

Neural Stem Cell (NSC) Secretome and Wnt/ β -catenin Signaling Pathway in Alzheimer's Disease
(AD): A Systematic Literature Review and Bioinformatics Analysis

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by neuronal loss, synaptic dysfunction, and cognitive impairment, with no current cure (DeTure & Dickson, 2019; Kumar et al., 2023). The Wnt/ β -catenin signaling pathway plays a crucial role in neurogenesis and synaptic plasticity; however, its dysregulation in AD, due to pathological factors such as amyloid- β (A β) plaques and neurofibrillary tangles (NFTs), contributes to disease progression (Jia et al., 2019; Priya et al., 2024). Neural stem cell (NSC) secretome, a collection of bioactive molecules secreted by NSCs, has shown potential in activating the Wnt/ β -catenin signaling pathway and promoting neurogenesis in AD (Bahlakeh et al., 2022; Hijroudi et al., 2022). However, the specific components within the NSC secretome that contribute to Wnt/ β -catenin activation and their underlying mechanisms remain unknown. This study aimed to identify the components of the NSC secretome, specifically growth factors and neurotrophic factors, that are responsible for activating the Wnt/ β -catenin signaling pathway in AD, thereby enhancing neurogenesis, and to investigate their potential mechanisms of action through a comprehensive literature review and bioinformatics analysis. A systematic literature review identified eight growth factors – epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), fibroblast growth factor 8 (FGF8), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), stem cell factor (SCF), vascular endothelial growth factor (VEGF) – and three neurotrophic factors – brain-derived neurotrophic factor (BDNF),

glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF) – within the NSC secretome that may contribute to Wnt/ β -catenin activation in AD.

Bioinformatics analysis categorized these proteins using UniProt database, assessed their molecular and biological functions through Gene Ontology (GO) analysis using PANTHER database, and identified their pathway involvement through KEGG database. STRING-based protein-protein analysis (PPI) network analysis provided insight into the intricate crosstalk between PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin signaling pathways. Then a targeted literature review was conducted to validate bioinformatics predictions, identifying two direct and one indirect crosstalk mechanism of Wnt/ β -catenin signaling pathway activation. The first direct mechanism involves growth factors and neurotrophic factors activating the PI3K/Akt signaling pathway, leading to Akt-mediated phosphorylation and inhibition of glycogen synthase kinase-3 β (GSK-3 β), which stabilizes β -catenin and promotes its nuclear translocation to activate Wnt target genes. The second direct mechanism involves EGF binding to epidermal growth factor receptor (EGFR) triggering MAPK/ERK activation, where extracellular signal-regulated kinase (ERK) phosphorylates casein kinase 2 (CK2), which subsequently phosphorylates α -catenin. This phosphorylation event disrupts the β -catenin-E-cadherin complex, allowing β -catenin to dissociate and translocate into the nucleus to drive Wnt target gene transcription. The indirect mechanism involves MAPK/ERK signaling activation by growth factors and neurotrophic factors, where rat sarcoma (Ras) activation converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), reinforcing PI3K/Akt pathway activation and enhancing β -catenin stabilization. A final pathway interaction map was constructed to visualize the complex interplay

between these signaling pathways and their potential therapeutic implications in AD. Collectively, these findings highlight the potential of NSC secretome components in restoring Wnt/ β -catenin signaling in AD through intracellular crosstalk with PI3K/Akt and MAPK/ERK signaling pathway. This study provides a molecular framework for future exploration of NSC secretome-based therapies in AD.

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Chapter I.

Introduction

The objective of this chapter is to introduce the thesis. This chapter begins with a literature review, providing in-depth background information, analyzing key concepts, and identifying gaps in existing research. This is followed by the problem statement, defining the research problem and its significance. It then states the hypothesis of the study, providing a testable prediction based on existing information and preliminary data. Following this, the objectives of the study are outlined, detailing the specific goals the study aims to achieve. Lastly, this chapter includes an overview of the organization of the thesis, explaining the structure and content of the subsequent chapters.

Literature Review

This section provides a comprehensive overview of the background information pertaining to the main themes of the thesis. It offers a detailed and critical analysis of previous scientific studies, focusing on the key concepts central to the study. This section surveys the current state of knowledge, identifies gaps in the literature, and situates the current study within the broader research field. By examining existing research related to the thesis topic, it establishes essential context and background information for the study, demonstrating the significance and relevance of the research problem. This comprehensive literature review ensures a thorough understanding of the thesis topic and

emphasizes the importance of addressing the identified gaps in the existing literature that this study intends to accomplish.

Neural Stem Cells in Regenerative Medicine

Neural stem cells (NSCs) hold significant promise in regenerative medicine due to their ability to self-renew and differentiate into multiple neural lineages, playing a critical role in neurogenesis and brain repair. As research advances, the focus has shifted from understanding their fundamental biology to exploring their therapeutic potential in treating neurodegenerative diseases. While NSC transplantation was initially believed to work primarily through cell replacement, emerging evidence highlights their significant paracrine effects, wherein secreted bioactive molecules influence the cellular microenvironment, promote neuroprotection, and enhance endogenous repair mechanisms. This section provides an overview of NSCs, their role in adult neurogenesis, their application in neurodegenerative disease therapy, and the mechanisms underlying their regenerative effects.

Introduction to Neural Stem Cells and Adult Neurogenesis

NSCs are multipotent cells of the central nervous system (CNS) defined by: 1) their ability to indefinitely self-renew, 2) the ability to give rise to cell types other than themselves through asymmetric cell division, and 3) their ability to differentiate into all neural lineages, including neurons, astrocytes, and oligodendrocytes (Gage, 2000). In mammals, including humans, these cells play a vital role in both embryonic development and adult neurogenesis – the process by which NSCs generate new neurons. Neurogenesis is a multi-phase process that includes NSCs proliferation, fate specification, differentiation, maturation, migration, and the functional integration of

newborn cells into the neuronal circuitry (Faigle & Song, 2012). During embryonic development, NSCs give rise to the multitude of neuronal and glial lineages that populate the mature brain, contributing to the diverse functions of the CNS. In adulthood, these cells persist as small populations in two discrete regions of the adult brain known as neurogenic niches: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Ma et al., 2009). These neurogenic niches are crucial for NSCs maintenance and adult neurogenesis, as they provide a specialized microenvironment that maintains a delicate balance between NSCs quiescence and activation. This balance is essential for brain plasticity – also known as neuroplasticity, defined as the ability of the brain to change and reorganize structure and function of neural networks in response to intrinsic or extrinsic stimuli – and for inherent repair mechanisms in response to a brain injury or a disease (Llorens-Bobadilla & Martin-Villalba, 2017; Puderbaugh & Emmady, 2024).

