The development of a small molecule neuroserpin inhibitor towards the restoration of NGF metabolism in Alzheimer's disease

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English Abstract

In Alzheimer's Disease (AD) the atrophy and degeneration of the cholinergic neurotransmitter system, beginning with the basal forebrain cholinergic neurons (BFCNs), is central to the onset of cognitive symptoms. These BFCNs rely solely on mature nerve growth factor (mNGF) for their trophic support. Our McGill lab has discovered a metabolic system controlling the availability of mNGF and found this brain metabolic pathway compromised in AD at clinical as well as preclinical stages, resulting in a substantial loss of mNGF. Thus, a dysregulation of this NGF metabolic cascade leads to the deprivation of adequate trophic support of BFCNs, therefore resulting in the well-established atrophy of these neurons and loss of their synaptic terminations in the cerebral cortex and hippocampus. Our lab hypothesizes that a pharmacological correction of this metabolism, through neuroserpin inhibition, could serve as a therapeutic route for normalizing the rate of endogenous mNGF production. Thus, restoring trophic support to the BFCNs and ameliorating the downstream cognitive decline incited by their atrophy. This Thesis covers the *in silico* identification, and *in vitro* testing (applying an in-house neuroserpin activity assay), of small molecule candidate neuroserpin inhibitors. It also covers the initial exploration of the structural activity relationships between successful neuroserpin inhibitors and their respective binding pocket.

French Abstract

Dans la maladie d'Alzheimer (MA), l'atrophie et la dégénérescence du système de neurotransmetteurs cholinergiques, à commencer par les neurones cholinergiques dans le cerveau antérieur basal (BFCNs), sont au cœur de l'apparition des symptômes cognitifs. Ces

BFCNs dépendent uniquement du facteur de croissance nerveuse mature (mNGF) pour leur soutien trophique. Notre laboratoire de McGill a découvert un système métabolique contrôlant la disponibilité du mNGF et a constaté que cette voie métabolique cérébrale était compromise dans la MA aux stades cliniques et précliniques, entraînant une perte considérable de mNGF. Selon nos conclusions donc, une dérégulation de cette cascade métabolique du NGF se solde par la privation d'un support trophique adéquat des BFCNs, entraînant ainsi une atrophie bien établie de ces neurones et une perte de leurs terminaisons synaptiques dans le cortex cérébral et l'hippocampe. Notre laboratoire émet l'hypothèse qu'une correction pharmacologique de ce métabolisme, via l'inhibition de la neuroserpine, pourrait servir de voie thérapeutique pour normaliser le taux de production endogène de mNGF, et ainsi restaurer le soutien trophique des BFCNs et ralentir le déclin cognitif provoqué par leur atrophie. Cette thèse couvre l'identification in silico, ainsi que les tests in vitro (en appliquant en interne un test d'activité de la neuroserpine) de petites molécules potentiellement capables d'inhiber la neuroserpine. Elle couvre également l'exploration initiale des relations structure-activité entre les inhibiteurs efficaces de la neuroséprine et leur poche de fixation respective.

Acknowledgments

I began my master's during the peak of the Covid pandemic. As such there were many hurdles: I had an 8-week quarantine within the first 6 months of my grad studies, the laboratory was in a 'shift' system, delayed shipments of reagents were a common occurrence, and I once had to drive 15 hours in a day as my covid test results hadn't been uploaded by the pharmacy by the time I made it to the border. The fact that I was able to

achieve the work detailed in this Thesis, despite the more than rocky start, is an achievement I cannot take sole responsibility for. Thus, I have many people to thank in this acknowledgements section.

I would first like to thank my committee, Dr. Edith Hamel, and Dr. Paul Clarke, for their insight into the best ways of realizing the goals of my project. I would like to thank my supervisor, Dr. A. Claudio Cuello, whose immense legacy set the stage for my project, and whose mentorship was key to its success. My co-supervisor Dr. Sonia Do Carmo, whose constant presence, willingness to help, and breadth of knowledge both scientific and pragmatic helped me become the scientist I am today.

Indeed, I am grateful to all the members of the Cuello lab, past and present. Firstly, Quentin Bonomo and Ai Liu, who started with me in the tumultuous time of Covid, and like me had to adapt to not only a new work environment, but a new country of living in a time of limited social interactions. Further I would like to thank Dr. Joshua Emmerson, Dr. Chiara Orciani, and Dr. Morgan Foret who provided me with constant support and advice as the senior members of the lab and helped me both with my development of scientific abilities, and my development as a person. And I would like to thank Adriana Ducatenzeiler, Chunwei Huang, and Augustina Lavagna for their continued advice, assistance, and knowledge. As well as Dr. Rowan Pentz, while our time in lab never coincided, his blueprints and initial work set my project in motion. I'd also like to thank all my friends at McGill, especially Simran Dhir, who kept me sane along this journey, and kept me fed on days I was working so hard I forgot to eat.

I would like to thank my parents and my family who allowed me to move to a new country during such a tumultuous time and provided me with constant support. Lastly, I would like to thank Molly, who gave me something to look forward to after long days of work, and whose sacrifice in moving to Canada with me made all of this possible.

Contribution of authors

Each section of this traditional thesis was written solely by myself.

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List of abbreviations:

tPA = tissue Plasminogen Activator

AB = Amyloid beta

AD = Alzheimer's disease

APP = Amyloid Precursor Protein

BFCN = Basal Forebrain Cholinergic Neuron

CNS = Central nervous system

GCS = Global cognitive score

HA-NCI = High Amyloid, No Cognitive Impairment

LA-NCI = Low Amyloid, No Cognitive Impairment

MCI = Mild Cognitive Impairment

MMP-3/9 = Matrix Metalloproteinase-3/9

MMSE = Mini-mental state examination

MoCA = Montreal Cognitive Assessment

mNGF = mature Nerve Growth Factor

NCI = No Cognitive Impairment

NGF = Nerve Growth Factor

proMMP-3/9 = precursor to MMP-3/9

proNGF = the precursor to Nerve Growth Factor

SEM = Standard Error of the Mean

TIMP1 = Tissue Inhibitor of Metalloproteinases-1

CSF = cerebro-spinal fluid

MMP = matrix metallo-protease

FDA = U.S.A Food and Drug Administration

NMDA = N-methyl-D-aspartate

Serpin = Serine protease inhibitor

RCL = Reactive center loop

CS = Consensus site

MM = Michealis Menten

SAR = Structural activity relationships

Introduction

Historical perspective

Dementia is a condition that has been with humanity since the onset of recorded history. Commentary on the cognitive loss associated with aging is seen as early as 25th century BCE hieroglyphs, and was discussed by classical thinkers such as Pythagoras, Plato, and Hippocrates [7, 8]. Later, Cicero proposed that dementia was not an inevitable aspect of growing old. In his work "De Senectute" (On Old Age), Cicero argued that age-related memory loss was a pathology in and of itself, a deviation from the course of normal aging. He believed that this condition could be prevented through a combination of healthy living and mental activity as discussed in [9].

Medieval scholars continued to explore dementia. The famous physician Galen utilized terminology like "the rarefaction and diminution in quantity of the animal spirits" and "the coldness and humidity of the brain" to explain dementia as discussed in [7]. As Europe entered the early modern period, the empiricist Roger Bacon wrote a treatise on preventing senility. Importantly, he associated dementia with a defect in the posterior part of the brain again discussed in [7]. This period marked the transition in the west from purely philosophical and theoretical discussions of dementia to more empirical approaches.

The late 19th century brought about significant advancements in histology, providing the means for more in-depth investigations into dementia. In particular, the pioneering work of Oskar Fischer, a Czech neuropathologist, marked a turning point in the history of dementia. Fischer identified a specific form of dementia, which he called "presbyophrenic dementia" characterized by confusion and episodic memory disturbance. Importantly, Fischer, aided by Bielchowsky's novel silver staining technique, showed the existence of plaques specific to cases of presbyophrenic dementia [10]. This breakthrough provided the first specific, albeit incomplete, characterization of what would later be known as Alzheimer's disease (AD).

Dr. Alzheimer's contribution

The same year that Fischer published his groundbreaking report, another pivotal moment occurred in the history of dementia. In 1906, Auguste Dieter, a woman only fifty-one years old, began to experience severe and progressive memory loss, accompanied by paranoia, confusion, and aggression. Her husband took her to the Psychiatric Hospital in Frankfurt when her symptoms made cohabitation impossible. Dr. Alois Alzheimer was the

attending psychiatrist for Auguste. Dr. Alzheimer took detailed notes of his conversations with Auguste, who had severe memory impairments. She couldn't recall her address, her husband's name, and couldn't even name the eleventh month. After Auguste passed away from pneumonia, Dr. Alzheimer was granted permission to analyze her brain histologically. Dr. Alzheimer was working with a renowned group of expert pathologists, histologists, and psychiatrists in Munich, led by Emil Kraepelin. Alzheimer also worked with Franz Nissl as well as Bielchowsky, whose silver stain technique proved crucial to Alzheimer's work, just as it had been for Fischer's [11, 12].

In Auguste's brain, Dr. Alzheimer detailed extracellular plaques throughout the brain parenchyma, a finding consistent with earlier observations by other neuropathologists. However, Dr. Alzheimer's most critical discovery was a second pathology, neurofibrils. He saw neurofibrils in different stages of their development and concluded that neurofibrils became pathological, forming progressively thicker bundles until they filled the neuron as a "neurofibrillary tangle" destroying it. In 1906, Dr. Alzheimer presented his groundbreaking work to the South German Psychiatric Association, who were seemingly uninterested in his findings. Nevertheless, he published his case report in 1907, a crucial milestone in the history of dementia [11, 12].

Confirmation and the naming of Alzheimer's disease

Initially, Dr. Alzheimer put forth the possibility that Auguste's case might have been unique. Nonetheless, reports by the Italian researchers Sarteschi and Bonfiglio soon followed describing similar dementia cases in young people featuring plaques, tangles, and memory impairment [13, 14].

Alzheimer, along with his student Gaetano Perusini, expanded the research by incorporating two additional cases and conducting a thorough reevaluation of Auguste's case. The reexamination posited that the vasculopathy observed in her brain could not adequately explain her early-onset dementia. This seminal investigation marked the first differentiation between Alzheimer's disease and vascular dementia [15].

The term "Alzheimer's disease" as a distinct clinical entity involving early dementia, plaques, and tangles began to take shape a bit later. In 1910, it was first introduced in a popular textbook of psychiatry published by Emil Kraepelin [16].

Dementia classifications and terminologies

Following these developments, "Alzheimer's disease" initially referred primarily to "pre-senile" dementia, which we now know to be relatively rare. In 1910 and 1912 however, Fischer elaborated on his previous paper by illustrating that presbyrophrenic dementia was associated with both tangles and plaques in a broader sample of brains from individuals with senile dementia. He put forth the term "Sphaerotrichia multiplex cerebri" to include all cases of presbyophrenic dementia characterized by the presence of tangles and plaques, distinguishing them from other instances of senile dementia, and indicating they may represent a large portion of senile dementia cases [17, 18]. Despite this attempt to put forth a standard definition, for much of the 20th century different terms and classifications were used interchangeably [7].

Modern era and advances in understanding

In the 1970s, an increase in United States federal funding brought renewed focus to age related diseases. Dr. Robert Katzman, in a 1976 paper, put forth that senile dementia

might be largely identical to Alzheimer's disease. He suggested that Alzheimer's disease might thus be one of the most prevalent and lethal diseases in all of society [19]. Although this assertion didn't expand upon the viewpoint Fischer had already proposed fifty years prior, Fischer's research had tragically fallen into obscurity due to him being a Jewish scientist in an era marked by widespread anti-Semitism [20].

As the understanding of Alzheimer's disease evolved, it continued to be closely associated with dementia and the presence of amyloid-beta plaques and neurofibrillary tangles. By the 1980s, the modern concept of "Alzheimer's clinical syndrome" emerged [21].

The scale of the Alzheimer's problem

Nowadays, we understand Alzheimer's Disease (AD) to be the leading cause of dementia worldwide, and it is currently defined as a progressive neurological disorder characterized by memory loss, cognitive decline, and behavioral changes, ultimately leading to severe impairment in daily functioning. With the presence of extracellular amyloid beta-plaques and intraneuronal neurofibrillary tangles composed of abnormally phosphorylated tau isoforms serving as the key pathological hallmarks.

As the most common neurodegenerative illness it presents a problem of immense scale to humanity. A problem that is only getting worse. Indeed, from 2006 to 2016, there was a significant decline in global age-standardized mortality rates across various categories. Maternal, developmental, and neonatal deaths saw a reduction of twenty-five percent, communicable diseases thirty percent, and cancer-related deaths ten percent. Cardiovascular disease mortality rates also declined by fifteen percent, and there was a fifteen percent decrease in mortality rates due to injuries. Additionally, chronic respiratory

disease mortality rates decreased by twenty percent during this period. However, there was a concerning 45% increase in deaths attributed to Alzheimer's disease during the same period, which was higher than any other disease considered, with the exception of Dengue fever. Ultimately resulting in remarkably unchanged age-standardized death rates [22]. As we continue to address various global health challenges, with the aging of our population ages, the burden of Alzheimer's disease is expected to rise steadily.

Indeed, with such growing numbers the burden of AD is great. AD sufferers typically die within ten years of the onset of cognitive symptoms, and spend those years in increasing states of disability [23]. This, however, is just for those suffering from the disease directly, it says nothing of the massive burden on healthcare systems, or on caretakers. It is estimated that those with dementia in Canada will receive two billion hours of care from friends and neighbors in 2023. This is not an insignificant burden, those who provide care to a person with dementia have greater likelihoods of medication use, hospitalization, and mortality, than demographically-matched controls [24]. Therefore, this problem extends beyond just symptomatic individuals, it is truly a problem affecting all society, demonstrating the importance of AD research.

Definition of the stages of Alzheimer's disease

Preclinical AD:

Preclinical Alzheimer's Disease refers to the initial, asymptomatic phase of the disease. During this stage, individuals exhibit no overt cognitive or functional impairments but may demonstrate underlying pathological hallmarks of the disease, such as the accumulation of beta-amyloid oligomers and abnormal tau isoforms in the brain. Detection

and characterization of preclinical AD in the general population is primarily based on neuroimaging, cerebrospinal fluid (CSF) biomarker assessments, and the reports of subtle cognitive and behavioral changes by a relative of the patient as discussed in [25].

Prodromal AD:

The prodromal phase of AD, also recognized as mild cognitive impairment (MCI) due to AD, is marked by observable cognitive deficits that surpass age-appropriate norms but do not meet the formal criteria for diagnosing dementia. For a prodromal AD diagnosis individuals must meet the criteria for MCI and also have a clinicians assessment identify that the MCI is likely due to progressing AD pathology as discussed in [26]. The standard MCI criteria include the presence of concerns regarding cognitive changes, impairment in one or more cognitive domains beyond what is expected based on the individual's age and educational background, the loss of ability to maintain independence in activities of daily living, and the absence of notable impairments in social or occupational functioning (i.e., the absence of dementia) [27]. The early detection of prodromal AD facilitates timely diagnosis and intervention for individuals at risk of progressing to clinically overt AD [25].

Clinical AD:

Clinical AD refers to the symptomatic and diagnosable stage of AD. At this stage, individuals exhibit significant cognitive decline, particularly in memory, reasoning, and other higher order cognitive functions, which impairs their daily activities and functioning [25]. This diagnosis is based on clinical assessments surrounding measures of underlying neuropathology (AB, Tau, neuronal loss…etc.) as well as cognitive measures such as a score

below 23 on the Mini-Mental State Exam (MMSE), or a score below 26 on the Montreal Cognitive assessment (MoCA), as also discussed in [25].

Current treatment landscape

The immense problem of Alzheimer's disease has generated much effort in the scientific community over the last 50 years to provide therapeutics capable of 'curing' the disease. Today however, the U.S. Food and Drug Administration (FDA) approved commercial treatment options available for those suffering with AD remain merely symptom relief drugs. Currently these are acetylcholinesterase inhibitors (ACheIs) (a topic discussed in more detail in the following section), with the three orally administered ones being the small molecule (<1000 Daltons) pharmacologics galantamine, donepezil and rivastigmine [28]. ACheIs have been available since the 1980s and relieve some of the major cognitive symptoms of clinical dementia but do nothing to prolong the lifespan of those afflicted, or reverse the disease [29-32].

The other FDA approved AD drugs are the small molecule N-methyl-D-aspartate (NMDA) receptor antagonist Memantine which has been shown to delay, but not prevent, the worsening of cognitive performance and neuronal loss [33-35], as well as two anti-AB monoclonal antibodies Aducanumab and Lecanemab. Aducanumab had positive trends on cognition (with the highest dose) in the EMERGE trial of patients with Mild Cognitive Impairment (MCI) but no such effect was seen in the identical study, ENGAGE [36], and Lecanemab has been shown to moderately slow mild cognitive decline and reduce AB plaques in patients with early AD [37]. There is also the FDA approved AD drug Namzaric™ which includes both Memantine and Donepezil[38].

In truth, hope is low in the AD scientific community that curing AD dementia is even possible. The major pathologies thought to underlie Alzheimer's disease, namely AB and tau pathology, begin years or decades prior to any detectable cognitive impairment and have reached or are approaching their maximal levels by the time clinically diagnosable cognitive impairment appears (figure 1) [2]. This, combined with the repeated failures of late-stage AD clinical trials, has generated a strong consensus in the field that AD is something that is going to be easier to prevent or delay at early disease or preclinical stages, then to cure after clinical stages are reached.

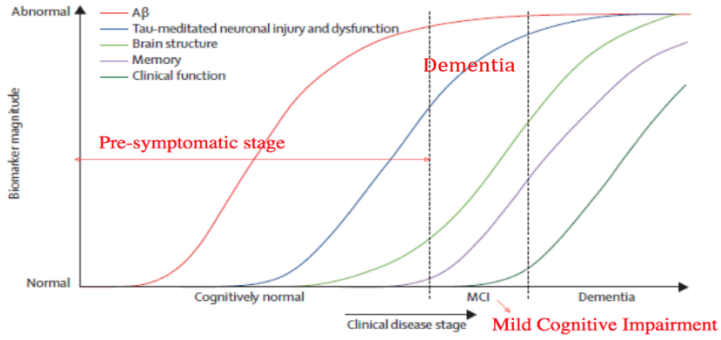


Figure 1: Dynamic biomarkers of the AD pathological cascade model – 2010: Aβ is identified by CSF Aβ42 or PET amyloid imaging. Neuronal injury and dysfunction are identified by CSF tau or FDG-PET. Neurodegenerative atrophy is measured by structural MRI. Adapted from Jack et al., 2013 [2].

