (RMT) system receptor fused to a therapeutic biologic, to shuttle the therapeutic biologic through the BBB [88]. The transferrin receptor-1 (TfR1) is a transcytosis receptor, which is highly expressed at the BBB [116, 117]. An MTH-EPO fusion protein was engineered, with the MTH domain being a rat/mouse chimeric
antibody against the mouse TfR1 (TfRMAb). The TfRMAb-EPO fusion protein has two binding domains: a mouse TfR1 binding domain, TfRMAb, for brain delivery, and an EPO receptor (EPOR) binding domain, EPO, for neuroprotective effects. The TfRMAb-EPO has a high binding affinity to both the TfR1 and EPOR with low nanomolar dissociation constants (KD) [1, 91].

A single injection of a TfRMAb comprising a human Fc-domain in mice at high doses (20-50 mg/kg) induces adverse-effects including lethargy, spastic movements, and suppression of reticulocytes [98]. Similarly, our recent work showed reticulocyte suppression in mice with a single therapeutic dose (3 mg/kg) of TfRMAb-EPO comprising a mouse Fc-domain [1]. These effects of TfRMAb are attributed in part to the TfRMAb Fc-effector function ensuing from the binding of TfRMAbs to reticulocyte TfR and resultant antibody-dependent cell-mediated cytotoxicity [98, 101].

Administration of ‘effectorless’ TfRMAbs comprising mutations in the Fc N-linked glycosylation partially rescues the suppression of reticulocytes in mice and eliminates this adverse effect in non-human primates [97, 118]. Studies show that such Fc N-linked glycosylation site mutations do not alter plasma clearance of a low-affinity TfRMAb in mice following a 50 mg/kg intravenous (IV) injected dose (ID) [118], and do not affect the plasma clearance of a non-TfR-targeting monoclonal antibody in mice and non-human primates [119, 120].

Based on this previous work, we recently engineered an effectorless IgG-EPO fusion protein, cTfRMAb-N292G-EPO, which has a single mutation in the Fc N-linked glycosylation site with a substitution of asparagine (N) with glycine (G) at position 292 [1]. While this N292G mutation does not impact the binding of the IgG to the neonatal
Fc receptor (FcRn), binding of the IgG to the Fcγ receptor (FcγR) is abolished [121]. We recently reported the plasma pharmacokinetics (PK) and effects on reticulocytes of therapeutic doses (0.3-3 mg/kg) of cTfRMAb-N292G-EPO following IV, intraperitoneal (IP), and subcutaneous (SQ) injection in mice [1]. Compared with the nonmutant TfRMAb-EPO, mutant cTfRMAb-N292G-EPO mitigated reticulocyte suppression at these doses. cTfRMAb-N292G-EPO, however, showed a profound increase in plasma clearance following IV administration and a 37- to 114-fold reduction in plasma Cmax following IP and SQ administration at therapeutic doses compared with the nonmutant TfRMAb-EPO [1]. Further, the plasma PK of the cTfRMAb-N292G-EPO fusion protein displayed the characteristics of non-linear PK showing a reduction in clearance with an increase in dose [1]. This would mean that higher doses of the N292G mutant will be needed to achieve plasma concentrations and therefore therapeutic effects comparable to the nonmutant TfRMAb-EPO. However, it is unclear if the rescue of reticulocyte suppression can be maintained at higher doses of the N292G mutant TfRMAb-EPO.

Given the above information, this study aimed to determine if increasing the doses of the mutant cTfRMAb-N292G-EPO can saturate its accelerated clearance to yield higher plasma concentrations while maintaining the rescue of reticulocyte suppression following IP and SQ administration, in mice.

**Materials & Methods:**

*cTfRMAb-N292G-EPO Fusion Protein:*
The cTfRMAb-N292G-EPO fusion protein was produced by Genscript (Piscataway, NJ) in ExpiCHO cells as described previously [1]. The formulation buffer of the fusion protein was 98 mM glycine, 148 mM NaCl, 28 mM Tris, 0.01% Polysorbate 80, pH=5.5.

Mouse Animal Study:

The animal studies were approved by the University of La Verne Institutional Animal Care and Use Committee. Male C57BL/6J mice (8 weeks old and weighing 22–27g) (Jackson Laboratory, Bar Harbor, ME) were provided constant access to food and water, and were maintained on a 12 h light/12 h dark cycle. The mice were randomized to either IP or SQ route of administration and received a single injection at a dose of 3, 6, 9, and 20 mg/kg of cTfRMAb-N292G-EPO. The average injection volume of the cTfRMAb-N292G-EPO was 100 μL, 200 μL, 300 μL, and 667 μL for the 3, 6, 9 and 20 mg/kg IP and SQ doses, respectively, all within the recommended administration volumes in a mouse [122]. The control mice were treated with an equivalent volume of saline or 3 mg/kg isotype control mouse IgG1 (Bio X Cell, West Lebanon, NH) via the SQ route. Whole blood was collected via the retro-orbital sinus at 3, 6, and 24 h following the initial injection. These sampling times were selected based on our previous work with the cTfRMAb-N292G-EPO15 and other TfrMAb-based fusion proteins [99] showing a Tmax of 3-6 hours following IP and SQ dosing. Plasma was collected from whole blood, and plasma concentrations of the fusion protein were measured as described previously [1]. Terminal whole blood aliquot was used for a complete blood count (CBC) (Molecular Diagnostic Services, Inc., San Diego, CA).
Plasma and Brain Concentration Measurements:

The EPO receptor (EPOR) ELISA was used to determine the plasma- and brain-concentrations of the fusion proteins, as described previously [1]. Perfused frozen hemi-brains of the mice treated with either the wild-type (WT) nonmutant or N292G mutant fusion protein were homogenized in 7 brain volumes (7µL/mg brain) of the ice-cold Tissue Protein Extraction Reagent (T-PER) (ThermoFisher Scientific, Waltham, MA) and EDTA-free Protease Inhibitor Tablet (MilliporeSigma, Burlington, MA). The brain homogenates were shaken at 4°C for 1h, centrifuged at 14,000xg for 20 min at 4°C, and the supernatant was then collected. The supernatant was concentrated using the Amicon Ultra Protein Purification and Concentration Filter Tubes (4 mL and 50 kDa cut-off; MilliporeSigma, Burlington, MA) and centrifuged at 4°C at 3000xg for 40 min. The brain concentrates were used to determine brain concentrations of the fusion proteins.

Nunc Maxisorp plate wells (Fisher Scientific, Waltham, MA) were coated with 2 µg/mL of the EPOR/Fc fusion protein (R&D system, Minneapolis, MN) at 4°C overnight. The wells were then washed with tris-buffered saline containing 0.05% Tween-20 (TBST) and blocked with tris-buffered saline containing 1% bovine serum albumin (TBSB). Plasma samples, brain concentrates, and standards (0.03-9000 ng/well of fusion proteins) were incubated for 2 h at room temperature. After washing with TBST, wells were incubated with 100 ng/well of alkaline-phosphatase conjugated goat anti-mouse light chain (kappa) antibody (Bethyl Laboratories, Inc., Montgomery, TX) for 45 min at room temperature. The wells were incubated with P-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO) for 16-30 min in the dark, and 1.2M NaOH was added to
halt the reaction. The optical density (OD) was measured at 405 nm and nonlinear regression analysis was used to determine Bmax and KD from standard curves (GraphPad Prism, San Diego, CA). TBSB blank corrected OD values were used. Plasma concentrations are reported as ng of fusion protein per mL plasma (ng/mL) and the percentage of injected dose per mL of plasma (%ID/mL). Brain concentrations in ng/g brain tissue were normalized to saline-control values.

Plasma data was analyzed using the Thermo Scientific Kinetica 5.0 (ThermoFisher Scientific, Waltham, MA) software to calculate the following PK parameters: maximum plasma concentration (Cmax), mean residence time (MRT), area under the plasma concentration-time curve (AUC), time to reach maximum plasma concentration (Tmax), clearance/bioavailability (apparent clearance), and the volume of distribution at steady-state (Vss) [1].

In vitro Stability of the Fusion Protein:

The stability of the cTfRMAb-N292G-EPO in saline and mouse plasma was determined in vitro. For this, the cTfRMAb-N292G-EPO fusion protein (9000 ng/mL) was incubated in saline and mouse plasma at 37°C, and aliquots were collected at 0, 3, and 24 h. Concentrations of the fusion protein were determined using the EPOR ELISA described above which detects the intact fusion protein, and blank (saline and mouse plasma) corrected OD values were used. Data was presented as % of 0-hour concentration to determine changes in the concentration of the fusion protein over 24 hours, in vitro.
**Statistical Analysis:**

All data are represented as mean ± SEM, and all statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA). A one-way ANOVA with Holm-Sidak’s post-hoc test was used to compare more than 2 groups. Spearman correlation coefficient \( r \) was used to determine the relationship between AUC and reticulocyte count. Two-way ANOVA with repeated-measures with Holm-Sidak’s post-hoc test was used to compare data with 2 independent variables (e.g. stability study). A two-tailed p-value of <0.05 was considered statistically significant.

**Results:**

**Plasma Pharmacokinetics after SQ Injection:**

Plasma concentration-time curves after SQ injections are shown in Figure 2.1. The Cmax was 15.0 ± 2.0 ng/mL (0.02 ± 0.003 %ID/mL), 21.3 ± 4.1 ng/mL (0.015 ± 0.003%ID/mL), and 21.3 ± 6.4 ng/mL (0.010 ± 0.003 %ID/mL) for the 3, 6 and 9 mg/kg SQ dose, respectively (Table 2.1, Figure 2.1A & B). The 20 mg/kg SQ dose resulted in a Cmax of 52.8 ± 27.9 ng/mL (0.011 ± 0.006 %ID/mL), which was 2.5-fold higher than the Cmax following the 9 mg/kg SQ dose. At 6 hours following SQ administration, the plasma concentration of cTfRMAb-N292G-EPO decreased to 9.7 ± 1.2 ng/mL (0.013 ± 0.002 %ID/mL), 12.7 ± 2.1 ng/mL (0.009 ± 0.002%ID/mL), 15.5 ± 3.9 ng/mL (0.007 ± 0.002%ID/mL), and 27.8 ± 17.1 ng/mL (0.006 ± 0.003%ID/mL) for the 3, 6, 9, and 20 mg/kg doses, respectively (Figure 2.1A & B). These plasma Cmax values of cTfRMAb-N292G-EPO for doses between 3-20 mg/kg following SQ injection are 90- to 317-fold
lower than the previously reported plasma Cmax of the nonmutant WT TfrMAb-EPO following a 3 mg/kg SQ dose (Table 2.1) [1].