The discovery of adult neurogenesis has significantly improved our understanding of the brain's regenerative capabilities. Historically, the presence of adult neurogenesis was an area filled with controversy in the scientific community due to a long-standing dogma in the neuroscience community that no new neurons are formed after birth. This dogma was established by Santiago Ramón y Cajal (1852-1934), a Spanish neuroanatomist and Nobel laureate commonly referred to as the 'father of neuroscience', who stated that:

“Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated.” (Ramón y Cajal, 1928)

Although some scientists held different opinions, this has been the central dogma in neuroscience for many decades, given the limitations of research methods available at the time to prove that postnatally generated cells were new neurons and not glia (Yang et al., 2008).

However, this dogma was challenged in the 1960s when Joseph Altman provided the first evidence of neurogenesis in the brain of an adult mouse, using the newly developed labeling technique (Altman, 1962). This groundbreaking discovery was later substantiated and expanded upon by further studies with the advancement in various methodologies, such as biomarker analysis, immunohistological techniques, stereological techniques, and imaging technologies. In the 1980s, Goldman and Nottebohm demonstrated that adult songbirds undergo seasonal neurogenesis, with new neurons being generated in the brain regions responsible for seasonal song learning and memory. Their study suggested neuroplasticity, revealing a functional role for these new neurons in learning and memory, as the observed adult neurogenesis was linked to the acquisition and modification of seasonal songs (Goldman & Nottebohm, 1983). Then, in the 1990s, Reynolds and Weiss identified and isolated NSCs from the adult mouse brains that proliferated and differentiated into neurons and astrocytes in vitro, demonstrating self-renewal and the differentiation capacity of NSCs in adult mammalian brains (Reynolds & Weiss, 1992). Finally, in 1998, Eriksson and colleagues demonstrated the first report of hippocampal neurogenesis in the human adult brain (Eriksson et al., 1998). Adult neurogenesis in the human brain is now a widely accepted neuroplastic process that involves adaptive structural and functional changes to the brain in response to internal or external stimuli (Puderbaugh & Emmady; 2024). These findings not only revolutionized

the understanding of the brain's plasticity and regenerative potential, but also paved the way for exploring the potential therapeutic application of NSCs in regenerative medicine, particularly in treating neurodegenerative diseases.

Neural Stem Cell Transplantation in Neurodegenerative Diseases

NSC transplantation have been studied as a potential therapeutic strategy to promote tissue regeneration in wide range of neurodegenerative diseases.

Neurodegenerative diseases are a heterogeneous group of disorders that are characterized by progressive loss of structure or function of neurons, resulting in sensory, motor, and cognitive impairment (Lamprey et al., 2022). For example, Alzheimer's disease (AD) is associated with the widespread loss of neurons and synapses, particularly involving the loss of cholinergic neurons, leading to memory impairment and cognitive decline, while Parkinson's disease (PD) primarily involves the degeneration of dopaminergic neurons, resulting in motor impairments such as tremors and bradykinesia. Other examples include Huntington's disease (HD), characterized by the loss of medium spiny GABAergic neurons, causing motor control issues and cognitive impairment; amyotrophic lateral sclerosis (ALS) with its degeneration of cholinergic motor neurons, leading to progressive muscle weakness and paralysis; and multiple sclerosis (MS) marked by oligodendrocyte loss, resulting in various sensory and motor deficits such as visual disturbances, muscle weakness, and coordination problems (Nie et al., 2023). Currently, all known neurodegenerative diseases are incurable and treatment options are non-existent or relatively limited, most of which can only alleviate symptoms or delay the progression of disease (Pasteuning-Vuhman et al., 2020).

Neurodegenerative diseases are characterized not only by the progressive loss of neurons but also by the failure of the brain's inherent repair mechanisms to restore lost neural tissue and regain lost functions. Under normal physiological conditions, NSCs in the neurogenic niche largely remains in a state of reversible cell cycle arrest, known as quiescence (Urbán et al., 2019). Nevertheless, they maintain high sensitivity to both intrinsic and extrinsic signals from the environment, enabling them to retain the ability to transition into an active state that support, when required, the formation of new neurons that repopulate and regenerate the damaged neural tissues (Sun, 2014; Urbán et al., 2019). In this active state, NSCs initiate cell cycle division, proliferating for self-renewal and to produce neuronal progenitor cells (NPCs) or glial progenitor cells (GPCs), also known as transit amplifying cells (Kaise & Kageyama, 2024). These progenitor cells then differentiate into specialized cell types, such as neurons, astrocytes, or oligodendrocytes, in response to complex arrays of signals, which eventually mature and integrate into the existing neuronal circuitry, replacing damaged or lost neural cells and thereby supporting the natural regenerative capacity of the brain (Tang et al., 2017). Although the mechanisms governing NSC state transitions are not yet fully understood, the delicate balance between NSC quiescence and activation is crucial for brain tissue repair and maintaining homeostasis, as it determines the rate of neurogenesis and ensures the preservation of the NSC reservoir for long-term neurogenic capacity. However, in neurodegenerative conditions, this intricate balance is disrupted, leading to impaired neurogenesis and limited capacity to repair neural tissue damage contributing to the progression of neurodegenerative diseases (Urbán et al., 2019). Furthermore, studies have shown that impaired adult neurogenesis is an early critical event in the course of AD that

may underlie or exacerbate learning and memory deficits. Moreno-Jiménez et al. studied the persistence of impaired adult hippocampal neurogenesis (AHN) in healthy aging and its significant impairment in AD, examining human brains of 13 neurologically healthy patients and 45 patients with AD. Although a gradual decline in the neurogenesis was observed with normal aging, the study identified thousands of doublecortin-expressing (DCX+) neurons, a neurogenesis marker, in neurologically healthy patients that exhibited various degrees of maturation along different stages of neurogenesis. In contrast, the number and maturation of DCX+ cells progressively declined in the AD patients as the disease advanced. Interestingly, alterations in AHN were detected at early stages of AD, even before the presence of AD-associated pathological markers in the dentate gyrus, and notably before the manifestation of the first clinical symptoms (Moreno-Jiménez et al., 2019). This result suggests that impaired neurogenesis may be a contributing factor in AD pathology, rather than a consequence of normal aging or the disease's neuropathological changes, which may potentially underlie or exacerbate cognitive deterioration and make neurons more susceptible to damage in AD (Demars et al., 2010). Similarly, in PD, the rapid loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) is not sufficiently replaced by endogenous neurogenesis, suggesting alterations of adult neurogenesis in PD. Consequently, the progressive loss of dopaminergic neurons and impaired neurogenesis leaves the nigrostriatal pathway compromised in PD, causing motor deficits to worsen over time (Rodriguez et al., 2014; Zhao et al., 2003). A recent study by Jiang et al. has provided evidence of impaired neurogenesis in a mouse model of PD featuring the vacuolar protein sorting protein 35 (VPS35) Asp620Asn (D620N) mutation. They observed a reduction in NSC reserves and