Another strong consensus in the field is that a true AD preventative treatment will likely be a combination therapy addressing multiple underlying pathologies, rather than purely focusing on AB which has remained been the major focus of most AD clinical trials [39]. Thus, we need to explore more pharmaceutical targets outside of amyloid, and with this in mind there is another key pathology, directly tied to symptom onset, that is receiving less attention, that of cholinergic involvement in AD.

Cholinergic involvement in AD

The core pathology related to the present Thesis is cholinergic dysfunction: more specifically, the degeneration of the basal forebrain cholinergic neurons (BFCNs). BFCNs in

the mature central nervous system (CNS), while modulated by other neurotrophic factors, rely exclusively on mature nerve growth factor (mNGF) for maintenance of their phenotype and function, as reviewed in both [4, 40], and, as explained in the following section, trophic support is lost in the pathological progression of AD providing a target for therapeutic intervention.

Cholinergic dysfunction and basal forebrain cholinergic neuron degeneration in AD:

Cholinergic signaling in the CNS is essential for a wide array of cognitive processes via regional modulation of neuronal inhibition and excitation [41-43]. The identification of a CNS cholinergic system significant to a broad spectrum of cognitive processes led scientists to suggest that this system may be compromised in the widespread cognitive deterioration of aging. This led to multiple researchers demonstrating that cognitive insult induced by impairing CNS cholinergic signaling (to reduce cholinergic tone) mirrored the cognitive impairment seen in aging [44-51]. This lead Bartus and his collaborators to suggest the *"Cholinergic hypothesis of geriatric memory dysfunction",* which posited that a loss of central cholinergic tone underlay memory decline in aging [52]. As findings indicating cholinergic loss of tone were also found in human post-mortem AD samples [51, 53-59], Bartus applied this hypothesis as a symptomological account for cognitive decline in AD as well [52]. Neuroimaging demonstrating this severe loss of cholinergic innervation (presumptive cholinergic terminals) over the course of AD is available in figure 2.

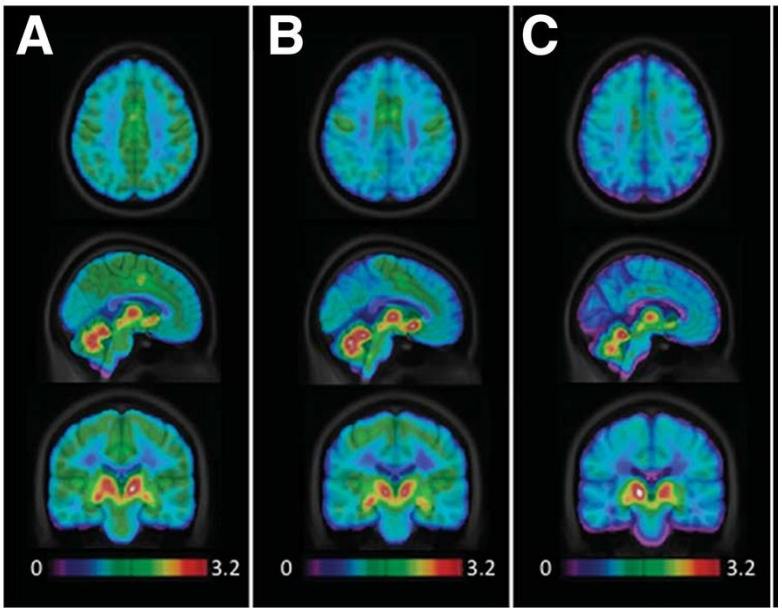


Figure 2: 18F-FEOBV Vesicular acetylcholine transporter (VAChT) binding (SUV ratio) using PET in 1 representative HC (A), mild-AD patient (B), and severe-AD patient (C) showing lower cortical VAChT binding depending on severity of AD. Statistical parametric analysis indicates significant clusters of lower cortical VAChT binding in AD compared with HC from [3].

Evidence for the central role of lost cholinergic signaling in AD symptomatology can be seen in how acetylcholinesterase inhibitors (ACheIs) are 4 of the now 8 FDA approved AD drugs. These ACheIs achieve major AD symptom relief by delaying acetylcholines breakdown in the synaptic cleft by acetylcholinesterase allowing for increased cholinergic signaling even at late disease stages where the number of cholinergic synapses is greatly diminished [60-63]. However, while initially effective in preserving cognition, the effects of anticholinesterase treatment are of limited duration as they do nothing to protect the synapses that are left from further degeneration [64]. These drugs' ability to provide transient symptomatic relief through boosting the diminished cholinergic signaling within AD demonstrates that cholinergic signaling is directly utilized in the types of cognitive circuitry that are hindered in AD.

As of now, due to this and other lines of evidence, it has become well accepted that, in AD, the atrophy and degeneration of the cholinergic neurotransmitter system is central to the onset of symptoms [53, 56, 65-70]. In particular the degeneration of the BFCNs appear to

play an important role [42, 71]. These are the cholinergic inputs to the cortex and hippocampus which are important for multiple higher order CNS functions such as learning, attention, and memory [44, 72, 73]. Further, their potentiated signaling in particular is key to the effect of ACheIs [60-62].

For a long time, a relatively early and severe atrophy of BFCNs has been consistently observed in AD [53, 56, 70]. This is the earliest cholinergic degeneration in the AD continuum [74], and further, degeneration of BFCNs predicts atrophy of the brain regions innervated by their projections. Moreover, BFCNs early degeneration associates with advancing preclinical AD pathology [75, 76], as reviewed in both [63, 77].

Could a lack of NGF be responsible for BFCN degeneration in AD?

Early findings on this BFCN atrophy in AD led Dr. Stanley Appel to hypothesize that a loss of trophic support to BFCNs is present in AD, and the cause of a good portion of its symptomatology [78]. As BFCNs solely rely on nerve growth factor (NGF) for maintenance of their phenotype and function, this would mean that there must be some change to NGF availability. However, initial studies showed the levels of NGF transcripts are not found to be changed in the AD continuum [79-81], and that the protein levels of the NGF are greatly elevated in AD post-mortem brain samples [81-86]. These findings seemed paradoxical to scientists at the time, because if this were the case and there was plenty of NGF, why were these cells becoming atrophic?

A number of ideas were proposed, such as deficits in axonal transport [87] or a loss of NGF's TrkA receptors [67]. Then, the Cuello lab offered a new paradigm of understanding, resolving the paradoxical findings with the discovery of an extracellular NGF metabolic

pathway and its dysregulation in AD as the source of the trophic disconnect of BFCNs in the disease [88].

The NGF metabolic cascade and its failure in AD

Work done by Dr. Martin Bruno and Dr. Claudio Cuello discovered that mNGF is produced transiently in the synaptic cleft via an NGF metabolic cascade in which proNGF, zymogens, convertases, and endogenous regulators are co-secreted from BFCN-target neurons in an activity-dependent manner [88].

In brief, upon the extracellular release of these factors, the inactive zymogen plasminogen is cleaved into its active enzyme form plasmin by tissue plasminogen activator (tPA), in a process regulated by the tPA inhibitor, neuroserpin [88]. Plasmin is then responsible for the cleavage of the pro-domain off proNGF resulting in the production of mNGF. This mNGF then binds to its high affinity receptor TrkA and low affinity receptor p75NTR on the presynaptic cell where it is internalized and retrogradely transported to the cell body where it stimulates multiple pathways centered around synaptic maintenance. These include the control of the expression of housekeeping proteins, transcription factors, ribosomal proteins, and cytoskeletal proteins responsible for axonal transport [4, 89, 90].

Degradation of the receptor-unbound mNGF is accomplished by the metalloproteases MMP-3 and MMP-9. Their precursors, proMMP-9 and proMMP-3, are released from the BFCN target cells alongside tissue inhibitor of metalloproteinases-1 (TIMP-1) which regulates the cleavage of these precursors into their mature forms [88, 91].

Initial studies by the Cuello lab following the discovery of the extracellular metabolic NGF pathway illustrated that in AD post-mortem brain tissue, the NGF metabolic pathway is

dysregulated in a dual pronged nature. Specifically, that there is reduced maturation of proNGF to mNGF, as well as excessive degradation of mNGF. The decreased maturation is caused by increased levels of neuroserpin which drive down tPA activity which, combined with the reduced plasminogen levels in the AD brain, result in a lack of plasmin availability to cleave proNGF into mNGF. The increased degradation of receptor unbound mNGF is caused by increased levels of MMP-3 and MMP-9. This two-pronged attack on the NGF metabolic cascade in the AD pathology, leads to a substantial decrease in mNGF bioavailability, and thus a substantial decrease in the trophic support of BFCNs, leading to their atrophy [86, 92]. A schematic representation of this healthy metabolism and pathological dysmetabolism is seen in figure 3.

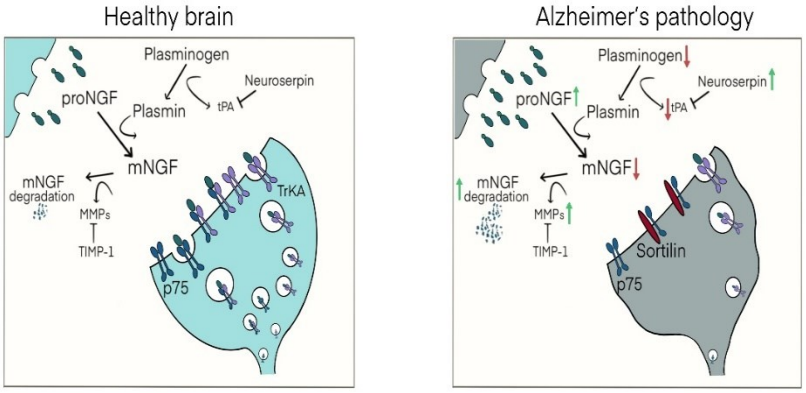


Figure 3: The NGF pathway and its dysregulation in Alzheimer's pathology. In the healthy brain (**left**), upon neuronal stimulation, proNGF is secreted into the synaptic cleft along with the zymogens and convertases involved in its maturation and degradation. proNGF is converted to mNGF by plasmin, itself produced from the cleavage of plasminogen by tPA, under the control of neuroserpin. mNGF then dimerizes and binds to p75NTR/TrkA receptor complexes on presynaptic terminals of BFCNs, to be internalized and retrogradely transported to their neuronal soma in the basal forebrain to fulfill its trophic functions. Receptor-unbound mNGF is rapidly degraded by metalloproteinases, produced under the control of TIMP-1. In the brain of individuals with Alzheimer's pathology (**right**), increased neuroserpin and decreased tPA limit the conversion of plasminogen into plasmin. As plasmin is responsible for the maturation of proNGF to mNGF, this results in a build-up of proNGF. In parallel, increased MMP-9 and MMP-3 and decreased TIMP-1, their natural inhibitor, resulted in the excessive degradation of free, receptor-unbound mNGF. Arrows indicate the direction of the alterations for each important member of the NGF pathway. Red represents a reduction in the protein levels and green an elevation. Adapted from [4]

More recently, work from our lab has revealed the existence of this dual pronged NGF dysmetabolism at preclinical stages as assessed by human post-mortem brain tissue, plasma, and CSF. Preclinical AD patients were defined as those having a high amyloid

burden (within 2 SD of the average AB scores of the MCI group from the same study), but no diagnosed cognitive impairment (HA-NCI) [5]. Specifically at this preclinical stage, as seen in figure 4, while neuroserpin levels are not yet elevated, there is decreased tPA expression and protein levels, which nonetheless result in a lack of plasmin activation and thus a lack of proNGF→mNGF maturation. Further, much like the MCI and clinical AD dementia stages, there are increased MMP-9 and MMP-3 levels and activity at this preclinical stage, increasing the degradation of mNGF and further exhausting this already smaller than normal supply of mNGF in the synaptic cleft [5 91].

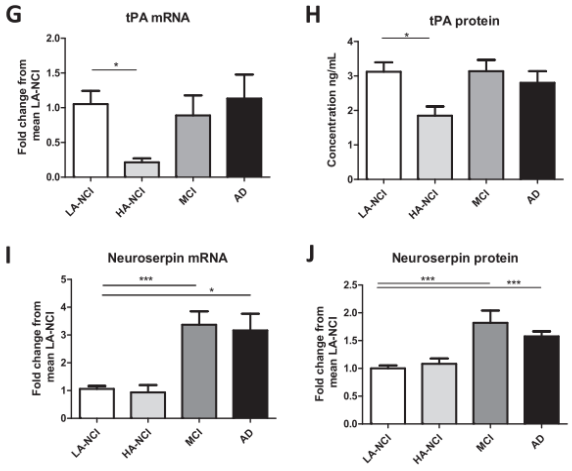


Figure 4: The differential expression and abundance changes to tPA and neuroserpin across the AD continuum. tPA mRNA in dorsolateral/medial prefrontal cortex homogenates is decreased in HA-NCI and unchanged in MCI and AD versus LA-NCI (G). tPA protein is likewise solely decreased in HA-NCI vs. LA-NCI (H). Neuroserpin mRNA is increased in dorsolateral/medial prefrontal cortex homogenates in AD and MCI versus LA-NCI (I). levels of neuroserpin protein are higher in dorsolateral/medial prefrontal cortex homogenates from individuals diagnosed with MCI/AD (J). Representative Western blots are shown for. All comparisons performed with a one-way ANOVA and Bonferroni post-hoc tests or a Kruskal-Wallace test and Dunn's post-hoc tests. All bars indicate mean + SEM. Adapted from [5].

Importantly, our lab has demonstrated that NGF dysmetabolism in patients with HA-NCI correlates to decreased cognition, as assessed by global cognitive score (GCS), and decreased cholinergic synapse number, as assessed by VAChT staining (figure 5). This demonstrates that the NGF dysmetabolism has relevance to even preclinical changes in cognitive ability, as well as preclinical changes in cholinergic tone.

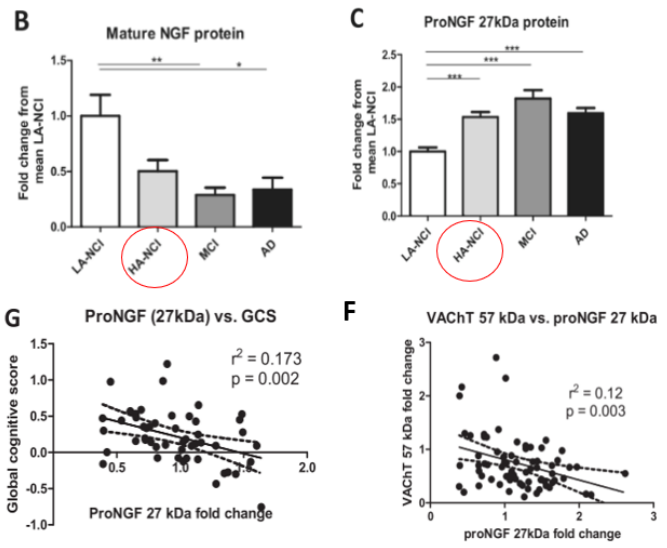


Figure 5: Normal NGF synthesis in the continuum of AD pathology is accompanied by an increase of proNGF and decrease of mNGF, beginning at preclinical stages, as well as abnormal expression of proteins participating in proNGF maturation. Decreased mNGF immunoreactivity at 13 kDa in MCI/AD brains (B). Increased proNGF immunoreactivity at 27 kDa in HANCI/MCI/AD brains (C). VAChT immunoreactivity at 57 kDa significantly correlates with proNGF immunoreactivity at 27 kDa (F). ProNGF 27 kDa protein correlates negatively to Global Cognitive Score z-scores (Gad). Adapted from [5]

Lastly, our lab has validated this pathway pharmacologically, demonstrating in rodent models that applying neuroserpin or blocking plasmin activity compromises the conversion of proNGF to mNGF leading, as in the AD pathology, to increased proNGF brain levels, atrophy of BFCNs, loss of cholinergic synapses, and cognitive impairment [88, 93, 94]. As such, our lab hypothesizes that it is this exhausted supply of mNGF which results in the progressive atrophy of cholinergic synapses within the cortex, as well as the somatodendritic atrophy of those same BFCNs.

History of NGF as a therapeutic target in AD

Prior to the discovery of the NGF metabolic cascade, findings that atrophy of BFCNs were central to the symptomatology of AD led to attempts to utilize the BFCNs sole trophic factor NGF to restore BFCN maintenance in hopes of restoring cognition. Indeed, exogenous application of NGF recovered atrophic BFCNs and rescued cholinergic tone in animal lesion models and in models of aging and AD [95-106].

In fact, due to this abundant experimental evidence that the application of exogenous NGF recovered cholinergic tone, in 1992, Olson and collaborators published a

case report where NGF brain infusions in an AD patient caused a transient upregulation of cortical nicotinic binding sites (presumptive cholinergic terminals) and maintained enhanced blood flow, accompanied later by improved verbal episodic memory [107]. This created much expectation that this was going to be a major success. However, consecutive studies with three patients revealed back and muscle pain as well as a loss of weight. Results which sparked caution in view that the intracerebral infusion of NGF in the AD therapy could have "off target" effects such as the stimulation of primary nociceptive terminations as well as other unknown receptor sites [108, 109].

From this evidence it was clear that an exogenous application of NGF, while able to restore BFCN trophic support, was undesirable due to the vast known and unknown other roles of NGF, thus a targeted approach is needed.

More recently, approaches have been attempted trying to circumvent these issues. For example, Dr. Capsoni and collaborators have attempted to replace NGF with a mutant 'painless' NGF which maintains its trophic capabilities, and intra-nasal application of this mutant NGF has been shown to be neuroprotective and reduce AB plaque burden in the 5xFAD mouse model [110].

Further attempts include the work of Dr. Maria Eriksdotter and colleagues to target NGF delivery to the relevant brain circuits via the targeted implantation of genetically modified cells releasing NGF, or the intracerebral gene delivery of NGF to relevant sites as discussed in both [63, 111]. Clinical trials utilizing such strategies revealed moderate improvements in cognitive outcomes and cholinergic markers [112].