**Figure 2.1:** cTfrMAb-N292G-EPO plasma concentrations after SQ (A, B) or IP (C, D) dosing represented in ng/mL (A, C) or %ID/mL (B, D). Data are shown as mean ± SEM of n = 3-6 per dose per route of administration.

**Plasma Pharmacokinetics after IP Injections:**

Plasma concentration-time curves after IP injections are shown in Figure 2.1. The Cmax was 288 ± 47 ng/mL (0.38 ± 0.062 %ID/mL), 389 ± 154 ng/mL (0.26 ± 0.11
%ID/mL), and 633 ± 194 ng/mL (0.28 ± 0.083 %ID/mL) for the 3, 6 and 9 mg/kg dose, respectively (Table 2.1 & Figure 2.1C & D). The Cmax following the 20 mg/kg IP dose was 10,066 ± 7,059 ng/mL (2 ± 1.4 %ID/mL), which was 16-fold higher than the 9 mg/kg IP dose. At 6 hours following IP administration, the plasma concentration decreased to 31.0 ± 11.9 ng/mL (0.041 ± 0.016 %ID/mL), 21.1 ± 10.1 ng/mL (0.014 ± 0.007 %ID/mL), 76.1 ± 40.9 ng/mL (0.033 ± 0.017%ID/mL), and 568 ± 227 ng/mL (0.11 ± 0.043%ID/mL) for the 3, 6, 9, and 20 mg/kg doses, respectively (Figure 2.1C & D). These plasma Cmax values of cTfRMAb-N292G-EPO for doses between 3-9 mg/kg are 2- to 5-fold lower than the previously reported plasma Cmax of the nonmutant WT TfRMAb-EPO following a 3 mg/kg IP dose (Table 2.1). The plasma Cmax of cTfRMAb-N292G-EPO following a 20 mg/kg IP dose is 190-fold higher than that following SQ administration of the same dose, and is 7-fold higher than the previously reported plasma Cmax of the nonmutant WT TfRMAb-EPO following a 3 mg/kg IP dose (Table 2.1) [1].

The time to maximum plasma concentration (Tmax) was 180 minutes for all the groups (Table 2.1). The AUC(0-24h) increased with dose, with IP AUC(0-24h) being 5- to 78-fold greater than the SQ AUC(0-24h) for 3-20 mg/kg doses, respectively. Similarly, the AUC(0-∞) increased with dose, with the IP AUC(0-∞) being 2- to 59-fold greater than the SQ AUC(0-∞) for 3-20 mg/kg doses, respectively. At the highest dose administered (20 mg/kg), the apparent clearance (Cl/F) was 31-fold higher following the SQ route compared with the IP route. The MRT for the SQ route was 1.5- to 9-fold higher compared with the IP route for each dose, which can be attributed to the high Vss associated with the SQ route (10- to 42-fold higher than the Vss at the respective IP dose) (Table 2.1).
Brain Fusion Protein Concentrations:

The brain concentrations of the WT nonmutant TfrMAb-EPO fusion protein were significantly higher than those of the mutant cTfrMAb-N292G-EPO fusion protein following a 3 mg/kg SQ, IP and IV dose (Figure 2.2). The brain concentrations of the N929G mutant fusion protein following both the IP and SQ routes of administration were comparable to the background value (brain concentrations in mice treated with saline) of the EPOR ELISA assay used to detect the intact fusion protein in the brain (Figure 2.2).

Figure 2.2: Brain concentrations of the WT TfrMAb-EPO and mutant cTfrMAb-N292G-EPO fusion protein 24 hours following SQ, IP and IV administration of a 3 mg/kg dose in mice. The black dotted line shows the background signal in saline treated mice. The plasma data for the corresponding brain WT TfrMAb-EPO fusion protein data are published in Sun et al., 2019.15 Data are shown as mean ± SEM of n = 3-6 per dose per route of administration. ***P<0.001 and ****P<0.0001 compared to brain concentrations in mice treated with the WT TfrMAb-EPO fusion protein.
Complete Blood Count:

No significant difference was observed in the RBC count and hemoglobin of the mice treated with either IP or SQ administration of cTfRMAb-N292G-EPO compared with saline-treated mice at 24 hours after a single injection of 3, 9 and 20 mg/kg doses (Table 2.2). The WBC count was significantly elevated for the 9 mg/kg IP, 9 mg/kg SQ, and 20 mg/kg SQ injected mice compared to saline-treated mice by 91%, 61%, and 103%, respectively. However, no significant difference was observed in the WBC count between the isotype control mouse IgG1 and cTfRMAb-N292G-EPO treated mice (Table 2.2).

The total reticulocytes (%) in the saline-treated mice was 5.5 ± 0.4 (Table 2.2), and the reticulocyte counts decreased significantly with an increasing dose following both IP and SQ routes of administration of cTfRMAb-N292G-EPO compared with saline-treated mice (Table 2.2). Following IP injection, the total reticulocyte percentage was 2.2 ± 0.3 (p<0.05), 1.6 ± 0.2 (p<0.001), and 0.5 ± 0.03 (p<0.001) for the 3 mg/kg, 9 mg/kg, and 20 mg/kg doses, respectively (Table 2.2). No significant reduction in reticulocyte counts was observed between the 3 mg/kg SQ dose compared with saline-treated mice, and the total reticulocyte percentage was 3.6 ± 1.3, 3.1 ± 1.0 (p<0.05), and 1.2 ± 0.2 (p<0.01) for the 3 mg/kg, 9 mg/kg, and 20 mg/kg SQ doses, respectively (Table 2). Overall, there was a strong negative correlation (r = −0.84, p<0.0001) between AUC and reticulocyte count (Figure 2.3A).

In vitro Stability of the Fusion Protein:
No significant loss of the fusion protein was observed over 24 hours at 37°C in normal saline and mouse plasma. Greater than 95 ± 2% and 99 ± 12% of the fusion protein was recovered following a 24-hour incubation in saline and mouse plasma, respectively (Figure 2.3B).

**Figure 2.3:** Reticulocyte count is inversely correlated with AUC. The 3 mg/kg WT data is taken from Sun et al., 2019 and represents the data for the nonmutant TfRMAb-EPO [1]. Spearman correlation coefficient $r = -0.84$, $p<0.0001$ (A). No significant loss of the fusion protein in saline and mouse plasma over 24h at 37°C, in vitro (B). Proposed pathways for cTfRMAb-N292G-EPO transit from the site of injection to systemic circulation following SQ and IP administration. The figure was created using BioRender.com (C).
Discussion:

Antibodies directed against the TfR1, which is expressed at the BBB, act as vectors to shuttle neurotherapeutics into the brain across the BBB [117]. Recent work has revealed Fc-dependent effector function adverse effects associated with TfRMAbs, which are partially mitigated by eliminating Fc-N-linked glycosylation [98]. We recently engineered a brain-penetrable analog of EPO by fusing EPO to a high-affinity TfRMAb with an effectorless Fc-region by mutating the asparagine (N) at position 292 to glycine (G). This mutant brain-penetrating EPO fusion protein, designated cTfRMAb-N292G-EPO, shows a 6- to 8-fold faster plasma clearance, 37- to 114-fold lower plasma Cmax, and partial rescue of reticulocyte suppression compared to the WT nonmutant TfRMAb-EPO at low therapeutic doses (0.3-3 mg/kg) [1]. Such differences in the plasma PK between the WT and mutant TfRMAb-EPO would necessitate higher doses of the mutant fusion protein to achieve plasma concentrations and therapeutic benefits comparable to that of the WT fusion protein. The aim of the current study was, therefore, to determine if an increase in administered dose would saturate the accelerated clearance of the N292G mutant to increase its plasma exposure while maintaining the rescue in the Fc-effector function adverse effects.

For this, we examined the plasma PK of cTfRMAb-N292G-EPO at doses ranging from 3 mg/kg to 20 mg/kg following IP and SQ administration in adult male C57BL/6J mice. We showed that the IP route of administration overall resulted in a higher plasma exposure (plasma Cmax and AUC) compared to the SQ route, for all the doses studied. Despite a 7-fold dose escalation of the N292G mutant from 3 mg/kg to 20 mg/kg, plasma Cmax increased only 3.5-fold and remained low (between 15.0 ± 2.0 ng/mL and
52.8 ± 27.9 ng/mL) following SQ administration. However, the plasma Cmax increased 35-fold (from 288 ± 46.9 ng/mL to 10,066 ± 7,059 ng/mL) following IP administration as the dose was increased from 3 mg/kg to 20 mg/kg. The non-linear increase in plasma Cmax of the mutant fusion protein following the IP and SQ injections suggests involvement of saturable processes. For e.g. saturable absorption may contribute to less than proportional increase in plasma concentrations and saturable clearance may contribute to a more than proportional increase in plasma concentrations. Such a non-linear PK behavior is not unlikely, and has been reported for other TfRMAb-based fusion proteins [1, 123]. The extremely low plasma bioavailability of the mutant fusion protein following SQ administration becomes very evident when the plasma exposure of the mutant is compared to that of the WT nonmutant fusion protein. The plasma Cmax of the WT nonmutant TfRMAb-EPO fusion protein following a 3 mg/kg SQ dose is 4,748 ± 290 ng/mL (Table 2.1) [1]. However, following a 7-fold higher 20 mg/kg dose of the mutant cTfRMAb-N292G-EPO, the Cmax is 90-fold lower at 52.8 ± 27.9 ng/mL. The plasma bioavailability following IP administration is higher than that following SQ administration of the mutant fusion protein but still lower than that of the nonmutant fusion protein. The plasma exposure of the mutant fusion protein was 2- to 5-fold lower for doses between 3-9 mg/kg, but was 7-fold higher for the 20 mg/kg dose than the plasma Cmax after a 3 mg/kg dose of the nonmutant fusion protein (Table 2.1) [1]. The 35-fold increase in the plasma Cmax following IP dosing with an increase in dose from 3-20 mg/kg suggests a saturation of peripheral clearance of the N292G mutant at higher doses following the IP route.
The fusion protein was stable in the mouse plasma over 24 hours, in vitro (Figure 2.3B), and the reduced bioavailability of the mutant fusion protein following SQ injection compared to IP injection may be caused by the anatomical differences in the sites of injection (Figure 2.3C). Both parenteral routes have different avenues for entering the systemic circulation. Drugs administered through the IP route enter the peritoneal cavity, are absorbed via the mesenteric vasculature before being emptied into the hepatic portal vein [124, 125]. Lymphatic absorption from the peritoneal cavity contributes only modestly to the overall absorption process even for macromolecules [126, 127]. Following SQ administration, drugs are absorbed at a slower rate compared to other parenteral routes, and drugs reach the systemic circulation from the subcutis either directly by absorption into the blood capillaries underlying the skin or indirectly via the subcutis lymphatic circulation [128, 129]. Drug molecular weight and the proportion absorbed via the lymphatics following SQ administration are linearly related, and macromolecules with a molecular mass greater than 16 kDa are absorbed primarily by the lymphatic system [130]. The extent of lymphatic accumulation and catabolism after SQ administration is further dependent on the physicochemical characteristics of the molecule. Recent work showed that modification of surface glycosylation of extracellular vesicles increases lymphatic accumulation following SQ administration in mice [131]. Similarly, minor modifications to the glycan structure of EPO increased its lymphatic catabolism following SQ administration in rats [87]. Based on the role of lymphatic absorption and catabolism following SQ administration, and given that the mutant fusion protein is a large molecule (~200 kDa) and lacks the Fc-N-linked glycosylation [1], lymphatic catabolism of the mutant fusion protein perhaps results in the low
bioavailability following SQ administration in the current study. The degradation of the fusion protein at the site of injection and in lymph nodes was however not assessed in the current study. Future studies are needed to delineate the definitive role of lymphatic catabolism in reducing plasma bioavailability of the N292G mutant after SQ injection.