disruptions in NPC proliferation, differentiation, migration, and neurite development, possibly due to the accumulation of amyloid precursor protein (APP), a VPS35 D620 mutation-associated pathological changes in PD (Jiang et al., 2021). Research on other neurodegenerative diseases, such as HD and MS, further corroborates the connection between impaired neurogenesis and the progression of these diseases (Gil-Mohapel et al., 2011; Zhang et al., 2020). Despite emerging evidence of impaired neurogenesis in neurodegenerative diseases, the exact factors that cause the failure of adult neurogenesis, whether it occurs before or during disease progression, is unknown (Wakhloo et al., 2022). Research on ischemic stroke has revealed that the failure of the endogenous repair mechanisms and impaired neurogenesis stems from an inadequate supply of NSCs, unable to meet the demands necessary for repairing neurological damage (Willis et al., 2020). However, whether this is the case in other neurodegenerative diseases remains unanswered. Nevertheless, these examples suggest that impaired adult neurogenesis, in addition to the progressive loss of neurons, plays a critical role in the pathophysiology of various neurodegenerative diseases, highlighting the failure of the brain's endogenous repair mechanisms to compensate for the ongoing loss of different neural populations as a key factor in the progression of these diseases. Therefore, developing strategies aimed at reinstating the brain's endogenous repair mechanisms or replacing lost neuronal populations may be a potential novel therapeutic approach for treating neurodegenerative diseases.

Given the substantial loss of neurons and impaired neurogenesis in neurodegenerative diseases, there has been a significant focus over the past decade on developing therapeutic strategies aimed at replacing the damaged or lost neurons. These

types of therapeutic strategies are collectively referred to as regenerative medicine, which seeks to replace, repair, or regenerate damaged tissues or organs to restore normal functions. Among these, stem cell-based therapy, specifically NSC-based therapy, has emerged as a promising approach for treating neurodegenerative diseases. NSC-based therapy involves the direct transplantation of exogenous neural stem/progenitor cells, which are then expected to migrate into the diseased regions, proliferate and differentiate to replace the damaged or lost cells, functionally integrate into existing neural circuits, and thereby potentially restore neurological functions (Latchney & Eisch, 2012). NSCs can be derived from fetal, postnatal, and adult brain tissue, reprogrammed from somatic cells, or differentiated from pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Vogel et al., 2018). These NSCs can then be cultured in vitro for transplantations studies in animal models of neurodegenerative diseases.

The restorative, regenerative, and neuroprotective potentials of NSCs are evident from numerous transplantation studies employing differentiated or undifferentiated NSC grafts in animal models of different neurodegenerative diseases (Willis et al., 2020). For example, several studies in animal models of AD have revealed that transplanted NSCs demonstrate the ability to migrate to damaged areas, proliferate and differentiate into functional cholinergic neurons, stimulate neurogenesis, inhibit inflammation, secrete neurotrophic factors, restore synaptic impairment, promote neurogenesis, and improve spatial learning and memory to some extent (Ager et al., 2015; Chen et al., 2014; Lilja et al., 2015; McGinley et al., 2018; Zhang et al., 2017; Zhu et al., 2020). However, despite promising results of preclinical studies using AD animal models, human clinical trials in

AD patients have had unfavorable outcomes (Chen et al., 2023). Furthermore, the clinical application of NSC transplantation in AD is currently limited by several challenges, including the low survival rate of transplanted NSCs, their low and inconsistent differentiation rate into functional cholinergic neurons, and the inability of newly differentiated neurons to integrate into the existing neuronal circuits for therapeutically significant functional recovery (Madhavan & Collier, 2010; Zhang et al., 2020).

These successes and limitations are similarly reflected in NSC-based transplantation studies of other neurodegenerative diseases. In PD research, numerous studies have shown that transplantation of NSCs or dopaminergic neurons, derived from the in vitro differentiation of human fetal NSCs, can achieve long-term survival, differentiate into functional dopaminergic neurons, form synaptic connections, secrete neurotrophic factors, and thereby alleviate motor deficits in rodent and nonhuman primate models (Li et al., 2016; Wianny & Vezoli, 2017). However, despite the compelling therapeutic benefits, the concentrations of mature dopaminergic neurons in the graft are often too low to provide optimal benefits, and a high incidence of graft-induced dyskinesias has been reported, possibly related to the grafts containing aberrant cell types such as serotonergic neurons alongside the desired dopaminergic neurons (Hiller et al., 2022). Additionally, the formation of dense clusters of undifferentiated NPCs at the site of implantation poses a risk of graft-associated tumor formation, and limited migration of graft-derived neurons into the host brain tissue remains a key challenge in NSC-based transplantation studies (Ladewig et al., 2014; Roy et al., 2006). In the field of HD, preclinical transplantation studies using different types of NSCs in rodent models have shown promising results, such as increased survival of host striatal

neurons, reduced inflammation, enhanced endogenous neurogenesis, partial replacement of lost medium spiny GABAergic neurons, and improvement in motor function (Drew & Busse, 2022; Yoon et al., 2020). It is hypothesized that the observed motor function recovery may be attributed to the secretion of neurotrophic factors such as glial cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and BDNF released from the grafted NSCs, rather than the restoration of neural circuit connectivity by new GABAergic neurons (McBride et al., 2004; Ryu et al., 2004). This speculation arises from the fact that a significant percentage of the transplanted NSCs failed to migrate to the lesioned areas, and of those that did, the number of NSCs that differentiated into the specific neuronal cell types was too small to produce the observed therapeutic benefits (McBride et al., 2004). Nevertheless, these successes in animal models of HD prompted several phase I and II human clinical trials. Unfortunately, all trials to date, including the largest and most significant Multicentric Intracerebral Grafting in Huntington's Disease (MIG-HD) trial, have failed to demonstrate clinical benefits associated with stem cell-based transplantation in HD, indicating the need for further research to bridge the gap between laboratory findings and clinical applications in humans (Bachoud-Lévi et al., 2020; Conner et al., 2023). Although there is limited understanding of the exact causes behind graft failure in these trials, the failure in the MIG-HD trial was believed to result from a combination of common challenges associated with stem cell-based transplantation, including the viability of cells upon implantation, a non-permissive host environment affecting cell survival, ectopic grafts, and graft rejections (Bachoud-Lévi et al., 2020). Overall, these NSC-based graft studies in various neurodegenerative diseases demonstrate both the inherent challenges of stem

cell-based transplantation that must be addressed and the promising potential of NSCs in developing treatments for neurodegenerative diseases. Therefore, a comprehensive understanding of the multifaceted mechanisms through which NSCs exert their beneficial effects is crucial for developing therapies that effectively leverage NSCs in treating neurodegenerative diseases.