However, the therapeutic correction of the NGF dysmetabolism presents a route to normalize endogenous mNGF levels at physiological sites providing an even more targeted and less invasive intervention than these strategies.

Specific rationale for neuroserpin inhibition, aims and hypothesis

In the context of the NGF metabolic cascade and its alteration in AD described above, the inhibition of neuroserpin was chosen as an excellent point for intervention.

The rationale for inhibiting neuroserpin is that this serpin is elevated in the AD pathology where it blocks tPA's ability to convert plasminogen into plasmin, thus downregulating the cleavage of proNGF into mNGF. Such an inhibition of neuroserpin should therefore lead to a restoration of proNGF maturation into the trophically active mNGF.

In preclinical AD (HA-NCI) neuroserpin is not yet elevated, but there is decreased tPA levels, expression, and activity, as well as failed NGF maturation, which correlate with cholinergic degeneration [5]. Therefore, decreasing neuroserpin activity through inhibition, allowing for increased tPA activity, should lead to a restoration of mNGF levels and cholinergic trophic support at this stage. At MCI and AD stages tPA protein and mRNA levels normalize, however tPA activity is not normalized, as at these stages there is increased neuroserpin levels which brings down tPA activity. Again downregulating the maturation of NGF, and correlating to cholinergic degeneration [5]. This means that inhibiting neuroserpin at any of these stages (preclinical, MCI, or AD) should have a similar effect on elevating tPA activity in order to normalize NGF maturation.

Further support for neuroserpin as the choice of target, is that its expression (unlike the other altered factors in the NGF metabolic pathway) is confined to the brain regions most affected by AD pathology [113, 114], indicating a lower chance of off-target effects. Further still, preclinical experimentation demonstrates that a lower gene dosage of neuroserpin in rodent models of amyloid pathology has pro-cognitive effects [115]. These effects are likely achieved via restoration of NGF maturation.

Therefore, based on the above rationale, we **hypothesize** that inhibiting neuroserpin should lead to the restoration of NGF maturation, and a normalization of the proNGF/mNGF ratio.

As no neuroserpin inhibitor currently exists, the **specific hypothesis** of this Thesis is that *inhibition of neuroserpin's tPA inhibitory effect is achievable through small molecule pharmacological methods.*

Therefore, the **overarching aim** of my work has been *to validate small molecules capable of selectively inhibiting neuroserpin in vitro.* The specific aims are:

Aim 1: Identify target pockets on the neuroserpin protein amenable for small molecule inhibition of tPA inhibitory effect, and utilize *in silico* methods to identify molecules for *in vitro* testing.

Aim 2: **Develop and validate an assay to measure neuroserpin activity for the assessment of candidate inhibitors *in vitro*.**

Aim 3: Assess the capacity of inhibitor candidates identified *in silico* for the ability to selectively inhibit neuroserpin *in vitro*.

Aim 4: Elaborate the structural activity relationships (SAR) of each target pocket based on the differential capacity of molecules analogous to initial hits to bind to and inhibit neuroserpin.

Body Of Thesis

Aim 1: Identify target pockets on the neuroserpin protein amenable for small molecule inhibition of tPA inhibitory effect, and utilize *in silico* methods to identify molecules for *in vitro* testing.

Aim 1A: Pocket identification

Background

Our lab chose to focus efforts for a neuroserpin inhibitory therapeutic towards small molecules due to well recognized benefits for preclinical drug discovery. These include a low-likelihood of off-target interaction, high blood brain barrier (BBB) and cell membrane penetration, inexpensive synthesis storage and shipping, and access to a large library of known drug like molecules [116]. Indeed, as mentioned in the current treatment landscape section of the introduction, a majority of the FDA approved AD drugs are small molecules.

Also important to our lab is the concern of equity. We live in a time where many treatments for diseases are only available in wealthy countries. While focusing on how to effectively get already validated medications to those who need them is an important way to address medical equity around the world. Equity is easier to manifest in pharmacology if it is considered in the beginning of the drug development process. Here, focusing on small molecules as the route to neuroserpin inhibition ensures our drug, when/if validated, will likely not need proper temperature storage, expensive synthesis, or complex methods of administration.

Once a specific protein has been identified as a target for inhibitory small molecule therapeutics, the first step is to identify functionally significant portions of the protein which can serve as receptor sites to be targeted with small molecules in docking screens.

Neuroserpin belongs to the serpin family of proteins, the serpins are all SErine PRotease INhibitors and were one of the earliest defined protein superfamilies [114]. Serpins are understood to have two structural components which play an important role in the inhibition of their cognate proteases. First, is an exposed peptide loop (which in neuroserpin is 18 amino acids in length) termed the reactive center loop (RCL) that contains a protease cleavage site, and the second is a 5/6 stranded Beta sheet. With serpins the RCL serves as a false substrate for their cognate proteases, upon binding of the protease to the RCL, the RCL is cleaved leading to a conformational change of the serpin in which the RCL is internalized as a new strand in the center of the beta sheet complex. The internalization of the RCL traps the protease inside the serpin protein in a covalent serpin-proteinase acyl-enzyme complex, obscuring the protease active site, rendering it inactive [117-119]. Indeed, neuroserpin follows this mouse-trap strategy for inhibiting tPA, and as the RCL is exposed in the native form of neuroserpin [120], initial search for a receptor site was focused on the RCL.

As the RCL is thought to be relatively unstructured and does not resolve on crystallography, adjacent and accessory pockets were targeted. This should not be an issue for the ultimate goal of reducing neuroserpins ability in inhibiting tPA as there are findings that the high affinity displayed by serpins for cognate proteases is determined in part by regions neighboring RCL [121]. Additionally, the crystal structure of native human neuroserpin

has been solved at 3.15 Å resolution, yielding a good-quality model as judged by commonly accepted criteria amenable for receptor building for translation into *in vitro* pharmacology.

Methodology

Our lab utilized two separate crystal structures of human neuroserpin to generate models of the neuroserpin molecule within the softwares used to identify receptor sites (PDB codes 3FGQ and 3F5N) and pockets on the neuroserpin protein were identified utilizing two methods.

First, the program Apopdb2receptor (OpenEye) was employed to generate roughly 100 pockets randomly around the reactive center loop, then a small 10,000 molecule database of drug-like compounds (provided by ZINC15) was docked into these pockets (utilizing the software FRED) to assess their general binding capacity as a method of assessing plausibility before dedicating the necessary resources for larger screens. The use of Apopdb2receptor to identify pockets was done by previous lab member Dr. Rowan Pentz, prior to my joining the lab.

Alternatively, binding hot spots were identified blind to the RCL utilizing the Boston University system FTMAP. FTMAP distributes small organic probe molecules of varying size, shape, and polarity across the global surface of the protein of interest. Regions that bind several different probe clusters are called consensus sites (CSs), and the sites containing the largest number of probe clusters are considered hot spots [122]. These hot-spots detail the sites on the proteins surface with the most ligand binding free energy, identifying sites likely to have higher affinity tom various kinds of small molecules, and thus the sites most amenable to small-molecule pharmacology. After the design of several

receptor sites around 'hot-spots' with proximity to the RCL a small 10,000 molecule database of drug-like compounds (provided by ZINC15) was docked into these pockets (utilizing the software FRED), again, to assess their general binding capacity. The FRED software will be further explained in Aim 1B.

Results and discussion

Out of 100 pockets designed with Apopdb2receptor (method one), two promising pockets were identified.

Pocket defined by Alanine 54. This pocket has good binding properties and inserts behind the parallel beta-sheets that form the backbone of the neuroserpin molecule and are paramount to the trapping of tPA; as such it may have a significant impact on the overall geometry of the neuroserpin molecule and its binding affinities, as well as the specific ability to internalize tPA. The 10,000 molecule initial screens identified chemgauss4 scores (for top hits) of -10 to -11, scores high enough to provoke larger molecular screens.

Pocket defined by Glutamine 227. This pocket has excellent binding properties and inserts near to the unstructured reactive center loop that forms the false substrate for tPA; as such it may have a significant impact on the presentation of the RCL, as well as the geometry of the neuroserpin molecule and its binding affinities. The 10,000 molecule initial screens identified chemgauss4 scores (for top hits) of -14 to -15, scores high enough to provoke larger molecular screens.

In method two, FTMAP identified numerous binding hotspots on the neuroserpin protein. Of these hotspots the one with the most hydrogen and non-hydrogen bonded probe clusters with adjacency to the RCL is defined by a Glutamine 220 residue (figure 10). This

pocket had similar positive attributes to that of Glutamine 227. Further, the 10,000-molecule initial screen identified chemgauss4 scores (for top hits) of -12 to -14, scores high enough to provoke larger molecular screens.

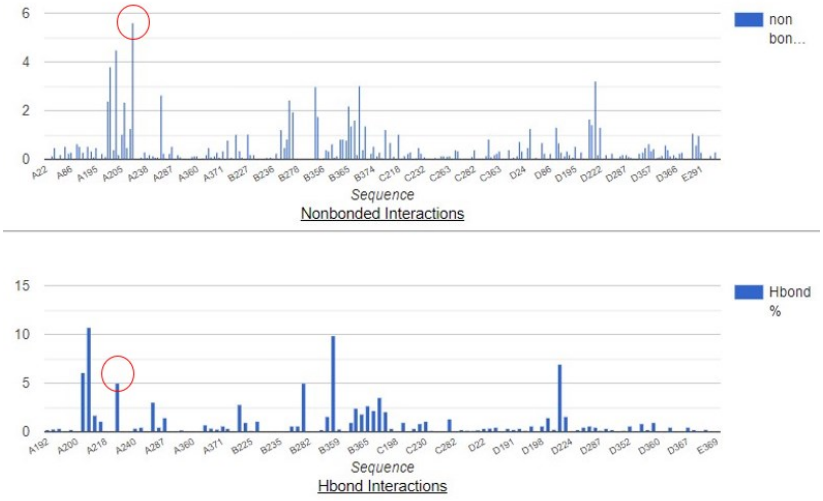


Figure 10: The FTMAP output identifying the percentage of total probes which bound to a given part of the neuroserpin protein sequence with either a non-hydrogen, or hydrogen bond. GLU220 residue circled in red.

Using neuroserpin crystallography PDB3fgq

All identified pockets had receptor sites designed in the OpenEye software MAKERECEPTOR to form files amenable to docking screens (Aim 1B).

In summary, this work successfully identified 3 pockets on the neuroserpin protein with both confirmed plausibility to act as the ligand-site for small molecules, and high functional relevance to neuroserpins mechanism of tPA inhibition.

Aim 1B: Screening compounds for predicted affinity

Background

Now that the work in aim 1A identified promising target pockets on the neuroserpin protein where small molecule binding could lead to inhibition of neuroserpin's tPA inhibitory activity. We next ran virtual docking screens to identify candidates promising

enough to take into *in vi*tro assessment. Specifically, in collaboration with Dr. Grant Churchill we used both ligand-based and structure-based virtual screening to identify small-molecule inhibitor candidates of neuroserpin. This approach has been successfully used by Dr. Churchill both academically [123-125] and commercially (Intrabio.com).

Methods results and discussion

For both structure-based and ligand-based virtual screening, we utilized the drug-like subset of 350 million purchasable molecules from the ZINC15 database. Drug-like means that through *in silico* work the molecules were determined to likely be orally bioavailable and absent of any known pan-reactive elements or pan-assay interference compounds, and thus amenable for taking directly into *in vitro* work and further translational studies [116]. This ZINC15 database was further concentrated into a cohort of 10 million small molecules utilizing criteria such as a lack of color (as to not confound the absorbance based assay developed in aim 2), synthetic accessibility (inexpensive to synthesize and store) and high predicted blood brain barrier (BBB) permeability (logP value between 1.5 and 2.7) [126].

Specifically for the ligand-based screening, this cohort was docked against the ALA54, GLU220, and GLU227-defined pockets using the 3D docking software FRED (OpenEye) to assess their affinity to each pocket. The FRED tool docks molecules (each in multiple confirmations) into a single receptor using an optimized and exhaustive search, and evaluates the steric and electrosteric complementarily between a ligand (small molecule) in a given orientation and a target pocket and then ranks the input database of molecules by predicted affinity via Chemgauss4 score, with the highest scoring molecules

being recommended for biological or *in vitro* testing.

Exhaustive search:

The exhaustive search aspect of the FRED system enumerates every possible rotation and translation of each conformer of the ligand being docked within a box enclosing the active site, these spatial variations of a conformer are dubbed 'poses'. It then discards poses that either overlap with the protein in 3D space, or alternatively extend too far from the binding site as these poses would be unable to bind. FRED then scores all remaining poses by Chemgauss4 score and passes the top scoring poses to optimization.

Optimization:

The optimization aspect of the FRED system enumerates nearby positions of each successful pose by having the initial pose take one positive and one negative step for each translational and rotational degree of freedom, and redocks them. The best scoring of this increased pool of poses are retained, becoming the overall score of a given molecule, used to rank the molecule against other molecules in the docking database for predicted affinity.

The Chemgauss4 score used to rank the molecule measures the complementarity of ligand poses within the active site based on the following types of interactions:

1. Shape
2. Hydrogen bonding between ligand and protein
3. Hydrogen bonding of protein/molecule interactions with implicit solvent
4. Metal-chelator interactions.

The implicit solvent is a complicated mathematical representation of the mean force of many highly dynamic solvent molecules that is commonly used in this sort of molecular

dynamic simulations geared towards pharmaceutical applications. While the brain is an appreciably different environment than the rest of the body. There is limited rationale to believe this type of simulation hinders the drug discovery of neuropharmaceuticals.

The ranked output of all the FRED screens were manually inspected and the top 50 compounds per pocket were purchased (minimum purity 90%) and tested *in vitro*, as will be detailed in aim 3.

For the structure-based screening our lab attempted to find molecules with a similar 3D shape but with a different 2D chemical scaffold to known neuroserpin modulators. Our lab utilized the program ROCS (OpenEye) with the query molecule embelin, which binds neuroserpin [127], but as a Michael acceptor capable of binding to numerous off-target molecules, is chemically unsuited for therapeutic use [128]. Our lab also queried with other reported modulators such as beta-sheet insertion peptides with homology to the neuroserpin RCL, as well as using truncated versions of the bound conformation [129], or known inhibitors of other serpins.

The ROCS program screens an input library of molecules against a queried compound and ranks them for predicted bioactive similarity utilizing the Tanimoto composite score [130]. I utilized the same input database containing more than 10 million drug-like compounds to screen for molecules with high predicted bioactive similarity to known neuroserpin (or other serpin) modulators.

The Tanimoto composite score is a mathematical measure commonly used in cheminformatics and bioinformatics to assess the similarity between two sets, particularly in the context of chemical compounds, genes, or other molecular entities. It quantifies the

degree of overlap or similarity between these sets as determined by their physical and chemical properties.

The top 2000 Tanimoto-ranked hits from ROCS were screened for further electrostatic similarity with EON [131], to further identify candidates which mimicked the electrostatic properties of known neuroserpin (or other serpin) modulators. The ranked output of these screens were manually inspected, and the top 25 compounds were purchased for *in vitro* testing alongside those identified via the ligand-based screening.

Aim 2: Develop and validate an assay to measure neuroserpin activity for the assessment of candidate inhibitors *in vitro*.

Background:

As there is no direct neuroserpin activity assay, in the effort to validate small molecule neuroserpin candidate inhibitors identified *in silico* (Aim 1) I developed an in-house (indirect) high-throughput neuroserpin activity assay, modified from [132] in which the ability of neuroserpin candidate inhibitors to normalize tPA activity in a neuroserpin dependent manner was taken as evidence of the small molecules ability to inhibit neuroserpin.

Briefly, in a structural elaboration study to assess the structural importance of the hydrophobic patch between helix F and B-Sheet A on the neuroserpin protein to its tPA inhibitory function, Dr. Mohammad Farhan Ali and collaborators invented and validated a tPA activity assay where they took a measurement of neuroserpin inhibitory capacity. In this case, to measure how tPA activity is differentially affected by mutant neuroserpin isoforms with mutations surrounding this hydrophobic patch [132]. Our lab felt that we could

utilize this as a starting point to develop an indirect neuroserpin activity assay where we can test if small molecules impact neuroserpin's ability to downregulate tPA activity, providing a measure of inhibition.

The assay was generated surrounding the kinetic reading of the cleavage of plasminogen into plasmin, by tPA, utilizing a chromogenic version of plasminogen (T2943 Sigma-Aldrich) which releases a fluorophore upon cleavage, to give a measure of tPA reaction velocity. With this we obtain a baseline level of tPA activity. Next, we downregulate this tPA activity with a standardized dosage of neuroserpin to form a control value for the effect of such a dosage of neuroserpin on tPA activity. Finally, we see if co-incubating the neuroserpin, tPA, and plasminogen interaction, with a candidate inhibitor results in a change to tPA rate of reaction (RoR) towards that of the non-neuroserpin inhibited tPA activity.

These measurements were taken along with the following negative controls:

- Application of candidate inhibitor to wells containing just buffer to control for any inherent absorbance of the compound.
- Application of candidate inhibitor to wells containing tPA and plasminogen, to ensure any effect on tPA activity is dependent on the presence of neuroserpin.

A graphical representation of the assay's principles can be seen in figure 6.

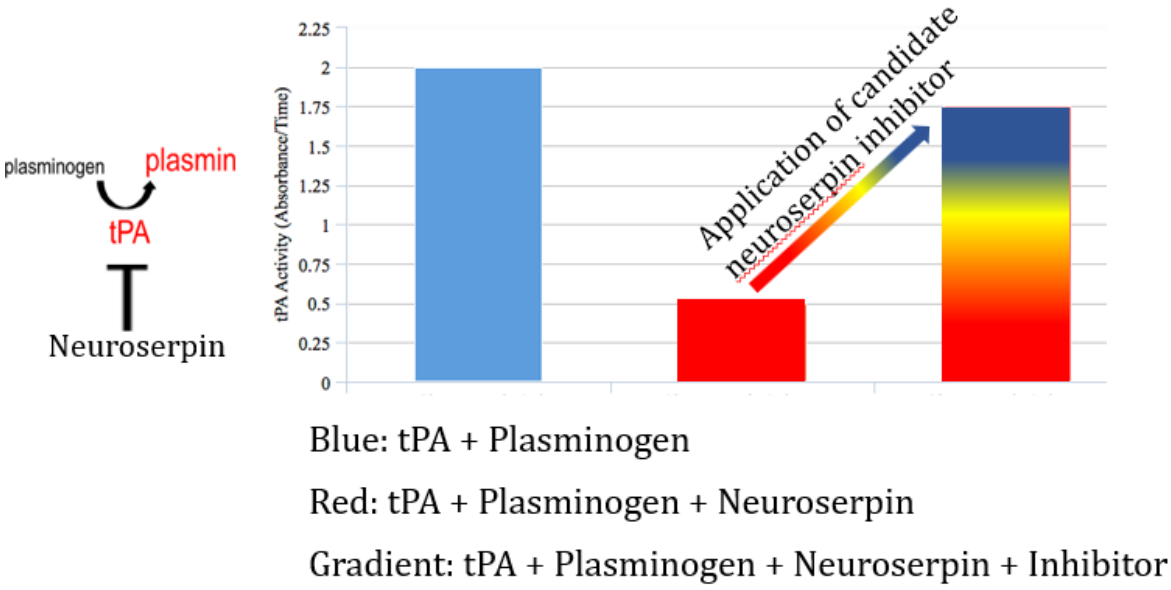


Figure 6: A graphical representation of the principle behind the in-house developed neuroserpin activity assay.