The binding of the Fc-domain of a therapeutic antibody to the FcγR is important, in many cases, to elicit antibody-dependent cell-mediated toxicity [132]. However, for TfRMAbs that are used as delivery vectors, such an Fc-effector function is not desirable. Studies using different variants (high- and low-affinity) of human TfRMAbs with a fully active effector function report acute clinical signs and reticulocyte suppression following IV administration in mice; adverse effects that were partly rescued by eliminating the Fc-N-linked glycosylation [98]. Using a high-affinity rat/mouse chimeric TfRMAb-EPO with a fully active effector function, we recently showed a similar suppression of reticulocytes 24 hours following IV, SQ, and IP administration [1]. Reticulocyte suppression or alteration of other hematology indices with TfRMAb-based treatments is an acute but reversible occurrence, and long-term treatment with the high-affinity TfRMAb-EPO with a fully active effector function has no significant long-lasting hematologic adverse effects in mice [1].

Nevertheless, one approach to reduce the acute suppression of reticulocytes is to use effectorless TfRMAbs, and both high- and low-affinity effectorless TfRMAbs rescue reticulocyte suppression at low doses (3-5 mg/kg) [1, 98]. In the current study, we found that the rescue of reticulocyte suppression is a function of plasma exposure of the effectorless TfRMAb-EPO such that an increase in AUC of the effectorless TfRMAb-EPO increases reticulocyte suppression. The lowest AUC in the current study was
observed with the 3 mg/kg SQ dose of the effectorless mutant cTfRMAb-N292G-EPO fusion protein, and no significant reticulocyte suppression was observed at this dose, consistent with our previous work [1]. However, despite the effector-silencing N292G mutation, the cTfRMAb-N292G-EPO fusion protein significantly reduced reticulocytes as the plasma exposure increased. At the highest dose tested (20 mg/kg), the reticulocyte suppression was comparable to that with the fully effector-functional WT TfRMAb-EPO [1]. This is consistent with previous work showing only a partial rescue of reticulocyte suppression at high doses (25 mg/kg), but a complete reversal of reticulocyte suppression at low doses (5 mg/kg) of an effectorless low-affinity TfRMAb [98]. Therefore, the rescue of reticulocyte suppression is lost at high doses of the effectorless mutant fusion protein.

We recently showed that both the WT and mutant TfRMAb-EPO fusion proteins bind to the TfR1 and EPOR with similar high-affinity, and result in comparable biological effect when given at the same concentration in vitro [1]. Therefore, similar brain concentrations of the fusion proteins are expected to result in comparable therapeutic efficacy, in vivo. Chronic treatment with a 3 mg/kg IP and SQ dose of the WT fully effector-functional TfRMAb-EPO resulted in therapeutic effects in a transgenic mouse model of AD [3, 77]. With this IP and SQ dose of the WT TfRMAb-EPO, the plasma Cmax is 1380 ng/mL and 4748 ng/mL, respectively [1]. In the current study, we show that the Cmax of the mutant fusion protein is 5-fold lower at 288 ng/mL, and 316-fold lower at 15 ng/mL following the same 3 mg/kg IP and SQ dose, respectively (Table 2.1). This reduction in plasma exposure of the mutant fusion protein is expected to have a significant impact on the brain concentrations of the fusion protein. Accordingly, we
found a profound reduction in the brain concentrations of the mutant fusion protein compared to the WT fusion protein 24 hours following a single 3 mg/kg injection in mice, which is predicted to reduce the therapeutic efficacy of the mutant fusion protein. In fact, the brain concentrations of the mutant fusion protein were comparable to background values (brain concentrations in saline-treated mice) following IP and SQ administration (Figure 2.2). Notably, even at the highest SQ dose (20 mg/kg) in the current study, the plasma Cmax was lower than the plasma Cmax following the 3 mg/kg IP dose. Such low plasma bioavailability following the SQ route is therefore expected to result in low brain bioavailability of the mutant fusion protein even at the highest dose administered in the current study. SQ administration, which is the ideal route for repeated injections in humans, is therefore not recommended for therapeutic studies using the mutant fusion protein for doses ranging between 3-20 mg/kg. Additionally, at such high doses, the beneficial rescue of reticulocyte suppression with the mutant fusion protein is lost. Notably, the brain concentrations were measured 24 hours after injection, and the possibility that the brain concentrations of the mutant fusion protein are higher at an earlier sampling time cannot be ruled out. Taken together, the prohibitively low plasma bioavailability and negligible brain uptake of the effectorless N292G mutant compared to its WT fully effector-functional counterpart will necessitate high doses of the mutant fusion protein so that a therapeutic response similar to the WT fusion protein can be achieved. However, the beneficial rescue of reticulocyte suppression with the effectorless cTfRMAb-N292G-EPO fusion protein is lost at high doses.

Another interesting observation was the elevation in the WBC counts following higher injection doses of cTfRMAb-N292G-EPO. This elevation is more pronounced
following the SQ route compared to the IP route. Whether this response is acute and
reversible, and relates to lymphatic absorption following the SQ administration needs
further investigation. However, no significant difference was observed between the
WBC counts of mice treated with the isotype control mouse IgG1 and cTfRMAb-N292G-
EPO treated mice, and the elevation in WBC counts in the current study is most likely
associated with the IgG1 (TfRMA) domain of the cTfRMAb-N292G-EPO fusion protein.

Overall, elimination of the Fc N-linked glycosylation, to mitigate TfRMAb effector
function side-effects, has a profound effect on the plasma PK and brain concentrations
of cTfRMAb-N292G-EPO. These effects on plasma PK were more pronounced
following the SQ injection, which is also the preferred injection route for repeated dosing
of biologics in humans. Plasma Cmax following the SQ route was 19- to 190-fold lower
compared with the IP route for doses between 3-20 mg/kg, illustrating the profound
impact the N292G mutation can have on the choice of the route of administration and
the bioavailability of the fusion protein. Further, the beneficial rescue of reticulocyte
suppression by the N292G mutation is a function of AUC and is negated at high doses
of the N292G mutant that are required to achieve plasma concentrations comparable to
that of the WT fusion protein with a fully active effector function. These studies,
therefore, shed light on important dosing considerations for future studies with
effectorless TfRMAb-based fusion proteins to mitigate effector function adverse effects.
The significant impact this modification may have on the dosing regimen (choice of
route of administration and dose), plasma exposure and resulting brain concentrations,
and resultant Fc-effector function may have to be considered before an effectorless
variant of the WT TfRMAb-based fusion protein is used in therapeutic studies.
Conclusion:

In the current PK study, a high dose of 20 mg/kg of the cTfRMAb-N292G-EPO fusion protein through the parenteral route of administration was needed to saturate the clearance mechanisms in the plasma of WT mice. These results indicated that a higher ID of the mutant fusion protein was needed to obtain similar plasma exposure to that of the non-mutant fusion protein. As a result, these high doses also led to adverse Fc effector function effects related to reductions in RBC indices.

The current study also showed differences of the cTfRMAb-N292G-EPO fusion protein in the plasma profile between IP and SQ parenteral routes of administration. The higher plasma exposure of the IP administration of the cTfRMAb-N292G-EPO fusion protein compared to SQ administration indicate that the protein is cleared faster during SQ administration. These considerations are important for designing experiments based on dosage in utilizing parenteral routes of administration for the cTfRMAb-N292G-EPO fusion protein. The future direction of this research will be to study why these differences in the plasma profile exist for the cTfRMAb-N292G-EPO fusion protein for different parenteral routes of administration and to utilize this PK data for studying the effects of the fusion protein on other AD animal models.

Acknowledgments

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<table>
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**Table 2.1:** Non-compartmental PK Parameters for TfrMAb-N292G-EPO in mice following IP and SQ administration

Data are presented as mean ± SEM.

††AUC, area under the plasma concentration curve; MRT, mean residence time; F, Bioavailability; Vss, volume of distribution at steady-state. Non-mutant TfrMAb-EPO data taken from Sun et al., 2019 [1]:

IP TfrMAb-EPO (3 mg/kg; n=3): Cmax (ng/mL): 1,380 ± 213; Tmax (min): 180; AUC(0-24h) (min)(ng/mL): 705,390 ± 46,814; Clearance/F: 4.3 ± 0.5 (mL/min/kg)

SQ TfrMAb-EPO (3 mg/kg; n=3): Cmax (ng/mL): 4,748 ± 290; Tmax (min): 360; AUC(0-24h) (min)(ng/mL): 4,198,080 ± 234,375; Clearance/F: 0.72 ± 0.07 (mL/min/kg)
### Table 2.2: Hematology indices and white blood cell count at 24 hours after a single dose of TfRMAb-N292G-EPO in mice.

Data are presented as mean ± SEM.

*P<0.05, **P<0.01, ***P<0.001 compared to Saline  
$P<0.05$ compared to IgG1  
††RBC, red blood cell; HGB, hemoglobin; WBC, White blood cell; Retic, reticulocytes  
CBC was not performed at the 6 mg/kg dose.
Introduction:

Alzheimer’s disease (AD) is a progressive chronic neurodegenerative disease that is characterized by the deposition of fibrous senile amyloid-beta (Aβ) plaques and intraneuronal aggregates of hyperphosphorylated tau known as neurofibrillary tangle (NFTs) [133]. Erythropoietin (EPO) is a 30.4 kDa hematopoietic growth factor that has neuroprotective effects such as reducing inflammation, oxidative stress, and neuronal loss while promoting neurogenesis and angiogenesis [108-110]. However, due to its size and polarity, EPO cannot readily enter the brain parenchyma due to the blood-brain barrier (BBB) [86, 114, 115], requiring high doses that can increase hematopoiesis [134].
The primary function of the BBB is to protect the brain against circulating toxins and pathogens from the bloodstream while selectively allowing certain nutrients and molecules to cross into the brain's extracellular fluid [113]. The BBB also mediates communication between the Central Nervous System (CNS) and the periphery through different receptors and signaling molecules [135]. One such receptor that is highly expressed at the BBB is the transferrin receptor-1 (TfR1) [116]. TfR1 through its binding to the iron-binding protein transferrin regulates intracellular iron and iron transport across the BBB [96]. The binding of iron-bound transferrin to the TfR1 leads to receptor-mediated transcytosis (RMT), shuttling iron into the brain parenchyma [96]. Accordingly, an antibody directed against the TfR1 can be used as a molecular Trojan horse (MTH) to deliver biologics into the brain parenchyma via the transvascular route of administration using this RMT approach [136]. Notably, the MTH binds to different epitopes distinct from the transferrin binding site of TfR1 to avoid interference with the physiological iron transport into the cell [92].