The Paracrine Effect

Recent studies suggest that the therapeutic benefits of NSC transplantation extend beyond the replacement of damaged or lost neurons. In fact, stem cells secrete a wide array of bioactive molecules, known collectively as the secretome, that have been shown to ameliorate neurodegenerative disease pathology and promote recovery through influencing cellular microenvironment, modulating signaling pathways, inducing immunomodulation, enhancing neuroprotection, and promoting endogenous tissue repair and regenerative processes (Adamowicz et al., 2014; Boomsma & Geenen, 2012; Hathout, 2014). The NSC-derived secretome comprises diverse signaling molecules, including growth factors, chemokines, cytokines, bioactive lipids, and microRNAs (miRNAs), which exert profound effects on the cellular dynamics of neighboring cells through paracrine signaling, commonly referred to as the paracrine effect or bystander effect (Tran & Damaser, 2015; Zhang et al., 2020). Contrary to previous assumptions, compelling evidence now suggests that this paracrine effect, rather than cell replacement, is the primary mechanism driving the therapeutic benefits observed in NSC transplantation studies (Foo et al., 2021). Initially, the therapeutic benefits of NSC transplantation were thought to be derived from their inherent ability to differentiate into specialized cell types to replace cells that are lost during disease progression, thereby

restoring the structure and function of damaged tissue (Latchney & Eisch, 2012).

However, this initial hypothesis has been challenged by numerous studies revealing that transplanted NSCs exhibit a relatively low survival rate after engraftment and do not differentiate in sufficient quantity to explain their therapeutic benefits (Sun et al., 2020; Zhang et al., 2020). Moreover, studies have also observed that grafted NSCs demonstrate apparently limited migration ability to the lesioned area, often display irrational differentiation, and often fail to integrate newly differentiated neurons into the local neural circuitry (Gioia et al., 2020; Nie et al., 2023; Zhang et al., 2020). Nevertheless, grafted NSCs still exert their beneficial effects of tissue repair and regeneration, as well as functional improvements (Sun et al., 2020). These findings, and others, have led researchers to reconsider the initial hypothesis of stem cell-based therapy focused solely on the replacement of damaged or lost cells.

Additional studies have further substantiated the alternate hypothesis that the therapeutic potential of transplanted NSCs is predominantly attributed to the paracrine effect, rather than the direct cell replacement. A recent study by Hu et al. observed that transplantation of human NSCs (hNSCs) into a rodent model of traumatic brain injury have been found to exert therapeutic benefits into regions distant from the site of engraftment, even before the migration of grafted hNSCs, suggesting that the cell replacement may not directly contribute to the hNSC-mediated neuroprotection and regeneration (Hu et al., 2020). Moreover, Ryu et al. demonstrated that transplantation of hNSCs into the subventricular zone of the rat's brain 24 hours after induced cerebral ischemia stimulated the proliferation and differentiation of endogenous NSCs, despite the complete absence of grafted hNSCs in these brain regions. An increase in the astrocyte

marker glial fibrillary acidic protein (GFAP) and enhanced angiogenesis were also observed in the ischemic hemisphere, which suggests that the transplantation of hNSCs provides a microenvironment, likely through paracrine effects, that activates endogenous restorative mechanisms to promote endogenous neurogenesis in the ischemic brain (Ryu et al., 2016). Similarly, a study by Chen et al. found that intranasal transplantation of hNSCs into a mouse model of MS, induced by viral infection, led to sustained functional and neurologic recovery for up to 6 months following transplantation, despite the hNSCs not being detectable beyond 8 days post-transplantation. This recovery was associated with reduced neuroinflammation, decreased demyelination, enhanced remyelination, and an increase in regulatory T cells (Tregs). Cultured hNSCs were found to secrete anti-inflammatory cytokines, including TGF- β 1 and TGF- β 2, which are known for regulating immune tolerance and T cell homeostasis. The findings indicate that the therapeutic effects of hNSC transplantation are mediated by the secretome, including modulation of the immune response and promotion of remyelination processes, rather than through the direct replacement of damaged neural cells by the transplanted hNSCs. Additionally, this study suggests that the hNSCs secretome can have a prolonged influence on the microenvironment, even in the absence of the transplanted hNSCs prolonged survival within the host (Chen et al., 2014). Together, these findings collectively emphasize the significance of the paracrine effects exerted by the transplanted NSCs in promoting neurological recovery across various animal models of brain injury and neurodegenerative diseases. The observed therapeutic benefits support the alternate hypothesis that the therapeutic benefits of transplanted NSCs are not mainly derived from the direct cell replacement but rather from the effects of the secretome that activate the

brain's intrinsic repair and regenerative mechanisms via modulating the lesioned microenvironment. This paradigm shift opens new avenues for developing therapeutic interventions that harness the potent paracrine effects of the NSC secretome for the effective treatment of neurodegenerative diseases.

Neural Stem Cell Secretome

NSCs exert their therapeutic effects not only through direct differentiation into neuronal and glial cells but also by secreting bioactive molecules collectively known as the secretome. The NSC secretome consists of a diverse array of proteins, growth factors, cytokines, and microRNAs that influence cellular processes such as neurogenesis, neuroprotection, synaptic plasticity, and immune modulation. Recent research suggests that the paracrine effect, where secreted molecules modulate the microenvironment and promote endogenous repair, is a primary mechanism underlying NSC-mediated regeneration, rather than direct cell replacement alone. The therapeutic potential of the NSC secretome has been demonstrated in various preclinical models of neurodegenerative diseases, where it enhances neuronal survival, reduces inflammation, and stimulates synaptic repair. As a cell-free alternative to NSC transplantation, secretome-based therapies offer several advantages, including reduced risks of tumor formation, immune rejection, and ethical concerns associated with stem cell use. Understanding the composition and molecular mechanisms of the NSC secretome is essential for optimizing its therapeutic applications in treating neurodegenerative disorders.