The following section addresses the initial testing and standardization of this assay.

Methodology, results, and discussion

First, I tested whether I could reliably read baseline tPA activity levels utilizing the protocol developed by Ali and collaborators [132], with their listed concentrations of 100nM tPA (>85% sctPA, Molecular Innovations, USA), 1mM chromogenic substrate (T2943 [CH3SO2-D-HHT-G-R-pNA.AcOH] from Sigma-Aldrich, St. Louis MO, USA) in kinetic buffer (50 mM HEPES [pH 7.4], 150mM NaCl, and 0.1% Tween-20) at 25 °C [132]. Then, in order to be more in-line with the end-goal of this endeavor we changed the temperature and pH to a more physiologically relevant 37^0C and 7.4.

I then noticed that there was a high variability in the slope of the tPA-plasminogen reaction, beyond the inherent variability expected in such a kinetics experiment (figure 7). The variability, across seven replicates, included a 500% difference in RoR between the replicate with the smallest slope, and the replicate with the largest. This was deemed high

enough that it would interfere with analysis. After intensive troubleshooting, we deemed that the solution was to utilize a 2mM concentration of the substrate (chromogenic plasminogen (T2943 [CH3SO2-D-HHT-G-R-pNA.AcOH] from Sigma-Aldrich, St. Louis MO, USA). This greatly reduced RoR variability to 50% across seven replicates utilizing different batches of substrate (figure 8), which was deemed sufficient to not compromise the ability to measure slope normalization upon co-incubation of neuroserpin and inhibitor candidate.

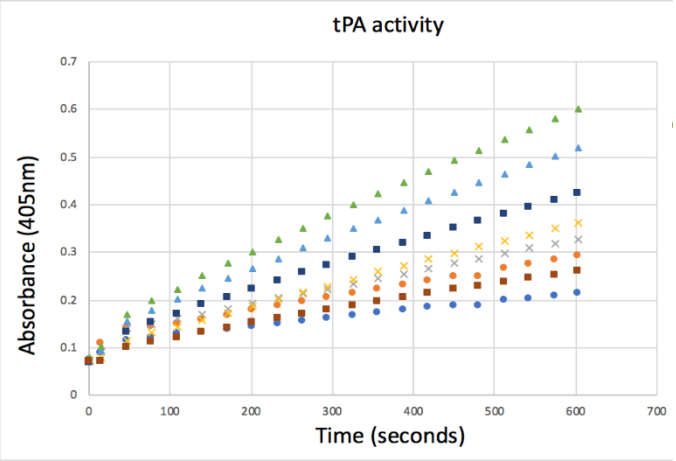


Figure 7: The slope of tPA activity in replicate wells containing 100 nM tPA (>85% sctPA, Molecular Innovations, USA), 1 mM chromogenic substrate (T2943 [CH3SO2-D-HHT-G-R-pNA.AcOH] from Sigma-Aldrich, St. Louis MO, USA) in kinetic buffer 50 mM HEPES [pH 7.4], 150 mM NaCl, and 0.1% Tween-20 at 37 °C, all with 0 nM of neuroserpin. The variability in the initial rate of reaction (first 10 minutes) was a multiplicative factor of 5 between the smallest slope and largest.

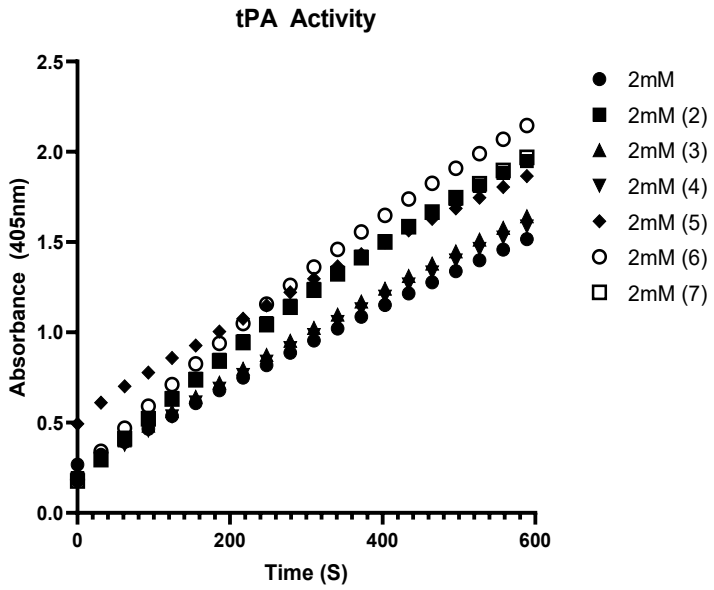


Figure 8: The slope of tPA activity in replicate wells containing 100 nM tPA (>85% sctPA, Molecular Innovations, USA), 2 mM chromogenic substrate (T2943 [CH3SO2-D-HHT-G-R-pNA.AcOH] from Sigma-Aldrich, St. Louis MO, USA) in kinetic buffer 50 mM HEPES [pH 7.4], 150 mM NaCl, and 0.1% Tween-20 at 37 °C, all with 0 nM of neuroserpin. The variability in the initial rate of reaction (first 10 minutes) was a multiplicative factor of 1.5 between the smallest slope and largest.

Finding the ideal concentration of neuroserpin

In order for the assay to give us an appreciable range of data for the comparison of different candidate inhibitors, the gap in tPA activity rate between uninhibited and neuroserpin application conditions needed to be large enough such that the incubation of neuroserpin with a neuroserpin inhibitor would reveal a level of recovery of tPA activity.

Towards this end, I tested differential neuroserpin concentrations of 100, 200, 400, 600, and 750μM to bring down tPA activity. As expected, tPA activity decreased with increasing neuroserpin concentrations in a consistent manner. Further it was determined that 600nM neuroserpin was the ideal concentration to bring down tPA activity effectively for our purposes, while still maintaining cost concerns. As the slope of the tPA curve, once concentrations of neuroserpin reached 600nM, trended quite close to 0 and the application of further neuroserpin concentrations provided minimal increase in the range between uninhibited and neuroserpin reduced tPA levels (figure 9).

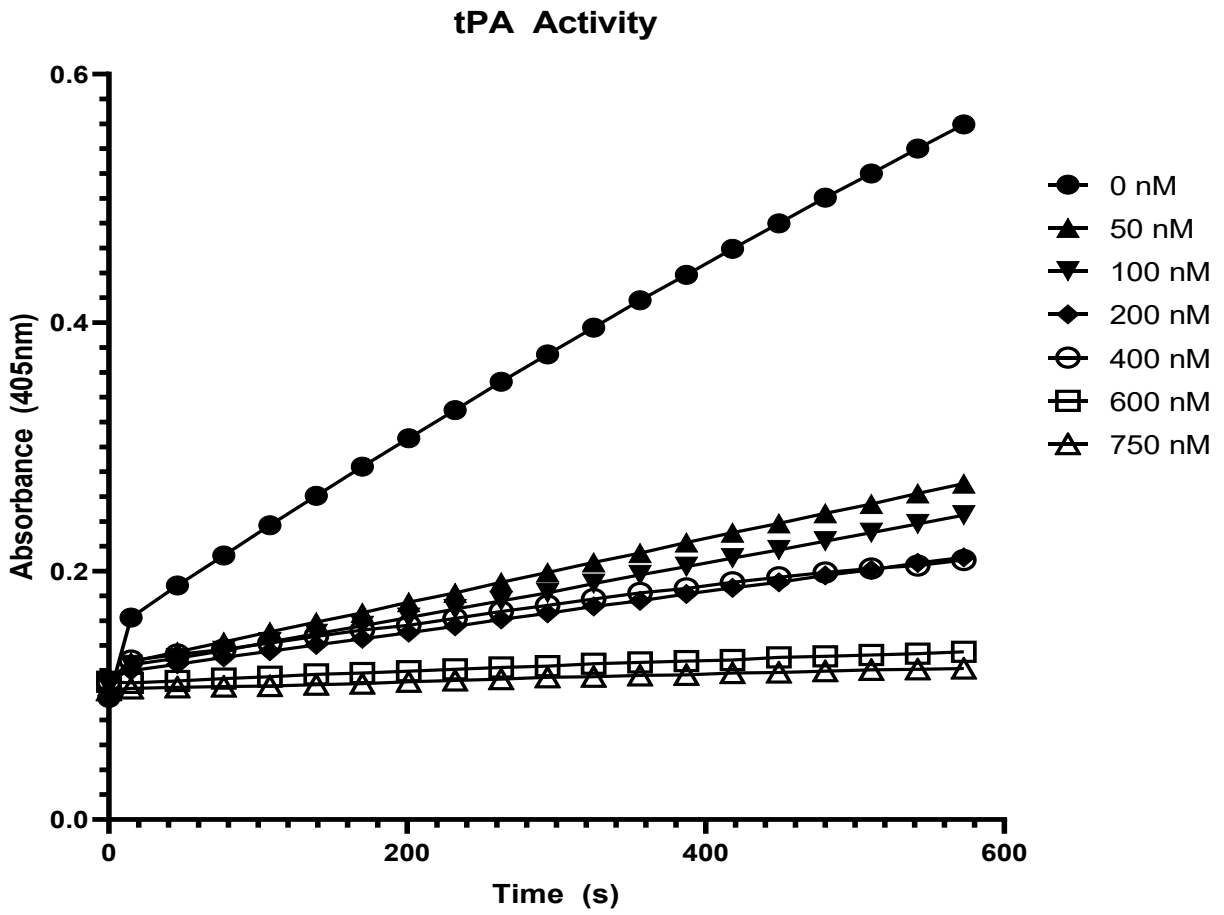


Figure 9: tPA activity (absorbance) over time for plate wells containing 100nm tPA (Innovative Research), 2mM tPA chromogenic substrate (T2943), and differing concentrations of neuroserpin ranging from 0nm-750nM (ProSpec SERPIN1). Rate of reaction and total absorbance over time decrease the higher the concentration of neuroserpin present in the wells, with this effect plateauing around 600nM.

Additionally, I validated that the negative controls were indeed negative, i.e., that lanes of just neuroserpin (at the highest concentration), just tPA, just substrate, just DMSO, or any combination of which is not uninhibited tPA control or experimental, produced no signal *per se*.

I then determined the time necessary to read differential tPA activity by assessing how long it took the 600nM neuroserpin infused wells to exhaust their substrate and

plateau with the same amount of absorbance as the tPA-plasminogen wells, determining it to be 3 hours.

The resulting assay was applied for the *in vitro* testing of candidates identified *in silico* for neuroserpin inhibitory capacity. A more streamlined version of this protocol is seen in the aim 3 methodology section.

Aim 3: Assess the capacity of inhibitor candidates identified *in silico* for the ability to selectively inhibit neuroserpin *in vitro*.

Background

Utilizing the assay generated in aim 2, we first tested the inhibitory capacity of the small molecule candidate neuroserpin inhibitors identified *in silico* through aim 1. Further, as cross-reactivity of validated neuroserpin inhibitory molecules (hit compounds) with other serpins would compromise efficacy, we then assessed the specificity of hit compounds by testing if their presence altered the activity of closely related serpins thrombin and trypsin. Most known for its procoagulant function, thrombin is a serine protease with both procoagulant and anticoagulant roles, mainly expressed in the liver and brain [133, 134]. Trypsin on the other hand, which is primarily expressed in the pancreas, is a major digestive proteinase. While serpins in general have poor sequence homology between family members, these two serpins have greater homology to neuroserpin then other family members [117]. Thus, it was determined that this was the optimal initial set of proteins to use to assess specificity of hit compounds.

Methodology

Neuroserpin activity assay:

Uninhibited tPA wells contained 100nM tPA (>85% sctPA, Innovative Research) and 2mM tPA chromogenic substrate (T2943 Sigma-Aldrich) suspended in a buffer of 50mM HEPES (pH 7.4), 150mM NaCl, 0.1% Tween-20. Neuroserpin control wells (i.e., wells containing neuroserpin downregulated tPA activity with no candidate inhibitor) contained the same ratio of tPA to substrate but with the addition of a 600nM concentration of recombinant human neuroserpin (SERPIN1 HUMAN, ProSpec). Initial screening of candidate inhibitors was done at 100μM concentrations utilizing a 10mM stock solution of the molecules dissolved in DMSO. Dose response curve testing was done with various ranges of small molecule concentrations, some of which required serial dilutions of these stock solutions (in assay buffer). All reagents came lyophilized and were dissolved and stored in either ddH_20 or a buffer of 50mM HEPES (pH 7.4), 150mM NaCl, depending on manufacturer instructions. Utilizing a minimum of 3 replicates per condition, the reaction was initiated via the injection of the 2mM substrate, and the absorbance was read at 405nm every minute for 3 hours utilizing a SPECTROstar OMEGA plate reader by BMG LABTECH. Negative controls included the application of candidate inhibitors to wells containing just buffer to control for any inherent absorbance of the compound, and application of candidate inhibitors to wells containing tPA and plasminogen, to ensure any effect on tPA activity is dependent on neuroserpin. All wells were completed to a final volume of 100uL with buffer.

Analysis was done utilizing an in-house developed Excel template to convert the files generated from the SPECTROstar plate reader into temporally stratified values arranged by

well classification. The initial identification of candidates as having neuroserpin inhibitory capacity was done by measuring the fold change in tPA RoR between the small molecule inhabited and intra-run neuroserpin control wells. A value of 1 would indicate no change in tPA RoR when small molecules were applied, and thus no effect on neuroserpin. A value over 1 would indicate the molecule inhibited neuroserpin's tPA inhibitory effect (for example a value of 1.45 would be equivalent to a 45% increase in the tPA RoR when a small molecule was applied), and a value under 1 would indicate a neuroserpin activation effect by the small molecule. Molecules which affected tPA activity in wells without neuroserpin, or had appreciable absorbance values in wells of purely buffer and small molecule were discounted. Validated neuroserpin inhibitors then underwent dose response testing as outlined above.

Due to these being all technical replicates with no biological component, significance testing was not done. Instead, molecules were validated as successful neuroserpin inhibitors if, at 100μM, they increased the tPA RoR by at least 15% (within the lower bound of the SEM) in comparison to the neuroserpin reduced tPA activity.

As the indirect nature of our assay provides no standard measurement of percent inhibition, we thus decided not to use the traditional IC50 analysis for graphing of our dose response curves. Instead, analysis of dose response was done using Michealis Menten (MM) kinetics. By substituting substrate concentration for small molecule concentration in the MM kinetics curve we can analyze how each candidate increases tPA RoR as its concentration increases. This allows us to take a MM constant value (K_M) which details the substrate concentration at which half the maximum tPA slope recovery occurs (i.e., half the

total neuroserpin inhibition capacity of that candidate) which we can use to compare candidates. This strategy of utilizing MM kinetics has limitations such as ignoring the uninhibited tPA curve data and therefore providing no information as to the specific degree of normalization, as well as not providing an IC50 value which can be utilized to compare the strength of our candidates to standard inhibitory therapeutics. Despite this it was deemed enough for the initial validation and comparison of candidate inhibitors identified *in silico*.

Thrombin activity assay:

The specificity of validated neuroserpin inhibitory compounds to neuroserpin was assessed in part by testing their effect on the closely related serpin thrombin. For this, a commercially available thrombin activity kit was used (ab197006). 12ng of the thrombin standard was utilized per well along with 0.2ng of the thrombin substrate suspended in the proprietary assay buffer to achieve a baseline of thrombin activity. Parallel to this, identical wells were composed to which 600µM of small molecule were added. These molecules were suspended in the assay buffer and all wells were completed to 100µL with buffer. Plates were read on a BioTek Synergy 2 Microplate reader with fluorescence at Ex/Em = 350/450 nm in a kinetic mode, every 2 – 3 minutes, for 60 minutes at 37°C.

Due to a change in availability between specificity testing of different compounds a second, absorbance based, thrombin activity assay was used (ab234620). For this assay 5ng of Human Thrombin Standard was used per well alongside 0.4ng of thrombin substrate suspended in 65µL of the assay buffer to get a baseline for thrombin activity. Then, as in the previous assay, identical wells with the addition of 600µM of small molecule suspended in

assay buffer (with the equivalent volume of buffer removed) were composed. The plate was then read on a Spectrostar Omega microplate reader every 2-3 minutes for 1 hour and 40 minutes at 405nm at 37*C.

Trypsin activity assay:

The specificity of identified neuroserpin inhibitory compounds to neuroserpin was assessed in part by testing their effect on the closely related serpin trypsin. For this, a commercially available trypsin activity kit was used (ab10253). Baseline trypsin levels were measured via wells containing 12µL of the 2mM trypsin standard was utilized alongside 0.2ng of the trypsin substrate, suspended in 86µL of assay buffer to achieve a 100µL final volume. Identical wells were composed with the addition of 600µM concentrations of small molecule suspended in assay buffer, again completed to 100µL final volume. The plates were incubated for 1 hour at 25*C and then read every 2 minutes for 60 minutes at 405nm on a Spectrostar Omega microplate reader.