To study the effects of EPO on AD pathology in mouse models via a non-invasive route of administration, an MTH-EPO fusion protein was engineered with a rat/mouse chimeric antibody directed against the mouse TfR1 (cTfRMAb) and EPO [91]. The cTfRMAb-EPO fusion protein binds to the BBB TfR and brain EPO receptors (EPOR) with high-affinity; the former enabling brain delivery of EPO and the latter resulting in neuroprotective effects of EPO in the brain parenchyma [1, 91]. Further, the cTfRMAb-EPO also binds to the peripheral TfR enhancing its clearance from the systemic circulation which is expected to reduce the hematopoietic adverse effects associated with high circulating concentrations of EPO [3]. Our previous work has
shown that the cTfRMAb-EPO reduces Aβ plaques and insoluble Aβ (1-42) levels in the APP/PS1 transgenic mouse model of amyloidosis [3, 77]. However, the effect of cTfRMAb-EPO on hyperphosphorylated tau, the primary constituent of NFTs which are a characteristic neuropathologic marker of tauopathies, including AD [137], has not been studied. While there is a growing body of literature reporting the protective effects of EPO on amyloid pathology [78], studies reporting the effect of EPO on tau pathology are limited. In this respect, a recent study reported the use of a low molecular weight EPO-derived peptide that penetrates the BBB and showed that this EPO derivative can mitigate neurological deficits and neuropathological changes in the female PS19 mice [138] that express the P301S mutant human tau resulting in hyperphosphorylated tau and NFT-like inclusions with age [140], microgliosis and astrocytosis [141], and age-dependent brain atrophy and neuronal loss in the hippocampus, neocortex, and entorhinal cortex [141].

Given the potential protective effects of EPO on tauopathy, the current study aimed to investigate the effect of the BBB-penetrating EPO (cTfRMAb-EPO) on tau pathology in the PS19 mice. For this, 6-month-old male and female PS19 mice were treated intraperitoneally (IP) with cTfRMAb-EPO (10 mg/kg) for 8 weeks. Behaviorally, the mice were evaluated for hyperactivity, anxiety, and locomotion with the open-field test. After the mice were sacrificed, the brain sections were harvested and stained for tau phosphorylation, microgliosis, and hippocampal neuronal cell density.

**Materials & Methods:**
cTfRMAb-EPO Fusion Protein:

The cTfRMAb-EPO fusion protein was synthesized and formulated from ExpiCHO cells grown in serum-free ExpiCHO Expression Medium (Gibco, Gaithersburg, MD) at a concentration of 0.78 mg/mL in 98 mM glycine, 148 mM NaCl, 28 mM Tris, 0.01% Polysorbate 80 (pH=5.5) and sterile filtered by Genscript (Piscataway, NJ) [1]. The current study utilized the cTfRMAb-EPO fusion protein with a single mutation in the Fc N-linked glycosylation site at position 292 where the amino acid asparagine (Asn) is substituted with glycine (Gly), making the fusion protein aglycosylated [1]. The TfRMAb-EPO fusion protein was affinity-purified with a Protein G column and further fractionated by Superdex 200 preparative grade size exclusion chromatography (SEC). The final cTfRMAb-EPO fusion protein molecular weight and purity were realized using reducing and non-reducing SDS-PAGE, SEC HPLC, and endotoxin.

Chronic Dosing in a Mouse Model of Tauopathy:

Both male and female 6-month-old hemizygous Tg (Prnp-MAPT*P301S) PS19Vle (PS19) mice (Jackson Laboratories, ME, USA) were utilized in the present study following University Laboratory Animal Resources under protocols approved by the University of California, Irvine, Institutional Animal Care and Use Committee. During the study, the mice were maintained under a 12-h light-dark cycle and had constant access to food and water. For 8 weeks, IP injections were performed on the mice two or three days per week alternatingly with cTfRMAb-EPO (PS19-cTfRMAb-EPO; 10 mg/kg,
n=10: female=5, male=5). IP injections of an equivalent volume of saline were performed on saline (PS19-Saline; n=9: female=5, male=4) or age-matched wild-type (WT) littermates (n=12: female=6, male=6). The current study was run concurrently with a previous study published from our lab and as a result, the control mice (PS19-Saline and WT) used in the current study belonged to the same cohort of mice used in our previous study and cryoprotected in increasing sucrose concentrations (10, 20, 30%) for immunostaining [142]. The mice's body weights were recorded weekly for dosing. Adverse effects and abnormal behavior were monitored in all the mice post-injection.

Euthasol was utilized to euthanize the mice (150 mg/kg, IP). After lethal injection, the mice were perfused with ice-cold phosphate-buffered saline (PBS). After perfusion, the mice brains were collected, and the brain hemispheres were fixed in 4% paraformaldehyde (PFA) and cryoprotected in increasing sucrose concentrations (10, 20, 30%) for immunostaining [142].

**Open-field Behavioral Test:**

The mice were tested in the open-field behavioral test after 8-weeks of injections. Locomotion, hyperactivity, and anxiety were assessed with the open-field behavioral test as described previously [142]. The mice were placed in the center square (36 cm by 36 cm) of an open box (72 cm x 72cm with 36 cm walls). The SMART Video Tracking Software (Panlab, Harvard Apparatus, MA, USA) tracked the mice for 5 min. Locomotion hyperactivity was evaluated by measuring the mean speed, resting time,
and total distance traveled by the mice. Anxiety was evaluated by measuring the amount of time the mice spent in the center.

*Cryosectioning:*

The right-cerebral hemi-brains were immersion-fixed with 4% paraformaldehyde (PFA) in PBS for 72h. Each hemi-brain was then serial incubated in 10%, 20%, and 30% sucrose solution in PBS containing 0.01% sodium azide at 4°C and then frozen using powdered dry ice followed by storage at -80°C. The fixed and cryoprotected hemi-brains were sliced into 20 μm-thick sagittal sections at −25°C using a cryostat (Micron Instruments, CA, USA). The pTau immunostaining, for detecting phosphorylated tau at Ser202 and Thr205, and Iba1 immunostaining, for detecting microgliosis, utilized 5 tissue sections that were 600 µm apart per mouse as described previously [142].

*Phospho-tau (pTau; Ser202, Thr205) and Ionized Calcium Binding Adapter Molecule-1 (IBA-1) Immunostaining:*

Briefly, tissue sections were washed in PBS and blocked with 0.5% bovine serum albumin (BSA) in PBS containing 0.3% Triton X-100 (TX100) for 60 min at room temperature (RT). For the pTau staining, the tissue sections were incubated with 0.2% biotin-conjugated pTau (AT8 antibody, Thermo Fisher Scientific, MA, USA) overnight at 4°C to detect tau phosphorylated at Ser202 and Thr205. For the Iba1 immunostaining, the tissue sections were incubated in 0.5 µg/mL anti-Iba1 rabbit antibody in PBS containing 0.3% TX100 and 0.5% BSA overnight at 4°C to detect microgliosis. Then the
pTau stained sections were incubated with 0.5% Alexafluor 594 conjugated streptavidin (Biolegend; CA, USA), and the Iba1-stained sections were incubated with 0.1% Alexafluor 488 donkey anti-rabbit IgG (Biolegend; CA, USA) in PBS containing 0.3% TX-100 and 0.5% BSA in the dark for 2 h at RT.

Stain-positive area (%) was quantified in the cerebral cortex, hippocampus, entorhinal cortex, and amygdala for each tissue section using NIH ImageJ Software (version 1.53e, MD USA) with a threshold setting, as described previously [142]. The overall stain-positive area was the average of the cerebral cortex, hippocampus, entorhinal cortex, and amygdala sections. All ImageJ images were analyzed by two blinded observers of the experimental groups.

Immunofluorescence Quantification:

Brain tissue sections were imaged on the Leica TCS SP5 Confocal Microscope (Leica, NJ, USA). For the ptau immunostaining, two regions in the cerebral cortex, one to two regions in the hippocampus, one region in the entorhinal cortex, and one region in the amygdala were analyzed at 10x magnification for each tissue section. For the Iba-1 immunostaining, for each tissue section, three regions in the cerebral cortex, two regions in the hippocampus, two regions in the entorhinal cortex, and one region in the amygdala were analyzed at 10x magnification with 3x manual zoom.

Each image was analyzed with the NIH ImageJ Software (version 1.53e, MD USA) with a threshold setting. Each immunofluorescent image was converted to 16-bit and converted to grayscale. Background noise was removed by manual calibration of
the threshold values, and the tissue was analyzed for the positive area for AT8 antibody or IBA-1. Stain-positive area was expressed as a percentage of the total analyzed area. The cerebral cortex, hippocampus, entorhinal cortex, and amygdala were averaged for each mouse to get the “overall” stain-positive area. All ImageJ images were analyzed by two observers blinded to the experimental groups.

*Hematoxylin & Eosin (H&E) Staining:*

The hippocampal neuronal health was assessed in six to seven randomly selected mice per treatment group as previously described [142]. Three 20 µm sagittal mouse brain sections about 600 µm apart were mounted onto glass slides and air-dried overnight. The H&E staining was performed by washes and incubation in acetal staining jars (Simport, Quebec, Canada) with water, Mayer’s Hematoxylin (Fisher Scientific, MA, USA), Scott’s tap water/bluing reagent (10 g anhydrous MgSO4, 2g NaHCO₃, 1L tap water), Eosin Y (0.5% aqueous solution; Sigma Aldrich, MO, USA), ethanol, and xylene (Sigma Aldrich, MO, USA). The slides were cover-slipped with Permount mounting media (Fisher Scientific, MA, USA).

The light microscope (Motic, British Columbia, Canada) was utilized to image each slide. Different regions in the hippocampus (Dentate Gyrus (DG), CA1, CA2, and CA3) were imaged at 10x magnification. The hippocampal neurons (µm²) in the granule cell layer in the DG and pyramidal layer in the CA1-3 regions were manually outlined, and all images were quantified with the NIH ImageJ software (version 1.53e, MD, USA).