Overview of the Neural Stem Cell Secretome

The stem cell secretome is defined as the complex set of bioactive molecules that are secreted by stem cells into the extracellular space. These secretory molecules play crucial roles in many biological functions, including cell growth, proliferation, differentiation, signaling, apoptosis, angiogenesis, and immunomodulation (Daneshmandi et al., 2020). The secretome can be broadly categorized into two forms: membrane-free and membrane-bound. The membrane-free secretome denotes molecules that are directly secreted into the extracellular space without any enclosing membrane (Willis et al., 2020). These include common secretory proteins, such as enzymes and hormones, that translocate from the endoplasmic reticulum lumen to the Golgi complex, where they are then excreted from the cell through exocytosis (Tran & Damaser, 2015). In contrast, the membrane-bound secretome indicates the packaging of a diverse array of molecules such as proteins, mRNAs, and miRNAs into small secretory extracellular vesicles (EV) or exosomes consisting of cytoplasm enclosed by a lipid bilayer before release into the extracellular space (Vogel et al., 2018).

The composition of the stem cell secretome is very dynamic, depending on the type of cell, developmental stage, and microenvironmental stimuli (Daneshmandi et al., 2020). The composition of the stem cell secretome reflects its adaptable nature in various biological contexts, contributing to the complexity and challenges involved in studying the secretome. In general, NSC-derived secretomes are known to contain a diverse array of proteins, growth factors, cytokines, chemokines, metabolites, morphogens, bioactive lipids, mRNAs, and miRNAs that are relevant for neurogenesis, neuroprotection, neuroplasticity, and neuroimmune response (Dause et al., 2022; Foo et al., 2021; Willis et al., 2020; Stevanato et al., 2016). However, studies identifying the exact molecular

composition of NSC secretomes and its functional roles in paracrine regulation are limited, highlighting a need for further investigation. To date, only a few studies have tried to characterize the hNSC secretome in an unbiased manner. Currently available unbiased methods to characterize the composition of hNSC secretome include quantitative proteomic analysis on hNSC conditioned media and hNSC-derived EVs in vitro utilizing mass spectrometry together with small RNA sequencing analysis (Willis et al., 2020). Stevano et al. used an unbiased technique to determine the extracellular miRNAs contents of exosomes derived from the conditioned media of cultured human embryonic cortex NSCs using next-generation sequencing (NGS), a small RNA sequencing tool. They identified five highly expressed miRNA, which included hsa-miR-1246, hsa-miR-4488, hsa-miR-4508, hsa-miR-4492, and hsa-miR-4516, in hNSC-derived exosomes that were previously undetected by microarray analysis in a similar type of stem cell (Stevano et al., 2016). Additionally, a recent study by Červenka et al. employed multiplex xMAP technology for unbiased secretome analysis in the conditioned media from cultured hNSCs differentiated from ESCs in vitro. They detected 28 secreted growth factors, chemokines, and cytokines, including vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) (Červenka et al., 2020). While these studies have started creating repositories of the NSC secretome, many other NSC secretome factors will likely remain to be discovered. Therefore, a more comprehensive investigation is necessary to generate NSC secretome libraries, which will help identify biologically relevant targets for therapeutic interventions in neurodegenerative diseases.

Therapeutic Potential of Neural Stem Cell Secretome

Despite the classical view that NSC transplantation only functions through structural cell replacement, compelling evidence from experimental studies have shown that transplanted NSCs act as a natural source of potent biologics, secretome, that modulate and promote the restoration of several vital functions in the damaged or diseased brain (Willis et al., 2020). To effectively examine the therapeutic potential of NSC secretome independent of cell replacement effects, researchers studied the effects of cell-free, NSC-derived conditioned medium and NSC-derived EVs in animal models of neurodegenerative diseases. For example, research conducted by Mendes-Pinheiro et al. revealed that injection of hNSC conditioned medium into the 6-hydroxydopamine (6-OHDA) mouse model of PD resulted in neuroprotective effects, significantly increasing dopaminergic neuron survival and enhancing performance in motor coordination tests, compared to the group transplanted with hNSCs and the untreated 6-OHDA group. They suggest that the minimal to no recovery in motor functions and dopaminergic neuron survival in the hNSCs-transplanted group is due to the low survival rate of hNSCs upon transplantation, possibly because of the lack of an immunomodulatory effect from neuroregulatory molecules that control oxidative stress levels in the lesioned microenvironment. In fact, their proteomic analysis reveals that the hNSC secretome contains crucial neuroregulatory molecules, such as 14-3-3 proteins, glia-derived nexin (GDN), cystatin-C, clusterin, semaphorin-7A (SEM7A), pigment epithelium-derived factor (PEDF), galectin-1, dickkopf-related protein 3 (DKK3), and cadherin-2, essential for cell survival, migration, differentiation, and neuroprotection mechanisms (Mendes-Pinheiro et al., 2018). Moreover, a study by Webb et al. investigated the beneficial effects of hNSC-derived EVs in a porcine model of ischemic stroke. This study involved the

induction of middle cerebral artery occlusion (MCAO) followed by the intravenous administration of hNSC-EVs at three time points after the stroke was induced. The effects of hNSC-EVs on tissue-level recovery and functional recovery were assessed at 1 and 84 days post-MCAO. The findings from this study revealed that treatment with hNSC-EVs notably enhanced behavioral and motor recovery and reduced brain damage, as the stroked pigs treated with hNSC-EVs showed a significant decrease in lesion volume, brain swelling, and edema, while also preserving white matter integrity. Additionally, hNSC-EVs were observed to promote a phenotypic switch in circulating macrophages from a pro-inflammatory M1 to an anti-inflammatory M2, and increased the number of Tregs resulting in a downregulation of pro-inflammatory effector T helper 17 (Th17) cells (Webb et al., 2018; Willis et al., 2020). This immunomodulatory effect might be attributed to anti-inflammatory cytokines like IL-4 and IL-10 found in NSC-EVs, which can promote a phenotypic switch (Vogel et al., 2018; Willis et al., 2020). Furthermore, the study's discovery of neuroprotective effects of hNSC-EVs in a large mammalian model, whose cerebral architecture and white matter composition closely resemble those of humans, highlights the significant promise the NSC secretome holds for the development of human stroke therapeutics (Webb et al., 2018; Willis et al., 2020). Overall, the findings from these two studies highlights the NSC secretome's therapeutic potential, demonstrating its ability to resist further degeneration, induce neurogenesis, promote repair, and alleviate inflammation in neurodegenerative conditions (Willis et al., 2020).