Results and discussion

Of the over 100 molecules assessed *in vitro* for neuroserpin inhibitory capacity, nine molecules displayed such capacity. This success rate of 9% when confirming in silico predicted hits in vitro is to be expected [116]. Of these 9 hit compounds, 5 inhibited neuroserpin in a dose-dependent manner and had properties which made them amenable to continued use in drug discovery. These properties included the presence of structural attributes amenable to increasing affinity, such as a modifiable alkyl chain or a fragment like nature, as well as properties related to the ease of use in the drug discovery process such as chirality, high ease of synthesis, high aqueous solubility, and the lack of open rings.

These 5 molecules are dubbed 31615, which (at 100μM concentration) increased tPA reaction velocity in the presence of 600nM neuroserpin by 37.5% (±3.12%), and upon testing at multiple dosages had a Michaelis Menten constant (K_M) of 288.7μM. 526, which (at 100μM concentration) increased tPA reaction velocity by 31.6% (±1.58%) and had a K_M of 176.1μM. 2746, which (at 100μM concentration) increased tPA reaction velocity by 31.4% (±2.91%) and had a K_M of 132.6μM. 392, which (at 100μM concentration) increased tPA reaction velocity by 34.8% (±3.26) and had a K_M of 287.6μM. As well as molecule 573 which increased tPA reaction velocity by 29.9% (±3.48) and had a K_M of 163.3μM. Importantly, these were neuroserpin dependent effects as none of these molecules had an effect on the activity of tPA when neuroserpin was not present in the well, nor had any non-zero absorbance values in wells of just molecule and buffer. Table 1 contains all these compounds, their structure, their MM curves, their Michaelis Menten constant (K_M) and the R^2 value for the MM regression. The high R^2 values (minimum 0.9476) indicate a high likelihood that these are single site inhibitors.

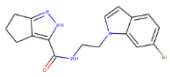
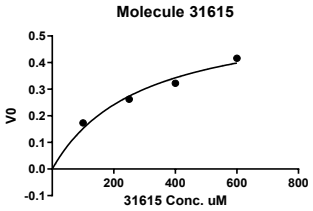
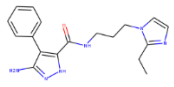
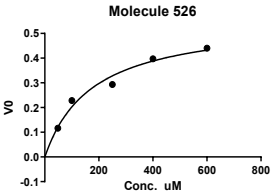
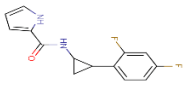
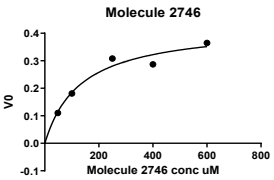
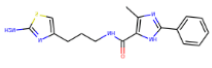
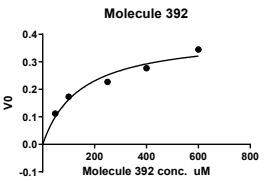
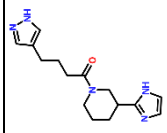
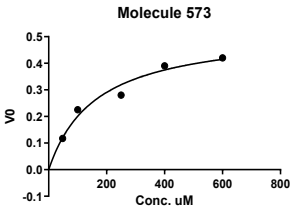
Molecule ID	Diagram	MM Curves	K_M	R^2
31615			288.7	0.9576
526			176.1	0.9701
2746			132.6	0.9476
392			287.6	0.9588
573			163.3	0.9605

Table 1: Table containing the ID, structure, Michealis Menten curve, K_M and R^2 values for molecules 31615, 526, 2746, 392 and 573.

To go more in depth on one example, figure 11 shows the tPA activity over time curves when multiple concentrations of molecule 573 are applied. There is a clear dose-

dependency as in the context of neuroserpin inhibition we have increasing levels of tPA activity with increasing dosages of 573.

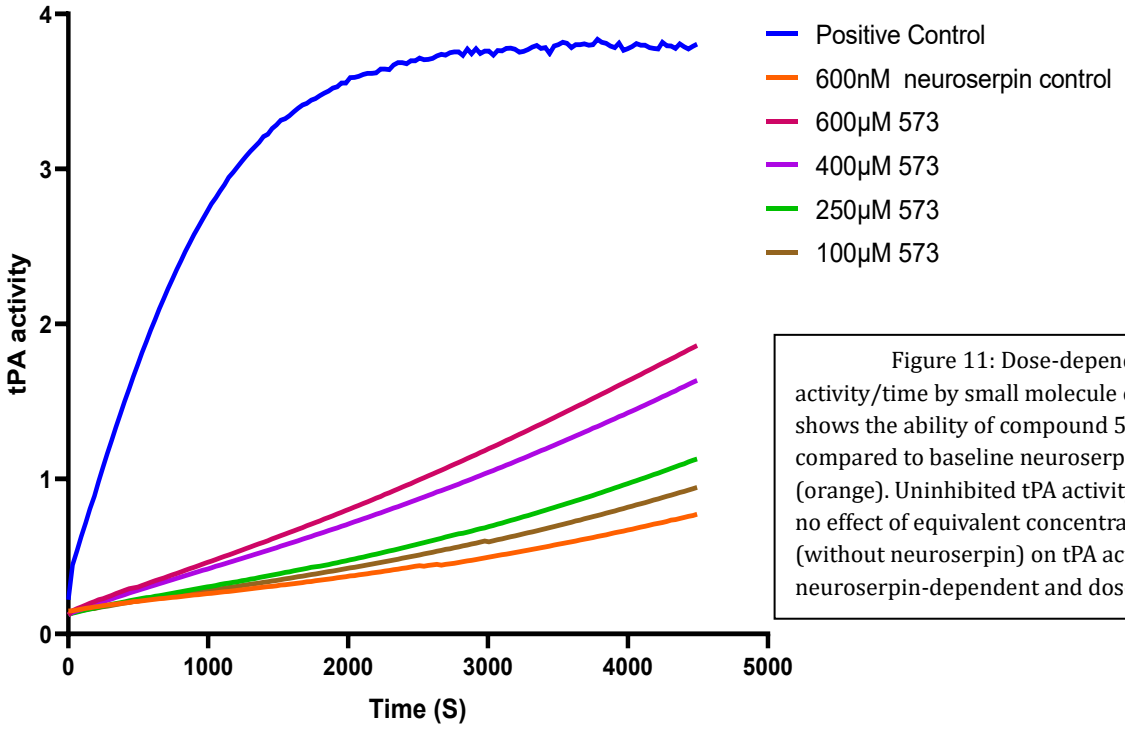


Figure 11: Dose-dependent normalization of tPA activity/time by small molecule candidate 573. This figure shows the ability of compound 573 to increase tPA activity as compared to baseline neuroserpin-inhibited tPA activity (orange). Uninhibited tPA activity appears in blue. There was no effect of equivalent concentrations of compound 573 alone (without neuroserpin) on tPA activity, demonstrating this is a neuroserpin-dependent and dose-dependent effect.

The total inhibition of the highest dosages of these initial hit compounds fails to reach a 50% slope normalization between neuroserpin reduced and uninhibited tPA conditions. Consequently, these molecules lack the potency required for immediate application to next steps such as primary target engagement studies (further elaborated on in general discussion). For this, we are aiming for molecules with a sub 10μM K_M. However, utilizing low potency hits as the basis for finding high potency leads through hit→lead optimization is at the core of small molecule drug development, and forms the basis of the following aim.

Still, before taking initial hits into hit→lead optimization, we must assess specificity as discussed in the background for this aim. Findings were that all initial hit compounds were specific to neuroserpin with the exceptions of 2746, which inhibited thrombin, and 392 which inhibited trypsin (figure 12).

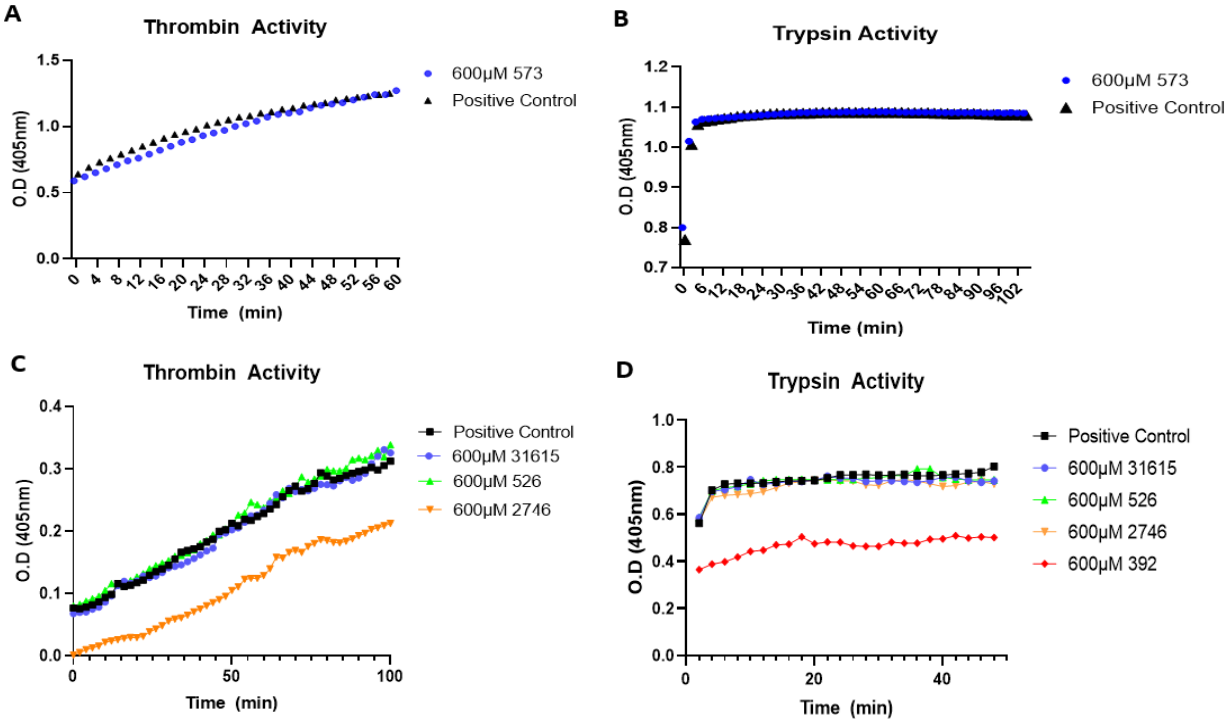


Figure 12: Cross-reactivity of hit compounds to closely related serpins. Thrombin activity kit shows thrombin activity is not affected by the application of 600µM compound 573 (A). Trypsin activity kit shows that trypsin activity is not affected by the application of 600µM compound 573 (B). Different activity kits were used for the same serpins and demonstrated that no hit compound effected thrombin activity at 600µM except for 2746 (C) and that no hit compound effected trypsin activity at 600µM except for 392 (D). The use of different assays was done due to availability issues in the time between testing specificity of different initial hit compounds. Trypsin testing was done first and thus the cross-reactive 392 was removed before thrombin testing occurred.

High blood brain barrier (BBB) permeability is also a necessary quality of hit compounds in this context. While *in vivo* confirmation of BBB penetration of neuroserpin inhibitory molecules will come when lead compounds have been identified, and is outside the scope of this Thesis, there is valuable information giving us confidence in the BBB penetration of these hit compounds. Indeed, as stated part of the parameters of our small molecule input database were that compounds had a logP value between 1.5 and 2.7. Meta-

analyses of studies related to neurological drugs and CNS active substances identify a logP value between 1.5 and 2.7 as optimal for BBB penetration [126]. According to our suppliers each of our specific neuroserpin inhibitory hit compounds have a logP between 2 and 2.5.

Aim 4: Elaborate the structural activity relationships (SAR) of each target pocket based on the differential capacity of molecules analogous to initial hits to bind to and inhibit neuroserpin.

Background

Based on the identification of an initial set of *in silico* derived molecules and subsequent testing for neuroserpin-specific inhibitory capacity as detailed in the previous aims, three neuroserpin specific inhibitory compounds were identified. All, interestingly, happened to be targeted to different pockets on the neuroserpin protein.

- Molecule 573 for pocket ALA54
- Molecule 526 for Glu220
- Molecule 31615 for Glu227

While these molecules were able to specifically inhibit neuroserpin in a dose dependent manner, as discussed in the previous section their potencies were too low for immediate applicability to *in vitro* (cell culture) or *in vivo* applications, therefore we will explore the structural activity relationships present in each pocket to gain a better understanding of how to build potent ligands in the effort of hit→lead optimization.

Exploring structural activity relationships (SAR) between a target pocket of a protein and its ligands is the main method of hit→lead optimization in small molecule drug design [135-138]. By testing compounds analogous to hit compounds but with slight structural variations across a number of different axes, and measuring the increased or decreased potency of these analogous molecules, we can explore the specific structural activity dynamics of a given pocket to determine what structures drive/reduce potency. The increased knowledge of what chemical properties drive the affinity between ligand and target receptor, combined with the cumulative knowledge of the small molecule drug discovery field, allows for the characterization of ideal traits both for ligand-receptor interaction and drug-like properties related to aqueous solubility, stability, permeability, and lack of toxicity. Thus, through exploring SAR we can derive the design of more potent drug-like molecules, in this case, for neuroserpin inhibition.

Methodology

To find commercially available molecules with similar 3D shapes but with a different 2D chemical scaffold, we used the program ROCS (OpenEye) with each of our three hit compounds as query molecules. Utilizing relevant ZINC15 databases of drug-like small molecules we ranked 1.5 million small molecules for their predicted bioactive similarity to each of our hit compounds by Tanimoto-composite score.

Concurrently, to find molecules with a similar 2D shape (>85% structural similarities) but different 3D structure we utilized the small molecule catalogues ZINC15 and Chembridge, which have built-in 2D structural similarity algorithms. From both methods a minimum of 15 analogous compounds per initial hit were purchased for *in vitro* testing of

neuroserpin inhibitory capacity. Measurement of neuroserpin inhibitory capacity was done as outlined in aim 3, and all the same negative controls were utilized.

Results

In the testing of 26 commercially available analogues of compound 573 (green) at 100μM we identified two candidates which had a stronger neuroserpin inhibitory capacity (>20% greater effect on tPA RoR increase than 573) (figure 13).

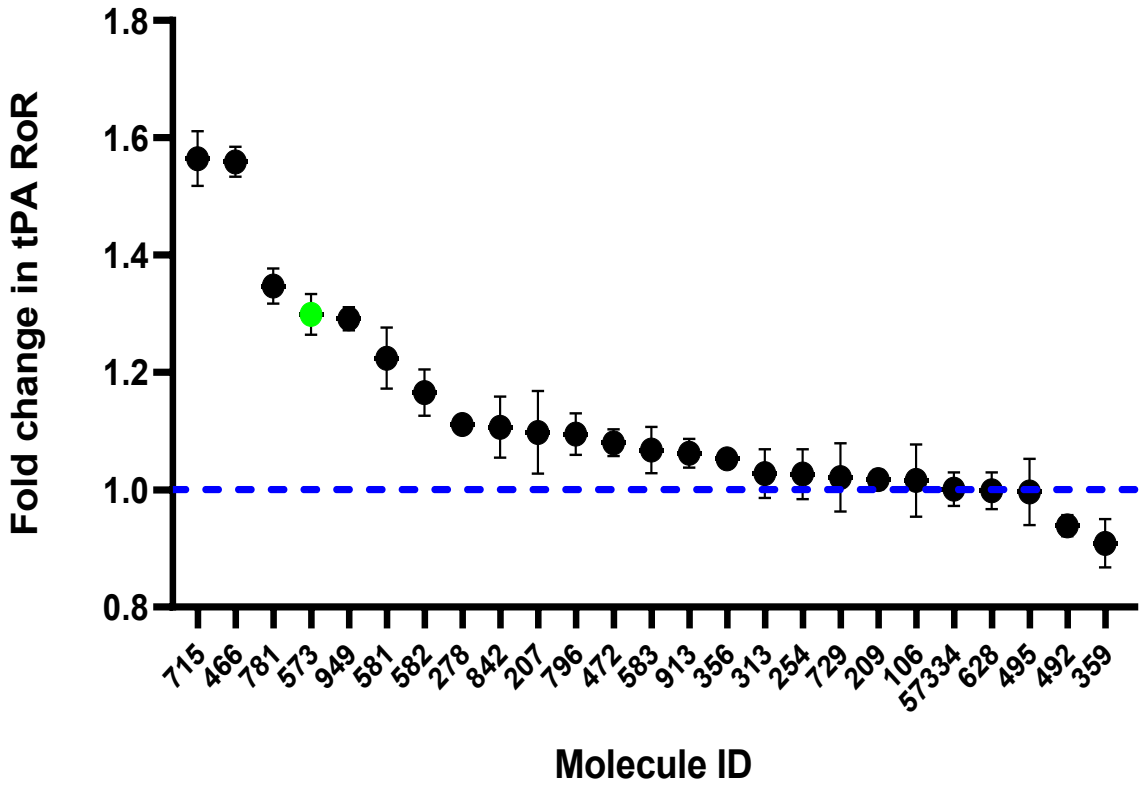


Figure 13: Fold change in the tPA rate of reaction when a small molecule candidate inhibitor is applied. All candidates identified as analogues to 573. The values are derived from taking the tPA activity slope of wells containing tPA, plasminogen, neuroserpin, and 100μM of a given compound, divided by the average of the intra-run control wells containing just tPA, plasminogen and neuroserpin. The value of 1, indicated as the dashed blue line, represents the value at which there is no change in tPA RoR from neuroserpin control when 100μM of candidate is applied (N=3, SEM).

In the testing of 15 commercially available analogues of compound 526 (green) at 100μM we identified 2 candidates which had a stronger neuroserpin inhibitory capacity (>10% greater effect on tPA RoR increase than 526) (figure 14).