*Statistical Analysis:*
All data are represented as mean ± SEM, and all statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc., CA, USA). Due to similar treatment trends, the male and female data were combined. The open-field and histochemistry data were analyzed with a one-way ANOVA with Holm-Sidak’s multiple comparisons test. A two-way ANOVA with Holm-Sidak’s multiple comparisons test was performed on weight measurements. Outliers were identified using Grubb’s test. A two-tailed $p<0.05$ was considered statistically significant.

Results:

**BBB-penetrating EPO Reversed the Altered Anxiety and Hyperactive Phenotype in PS19 Mice:**

The saline-treated PS19 mice spent significantly more time in the center of the open-field apparatus compared to the saline-treated WT ($p<0.05$). cTfRMAb-EPO treatment significantly reduced the time spent in the center by the PS19 mice to WT values ($p<0.05$; Figure 3.1A & E).

Concerning hyperactivity and locomotion, the saline-treated PS19 mice had a significantly reduced resting time compared to the saline-treated WT ($p<0.05$), and cTfRMAb-EPO treatment reversed the resting time of the PS19 mice back to WT values ($p<0.05$) mice (Figure 3.1B & E). A similar trend was observed in the overall mean speed and distance traveled by the mice, wherein the saline-treated PS19 mice trended towards a greater distance and a higher mean speed compared to the saline-treated WT
(p=0.054). There was a trend towards a reduction in increased distance and mean speed after cTfRMAb-EPO treatment of PS19 mice (p=0.054) (Figure 3.1C, D, & E).
Figure 3.1: Effect of cTfRMAb-EPO on anxiety and hyperactivity in the PS19 mice. The saline-treated PS19 mice spent more time in the center (A), had increased resting time (B), and trended towards a higher overall mean speed (C) and distance traveled (D) compared to the WT mice during the open-field testing. Chronic cTfRMAb-EPO-treated rescued these altered behaviors in the PS19 mice. Representative trajectory maps showing the movement of the WT and PS19 mice (E). One-way ANOVA with Holm Sidak’s post-hoc test was used to compare to the PS19-Saline mice. Data are shown as mean ± SEM of n = 8-12 per group. *p<0.05 compared to PS19-Saline.
**BBB-penetrating EPO Decreased Tau Phosphorylation at Ser202 and Thr205 by half in the PS19 Mice:**

The AT8-positive area was significantly higher (72-92% higher, p<0.01) in the saline-treated PS19 mice compared to the saline-treated WT mice in all the brain regions (Figure 3.2A-F). The AT8-positive area in the cTfRMAb-EPO-treated PS19 mice was significantly reduced in the cerebral cortex (51% reduction; p<0.01; Figure 3.2A & F), hippocampus (62% reduction, p<0.01; Figure 3.2B & F), entorhinal cortex (52% reduction, p<0.05; Figure 3.2C & F), amygdala (49% reduction, p<0.05; Figure 3.2D & F), and overall (average of all regions) (52% reduction; p<0.01; Figure 3.2E & F) compared to the saline-treated PS19 mice.

**BBB-penetrating EPO Reduced Microgliosis in the Entorhinal Cortex and Amygdala in the PS19 Mice:**

The Iba1-positive area was significantly higher (17-31% higher, p<0.01) in the saline-treated PS19 mice compared to the saline-treated WT mice (Figure 3.3A-F). cTfRMAb-EPO treatment lowered the Iba1-positive area in the entorhinal cortex (20% lower; p<0.05; Figure 3.3C & F) and amygdala (22% lower, p<0.05; Figure 3.3D & F). The resulting overall (average of all the brain regions) Iba1-positive area was also significantly lower (16% lower, p<0.05) in the cTfRMAb-EPO-treated PS19 mice compared with the saline-treated PS19 mice (Figure 3.3E & F).
Figure 3.2: Effect of cTfRMAb-EPO on the pTau-positive area in the PS19 mice with AT8 immunostaining. The AT8-positive area was significantly lower in the cTfRMAb-EPO-treated PS19 mice compared to the saline-treated PS19 mice in the cortex (A), hippocampus (B), entorhinal cortex (C), and amygdala (D). The overall AT8-positive area, which is the average of all the brain regions, was also significantly reduced by cTfRMAb-EPO (E). Representative images of AT8-positive pTau with thumbnail brain section images showing the cerebral cortex, hippocampus, entorhinal cortex, and amygdala adapted from the Allen Institute (F). Data are presented as mean ± SEM of 9 per treatment group. One-way ANOVA with Holm Sidak’s post-hoc test was used to compare to the PS19 saline group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar = 100 μm.
Figure 3.3: Effect of cTfRMAb-EPO on microgliosis in the PS19 mice with Iba1 immunostaining. The Iba1-positive area in the different brain regions (A-F). The Iba1-positive area was significantly lower in the cTfRMAb-EPO-treated PS19 mice compared to the saline-treated PS19 mice in the entorhinal cortex (C) and amygdala (D). The overall Iba1-positive area, which is the average of all the brain regions, was also significantly reduced by cTfRMAb-EPO (E). Representative images of Iba1-positive microglia with thumbnail brain section images showing the cerebral cortex, hippocampus, entorhinal cortex, and amygdala adapted from the Allen Institute (F). Data are presented as mean ± SEM of 9-10 per treatment group. One-way ANOVA with Holm Sidak’s post-hoc test was used to compare to the PS19 saline group. *p<0.05, **p<0.01. Scale bar = 100 μm.
*BBB-penetrating EPO Did Not Impact Hippocampal Neuronal Health in the PS19 Mice:*

Hippocampal neuronal health was determined by quantifying the area occupied by the pyramidal cell layer in the CA1-3 regions, and the granule cell layer in the dentate gyrus (DG) of the hippocampus. There was a trend towards a reduction in the CA1-CA2 pyramidal cell layer area in the PS19-saline mice compared to the WT mice (p=0.07, Figure 3.4A). There was no significant difference in the pyramidal cell layer area of the CA3 region between the experimental groups (Figure 3.4B). The hippocampal granule cell layer area in the PS19-saline mice was significantly lower in the DG (20% lower; p<0.05) compared to the WT mice (Figure 3.4C & D). Administration of cTfRMAb-EPO did not alter the pyramidal or granule cell layer area in the PS19 mice (Figure 3.4).
Figure 3.4: Effect of cTfRMAb-EPO on hippocampal pyramidal and granule cell layer with H&E staining. Pyramidal cell layer area in the CA1 and CA2 (A), CA3 (B), and dentate gyrus (DG) (C). The granule cell layer area was significantly higher in the saline-treated WT mice compared to the saline-treated PS19 mice in the DG (C). No significant effect of cTfRMAb-EPO treatment on the pyramidal (A, B) or granule cell layer (C) area in the PS19 mice compared to the PS19 saline-treated mice. Representative images of the H&E-stained hippocampal granule cell layer of the DG region (D). Data are presented as mean ± SEM of 5-7 per treatment group. One-way ANOVA with Holm Sidak’s post-hoc test was used to compare the PS19-saline mice. **p<0.01. Scale bar = 200 μm.
Discussion:

The hallmarks of AD pathology are caused by the accumulation of Aβ plaques, NFTs, and neuroinflammation [143]. In previous studies, we investigated the effects of the cTfRMAb-EPO fusion protein in the APP/PS1 transgenic mouse model of amyloidosis and found a significant reduction in amyloid pathology, neuroinflammation, synaptic loss, and spatial reference memory [3, 77]. The current study wanted to investigate the effects of the cTfRMAb-EPO in the PS19 mouse model of tauopathy. The PS19 mouse model is a transgenic line with the disease associated P301S mutation leading to a 5-fold overexpression of human tau compared to endogenous mouse tau [141]. The overexpression of mutant tau leads to the development of pTau and NFT pathology with neuroinflammation and microglial activation [144]. The current study primarily focused on key behavioral abnormalities observed in the PS19 mice (locomotion hyperactivity and anxiolytic behavior) using the open-field test, tau phosphorylated at serine 202 and threonine 205 using the AT8 immunostaining, and microgliosis associated with neuroinflammation detected by Iba1 immunostaining. Our results demonstrate that the BBB-penetrating cTfRMAb-EPO fusion protein results in a marked reduction in AT8-positive pTau, which correlated with a significant reduction in Iba1-positive microgliosis and locomotion hyperactivity in the PS19 mice.
PS19 mice exhibit behavioral abnormalities such as hyperactive locomotor activity and reduced anxiety caused by cognitive deficits and behavioral modifications from increased endogenous tau [145-147]. Endogenous tau can lead to subsequent tau-misfolding, disrupting neuronal connection and causing abnormalities in behavior [147]. WT mice and other rodents without behavioral modifications spend significantly more time exploring the periphery and walls of the open-field maze than the center of the maze [148]. Mice that spend more time in the center of the maze illustrate less anxiety-like behavior [148]. The saline-treated PS19 mice spent significantly more time in the center in the current study, demonstrating a reduced anxiety-like behavior (Figure 3.1A) which
is consistent with the PS19 mouse model of tauopathy (Figure 3.1A) [149]. The saline-treated PS19 mice also displayed increased locomotion hyperactivity which is also consistent with the PS19 mouse model of tauopathy (Figure 3.1B) [149, 150]. These mice had a significantly lower resting time compared to WT mice (Figure 3.1B). Chronic dosing of cTfRMAb-EPO both reduced locomotion hyperactivity and altered anxiety-like behavior in the PS19 mice, which suggests that administering the BBB-penetrating EPO analog had a positive therapeutic effect in this mouse model of tauopathy (Figure 3.1A & B).

Previous work from our group showed that the AT8 load was a significant correlate of the hyperactivity in the PS19 mice [142]; this was further corroborated in the current study. Chronic treatment with the cTfRMAb-EPO fusion protein markedly reduced the AT8-positive pTau immunoreactive area in PS19 mice compared to the saline-treated PS19 mice in all regions analyzed in the brain (Figure 3.2). This is consistent with a recent study showing that an EPO-derived peptide can reduce AT8-positive pTau in 10-month-old PS19 mice [138]. While the BBB-penetrating EPO did not completely reduce the AT8-positive area to the WT levels, the AT8-positive area was reduced by 50% or more compared to the saline-treated PS19 mice (Figure 3.2).

Although EPO has been shown to have neuroprotective effects by reducing pTau and oxidative stress, the exact mechanisms underlying the reduction in pTau are unknown [3, 151]. One potential mechanism by which EPO reduces pTau is by modulating microgliosis. Microglia are involved in maintaining brain homeostasis by surveilling and clearing any pathological proteins and debris from the brain extracellular space [152]. However, in disease states such as AD, abnormal and continuous activation of microglia can lead to impairments in tau phagocytosis and a pro-inflammatory state.
This pro-inflammatory state can lead to the hyperphosphorylation of tau [154]. Synaptic loss and microglial activation precede the emergence of NFTs in PS19 mice [141], and both the aggregation of the misfolded protein tau and microglia activation correlate with clinical AD and act as a key determinant in its progression [155-157]. Further, microglial activation is a significant correlate of pTau both in human tauopathy [158, 159] and PS19 mice [141, 154, 160, 161].