Some studies have pursued a targeted approach, identifying specific factors within the NSC secretome and then assessing their potential therapeutic potential in an animal

model of AD. Impaired neurogenesis and inadequate induction of neurogenesis is associated with defective motor or cognitive functions in AD (Hollands et al., 2017). Lee et al. employed Western blot analysis to identify the expression of growth factors relevant to AD in the conditioned media of cultured hNSCs derived from aborted fetal brain tissue at 13 weeks of gestation (Lee et al., 2015). They identified that hNSCs expressed growth factors including BDNF, NGF, VEGF, fibroblast growth factor 2 (FGF2), and GDNF in vitro that are believed to be associated with molecular signaling pathways associated with AD-like pathologies. Transplantation of hNSC conditioned medium into AD mouse model decreased neuropathological protein hallmarks of AD, increased synaptic plasticity, and reduced apoptosis via neurotrophic supplies (Lee et al., 2015). These NSC-derived conditioned medium transplantation studies demonstrate the neuroprotective and regenerative potential of the NSC in treatment of neurodegenerative diseases. However, the specific molecular interactions and pathways involved in mediating the observed therapeutic effects are still unknown and remains to be characterized.

A detailed understanding of the specific molecular mechanisms is essential for advancing NSC secretomes as a potential therapeutic option in treating of neurodegenerative diseases. A recurring theme in studies investigating NSC secretomes in regenerative medicine is that NSC secretomes provide neuroprotection, neuroplasticity, and immunoregulation. For example, neurotrophic growth factors, such as VEGF, GDNF, insulin-like growth factor (IGF-1), and BDNF, detected in the NSC secretome have been found to enhance the survival and regeneration of endogenous neurons and reduce apoptosis following NSC transplantation or NSC conditioned media treatment after traumatic brain injury, spinal cord injury (SCI), PD and AD (Hu et al.,

2020; Lee et al., 2015; Mendes-Pinheiro et al., 2018; Ziv et al., 2006). Additionally, cultured NSCs increased neuroplasticity in mouse models of SCI and ischemic stroke by secreting angiogenic growth factors, such as FGF, GDNF, and VEGF. These factors are known to stimulate axonal regeneration, dendritic growth, and angiogenesis, promoting axonal and dendritic connections (Hicks et al., 2013; Romanyuk et al., 2015). Moreover, NSCs conditioned media or NSC-derived EVs have been observed to decrease neuronal inflammation and suppress the immune cell activation in animal models of MS, SCI, and ischemic stroke through the secretion of neurotrophic factors, including BDNF, GDNF, and neurotrophin-3 (NT-3), and anti-inflammatory cytokines like TGF- β (Chen et al., 2014; Rong et al., 2019; Yang et al., 2018). These results clearly demonstrate the therapeutic capacity of the NSC secretome in regulating and enhancing the restoration of crucial functions in the CNS. Nevertheless, the majority of studies predominantly use indirect cause-and-effect correlations to indicate the neuroprotective, neuroplasticity, and immunomodulatory properties of the NSC secretome, rather than depicting molecular mechanisms involved. Therefore, understanding the implicated molecular mechanisms is crucial for advancing our knowledge of the NSC secretome profile and its potential clinical benefits.

Mechanism of Action of Neural Stem Cell Secretome

The precise mechanisms by which the NSC secretome transmits information and induces target cell responses remain unknown and require further investigation. Among those studies that have identified mechanisms, only a few have characterized the composition of the secretome and the target cells. One such study by Bian et al. found that NSC-derived EVs marked with CD63-red fluorescent protein (RFP) could be

absorbed by retinal microglia upon subretinal injection in a mouse model of retinal degeneration, which protected photoreceptors from apoptosis *in vivo* and *in vitro*. Microglia that internalized the NSC-derived EVs exhibited a ramified, homeostatic morphology in contrast to the round, activated morphology observed in microglia without NSC-derived EV internalization. Analysis of the NSC-derived EVs revealed a set of 17 miRNAs that inhibited several inflammatory-related signal pathways, mitogen-activated protein kinases (MAPKs), nuclear factor kappa B (NF- κ B), IL-17, and tumor necrosis factor (TNF) pathways, in microglia by TNF- α , IL-1 β , and cyclooxygenase-2 (COX-2), which are all known activators of microglia. The results of this study indicate that NSC-derived EVs have the ability to directly interact with immune cells, releasing miRNAs that have the potential to suppress activated microglia and restore a protective retinal microenvironment (Bian et al., 2020; Willis et al., 2020). Moreover, it is essential to recognize that the mechanism of action of NSC secretome is not limited to their mRNA, miRNA, lipids, and various protein contents that enter target cells, thereby altering their phenotype. Indeed, it also involves signaling receptor mechanisms of EVs on their membrane and the membranes of target cells, activating downstream pathways and inducing overall cellular changes (Willis et al., 2020). For example, a study by Cossetti et al. demonstrated that the exposure of NSCs to pro-inflammatory cytokines *in vitro* activated the interferon-gamma (IFN- γ)-induced signal transducer and activator of transcription 1 (Stat1) signaling pathway, a pathway that plays a crucial role in immune response and inflammation, which resulted in the sorting of proteins and mRNAs into EVs that mirrored the cellular contents. They observed the activation of Stat1-dependent signaling in target cells through the intracellular transfer of IFN- γ , following the binding

of IFN- γ to interferon-gamma receptor 1 (Ifngr1) on the surface of the NSC-derived EV. The findings reveal the mechanism by which EVs communicate with their target cells through membrane interactions. Their study challenged previous notions by demonstrating that the direct transfer of NSC-derived EVs enriched with proteins and mRNAs does not activate Stat1 signaling in target cells; instead, EVs use Ifngr1 to recycle free IFN- γ , which contributes to downstream signaling in target cells (Cossetti et al., 2014; Willis et al., 2020). A subsequent study expanded on this concept by showing that NSC-derived EVs act as independent metabolic units carrying L-asparaginase activity, which suggests that NSCs, through their secretome, exert a regulatory effect on the metabolic microenvironment of distant cells (Iraci et al., 2017). These studies suggest that the NSC secretome may shape the phenotype of neighboring immune cells by changing the metabolic conditions of the microenvironment (Drago et al., 2016). Further research is required to profile NSC secretome and to identify the mechanisms behind the beneficial effects of secreted biological factors (Willis et al., 2020). Therefore, understanding the mechanism of action of the NSC secretome is necessary to harness and utilize its regenerative and neuroprotective potential for developing therapies for the treatment of neurodegenerative diseases. Harnessing the NSC secretome will allow for more targeted therapeutic approaches, as opposed to relying on NSC transplantation, which is associated with many risks to long-term safety, especially concerning tumor formation and immune rejection.