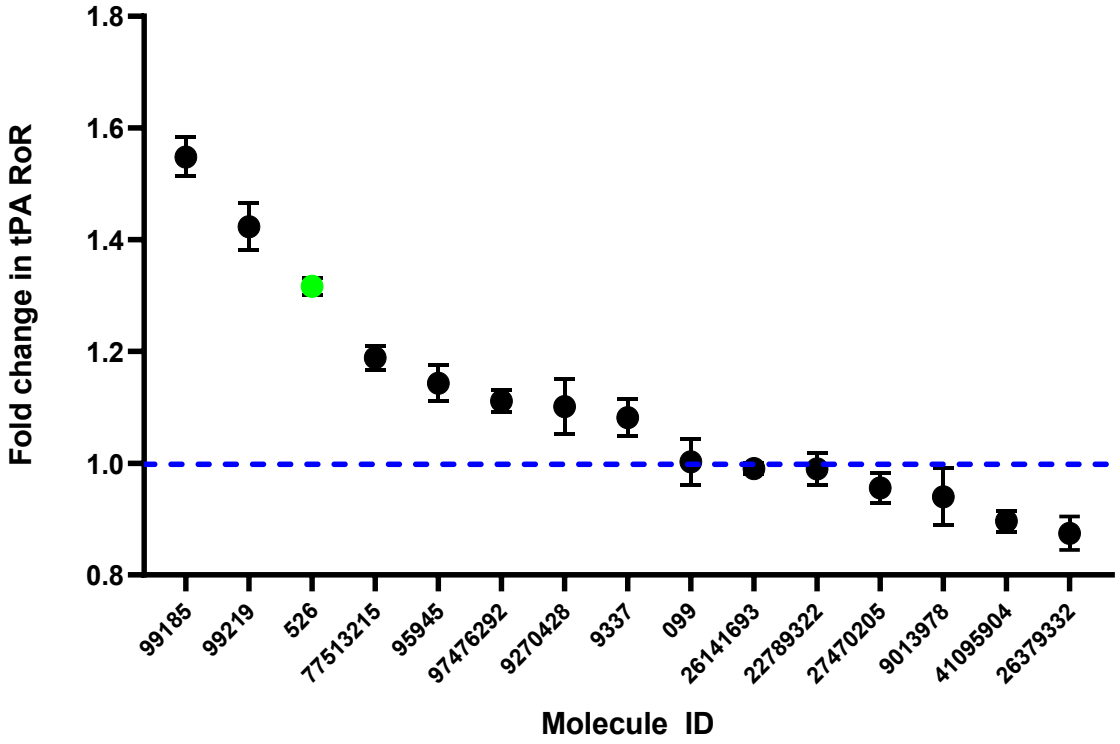


Figure 14: Fold change in the tPA rate of reaction when a small molecule candidate inhibitor is applied. All candidates identified as analogues to 526. The values are derived from taking the tPA activity slope of wells containing tPA, plasminogen, neuroserpin, and 100μM of a given compound, divided by the average of the intra-run control wells containing just tPA, plasminogen and neuroserpin. The value of 1, indicated as the dashed blue line, represents the value at which there is no change in tPA RoR from neuroserpin control when 100μM of candidate is applied (N=3, SEM).

In the testing of 15 commercially available analogues of compound 31615 (green) at 100μM we identified 1 candidate which had a stronger neuroserpin inhibitory capacity (>30% greater effect on tPA RoR increase than 31615) (figure 15).

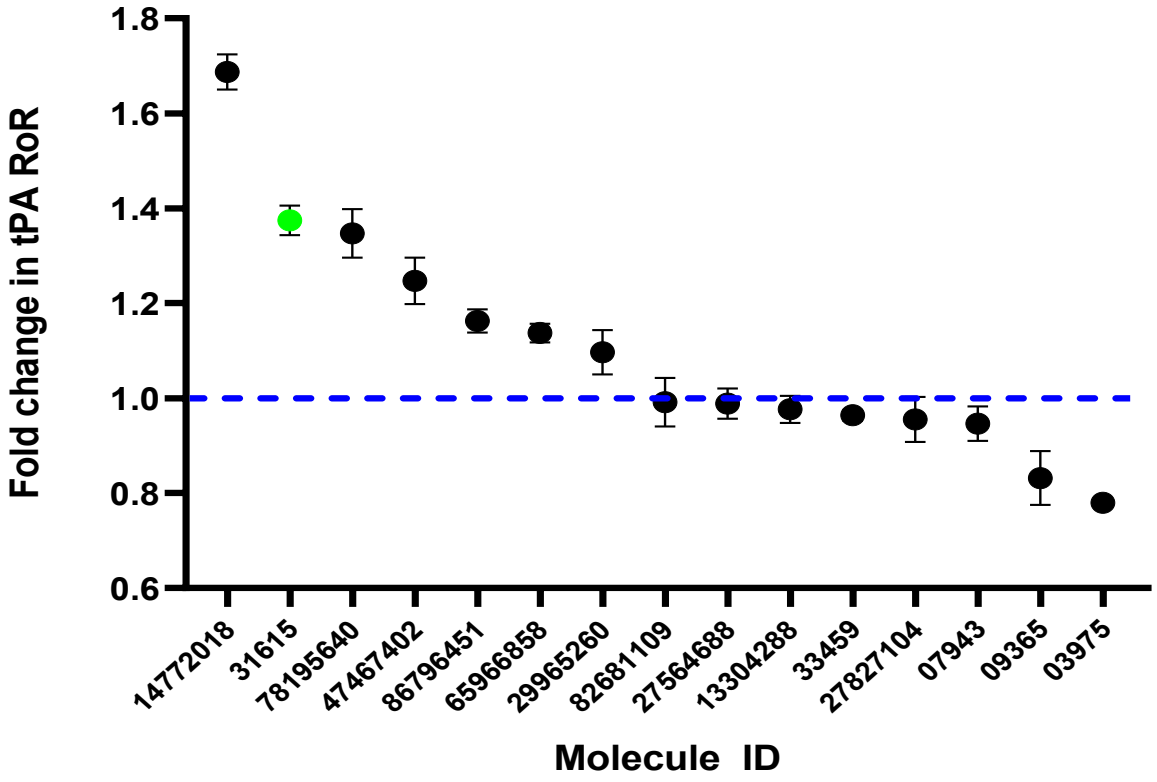


Figure 15: Fold change in the tPA rate of reaction when a small molecule candidate inhibitor is applied. All candidates identified as analogues to 31615. The values are derived from taking the tPA activity slope of wells containing tPA, plasminogen, neuroserpin, and 100μM of a given compound, divided by the average of the intra-run control wells containing just tPA, plasminogen and neuroserpin. The value of 1, indicated as the dashed blue line, represents the value at which there is no change in tPA RoR from neuroserpin control when 100μM of candidate is applied (N=3, SEM).

Discussion

This initial SAR work identified at least 1 molecule, per pocket, with greater neuroserpin inhibitory capacity than the initial hit from that pocket. This work also identified numerous compounds, analogous to initial hits, which had no effect on neuroserpin activity, and thus likely no affinity to neuroserpin. Further, this work identified molecules, analogous to initial hits, which upregulated neuroserpin activity, demonstrating maintained affinity but with a reversal of effect. While elaboration of these

'neuroserpin activators' is outside the scope of this Thesis, they may also have clinical relevance.

Due to our lab not having a resident medicinal chemist, a limitation of this work is that the analogues tested were restricted to those which were currently commercially available, which meant studying molecules which had multiple chemical changes to a given hit compound, obscuring interpretation of SAR. Future *in silico* work will elaborate on which specific changes are ultimately responsible for alteration of affinity and effect. Specifically, utilizing the software Flare (Cresset) following work can utilize data generated in the above results section to specifically perform sequential chemical changes between an initial hit and a commercial analogue which had greater neuroserpin inhibitory capacity, docking after each change to measure altered binding affinity, to assess what changes may have been responsible for the increased effect.

Further, to explore molecules in series with our hit compounds which are not available from commercially existing analogues, future work can utilize *in silico* elaboration and enumeration of the hit scaffold using the software Flare (Cresset). Examples of this structural elaboration could be using Flare to introduce heteroatoms at all possible positions in each hit compound to elaborate the structure, and dock and rank these new molecules for predicted target binding. Further, Flare can be used to perform *in silico* chemical reactions exploring several areas for each compound. For example, by disconnecting the central amide bond of hit compound 573 and then attaching panels of available amine/carboxylate fragments to generate new analogues for docking. The best performing analogues from this process could then be run in the neuroserpin activity assay

developed in aim 2 to confirm their increased neuroserpin inhibitory capacity. These strategies would allow for the exploration of a wide range of chemical space with a clear hypothesis for target interactions and will allow us to build a robust picture for SAR in each predicted binding site.

The lack of CRYO-EM confirmation of initial hit compounds to bind to their respective target pocket may serve as another limitation of the interpretation of SAR. It was simply outside the capacity of this project to perform CRYO-EM for each hit compound-neuroserpin interaction. However, our lab is confident in utilizing these initial hits and their analogues to explore the SAR of their respective pockets as this strategy, even without CRYO-EM confirmation, has been a key step in the drug development for clinically valuable small molecule medications currently applied in cancer, diabetes, arthritis as well as other diseases [129, 136, 139, 140].

Lastly, in the search for molecules with increased potencies to our original hits, due to the unfunded nature of the project, we are merely assessing trends and thus do not make statements regarding statistical significance. Statistical significance testing will come as the lab identifies lead compounds exhibiting potencies in the desired range and acquires the needed funding.

General Discussion

Summary and future directions

The work detailed in the above body of the Thesis represents a major step-forward towards the realization of a therapeutic aimed at maintaining cholinergic signaling in AD

via the modulation of mNGF availability. Not only has the neuroserpin protein been proved druggable by small molecule pharmacology [141, 142], validating our initial hypothesis, but the *in silico* and *in vitro* work utilized to discover and build the potency of neuroserpin inhibitory molecules takes our labs overarching hypothesis that the pharmaceutical inhibition of brain neuroserpin in the AD pathology should halt or delay the atrophy of basal forebrain cholinergic neurons, preserve cholinergic tone, and consequentially improve cognition, delaying clinical onset, out of the realm of purely theoretical and into something the lab is close to empirically testing.

While we do not yet have compounds sufficient for the assessment of our overarching hypothesis that neuroserpin inhibition should restore cholinergic trophic support and cognitive outcomes in the continuum of the Alzheimer's pathology, our initial findings, as described in this Thesis, offer solid grounds to generate such compounds.

Specifically, while we do not yet have lead compounds of sufficient potency for assessing primary target engagement, as discussed, the lab has strategies of hit→lead optimization through exploring SAR, and collaborations from which the lab is confident such lead compounds will be generated. Such compounds (3-5) would then undergo the necessary steps for evaluating/optimizing those leads for eventual *in vivo* application.

Namely, the laboratory intends to evaluate the effect of newly identified specific neuroserpin inhibitory lead compounds on NGF maturation in *ex vivo* Wistar rat brain tissue utilizing well validated lab protocols [88], as a measure of primary target engagement. While also monitoring the potential toxicity of lead compounds in these *ex vivo* blocks by applying LDH and MTT assays as described in [143]. These investigations should culminate

with the *in vivo* assessment of leading neuroserpin inhibitors effects on cognition, NGF metabolism, cholinergic synaptic density, and amyloid pathology in McGill-R-Thy1-APP transgenic rats exhibiting the AD-like amyloid pathology and replicating the human-like NGF dysmetabolism resulting in cholinergic synaptic losses following well-established lab protocols [144-148].

Larger context within AD

Briefly, while I discussed in the introduction the strong foundational link between loss of cholinergic tone and reduced cognitive capacity, in the following paragraphs I would like to discuss the role of NGF dysmetabolism in the larger context of the Alzheimer's disease pathology in order to drive home the impact a pharmaceutically viable neuroserpin inhibitory compound could have on AD. Importantly, our lab has generated solid evidence that NGF dysmetabolism is likely part of a basocortical feedback loop driving NGF dysmetabolism, AB pathology, and CNS inflammation in AD (figure 16), which will be discussed briefly in the following paragraphs.

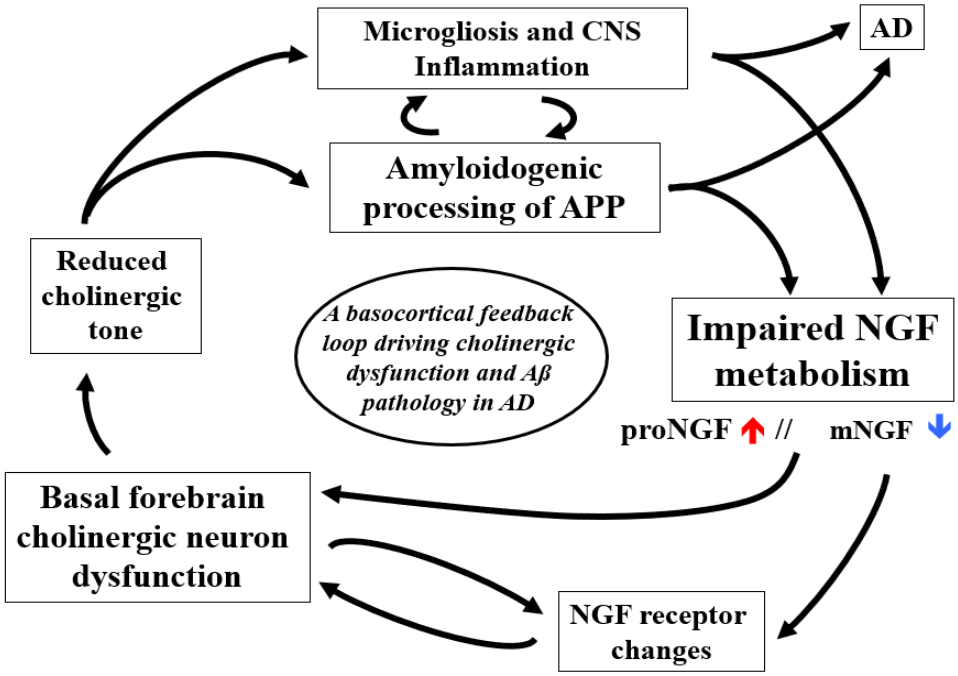


Figure 16: Early AD pathology is sufficient to impair NGF metabolism by facilitating proNGF accumulation and reduced mNGF availability. A compromised NGF metabolism leads to downregulation of NGF receptor expression and diminished cholinergic trophic support, resulting in reduced cholinergic tone. As a consequence of reduced cholinergic neurotransmission, the generation of toxic Aβ peptides, inflammation, increased excitatory/inhibitory balance, and vascular pathology will be favored, contributing to NGF dysmetabolism and therefore the self-propagation of this loop. Adapted from[6].

Our lab poses the hypothesis that NGF dysmetabolism follows primarily from amyloid pathology and an early stage proinflammatory process. This hypothesis is supported by the following findings:

A) The injection of soluble AB oligomers into the brain of naïve rats induced NGF dysmetabolism, showing competency [86].

B) That Down Syndrome individuals' (who inevitably develop AD due to the APP genes location on the trisomied 21st chromosome) foetal tissue shows concomitant amyloid oligomerization and NGF dysmetabolism, showing correlation within an AD population [149-156].

C) The induction of NGF dysmetabolism in the naïve rats was rescued by the application of minocycline, an anti-inflammatory drug [86]. Showing that amyloid beta oligomers are capable of inducing NGF dysmetabolism through their pro-inflammatory effect, which is likely how it is mediated in the Alzheimer's pathology. As this correlates well with the known pro/anti-inflammatory roles of many of the NGF metabolic factors which may have their expression and transcription changed in an environment of chronic neuroinflammation induced by AB pathology[157-159].

Also worth noting is that mNGF signaling with its high affinity receptor TrkA is responsible for the expression of all NGF "housekeeping" proteins including its own receptor TrkA. Therefore, this may constitute its own feedback loop where decreased mNGF-TrkA signaling from the NGF dysmetabolism leads to downregulation of TrkA expression and thus even less mNGF-TrkA signaling, further driving BFCN atrophy [89, 90, 160, 161].

As discussed in the introduction section, this NGF dysmetabolism results in a loss of cholinergic tone, and it is clear from the wider literature that reduced cholinergic tone induces both amyloidogenic APP processing and pathogenic neuroinflammation as well as cognitive decline [162-166]. Therefore, such cholinergic compromise would worsen AD pathology, CNS inflammation, and dementia.

Further, cholinergic signaling has been implicated as playing a role in other systems which have relevance to AD pathology. Such as being a mediator of vascular integrity and functional hyperperfusion [165], and as a neuroprotective signaler through α7-nAChRs [167] or M1-mAChRs [162]. Thus, cholinergic therapies, such as downregulating neuroserpin activity to restore BFCN trophic support, could impact multiple facets of AD pathology.

Mitigating and alternative strategies

Some research has indicated that neuroserpin is largely glycosylated *in vivo* [168-170]. To date, all published *in vitro* studies relating to neuroserpin utilized bacterially expressed neuroserpin, implicitly ignoring the role of glycosylation [168]. Indeed, the human recombinant neuroserpin used in aims 2, 3, and 4 in this Thesis is bacterially expressed and lacks glycosylation, as do the crystallized neuroserpins used in aim 1. The importance of neuroserpin glycosylation in respect to its tPA inhibitory function is currently unknown as is the degree to which glycosylation may affect the presentation of the targeted epitopes [168, 171]. This may complicate the translation of inhibitors from *in vitro* to *in vivo* conditions. If the best performing compounds *in vitro* do not work as well *in vivo* one mitigating strategy could be to start this process over and begin by assessing the ability of small molecule inhibitor candidates to inhibit neuroserpin in an assay utilizing human purified or recombinant glycosylated neuroserpin.

If, prior to *in vivo* application, neuroserpin inhibition by small molecules seems to plateau before the required potency needed for translatability, one alternative strategy could be the application of lysosomal targeting chimeras. This class of drugs utilize a small molecule with target affinity fused to a lysosomal attractor [172]. Thus, these drugs reduce a given proteins population by trafficking them for degradation. Therefore, neuroserpin attaching small molecules purely need high affinity rather than an ability to effect function per se, and thus we can explore a larger portion of neuroserpin binding hotspots (identified in aim 1) without restriction to hotspots where binding is likely to induce inhibition of tPA inhibitory function. While lysosomal targeting chimeras use in neurological therapeutics

has, until now, been impossible due to their size prohibiting BBB passage, novel strategies are being developed to circumvent this in hopes of applying this therapeutic strategy to neurological targets [173]. Therefore, in the near future this may present a viable route for neuroserpin overactivity reduction in the context of AD.

Applications outside of AD

Neuroserpin modulation via small molecules may have clinical and research relevance outside of AD. One such example is that it may offer a therapeutic opportunity in Familial Encephalopathy with neuroserpin inclusion bodies (FENIB). FENIB is caused by a mutation in the neuroserpin encoding gene, which produces a neuroserpin protein which polymerizes and forms inclusion bodies destroying neurons [174, 175]. An attached small molecule either to this polymerization site, or elsewhere affecting the appearance of this site, could prevent polymerization and thus be used to treat the disorder. This could be tested by utilizing the same *in silico* methods of drug candidate identification utilized in the present Thesis, except applied to the crystallized form of the mutant neuroserpin. Candidate drugs could then be initially verified *in vitro* by incubating the mutant neuroserpin in or outside of the presence of these candidates and then measuring the aggregation.