To determine if the reduction in AT8-positive pTau was accompanied by a reduction in microgliosis, the Iba1 antibody was utilized to stain the specific calcium-binding protein. This calcium-binding protein is unevenly distributed in aged and primed microglia and macrophages [162, 163], which are likely to be disease-associated [164]. Accordingly, we found a modest but significant reduction in the Iba1-positive area with chronic cTfRMAb-EPO treatment (Figure 3.3), which was consistent with the strong significant positive correlation between the overall AT8-positive area and the overall Iba1 positive area in the 8-month-old PS19 mice [142]. These findings are consistent with the reduction in phosphorylated tau aggregation and microglial activation observed with the 19’mer EPO-derived cyclic peptide in the PS19 mice [138].

The hippocampus is a structure that is vital for memory and an early target for neurodegenerative processes such as tau pathology, leading to age-dependent neuronal loss such as that found in the PS19 mice [141, 165]. In the current study, significant neuronal loss was observed only in the granule cell layer of the DG region of the hippocampus (Figure 3.4C) with a trend toward a reduction in the pyramidal cell layer in the CA1 and CA2 regions of the hippocampus. The modest hippocampal neuronal loss is possibly caused by the age of the mice at the time of sacrifice. The PS19 mice in the
current study were 8-months old at the time of sacrifice, and tau-induced neurodegeneration may not have progressed far enough to display significant losses in the other hippocampal regions at this age. These results are consistent with the progression of neuronal loss in 8-month-old PS19 mice [141]. Although 6-month-old PS19 mice do not experience significant neuronal loss, these mice have widespread tau-positive neuronal staining and microgliosis [141], implying that neuronal loss follows tau phosphorylation and microgliosis. Chronic treatment with the cTfRMAb-EPO did not reduce the small but significant hippocampal neuronal loss in the PS19 mice (Figure 3.4), despite the therapeutic effects in reducing hippocampal pTau (Figure 3.2), and despite our previous work showing an improvement in neuronal health with this BBB-penetrating EPO in an APP/PS1 mouse model of amyloidosis [3]. This observation was also contrary to the protective effect of the EPO-derived peptide on hippocampal neuronal loss in 10-month-old PS19 mice that display more robust hippocampal neuronal loss. The absence of a therapeutic effect of the BBB-penetrating EPO on hippocampal neuronal health may be attributed to limited hippocampal neuronal loss observed in the 8-month-old mice, and future studies on older PS19 mice that are expected to show more widespread neuronal loss will be needed to determine the effect of the BBB-penetrating EPO on phosphorylated tau lesion-induced neuronal loss.

In conclusion, chronic dosing of the cTfRMAb-EPO fusion protein reversed altered anxiety and hyperactive phenotype, and reduced phosphorylated tau and microgliosis, demonstrating protective effects in the PS19 mouse model of tauopathy. The results presented in this proof-of-concept study thus offer promise for the use of this BBB-penetrating EPO molecule for tauopathies, including AD.
Acknowledgments

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CHAPTER 4

THE CONCENTRATION OF BRAIN HOMOGENATES WITH THE AMICON ULTRA CENTRIFUGAL FILTERS


Introduction:

The Blood-Brain Barrier (BBB), formed by the brain microvascular endothelial cells that are sealed together by tight junctions, is a highly selective semipermeable membrane that regulates the movement of ions, molecules, and nutrients into the brain, maintaining homeostasis and protects the brain from toxins and pathogens [113]. The BBB also restricts the entry of drugs into the central nervous system (CNS) and acts as a major obstacle to brain drug delivery [85]. In vivo techniques provide the highest
sensitivity and more reliable reference information for testing and validating other BBB permeability models [166]. Determining the brain pharmacokinetic profile of a CNS permeant drug is dependent on accurately measuring the drug concentration in the brain. Over the last decade, there has been an increase in the number of studies using sandwich immunoassays to measure brain concentrations of BBB-penetrating antibody (IgG)-biologic fusion proteins [98, 167, 168]. These studies utilize sandwich immunoassays to measure transferrin receptor antibody-biologic fusion protein concentrations in brain homogenate-derived supernatants. However, homogenization of the brain tissue in the homogenization buffer dilutes the detection signal. Furthermore, the brain contains many proteins and other macromolecules that, if excluded before retrieving the supernatant containing the CNS therapeutic of interest, may increase the assay signal. These proteins and macromolecules can cause the matrix effect, adversely impacting the limit of the detection of the assay [169]. To increase the signal-to-noise ratio of the sandwich ELISA to measure BBB-penetrating transferrin receptor IgG- biologic fusion proteins, we developed a method to concentrate the brain homogenate supernatants using the Amicon Ultra Centrifugal filters. This method concentrates the sample volume and improves the overall signal in the ELISA. This entire process is shown in Figure 4.1.

This protocol was tested with the fusion protein of a chimeric transferrin receptor monoclonal antibody and erythropoietin (cTfRMAb-EPO) and a mutant form of the fusion protein (cTfRMAb-N292G-EPO). The cTfRMAb-N292G-EPO fusion protein was engineered by removing the Fc N-linked glycosylation site at position 292 in the heavy
chain of the TfRMAb with substitution of the amino acid asparagine (Asn) with glycine (Gly) [2].

**Materials:**

1. 4mL glass scintillation vials (#03-339-23B or #03-339-25B; Fisherbrand, Pittsburgh, Pennsylvania, USA).

2. 2mL Eppendorf® Safe-Lock microcentrifuge tubes (#EP022363344; Millipore Sigma, Burlington, Massachusetts, USA).
3. Pulverizer.

4. Handheld tissue homogenizer (#H100197; Waverly Scientific, Waverly, Iowa, USA).

5. T-PER TM Tissue Protein Extraction Reagent (#78510; Thermo Fisher Scientific, Waltham, Massachusetts, USA).

6. cOmplete TM Mini-EDTA-free protease inhibitor cocktail tablets (#4693159001; Sigma, St. Louis, Missouri, USA).

7. Shaker.

8. Dry Ice.

9. Ice.

10. Eppendorf® Centrifuge 5417R (Z366021; Sigma Aldrich, St. Louis, Missouri, USA).

11. Weighing scale for brain hemispheres.

12. Euthasol (stock concentration: 390 mg/mL. Each mL contains 390 mg pentobarbital sodium and 50 mg phenytoin sodium). Euthasol was diluted to 1:10 with sterile saline.

13. Perfused mice brain hemispheres.

14. Amicon Ultra 2 mL 50 kDa Protein Purification and Concentration Filters (#UFC205024; Millipore Sigma, Burlington, Massachusetts, USA).

15. Eppendorf® Centrifuge 5810R with the swinging bucket rotor A-4-62 (#EP022628168; Sigma Aldrich, St. Louis, Missouri, USA).

16. Tris Buffer Saline (TBS): 0.05 M Tris/0.15 M NaCl/pH 7.4.

17. TBST: TBS + 0.05% Tween-20.

18. Chimeric transferrin receptor antibody-biologic fusion protein therapeutic: cTfRMAb-EPO (0.7 mg/mL; Genscript, Piscataway, New Jersey, USA).
19. Chimeric transferrin receptor antibody-biologic fusion protein therapeutic: cTfRMAb-
N292G- EPO (0.78 mg/mL; Genscript, Piscataway, New Jersey, USA). 20. Transferrin
receptor antibody-biologic fusion protein therapeutic vehicle: 96 mM glycine, 28 mM
Tris, 148mM NaCl, 0.01% Polysorbate 80, pH = 5.5.
21. Recombinant human EPO receptor (EPOR)/Fc fusion protein (R&D System #963-
ER-050).
22. 0.1M NaHCO 3 /pH 8.5.
23. TBSB: 0.01M Tris/0.15M NaCl/pH 7.4/1% bovine serum albumin (BSA).
24. Goat anti-mouse light chain (kappa) antibody conjugated to alkaline phosphatase
(GAM-AP) (# A90-119AP; Bethyl, Montgomery, Texas, USA).
25. P-nitrophenyl phosphate (PNPP) (#P5994; Sigma Aldrich, St. Louis, Missouri,
USA).
27. 1.2M NaOH.
28. 96 well NUNC Maxisorp flatbottom 96 TS (#501123685; Fisher Scientific, Waltham,
Massachusetts, USA).
29. SpectraMax Plus 384 Microplate Reader (Molecular Devices; San Jose, California,
USA).
30. SoftMax Pro Software (Molecular Devices; San Jose, California, USA).
31. GraphPad Prism 8 Software (GraphPad Software Inc.; San Diego, California, USA).

Methods:

Mouse Animal Study:
All animal procedures were approved by the University of La Verne Institutional Animal Care and Use Committee (Protocol No. LV0012d). Eight-week-old male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

The mice were provided constant access to food and water and were maintained on a 12 h light/12 h dark cycle. The mice were injected intravenously with 0.3 mg/kg (n = 3) or 3 mg/kg (n = 4), or intraperitoneally with 3 mg/kg (n = 6) of cTfRMAb-EPO. A separate group of age-matched mice were injected intravenously with 0.3 mg/kg (n = 3) or 3 mg/kg (n = 6), or intraperitoneally with 3 mg/kg (n = 3) of the mutant cTfRMAb-N292G-EPO fusion protein. Control mice were dosed intraperitoneally with saline (n = 4). Twenty-four-hours after the initial injection, the mice were anesthetized with a lethal dose of Euthasol (150 mg/kg, intraperitoneally) and transcardially perfused with ice-cold phosphate buffer saline (PBS). The brain hemispheres were harvested and stored at -80 °C until use.

**Brain Pulverization Protocol:**

1. Prepare the 4 mL glass scintillation vials to store each individual pulverized brain hemisphere. Pre-weigh and label the scintillation vials clearly.

2. Place the handheld pulverizer in powdered dry ice for 3 min. This process ensures that the equipment is cold and prevents the frozen brain hemispheres from warming up and sticking to the pulverizer.

3. Place frozen brain hemisphere in the chilled pulverizer and pulverize into a fine powder with a hammer. This process is done over dry ice to maintain the cold temperature.
4. Separate the powdered brain hemisphere into two groups: the brain homogenate group and the Amicon Ultra Centrifugal Filter separated group.

NOTE: The brain homogenate group consists of the raw brain homogenate samples that have not been processed using the Amicon Ultra Centrifugal Filter but for which the brain homogenate supernatants are directly run on the ELISA (Figure 4.1). The brain homogenate group was compared to the Amicon retentate concentrate sample to compare the relative signal between the two samples.