Advantages of Cell-Free Secretome Therapy Over Stem Cell Therapy

Stem cells, including ESCs, iPSCs, and adult stem cells, have shown great promise for regenerative medicine due to their ability to self-renew and their potential to

differentiate into cells of different tissues. However, their clinical applications have been limited. Despite numerous stem cell-based therapy clinical trials registered in the US National Institutes of Health (NIH), only a handful of stem cell-based therapies have made it to clinical practice since the development of the first stem cell therapy in 1957 (Thomas et al., 1957). Currently, the only stem cell-based therapy approved by the Food and Drug Administration (FDA) for use in the United States is hematopoietic stem cell transplantation for hematopoietic and immunological reconstitution in patients with hematopoietic disorders (Golchin & Farahany, 2019). Additionally, a few stem cell products derived from tissue-specific stem cells or mesenchymal stem cells (MSCs) have received approval for clinical use in other countries. These include the limbal stem cell product (Holoclar®) by Europe to repair injured cornea, the autologous bone marrow-derived mesenchymal stem cell (BM-MSC) product (Neuronata-R®) by South Korea for ALS, the autologous BM-MSC product (Stemirac®) by Japan for SCI, the allogenic BM-MSC product (Stempeucel®) by India for osteoarthritis, and the allogeneic BM-MSC product (Prochymal) by Canada for graft-versus-host disease (GvHD) (Brianna et al., 2022). The challenges in translating stem cell-based therapies to clinical applications may be due to some inherent limitations associated with stem cell-based treatments. These inherent limitations include challenges related to tumorigenicity, immune compatibility, and the transmission of infections (Tran & Damaser, 2015). Furthermore, the long-term engraftment of transplanted stem cells may not be the primary mechanism for the observed beneficial effects, with paracrine action playing a significant role (Zhang & Cheng, 2023). These drawbacks highlight the need for alternative approaches, such as

cell-free, secretome-based therapies, which may offer similar benefits without these limitations.

The use of cell-free, secretome-based therapies presents several significant benefits over more conventional stem cell-based therapies in regenerative medicine. The key benefit of cell-free therapy is its ability to potentially bypass issues commonly associated with stem cell-based treatments such as immunogenicity and tumorigenicity. Unlike stem cells, secretome components are not subject to human leukocyte antigen (HLA) mismatch, a common cause of immune rejection in allogenic stem cell transplants. Additionally, secretome-based therapy eliminates concerns about potential tumor formation inherent in stem cell-based transplantation, as it does not involve the transfer of live cells that could proliferate uncontrollably. Another key strength of secretome-based therapy is its focus on paracrine effects, where therapeutic benefits are derived from biologics secreted by the stem cells rather than the cells themselves. This approach provides advantages such as customization of secretome for targeted treatment and easier production, storage, and handling compared to live cell cultures (González-González et al., 2020). Secretome use could also greatly reduce the cost and time involved in expansion and maintenance of clonal cell lines (Tran & Damaser, 2015). Furthermore, the administration methods of secretome are less invasive due to its small size, in contrast to stem cell-based therapy, where the large diameter of stem cells poses risk of lung accumulation and subsequent infusion toxicity following intravenous injection (Zhang & Cheng, 2023). In addition, secretome based-therapy circumvents the ethical concerns associated with the use of ESCs in stem cell-based therapy, especially as many of the studies utilize hNSCs generated from fetal tissue (Willis et al., 2020). These collective

attributes suggest that secretome-based therapy holds promising potential as an alternative to conventional stem cell-based therapy in the field of regenerative medicine, offering many advantages without the inherent drawbacks of stem cell-based treatments.

Wnt/ β -Catenin Signaling Pathway

The Wnt/ β -catenin signaling pathway is a highly conserved pathway essential for regulating various cellular processes, including neurogenesis, synaptic plasticity, and neuronal survival. In the CNS, this pathway plays a crucial role in brain development, maintaining NSC populations, and modulating neuroprotective responses in adulthood. Dysregulation of Wnt/ β -catenin signaling has been implicated in several neurodegenerative diseases, particularly AD, where its suppression contributes to synaptic loss, neuronal death, and cognitive decline. Increasing evidence suggests that restoring Wnt signaling could counteract the progression of neurodegeneration by enhancing neuroprotection, promoting synaptic integrity, and mitigating disease-associated pathologies. This section explores the fundamental mechanisms of the Wnt/ β -catenin signaling pathway and its involvement in AD pathology, highlighting its potential as a therapeutic target for neurodegenerative diseases.

Overview of Wnt/ β -Catenin Signaling Pathway

The Wingless/Integrated (Wnt) signaling pathway is an evolutionarily conserved pathway involved in the development of the CNS during embryogenesis and in the maintenance of tissue homeostasis in adulthood (Libro et al., 2016). Additionally, this pathway plays a pivotal role in regulating various cellular functions including growth, polarity, proliferation, differentiation, migration, genetic stability, stem cell renewal, and apoptosis, among others. Due to its multifaceted effects and significance in key biological

processes, disruptions or mutations in the physiological Wnt signaling pathway have been associated with birth defects, cancer, and neurodegenerative diseases, including AD and PD (Anand et al., 2023).

Wnt signaling is classically divided into the canonical Wnt/ β -catenin signaling pathway and the non-canonical Wnt signaling pathway, including the Wnt/planar cell polarity (Wnt/PCP) and the Wnt/calcium (Wnt/ Ca^{2+}) (Komiya & Habas, 2008). In the CNS, compelling evidence suggests that the Wnt signaling pathway regulates different aspects of adult neurogenesis: the canonical Wnt/ β -catenin signaling regulates the maintenance, proliferation, differentiation, and fate determination of NSCs, as well as neurite outgrowth and synapse formation (Clevers et al., 2014; Hirabayashi & Gotoh, 2005; Salinas & Zou, 2008); while the non-canonical Wnt signaling pathway regulates cell polarity, neuronal migration, dendritic development, and synaptic plasticity (Wu & Mlodzik, 2009; Varela-Nallar & Inestrosa, 2013; Ciani & Salinas, 2005). In the CNS, Wnt ligands, secreted by both immune cells and neurons, serve as the primary endogenous activators of the Wnt signaling pathways, which trigger the intracellular Wnt signaling (Libro et al., 2016; Marchetti & Pluchino, 2013). There are two subclasses of Wnt proteins: the Wnt1 and Wnt5a. The Wnt1 proteins, which include Wnt1, Wnt2, Wnt3, Wnt3a, Wnt7a, Wnt8b, and Wnt10b, are involved in the canonical Wnt/ β -catenin signaling pathway (He et al., 2015); whereas, the Wnt5a proteins, which include Wnt4, Wnt5a, and Wnt11, are involved in the non-canonical Wnt signaling pathway (Libro et al., 2016; Willert & Nusse, 2012). Additionally, a diverse evolutionarily conserved activators and inhibitors also contributes to the regulation of Wnt signaling pathway

(Cruciat & Niehrs, 2013). This study will specifically focus on the canonical Wnt/ β -catenin signaling pathway in neurodegenerative diseases, particularly in AD.