Final Conclusion and Summary

The work detailed in the above Thesis represents the accomplishment of the aims set out for the experimentation. This work details the *in silico* work of identifying candidate neuroserpin inhibitory molecules (Aim 1), the generation of an in-direct neuroserpin

activity assay (Aim 2), and the *in vitro* testing of molecules to serve as potent and specific neuroserpin inhibitors (Aims 3 and 4).

It is also worth noting that the reliable neuroserpin activity assay generated in this work has applicability not only for the Cuello lab in the continuation of this project, but also other projects with both research and clinical applications.

Lastly, as discussed in the introduction, neuroserpin inhibition could work at any stage (MCI/AD/preclinical) to normalize BFCN trophic support. However, a neuroserpin inhibition therapeutic would be most effective as a preclinical disease prevention drug, or an early-stage disease modification drug that delays the conversion of MCI to AD by prolonging cholinergic signaling, before significant neuronal death. Either of which would be impactful, as a treatment that can postpone the clinical onset of AD by five years, much like L-DOPA in Parkinson's disease, would result in a 41% reduction in the occurrence of AD worldwide [176]. Moreover, according to Canadian models, if dementia's onset could be postponed five years, then the Canadian AD population would rise only 10% by 2041, well behind population growth [177].

Therefore, the elaboration of this (and other) projects towards impactful pharmacologics aimed at delaying clinical onset, combined with the growing ability of biomarkers to diagnose individuals with preclinical AD could allow for a transformational impact on the clinical treatment of Alzheimer's disease.

References

1. Petersen, R.C., et al., *Alzheimer's disease neuroimaging initiative (ADNI): clinical characterization.* Neurology, 2010. **74**(3): p. 201-209.
2. Jack Jr, C.R., et al., *Update on hypothetical model of Alzheimer's disease biomarkers.* Lancet neurology, 2013. **12**(2): p. 207.
3. Tiepolt, S., et al., *PET imaging of cholinergic neurotransmission in neurodegenerative disorders.* Journal of Nuclear Medicine, 2022. **63**(Supplement 1): p. 33S-44S.
4. Do Carmo, S., B. Kannel, and A.C. Cuello, *The Nerve Growth Factor Metabolic Pathway Dysregulation as Cause of Alzheimer's Cholinergic Atrophy.* Cells, 2021. **11**(1): p. 16.
5. Pentz, R., et al., *The human brain NGF metabolic pathway is impaired in the pre-clinical and clinical continuum of Alzheimers disease.* Molecular Psychiatry, 2020: p. 1-15.
6. Pentz, R. and M.F. Iulita, *The NGF metabolic pathway: New opportunities for biomarker research and drug target discovery: NGF pathway biomarkers and drug targets.* Recent Advances in NGF and Related Molecules: The Continuum of the NGF "Saga", 2021: p. 31-48.
7. Berchtold, N.C. and C.W. Cotman, *Evolution in the conceptualization of dementia and Alzheimer's disease: Greco-Roman period to the 1960s.* Neurobiology of aging, 1998. **19**(3): p. 173-189.
8. Boller, F. and M.M. Forbes, *History of dementia and dementia in history: an overview.* Journal of the neurological sciences, 1998. **158**(2): p. 125-133.
9. Cicero, M.T. and W.A. Falconer, *Cicero. De senectute, De amicitia, De divinatione*. The Loeb classical library. 1923, LondonNew York: W. Heinemann; G.P. Putnamś Sons.
10. Fischer, O., *Miliare Nekrosen mit drusigen Wucherungen der Neuro-fibrillen, eine regelmässige Veränderung der Hirnrinde bei.* Monatsschr Psychiatr Neurol, 1907. **22**: p. 361.
11. Alzheimer, A., *Uber eine eigenartige Erkrankung der Hirnrinde. 37. Versammlung sudwestdeutscher Irrenarzte in Tubingen am 3. und 4. Nobember 1906.* Zentrallbl Nervenheilk Psychiatrie, 1907. **30**: p. 177-179.
12. Small, D.H. and R. Cappai, *Alois Alzheimer and Alzheimer's disease: a centennial perspective.* Journal of neurochemistry, 2006. **99**(3): p. 708-710.
13. Bonfiglio, F., *In un caso di probabile silifide cerebrale.* Riv. Sper. Fren. Med. Legale Alienaze Mentali, 1908. **34**: p. 196-206.
14. Sarteschi, U., *Contributo all'istologia patologica della presbiofrenia.* Riv Sper Freniatr, 1909. **35**: p. 464-84.
15. Perusini, G., *Über klinisch und histologisch eigenartige psychische Erkrankungen des späteren Lebensalters.* Histologische und histopathologische Arbeiten, 1909. **3**.
16. Kraepelin, E., *Psychiatrie: ein Lehrbuch für Ärzte und Studierende. 2. Klinische Psychiatrie*. 1899: Barth.
17. Fischer, O., *Die presbyophrene Demenz, deren anatomische Grundlage und klinische Abgrenzung.* Z Ges Neurol Psychiat, 1910. **3**: p. 371-471.

18. Fischer, O., *Ein weiterer Beitrag zur Klinik und Pathologie der presbyophrenen Demenz.* Zeitschrift für die gesamte Neurologie und Psychiatrie, 1912. **12**(1): p. 99-134.
19. Katzman, R., *The prevalence and malignancy of Alzheimer disease: a major killer.* Archives of neurology, 1976. **33**(4): p. 217-218.
20. Goedert, M., *Oskar Fischer and the study of dementia.* Brain, 2009. **132**(4): p. 1102-1111.
21. McKhann, G., et al., *Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA Work Group* under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease.* Neurology, 1984. **34**(7): p. 939-939.
22. Naghavi, M., et al., *Global, regional, and national age-sex specific mortality for 264 causes of death, 1980–2016: a systematic analysis for the Global Burden of Disease Study 2016.* The lancet, 2017. **390**(10100): p. 1151-1210.
23. Nichols, E., et al., *Global, regional, and national burden of Alzheimer's disease and other dementias, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016.* The Lancet Neurology, 2019. **18**(1): p. 88-106.
24. Szabo, S., et al., *The clinical and economic burden among caregivers of patients with Alzheimer's disease in Canada.* International journal of geriatric psychiatry, 2019. **34**(11): p. 1677-1688.
25. Scharre, D.W., *Preclinical, prodromal, and dementia stages of Alzheimer's disease.* Pract Neurol, 2019. **15**: p. 36-47.
26. Albert, M.S., et al., *The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease.* Alzheimer's & dementia, 2011. **7**(3): p. 270-279.
27. Hugo, J. and M. Ganguli, *Dementia and cognitive impairment: epidemiology, diagnosis, and treatment.* Clinics in geriatric medicine, 2014. **30**(3): p. 421-442.
28. Sarabia-Vallejo, Á., P. López-Alvarado, and J.C. Menéndez, *Small-molecule theranostics in Alzheimer's disease.* European Journal of Medicinal Chemistry, 2023: p. 115382.
29. Marucci, G., et al., *Efficacy of acetylcholinesterase inhibitors in Alzheimer's disease.* Neuropharmacology, 2020: p. 108352.
30. Haake, A., et al., *An update on the utility and safety of cholinesterase inhibitors for the treatment of Alzheimer's disease.* Expert opinion on drug safety, 2020. **19**(2): p. 147-157.
31. Kabir, M.T., et al., *Cholinesterase inhibitors for Alzheimer's disease: multitargeting strategy based on anti-Alzheimer's drugs repositioning.* Current pharmaceutical design, 2019. **25**(33): p. 3519-3535.
32. Sharma, K., *Cholinesterase inhibitors as Alzheimer's therapeutics.* Molecular medicine reports, 2019. **20**(2): p. 1479-1487.
33. McShane, R., et al., *Memantine for dementia.* Cochrane database of systematic reviews, 2019(3).
34. Reisberg, B., et al., *Memantine in moderate-to-severe Alzheimer's disease.* New England Journal of Medicine, 2003. **348**(14): p. 1333-1341.
35. Johnson, J.W. and S.E. Kotermanski, *Mechanism of action of memantine.* Current opinion in pharmacology, 2006. **6**(1): p. 61-67.

36. Kuller, L.H. and O.L. Lopez, *ENGAGE and EMERGE: Truth and consequences?* Alzheimer's & Dementia, 2021. **17**(4): p. 692-695.
37. Van Dyck, C.H., et al., *Lecanemab in early Alzheimer's disease.* New England Journal of Medicine, 2023. **388**(1): p. 9-21.
38. Greig, S.L., *Memantine ER/donepezil: a review in Alzheimer's disease.* CNS drugs, 2015. **29**: p. 963-970.
39. Cummings, J., et al., *Alzheimer's disease drug development pipeline: 2023.* Alzheimer's & Dementia: Translational Research & Clinical Interventions, 2023. **9**(2): p. e12385.
40. Cuello, A.C., R. Pentz, and H. Hall, *The brain NGF metabolic pathway in health and in Alzheimer's pathology.* Frontiers in neuroscience, 2019. **13**: p. 62.
41. Hasselmo, M.E. and M. Sarter, *Modes and models of forebrain cholinergic neuromodulation of cognition.* Neuropsychopharmacology, 2011. **36**(1): p. 52-73.
42. Mitchell, S., et al., *Medial septal area lesions disrupt theta rhythm and cholinergic staining in medial entorhinal cortex and produce impaired radial arm maze behavior in rats.* Journal of Neuroscience, 1982. **2**(3): p. 292-302.
43. Pinto, L., et al., *Fast modulation of visual perception by basal forebrain cholinergic neurons.* Nature neuroscience, 2013. **16**(12): p. 1857-1863.
44. Drachman, D.A. and J. Leavitt, *Human memory and the cholinergic system: a relationship to aging?* Archives of neurology, 1974. **30**(2): p. 113-121.
45. Drachman, D.A., *Memory and cognitive function in man: does the cholinergic system have a specific role?* Neurology, 1977. **27**(8): p. 783-783.
46. Pazzagli, A. and G. Pepeu, *Amnesic properties of scopolamine and brain acetylcholine in the rat.* International Journal of Neuropharmacology, 1965. **4**(5): p. 291-299.
47. Bartus, R.T., *Evidence for a direct cholinergic involvement in the scopolamine-induced amnesia in monkeys: effects of concurrent administration of physostigmine and methylphenidate with scopolamine.* Pharmacology Biochemistry and Behavior, 1978. **9**(6): p. 833-836.
48. Bartus, R.T. and H. Johnson, *Short-term memory in the rhesus monkey: disruption from the anti-cholinergic scopolamine.* Pharmacology Biochemistry and Behavior, 1976. **5**(1): p. 39-46.
49. Heise, G.A., R. Conner, and R.A. Martin, *Effects of scopolamine on variable intertrial interval spatial alternation and memory in the rat.* Psychopharmacology, 1976. **49**(2): p. 131-137.
50. Deutsch, J. and S.F. Leibowitz, *Amnesia or reversal of forgetting by anticholinesterase, depending simply on time of injection.* Science, 1966. **153**(3739): p. 1017-1018.
51. White, P., et al., *Neocortical cholinergic neurons in elderly people.* The Lancet, 1977. **309**(8013): p. 668-671.
52. Bartus, R.T., et al., *The cholinergic hypothesis of geriatric memory dysfunction.* Science, 1982. **217**(4558): p. 408-414.
53. Bowen, D.M., et al., *Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies.* Brain: a journal of neurology, 1976. **99**(3): p. 459-496.
54. Perry, E.K., et al., *Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia.* Br Med J, 1978. **2**(6150): p. 1457-1459.

55. Richter, J.A., E.K. Perry, and B.E. Tomlinson, *Acetylcholine and choline levels in post-mortem human brain tissue: preliminary observations in Alzheimer's disease.* Life sciences, 1980. **26**(20): p. 1683-1689.
56. Davies, P. and A. Maloney, *Selective loss of central cholinergic neurons in Alzheimer's disease.* The Lancet, 1976. **308**(8000): p. 1403.
57. Bowen, D. and A. Davison, *Changes in brain lysosomal activity, neurotransmitter-related enzymes, and other proteins in senile dementia.* Alzheimer's Disease, Senile Dementia and Related Disorders, R. Katzman, RD Terry, and KL Bick, eds, 1978: p. 421-424.
58. Perry, E.K., *The cholinergic hypothesis—ten years on.* British Medical Bulletin, 1986. **42**(1): p. 63-69.
59. Perry, E., et al., *Necropsy evidence of central cholinergic deficits in senile dementia.* The Lancet, 1977. **309**(8004): p. 189.
60. Richter, N., et al., *Effect of cholinergic treatment depends on cholinergic integrity in early Alzheimer's disease.* Brain, 2018. **141**(3): p. 903-915.
61. Pepeu, G. and M.G. Giovannini, *Cholinesterase inhibitors and memory.* Chemico-biological interactions, 2010. **187**(1-3): p. 403-408.
62. Birks, J.S. and R.J. Harvey, *Donepezil for dementia due to Alzheimer's disease.* Cochrane Database of systematic reviews, 2018(6).
63. Giacobini, E., A.C. Cuello, and A. Fisher, *Reimagining cholinergic therapy for Alzheimer's disease.* Brain, 2022.
64. Ibach, B. and E. Haen, *Acetylcholinesterase inhibition in Alzheimer's Disease.* Current Pharmaceutical Design, 2004. **10**(3): p. 231-251.
65. Whitehouse, P.J., et al., *Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis.* Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society, 1981. **10**(2): p. 122-126.
66. Whitehouse, P.J., et al., *Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain.* Science, 1982. **215**(4537): p. 1237-1239.
67. Mufson, E.J., M. Bothwell, and J.H. Kordower, *Loss of nerve growth factor receptor-containing neurons in Alzheimer's disease: a quantitative analysis across subregions of the basal forebrain.* Experimental neurology, 1989. **105**(3): p. 221-232.
68. Grothe, M., et al., *Reduction of basal forebrain cholinergic system parallels cognitive impairment in patients at high risk of developing Alzheimer's disease.* Cerebral Cortex, 2010. **20**(7): p. 1685-1695.
69. Kerbler, G.M., et al., *Basal forebrain atrophy contributes to allocentric navigation impairment in Alzheimer's disease patients.* Frontiers in aging neuroscience, 2015. **7**: p. 185.
70. Coyle, J.T., D.L. Price, and M.R. Delong, *Alzheimer's disease: a disorder of cortical cholinergic innervation.* Science, 1983. **219**(4589): p. 1184-1190.
71. Huerta, P.T. and J.E. Lisman, *Bidirectional synaptic plasticity induced by a single burst during cholinergic theta oscillation in CA1 in vitro.* Neuron, 1995. **15**(5): p. 1053-1063.
72. Hangya, B., et al., *Central cholinergic neurons are rapidly recruited by reinforcement feedback.* Cell, 2015. **162**(5): p. 1155-1168.
73. Ballinger, E.C., et al., *Basal forebrain cholinergic circuits and signaling in cognition and cognitive decline.* Neuron, 2016. **91**(6): p. 1199-1218.

74. Geula, C., et al., *Cholinergic neuronal and axonal abnormalities are present early in aging and in Alzheimer disease.* Journal of Neuropathology & Experimental Neurology, 2008. **67**(4): p. 309-318.
75. Grothe, M.J., et al., *Atrophy of the cholinergic basal forebrain in dementia with Lewy bodies and Alzheimer's disease dementia.* Journal of neurology, 2014. **261**(10): p. 1939-1948.
76. Schmitz, T.W. and R. Nathan Spreng, *Basal forebrain degeneration precedes and predicts the cortical spread of Alzheimer's pathology.* Nature communications, 2016. **7**(1): p. 13249.
77. Hampel, H., et al., *The cholinergic system in the pathophysiology and treatment of Alzheimer's disease.* Brain, 2018. **141**(7): p. 1917-1933.
78. Appel, S.H., *A unifying hypothesis for the cause of amyotrophic lateral sclerosis, parkinsonism, and Alzheimer disease.* Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society, 1981. **10**(6): p. 499-505.
79. Goedert, M., et al., *Nerve growth factor mRNA in peripheral and central rat tissues and in the human central nervous system: lesion effects in the rat brain and levels in Alzheimer's disease.* Molecular Brain Research, 1986. **1**(1): p. 85-92.
80. Jette, N., M. Cole, and M. Fahnestock, *NGF mRNA is not decreased in frontal cortex from Alzheimer's disease patients.* Molecular brain research, 1994. **25**(3-4): p. 242-250.
81. Fahnestock, M., et al., *Nerve growth factor mRNA and protein levels measured in the same tissue from normal and Alzheimer's disease parietal cortex.* Molecular brain research, 1996. **42**(1): p. 175-178.
82. Fahnestock, M., et al., *The nerve growth factor precursor proNGF exhibits neurotrophic activity but is less active than mature nerve growth factor.* Journal of neurochemistry, 2004. **89**(3): p. 581-592.
83. Peng, S., et al., *Increased proNGF levels in subjects with mild cognitive impairment and mild Alzheimer disease.* Journal of Neuropathology & Experimental Neurology, 2004. **63**(6): p. 641-649.
84. Pedraza, C.E., et al., *Pro-NGF isolated from the human brain affected by Alzheimer's disease induces neuronal apoptosis mediated by p75NTR.* The American journal of pathology, 2005. **166**(2): p. 533-543.
85. Al-Shawi, R., et al., *Neurotoxic and neurotrophic roles of proNGF and the receptor sortilin in the adult and ageing nervous system.* European Journal of Neuroscience, 2008. **27**(8): p. 2103-2114.
86. Bruno, M.A., et al., *Amyloid β-induced nerve growth factor dysmetabolism in Alzheimer disease.* Journal of Neuropathology & Experimental Neurology, 2009. **68**(8): p. 857-869.
87. Cooper, J.D., et al., *Failed retrograde transport of NGF in a mouse model of Down's syndrome: reversal of cholinergic neurodegenerative phenotypes following NGF infusion.* Proceedings of the National Academy of Sciences, 2001. **98**(18): p. 10439-10444.
88. Bruno, M.A. and A.C. Cuello, *Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease*