The Amicon Ultra Centrifugal Filter separated group consists of the raw brain homogenate supernatant samples that run through the Amicon Ultra 2 mL 50 kDa Protein Purification and Concentration Filter Tubes (Figure 4.1). This sample group is further separated between the relevant Amicon retentate concentrate samples and the extraneous Amicon filtrate samples. The retentate concentrate sample is the sample that is retained by the Amicon centrifugation process, while the filtrate sample is the sample that is filtered through and typically discarded.

1. Collect the powdered brain using a pre-chilled metal spatula and place it in pre-chilled scintillation vials.
2. Quickly weigh the scintillation vials again and note the weight. The weight difference in the pre-weighed vials and the vials containing the powdered brain is the total brain weight, which is important for the brain homogenization calculations below.
3. Place the 4 mL scintillation vials with the pulverized brains at -80°C until brain homogenization.
**Brain Homogenization Protocol:**

1. Make the homogenization buffer by mixing 10 mL of T-PER TM Tissue Protein Extraction Reagent with 1 tablet of cOmplete TM Mini-EDTA-free protease inhibitor cocktail tablet.

2. Take out the scintillation vials with the pulverized brains from -80°C and place them on ice.

3. Homogenize the pulverized brain in the homogenization buffer with the handheld tissue homogenizer for 30 sec. Wash the homogenizer with chilled ice-cold homogenization buffer between samples.
   
   a. For homogenizing the brain homogenate group, put 4 brain volumes (4 μL/mg of the brain) of ice-cold homogenization buffer (T-PER) into the chilled scintillation vial with the pulverized brain. Over-dilution of the samples will lead to a lower signal.

   b. For homogenizing the Amicon Ultra Centrifugal Filter separated group, put 7 brain volumes (7 μL/mg of the brain) of ice-cold homogenization buffer (T-PER) into the chilled scintillation vial with the pulverized brain. Dilution of the samples is not an issue because the sample will be concentrated with the Amicon Ultra 2 mL 50 kDa Protein Purification and Concentration Filters. NOTE: the Amicon Ultra 2 mL 50 kDa Protein Purification and Concentration Filters can hold a maximum volume of 2 mL. NOTE: the average weight of the pulverized hemisphere was about 200-220 mg and the average brain homogenate volume based on 7 μL/mg was 1400-1540 μL.

4. Mix the chilled scintillation vials on a shaker at 4°C for 1 h.


5. Pipette out all the brain homogenate into a chilled centrifuge tube. Collect everything to avoid protein loss and inaccuracies in the calculation.

6. Weigh the centrifuge tubes. Arrange and counterbalance the centrifuge tubes into weight-matched pairs in the rotor of the Eppendorf centrifuge 5417R. Centrifuge the brain samples at 14,000xg for 20 min at 4°C.

7. Collect and aliquot (to prevent repeated freeze-thaw cycles) all the supernatant in labeled 2 mL Eppendorf tubes and freeze all aliquots in the -80°C freezer for long-term storage. If the brain ELISA is run the following day, keep all tubes in the 4°C refrigerator to avoid freeze-thaw.

**Amicon Concentration & Purification Protocol:**

1. Pipette the Amicon Ultra Centrifugal Filter separated group samples into the Amicon Ultra 2 mL 50 kDa Protein Purification and Concentration Filters and assemble the Amicon Centrifugation Tubes, as described by the vendor.

2. Place and balance Amicon Centrifugation Tubes into the A-4-62 swinging bucket rotor of the Eppendorf® Centrifuge 5810R and centrifuge at 3000xg for 20 min at 4°C.

3. Take each individual Amicon Centrifugation Tube out and pipette up & down vigorously along the membranes of the Amicon Filter to detach any macromolecules sticking and blocking the membrane. NOTE: do this step carefully so as not to damage the membrane.

4. Place the Amicon Centrifugation Tubes back into the swinging bucket rotor and centrifuge at 3000xg for 7 min at 4°C.
5. Take each individual Amicon Centrifugation Tube out and pipette up & down vigorously again along the membranes of the Amicon Filter to detach any macromolecules sticking and blocking the membrane. Place the Amicon Centrifugation Tubes back into the A-4-62 swinging bucket rotor and centrifuge at 30 0 0xg for 5 min at 4°C. This step is repeated until the volume of the Amicon Centrifugation retentate is below 300 μL.

6. Once the volume of the collected Amicon retentate concentrate sample is below 300 μL, remove the assembled device from the centrifuge and separate the Amicon Ultra Filter from the filtrate collection tube.

7. To recover the Amicon retentate concentrate samples, invert the Amicon Ultra Filter device and concentrate collection tube. Place the inverted Amicon Ultra Filter device back in the Eppendorf® Centrifuge 5810R with rotor A-4-62 and counterbalance each sample. Spin for 2 min at 1000xg to transfer the Amicon retentate concentrate samples from the filter device to the concentrate collection tube.

8. Aliquot and freeze the final samples in the -80°C freezer to prevent repeated freeze-thaw cycles, unless you are running the brain ELISA the following day. If running the ELISA the next day, place the Amicon Ultra Centrifugal Filter separated samples in the 4°C refrigerator with the brain homogenate samples.

**Brain ELISA Protocol:**

1. Dilute the recombinant human EPOR/Fc fusion protein with 0.01M of PBS (pH 7.4) so that the stock is 100 μg/mL. For this, add 500 μL of PBS for the 50 μg of the lyophilized
powder of the recombinant human EPOR/Fc fusion protein. Split this stock solution into 3 aliquots of 167 μL and freeze it in the -80°C freezer until use.

2. To make the working solution, remove and thaw one aliquot (16.7 μg) and dilute it with 8.35 mL of 0.1M NaHCO₃ (pH 8.5). The concentration is 2 μg/mL and will be enough for 85 wells with each well containing 0.2 μg of the EPOR/Fc fusion protein. Plate 100 μL per well in the 96 well Nunc maxisorp ELISA plate and incubate overnight at 4°C.

3. Aspirate off the EPOR/Fc solution from the ELISA plate after overnight incubation. Then wash each well 3 times with 150 μL of TBST per well.

4. Block each well with 150 μL of TBSB for at least 30 min at room temperature (RT). Prepare the standards during this blocking step. Both transferrin receptor antibody-EPO fusion proteins are diluted to 90,000 ng/mL, 30,000 ng/mL, 3,000 ng/mL, 300 ng/mL, 30 ng/mL, and 3 ng/mL for the standards.

5. Aspirate the TBSB and add 100 μL of cTfRMAb-EPO and cTfRMAb-N292G-EPO standards, 200 μL of the brain homogenate samples including the T-PER brain homogenate blank, and all the Amicon retentate concentrate sample volume per well and incubate for 2 h at RT.

6. Aspirate the samples from the ELISA plate and wash each well 3 times with 150 μL of TBST.

7. Dilute 17 μL (8.5 μg) of GAM-AP with 8,483 μL of TBST and add 100 μL of the diluted GAM-AP working solution per well (100 ng/well). This volume is enough for 85 wells. Incubate the ELISA plate for 45 min at RT.
8. Aspirate off the GAM-AP working solution from the ELISA plate and wash 3 times with 150 μL of TBST per well.

9. Dilute a 40 mg PNPP tablet in 40 mL of glycine buffer (pH 10.4). Add 100 μL of the PNPP working solution in each well and incubate for 20 min at RT in the dark. The color of the solution typically changes immediately, and saturation of the reaction may be reached before 20 min.

10. Add 100 μL of 1.2M NaOH to each well to stop the color reaction. Monitor the color of the solution in each well carefully. The reaction needs to be stopped before 20 min if the color changes rapidly.

11. Place the plate on a plate reader and read the absorbance at 405 nm. Compute the mean blank with the blank T-PER brain homogenate samples. Subtract the blank from each individual test sample and standard, and compute the mean of the blank-corrected test samples and standard for each group.

**Brain Concentration Calculation:**

1. The blank-corrected standard curves (appropriate for the specific cTfRMAb-based fusion protein) were fit to the one site-specific binding nonlinear regression curve to determine the total density of binding receptor (Bmax) and the equilibrium dissociation constant (KD) values using GraphPad Prism 8.

2. The brain concentrations (ng/mL brain homogenate supernatant) of both TfRMAb antibody-based fusion proteins are calculated from the formula:

\[
\text{Concentration} = \frac{\text{OD} \times \text{K D}}{\text{B Max} - \text{OD}}
\]
The OD is the optical density measured from the plate reader at 405 nm that has been blank-corrected from the T-PER brain homogenate samples.

1. The amount (ng) of cTfRMAb-based fusion proteins (cTfRMAb-N292G-EPO and cTfRMAb-EPO) in the brain was calculated by multiplying the back-calculated concentration by the volume of the brain homogenate.

2. The amount of cTfRMAb-N292G-EPO and cTfRMAb-EPO per gram brain (ng/g brain) was calculated by dividing the amount (ng) of cTfRMAb-based fusion proteins in the brain by the weight of the brain (g).

Statistical Analysis:
All data are represented as mean ± standard error of the mean (SEM), and all statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA). To test the effect of two factors, two-way ANOVA with Holm-Sidak post-test was used. A two-tailed p < 0.05 was considered statistically significant.

Methods Validation:
In our experiment, we used two bivalent, BBB-penetrating cTfRMAb-based fusion proteins as model BBB-penetrating IgG-biologic fusion proteins and evaluated their brain concentrations after intravenous and intraperitoneal routes of administration. The reason for selecting these two TfRMAb-based fusion proteins for method validation was the profound difference in their plasma concentrations and expected brain concentrations [2]. Saline-treated mice were used to determine the background signal in
the brain homogenates (background of the assay) and limit of detection (LOD) of the assay as described previously [170].

Each set of Amicon Ultra Centrifugal Filter separated brain homogenates was paired with its brain homogenate group counterpart (Figure 4.2). As expected, the background concentrations (ng/mL of brain homogenate supernatant) obtained from saline-treated mice for both the Amicon Ultra Centrifugal Filter separated group (n = 4) and the brain homogenate group (n = 4) were comparable and ranged between 0-0.6 ng/mL and 0-0.4 ng/mL (designated as saline concentration threshold), respectively, and LOD of the assay was 0.8 ng/mL.

For mice treated with the cTfRMAb-EPO (n = 13), the concentrations (ng/mL of brain homogenate supernatant) resulting from the Amicon Ultra Centrifugal Filter separation ranged between 1-54 ng/mL, and those obtained using the regular brain homogenization method ranged between 0.1-4 ng/mL (Figure 4.2). All the brain concentrations of mice treated with the cTfRMAb-EPO and separated using the Amicon Ultra Centrifugal Filters were above the saline concentration threshold. While using the regular brain homogenate method, eight of the cTfRMAb-EPO-treated mice had brain concentrations above and five had brain concentrations below the saline concentration threshold (Figure 4.2). Amicon Ultra Centrifugal Filter separation elevated the brain homogenate concentration of the cTfRMAb-EPO in all thirteen brain samples tested.

For mice treated with the mutant cTfRMAb-N292G-EPO (n = 12), the concentrations (ng/mL of brain homogenate supernatant) resulting from the Amicon Ultra Centrifugal Filter separation ranged between 0-6 ng/mL, and those obtained using the regular brain homogenization method ranged between 0-1 ng/mL (Figure 4.2).
Seven of the brains separated using the Amicon Ultra Centrifugal Filters, but only two of the brains separated using the regular brain homogenization method, were above the saline concentration threshold (Figure 4.2). Notably, five of the brains treated with the cTfRMAb-N292G-EPO had brain concentrations within the saline concentration threshold while using either the Amicon or the regular brain homogenization separation method. This is an expected finding given the known profound differences in plasma clearance and thereby the resultant brain concentrations between the two cTfRMAb-based fusion proteins. The cTfRMAb-EPO has a higher plasma exposure and half-life with a lower clearance compared to that of the cTfRMAb-N292G-EPO [2]. As a result, the expected brain concentrations of the cTfRMAb-N292G-EPO are low at the doses used in the current study.

We then converted the ng/mL brain homogenate supernatant concentrations to brain concentrations in ng/g brain tissue. After intravenous administration, the Amicon Ultra Centrifugal Filter separated group displayed a significantly higher (p < 0.0001) brain concentration (ng/g brain) compared to the brain homogenate group for cTfRMAb-EPO, and a similar trend was seen for the cTfRMAb-N292G-EPO fusion protein, though these values did not reach statistical significance (Figure 4.3A). As seen in Figure 4.3A, as expected based on the plasma pharmacokinetics [2], the brain concentrations of the cTfRMAb-EPO were significantly (p < 0.001) higher than those of cTfRMAb-N292G-EPO and saline for the Amicon Ultra Centrifugal Filter separated group. The cTfRMAb-EPO group was 8-fold larger in brain concentration (ng/g brain) compared to the cTfRMAb-N292G-EPO group and was 642-fold larger in brain concentration compared to the saline group (Figure 4.3A). The regular brain homogenization method was unable
to detect any significant differences between the three groups (Figure 4.3A). These trends are also confirmed following the intraperitoneal route of administration.

After intraperitoneal administration, the Amicon Ultra Centrifugal Filter separated group displayed a trend ($p = 0.054$) towards a higher signal compared to the brain homogenate group for cTfRMAb- EPO. This trend was not observed for cTfRMAb-N292G-EPO because of the below-detection brain concentrations of cTfRMAb-N292G-EPO following intraperitoneal administration at the doses used in the current study (Figure 4.2). Again, as expected, the brain concentrations of the cTfRMAb-EPO were significantly ($p < 0.01$) higher than those of the cTfRMAb-N292G-EPO and saline for the Amicon Ultra Centrifugal Filter separated group (Figure 4.3 B). Similar to the intravenous administration, the regular brain homogenization method was unable to detect any significant differences between the three groups (Figure 4.3B).

Overall, while comparing the Amicon Ultra Centrifugal Filter separated method with the regular brain homogenization method, the Amicon separation method concentrated the brain homogenate supernatant for the cTfRMAb-based fusion proteins tested in the current study. This concentration of the brain homogenate supernatant significantly increased the signal for the ELISA which allowed the detection of expected differences in the brain concentrations of the two different cTfRMAb- based fusion proteins tested herein using the Amicon separation method; these differences were not detected by the regular brain homogenization method. We attribute this improvement of the signal with the Amicon Ultra Centrifugal Filters to 1) the concentration of the brain homogenate volume which results in the enrichment of the brain homogenate for the analyte of interest, and 2) clean-up of the brain homogenate supernatant owing to the
50kDa molecular weight cut-off filters and therefore an expected reduction in the samples matrix-effect. The use of centrifugal filtration devices for sample clean-up has been previously reported for small molecule quantification in biological samples prior to liquid chromatography coupled with mass spectrometry (LC-MS) [171]. Besides the use of the Amicon Ultra Centrifugal Filters, there are other sample clean-up techniques including solid-phase extraction (SPE) that are extensively used for sample preparation to measure large molecules such as antibody-drug conjugates (ADCs) prior to LC-MS quantification [172]. However, the use of SPE is more time consuming than simple centrifugation-based separation, and its use for sample preparation to measure the brain concentrations of IgG-biologic fusion proteins has not been reported and requires further investigation. Further, though LC-MS quantification is widely used to measure ADCs in biological samples and can have a reported limit of detection of 1 ng/mL [173], the measurement of IgG-biologic fusion proteins is further complicated due to their even larger size and difficulty in interpretation of the mass signal [172]. Furthermore, measurement of in vivo brain concentrations of IgG-biologic fusion proteins with this method has not been reported yet but may be a viable approach [174] that needs to be further investigated.

The most widely used technique to sensitively measure brain concentrations of IgG-biologic fusion proteins is radiolabeling of the fusion protein. The use of sandwich immunoassays such as ELISA and the electrochemiluminescence-Meso Scale Discovery (ECL-MSD) platforms to measure brain concentrations of IgG-biologic fusion proteins has increased considerably in the last decade and offers the advantage of being non-radioactive approaches. However, sample preparation for these
immunoassays can result in sample loss compared to using radioactive methods. Other non-radioactive approaches used to measure brain concentrations of IgG-biologic fusion proteins include near infrared fluorescence (NIRF) imaging and immunostaining techniques [175, 176], however these techniques are semi-quantitative. The current study shows promise in the use of Amicon Centrifugal Filters to enhance the signal of a quantitative sandwich ELISA to measure the brain concentrations of IgG-EPO fusion proteins, and future work is needed to see if this technique can be widely applied to other IgG-biologic fusion proteins.

Conclusion:

The Amicon Concentration Method developed in this manuscript is a potential new method for preparing samples for quantitative sandwich ELISA runs on brain homogenate samples. The brain contains many different proteins and macromolecules that can adversely impact the limit of detection of the assay. The Amicon Concentration Method not only concentrates the brain homogenate volume but also boosts the sample signals. This protocol offers better sensitivity to running raw brain homogenate samples on ELISAs and acts as a potential alternative method to utilizing radiolabeled proteins to measure brain protein concentration. The future direction of this research will be to improve upon the efficiency of this protocol to produce signals comparable to utilizing radiolabeled proteins. Once improved upon, this technique can be applied to measuring the brain protein concentration of other neurotherapeutics.

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Figure 4.2: The concentration of cTfRMAb-based fusion proteins in mouse brain homogenates. In the above-paired graph, each symbol-line-symbol represents the paired brain concentrations resulting post-ELISA by the brain homogenization and Amicon concentration method for the same brain. Each symbol-line-symbol represents an individual brain from a mouse treated either with cTfRMAb-EPO, cTfRMAb-N292G-EPO, or saline. The red dotted line represents the limit of detection of the assay (0.8 ng/mL).
Figure 4.3: The brain concentrations of the cTfRMAb-based fusion proteins following intravenous (A) and intraperitoneal (B) administration of a 3 mg/kg dose of each fusion protein comparing the Amicon Ultra Centrifugal Filter separated group and the regular brain homogenate group. The Amicon Ultra Centrifugal Filter data was presented in reference [2]. The data was analyzed using the two-way ANOVA with Holm-Sidak multiple comparisons test. All data are represented as mean ± standard error of the mean (SEM). **P < 0.01 and ****P < 0.0001 in A, $P < 0.0001$ in A and $p = 0.054$ in B represent comparisons to the corresponding Amicon Ultra Centrifugal Filter separated group.
CHAPTER 5

FUTURE DIRECTIONS

The studies done in this dissertation can be used as a template to investigate other potential neurotherapeutics for the treatment of AD. The current dissertation showed the therapeutic potential of the molecular Trojan horse technology in bypassing the BBB and entering into the brain parenchyma. The development of the cTfRMAb-N292G-EPO mutant fusion protein demonstrated that the glycosylation of the Fc region of the monoclonal antibody is important in maintaining the molecule’s plasma exposure. Removal of the Fc N-linked glycosylation site by substituting Asn with Gly not only reduced the Fc-effector function adverse effects of the cTfRMAb but also increased the clearance of the cTfRMAb-N292G-EPO fusion protein. This led to needing a higher ID of the mutant fusion protein to sustain a comparable plasma profile.

Utilizing the PK profile, chronic treatment of cTfRMAb-N292G-EPO in PS19 Tauopathic mice was effective in significantly reducing phosphorylated tau and microgliosis, two hallmarks of AD pathology. However, the increased dosage of cTfRMAb-N292G-EPO fusion protein also led to Fc-effector function adverse effects similar to that of the cTfRMAb-EPO at lower doses.

There are several directions that the development of a brain penetrating EPO can be taken to determine the efficacy in AD. cTfRMAb-EPOs are effective in in vivo mouse models of amyloidosis and tauopathy. However, the effects of cTfRMAb-EPOs on a triple transgenic model of AD mouse with both plaques and tangles have not yet been studied. One potential future direction in this research that can be utilized is to test
chronic dosing of cTfRMAb-EPO in the 3xTg-AD mice exhibiting PS1(M146V),
APP(Swen), and tau(P301L) transgenes as a high priority experiment [177]. This AD
model is a more complete model exhibiting more hallmarks of the disease, and
ultimately would be a better indicator of the efficacy of the cTfRMAb-EPO drug.

Another direction this research can be taken is investigating the integrity of
synaptic transmission and synaptic plasticity in cTfRMAb-EPO-treated mice in both WT
and AD mouse models. Although there are several studies investigating long-term
potentiation and long-term depression of hippocampal transmission [178], there have
not been studies exploring the effects of EPO and the MTH technology. These
experiments can be explored utilizing bipolar stimulating electrodes and single wire
recording electrodes placed in the dentate gyrus region of the hippocampus [179].

Ultimately, the utilization of cTfRMAb-EPO for AD treatment looks promising. The
neuronal lesion associated with AD is caused by senile plaques and neurofibrillar
tangles [180]. EPO’s erythropoietic effects on neurogenesis, angiogenesis, and the
reduction of plaques, phosphorylated tau, and inflammation can potentially be utilized to
treat other diseases. Coupled with the BBB transport capability of the TfRMAb, the
TfRMAb-EPO may be used to treat a spectrum of neurodegenerative diseases such as
stroke, Huntington’s disease, Parkinson’s disease, motor neuron diseases, traumatic
brain injury, and others.
LIST OF PUBLICATIONS:

Resulting Directly from Dissertation:


Indirectly Related to Dissertation:

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