cascade. Proceedings of the National Academy of Sciences, 2006. **103**(17): p. 6735-6740.
89. Berse, B., I. Lopez-Coviella, and J.K. Blusztajn, *Activation of TrkA by nerve growth factor upregulates expression of the cholinergic gene locus but attenuates the response to ciliary neurotrophic growth factor.* Biochemical Journal, 1999. **342**(2): p. 301-308.
90. Venero, J., et al., *Expression of neurotrophin and trk receptor genes in adult rats with fimbria transections: effect of intraventricular nerve growth factor and brain-derived neurotrophic factor administration.* Neuroscience, 1994. **59**(4): p. 797-815.
91. Pentz, R., et al., *A new role for matrix metalloproteinase-3 in the NGF metabolic pathway: proteolysis of mature NGF and sex-specific differences in the continuum of Alzheimer's pathology.* Neurobiology of disease, 2021. **148**: p. 105150.
92. Bruno, M.A., et al., *Increased matrix metalloproteinase 9 activity in mild cognitive impairment.* Journal of Neuropathology & Experimental Neurology, 2009. **68**(12): p. 1309-1318.
93. Allard, S., et al., *Compromise of cortical proNGF maturation causes selective retrograde atrophy in cholinergic nucleus basalis neurons.* Neurobiology of aging, 2018. **67**: p. 10-20.
94. Allard, S., et al., *Impact of the NGF maturation and degradation pathway on the cortical cholinergic system phenotype.* Journal of Neuroscience, 2012. **32**(6): p. 2002-2012.
95. Fischer, W., et al., *Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor.* Nature, 1987. **329**(6134): p. 65-68.
96. Kromer, L.F., *Nerve growth factor treatment after brain injury prevents neuronal death.* Science, 1987. **235**(4785): p. 214-216.
97. Hagg, T., et al., *Delayed treatment with nerve growth factor reverses the apparent loss of cholinergic neurons after acute brain damage.* Experimental neurology, 1988. **101**(2): p. 303-312.
98. Tuszynski, M.H., D.G. Amaral, and F.H. Gage, *Nerve growth factor infusion in the primate brain reduces lesion-induced cholinergic neuronal degeneration.* Journal of Neuroscience, 1990. **10**(11): p. 3604-3614.
99. TUSZYfiSKI, M.H. and H. Fred, *Potential use of neurotrophic agents in the treatment of neurodegenerative disorders.* Acta neurobiol. exp, 1990. **50**: p. 311-322.
100. Koliatsos, V.E., et al., *Human nerve growth factor prevents degeneration of basal forebrain cholinergic neurons in primates.* Annals of neurology, 1991. **30**(6): p. 831-840.
101. Garofalo, L., A. Ribeiro-da-Silva, and A.C. Cuello, *Nerve growth factor-induced synaptogenesis and hypertrophy of cortical cholinergic terminals.* Proceedings of the National Academy of Sciences, 1992. **89**(7): p. 2639-2643.
102. Garofalo, L. and A. Cuello, *Nerve growth factor and the monosialoganglioside GM1: analogous and different in vivo effects on biochemical, morphological, and behavioral parameters of adult cortically lesioned rats.* Experimental neurology, 1994. **125**(2): p. 195-217.
103. Garofalo, L. and A.C. Cuello, *Pharmacological characterization of nerve growth factor and/or monosialoganglioside GM1 effects on cholinergic markers in the adult lesioned brain.* Journal of Pharmacology and Experimental Therapeutics, 1995. **272**(2): p. 527-545.

104. Burgos, I., et al., *NGF-mediated synaptic sprouting in the cerebral cortex of lesioned primate brain.* Brain research, 1995. **692**(1-2): p. 154-160.
105. Albeck, D., et al., *Exogenous NGF restores endogenous NGF distribution in the brain of the cognitively impaired aged rat.* Brain research, 2003. **967**(1-2): p. 306-310.
106. Cuello, A., et al., *Gangliosides potentiate in vivo and in vitro effects of nerve growth factor on central cholinergic neurons.* Proceedings of the National Academy of Sciences, 1989. **86**(6): p. 2056-2060.
107. Olson, L., et al., *Nerve growth factor affects 11 C-nicotine binding, blood flow, EEG, and verbal episodic memory in an Alzheimer patient (case report).* Journal of neural transmission-Parkinson's disease and dementia section, 1992. **4**: p. 79-95.
108. Claudio Cuello, A. and H. Thoenen, *The pharmacology of neurotrophic factors.* Pharmacological sciences: perspectives for research and therapy in the late 1990s, 1995: p. 241-254.
109. Rocco, M.L., et al., *Nerve growth factor: early studies and recent clinical trials.* Current neuropharmacology, 2018. **16**(10): p. 1455-1465.
110. Cattaneo, A. and S. Capsoni, *Painless Nerve Growth Factor: A TrkA biased agonist mediating a broad neuroprotection via its actions on microglia cells.* Pharmacological research, 2019. **139**: p. 17-25.
111. Tuszynski, M.H., *Nerve growth factor gene delivery: animal models to clinical trials.* Developmental Neurobiology, 2007. **67**(9): p. 1204-1215.
112. Karami, A., et al., *Changes in CSF cholinergic biomarkers in response to cell therapy with NGF in patients with Alzheimer's disease.* Alzheimer's & Dementia, 2015. **11**(11): p. 1316-1328.
113. Krueger, S.R., et al., *Expression of neuroserpin, an inhibitor of tissue plasminogen activator, in the developing and adult nervous system of the mouse.* Journal of Neuroscience, 1997. **17**(23): p. 8984-8996.
114. Hastings, G.A., et al., *Neuroserpin, a brain-associated inhibitor of tissue plasminogen activator is localized primarily in neurons: implications for the regulation of motor learning and neuronal survival.* Journal of Biological Chemistry, 1997. **272**(52): p. 33062-33067.
115. Fabbro, S., K. Schaller, and N.W. Seeds, *Amyloid-beta levels are significantly reduced and spatial memory defects are rescued in a novel neuroserpin-deficient Alzheimer's disease transgenic mouse model.* Journal of neurochemistry, 2011. **118**(5): p. 928-938.
116. Irwin, J.J., et al., *ZINC: a free tool to discover chemistry for biology.* Journal of chemical information and modeling, 2012. **52**(7): p. 1757-1768.
117. Huntington, J., *Serpin structure, function and dysfunction.* Journal of thrombosis and haemostasis, 2011. **9**: p. 26-34.
118. Law, R.H., et al., *An overview of the serpin superfamily.* Genome biology, 2006. **7**(5): p. 1-11.
119. Gettins, P.G., *Serpin structure, mechanism, and function.* Chemical reviews, 2002. **102**(12): p. 4751-4804.
120. Ricagno, S., et al., *Human neuroserpin: structure and time-dependent inhibition.* Journal of molecular biology, 2009. **388**(1): p. 109-121.

121. Huber, R. and R. Carrell, *Implications of the three-dimensional structure of. alpha. 1-antitrypsin for structure and function of serpins.* Biochemistry, 1989. **28**(23): p. 8951-8966.
122. Kozakov, D., et al., *The FTMap family of web servers for determining and characterizing ligand-binding hot spots of proteins.* Nature protocols, 2015. **10**(5): p. 733-755.
123. Naylor, E., et al., *Identification of a chemical probe for NAADP by virtual screening.* nature chemical biology, 2009. **5**(4): p. 220-226.
124. Taylor, L., et al., *Discovery of Novel GPVI Receptor Antagonists by Structure-Based Repurposing.* plos one, 2014. **9**(6).
125. Vasudevan, S.R., N. Singh, and G.C. Churchill, *Scaffold Hopping with Virtual Screening from IP3 to a Drug-Like Partial Agonist of the Inositol Trisphosphate Receptor.* chembiochem, 2014. **15**(18): p. 2774-2782.
126. Carpenter, T.S., et al., *A method to predict blood-brain barrier permeability of drug-like compounds using molecular dynamics simulations.* Biophysical journal, 2014. **107**(3): p. 630-641.
127. Lin, Z., et al., *Structural insight into inactivation of plasminogen activator inhibitor-1 by a small-molecule antagonist.* Chemistry & biology, 2013. **20**(2): p. 253-261.
128. Saga, G., et al., *Embelin binds to human neuroserpin and impairs its polymerisation.* Scientific reports, 2016. **6**(1): p. 1-12.
129. Chang, Y.P., et al., *Small-molecule peptides inhibit Z α1-antitrypsin polymerization.* Journal of cellular and molecular medicine, 2009. **13**(8b): p. 2304-2316.
130. Hawkins, P.C., A.G. Skillman, and A. Nicholls, *Comparison of shape-matching and docking as virtual screening tools.* Journal of medicinal chemistry, 2007. **50**(1): p. 74-82.
131. Naylor, E., et al., *Identification of a chemical probe for NAADP by virtual screening.* Nature chemical biology, 2009. **5**(4): p. 220-226.
132. Ali, M.F., et al., *A hydrophobic patch surrounding Trp154 in human neuroserpin controls the helix F dynamics with implications in inhibition and aggregation.* Scientific reports, 2017. **7**(1): p. 1-11.
133. Davie, E.W. and J.D. Kulman. *An overview of the structure and function of thrombin*. in *Seminars in thrombosis and hemostasis*. 2006. Copyright© 2006 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New ….
134. Di Cera, E., *Thrombin.* Molecular aspects of medicine, 2008. **29**(4): p. 203-254.
135. Stegemann, S., et al., *Trends in oral small-molecule drug discovery and product development based on product launches before and after the Rule of Five.* Drug Discovery Today, 2022: p. 103344.
136. Xie, Z., et al., *Small-molecule kinase inhibitors for the treatment of nononcologic diseases.* Journal of Medicinal Chemistry, 2021. **64**(3): p. 1283-1345.
137. Rodon Ahnert, J., et al., *What it takes to improve a first-generation inhibitor to a second-or third-generation small molecule.* American Society of Clinical Oncology Educational Book, 2019. **39**: p. 196-205.
138. Khera, N. and S. Rajput, *Therapeutic potential of small molecule inhibitors.* Journal of Cellular Biochemistry, 2017. **118**(5): p. 959-961.
139. Warren, G.L., et al., *A critical assessment of docking programs and scoring functions.* Journal of medicinal chemistry, 2006. **49**(20): p. 5912-5931.

140. Warren, G.L., et al., *Essential considerations for using protein–ligand structures in drug discovery.* Drug Discovery Today, 2012. **17**(23-24): p. 1270-1281.
141. Fauman, E.B., B.K. Rai, and E.S. Huang, *Structure-based druggability assessment—identifying suitable targets for small molecule therapeutics.* Current opinion in chemical biology, 2011. **15**(4): p. 463-468.
142. Thangudu, R.R., et al., *Modulating protein–protein interactions with small molecules: the importance of binding hotspots.* Journal of molecular biology, 2012. **415**(2): p. 443-453.
143. Mewes, A., H. Franke, and D. Singer, *Organotypic brain slice cultures of adult transgenic P301S mice—a model for tauopathy studies.* 2012.
144. Hall, H., et al., *AF710B, an M1/sigma-1 receptor agonist with long-lasting disease-modifying properties in a transgenic rat model of Alzheimer's disease.* Alzheimer's & Dementia, 2018. **14**(6): p. 811-823.
145. Iulita, M.F., et al., *Intracellular Aβ pathology and early cognitive impairments in a transgenic rat overexpressing human amyloid precursor protein: a multidimensional study.* Acta neuropathologica communications, 2014. **2**(1): p. 1-17.
146. Iulita, M.F., et al., *Differential deregulation of NGF and BDNF neurotrophins in a transgenic rat model of Alzheimer's disease.* Neurobiology of disease, 2017. **108**: p. 307-323.
147. Wilson, E., et al., *BACE1 inhibition by microdose lithium formulation NP03 rescues memory loss and early stage amyloid neuropathology.* Translational psychiatry, 2017. **7**(8): p. e1190-e1190.
148. Leon, W.C., et al., *A novel transgenic rat model with a full Alzheimer's-like amyloid pathology displays pre-plaque intracellular amyloid-β-associated cognitive impairment.* Journal of Alzheimer's Disease, 2010. **20**(1): p. 113-126.
149. Florencia Iulita, M. and A. Claudio Cuello, *The NGF metabolic pathway in the CNS and its dysregulation in Down syndrome and Alzheimer's disease.* Current Alzheimer Research, 2016. **13**(1): p. 53-67.
150. Florencia Iulita, M., F. Caraci, and A. Claudio Cuello, *A link between nerve growth factor metabolic deregulation and amyloid-β-driven inflammation in down syndrome.* CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders), 2016. **15**(4): p. 434-447.
151. Teller, J.K., et al., *Presence of soluble amyloid β–peptide precedes amyloid plaque formation in Down's syndrome.* Nature medicine, 1996. **2**(1): p. 93-95.
152. Pentz, R., et al., *Nerve growth factor (NGF) pathway biomarkers in Down syndrome prior to and after the onset of clinical Alzheimer's disease: A paired CSF and plasma study.* Alzheimer's & Dementia, 2021. **17**(4): p. 605-617.
153. Do Carmo, S., B. Kannel, and A.C. Cuello, *Nerve Growth Factor Compromise in Down Syndrome.* Frontiers in Aging Neuroscience, 2021: p. 479.
154. Iulita, M.F., et al., *An inflammatory and trophic disconnect biomarker profile revealed in Down syndrome plasma: relation to cognitive decline and longitudinal evaluation.* Alzheimer's & Dementia, 2016. **12**(11): p. 1132-1148.
155. Iulita, M.F. and A.C. Cuello, *Nerve growth factor metabolic dysfunction in Alzheimer's disease and Down syndrome.* Trends in pharmacological sciences, 2014. **35**(7): p. 338-348.

156. Iulita, M.F., et al., *Nerve growth factor metabolic dysfunction in Down's syndrome brains.* Brain, 2014. **137**(3): p. 860-872.
157. Parks, W.C., C.L. Wilson, and Y.S. López-Boado, *Matrix metalloproteinases as modulators of inflammation and innate immunity.* Nature Reviews Immunology, 2004. **4**(8): p. 617-629.
158. Khokha, R., A. Murthy, and A. Weiss, *Metalloproteinases and their natural inhibitors in inflammation and immunity.* Nature Reviews Immunology, 2013. **13**(9): p. 649-665.
159. Nissinen, L. and V.-M. Kähäri, *Matrix metalloproteinases in inflammation.* Biochimica et Biophysica Acta (BBA)-General Subjects, 2014. **1840**(8): p. 2571-2580.
160. Gibbs, R.B. and D. Pfaff, *In situ hybridization detection of trkA mRNA in brain: distribution, colocalization with p75NGFR and up-regulation by nerve growth factor.* Journal of Comparative Neurology, 1994. **341**(3): p. 324-339.
161. Figueiredo, B., et al., *Differential expression of p140trk, p75NGFR and growth-associated phosphoprotein-43 genes in nucleus basalis magnocellularis, thalamus and adjacent cortex following neocortical infarction and nerve growth factor treatment.* Neuroscience, 1995. **68**(1): p. 29-45.
162. Fisher, A., *Cholinergic treatments with emphasis on m1 muscarinic agonists as potential disease-modifying agents for Alzheimer's disease.* Neurotherapeutics, 2008. **5**(3): p. 433-442.
163. Shen, H., et al., *Neuroprotection by donepezil against glutamate excitotoxicity involves stimulation of α7 nicotinic receptors and internalization of NMDA receptors.* British journal of pharmacology, 2010. **161**(1): p. 127-139.
164. Nizri, E., et al., *Anti-inflammatory properties of cholinergic up-regulation: a new role for acetylcholinesterase inhibitors.* Neuropharmacology, 2006. **50**(5): p. 540-547.
165. Claassen, J.A. and R.W. Jansen, *Cholinergically mediated augmentation of cerebral perfusion in alzheimer's disease and related cognitive disorders: the cholinergic–vascular hypothesis.* The Journals of Gerontology Series A: Biological Sciences and Medical Sciences, 2006. **61**(3): p. 267-271.
166. Nitsch, R.M., et al., *Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors.* Science, 1992. **258**(5080): p. 304-307.
167. M Hernandez, C. and K. T Dineley, *α7 Nicotinic acetylcholine receptors in Alzheimer's disease: neuroprotective, neurotrophic or both?* Current drug targets, 2012. **13**(5): p. 613-622.
168. Visentin, C., et al., *Glycosylation tunes neuroserpin physiological and pathological properties.* International Journal of Molecular Sciences, 2020. **21**(9): p. 3235.
169. Schipanski, A., et al., *The lectin OS-9 delivers mutant neuroserpin to endoplasmic reticulum associated degradation in familial encephalopathy with neuroserpin inclusion bodies.* Neurobiology of aging, 2014. **35**(10): p. 2394-2403.
170. Moriconi, C., et al., *Interactions between N-linked glycosylation and polymerisation of neuroserpin within the endoplasmic reticulum.* The FEBS Journal, 2015. **282**(23): p. 4565-4579.
171. Takehara, S., et al., *The 2.1-Å crystal structure of native neuroserpin reveals unique structural elements that contribute to conformational instability.* Journal of Molecular Biology, 2009. **388**(1): p. 11-20.

172. Pei, J., et al., *Targeting lysosomal degradation pathways: new strategies and techniques for drug discovery.* Journal of medicinal chemistry, 2021. **64**(7): p. 3493-3507.
173. Cai, Q. and Q.T. Shubhra, *Overcoming blood-brain barrier for targeted delivery of lysosome-targeting chimeras.* Neuron, 2023. **111**(18): p. 2778-2780.
174. Miranda, E., et al., *The intracellular accumulation of polymeric neuroserpin explains the severity of the dementia FENIB.* Human molecular genetics, 2008. **17**(11): p. 1527-1539.
175. Davis, R.L., et al., *Familial encephalopathy with neuroserpin inclusion bodies.* The American journal of pathology, 1999. **155**(6): p. 1901-1913.
176. Zissimopoulos, J., E. Crimmins, and P.S. Clair. *The value of delaying Alzheimer's disease onset*. in *Forum for Health Economics and Policy*. 2015. De Gruyter.
177. Manuel, D.G., et al., *Alzheimer's and other dementias in Canada, 2011 to 2031: a microsimulation Population Health Modeling (POHEM) study of projected prevalence, health burden, health services, and caregiving use.* Population health metrics, 2016. **14**(1): p. 1-10.