In the canonical Wnt/ β -catenin signaling pathway, the Wnt ligand binds to the extracellular N-terminal cysteine-rich domain (CRD) of the Frizzled (Fzd) receptor, a protein characterized by seven-transmembrane domains. This binding initiates the formation of a three-molecule complex with the co-receptor lipoprotein related protein 5/6 (LRP5/6) (Tmai et al., 2000). This receptor complex formation leads to the phosphorylation and activation of the intracellular scaffold protein Dishevelled (Dvl). Dvl then inhibits the destruction complex, which includes tumor suppressor protein Axin, adenomatous polyposis coli (APC), and two constitutively active serine-threonine kinases glycogen synthase kinase-3 β (GSK-3 β) and casein kinase 1 (CK1). This inhibition blocks the phosphorylation of β -catenin by the destruction complex, preventing its degradation by the proteasome. Consequently, unphosphorylated β -catenin accumulates in the cytoplasm and migrate into the nucleus. There, β -catenin binds with the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors, resulting in the transcription of more than 50 Wnt-target genes, such as c-myc, cyclin D1, survivin, Axin2, metalloproteinases, CD44, and PPAR γ . These genes are involved in neurogenesis-related processes, including NSC proliferation, differentiation, maturation, migration, and synaptic, as well as plasticity neuroprotection-related processes, such as apoptosis inhibition, inflammation reduction, and injury repair (Hooper et al., 2007). Conversely, in the absence of the Wnt ligand, CK1 and GSK-3 β within the destruction complex sequentially phosphorylate Axin-bound β -catenin. This phosphorylated β -catenin then undergoes ubiquitination by the E3 ubiquitin ligase, β -transducin repeat containing

protein (β -TrCP), marking it for subsequent proteasomal degradation. As a result, β -catenin nuclear translocation is blocked, leading to a suppression of Wnt-target gene expression (Nusse & Clevers, 2017).

Wnt/ β -Catenin Signaling Pathway in Alzheimer's Disease

AD is a progressive neurodegenerative disease, characterized by the progressive loss of cholinergic neurons and synapses, resulting in memory impairment and cognitive decline, progressing over time to affect behavior, speech, spatial navigation, and motor functions. The major pathological hallmarks of AD are intracellular formation of neurofibrillary tangles (NFTs) and extracellular accumulation of amyloid- β ($A\beta$) plaques (DeTure & Dickson, 2019). The NFTs are primarily composed of hyperphosphorylated microtubule-associated tau proteins in neurons. Normally, tau stabilizes microtubules within neurons, but in AD, it becomes abnormally phosphorylated, detaches from microtubules, and aggregates into insoluble tangles. Accumulation of NFTs inside the neurons disrupts the microtubule stability and cellular transport mechanisms, leading to impaired neurotransmission and synaptic degeneration (Gendron & Petrucelli, 2009). The $A\beta$ plaques are created from the aggregation of $A\beta$ 40 and $A\beta$ 42 peptides in the extracellular and nonvascular spaces of the brain, which are produced by the abnormal sequential cleavage by β -secretase and γ -secretase. A single protein called beta-site APP-cleaving enzyme 1 (BACE-1) is responsible for β -secretase activity, whereas γ -secretase activity involves at least four proteins, including the catalytic subunit presenilin-1 (PS1) (Giliberto et al., 2009). PS1 mutations have been implicated in the early onset familial AD development as it alters the activity of the γ -secretase leading to $A\beta$ overproduction and accumulation. The extracellular accumulation of $A\beta$ plaque in the brain ultimately

results in synaptic dysfunction, neuronal damage, oxidative stress, and neuroinflammation by microglial activation, leading to neuronal death and exacerbating the disease progression (Rischel et al., 2023). These pathological processes results in neurodegeneration characterized by the loss of neurons and synapses, ultimately causing cortical atrophy. Currently, there is no cure for AD, and existing treatments only alleviate the symptoms (Kumar et al., 2023).

The Wnt/ β -catenin signaling pathway regulates various essential cellular processes, including adult neurogenesis, cell migration, neuronal survival, synaptic plasticity, and many other brain functions. However, this pathway is significantly suppressed by multiple pathological mechanisms in AD brain (Figure 1), which contributes to A β -induced synaptic degeneration and subsequent cognitive deficit (Palomer et al., 2019). Folke et al., demonstrated impaired Wnt/ β -catenin signaling in the prefrontal cortex of postmortem AD patients, attributing this impairment to the dysregulation and malfunction of several Wnt pathway components, including LRP6 and GSK-3 β , as well as the upregulation of the Wnt antagonist Dickkopf-1 (DKK1) (Folke et al., 2019; Jia et al., 2019). Specifically, the downregulation of the Wnt co-receptor LRP6 and the consequent loss of LRP6-mediated Wnt/ β -catenin signaling function contribute to synaptic dysfunction and A β pathology in AD. Simultaneously, DKK1, the endogenous canonical Wnt antagonist, is upregulated in AD as studies suggest that the expression of DKK1 can be induced by A β peptides (Elliott et al., 2018; Purro et al., 2012). This DKK1's interaction with LRP6 blocks the assembly of the LRP-Wnt-Fzd ternary complex in response to Wnt ligands, thereby preventing the activation of Wnt/ β -catenin signaling pathway in AD. Unfortunately, a pathogenic positive-feedback loop exists

where the induction of DKK1 expression by A β peptides promotes synapse degeneration and exacerbates the production of more A β peptides. (Elliott et al., 2018). Moreover, elevated levels of GSK-3 β activity have been detected in the AD brain, which could be a result of both the downregulation of LRP6 and the upregulation of DKK1 expression. GSK-3 β is the main kinase in the phosphorylation of tau protein, and its overactivity in the AD brain facilitates the hyperphosphorylation of tau proteins, leading to the formation of NFTs (Hooper et al., 2008; Llorens-Martín et al., 2014). Furthermore, it is suggested that overexpression of GSK-3 β is linked to neurotoxicity, neuronal death, A β -associated microglial-mediated inflammatory responses, and eventually leads to cognitive impairment (Folke et al., 2019). Given the critical role of the Wnt/ β -catenin signaling pathway in maintaining various brain functions and its significant suppression in AD, targeting this pathway presents a promising therapeutic avenue. In fact, compelling evidence from animal models of AD have already demonstrated that activating the Wnt/ β -catenin signaling pathway can counteract neurodegeneration and cognitive impairments induced by A β plaques. Thus, therapeutic interventions that involve compounds that restore the Wnt/ β -catenin signaling pathway in AD, particularly those targeting the Wnt co-receptor LRP6, Wnt antagonist DKK1, and tau regulator GSK-3 β , suggests a promising strategy for AD treatment (De Ferrari et al., 2003; Jia et al., 2019; Liu et al., 2022).

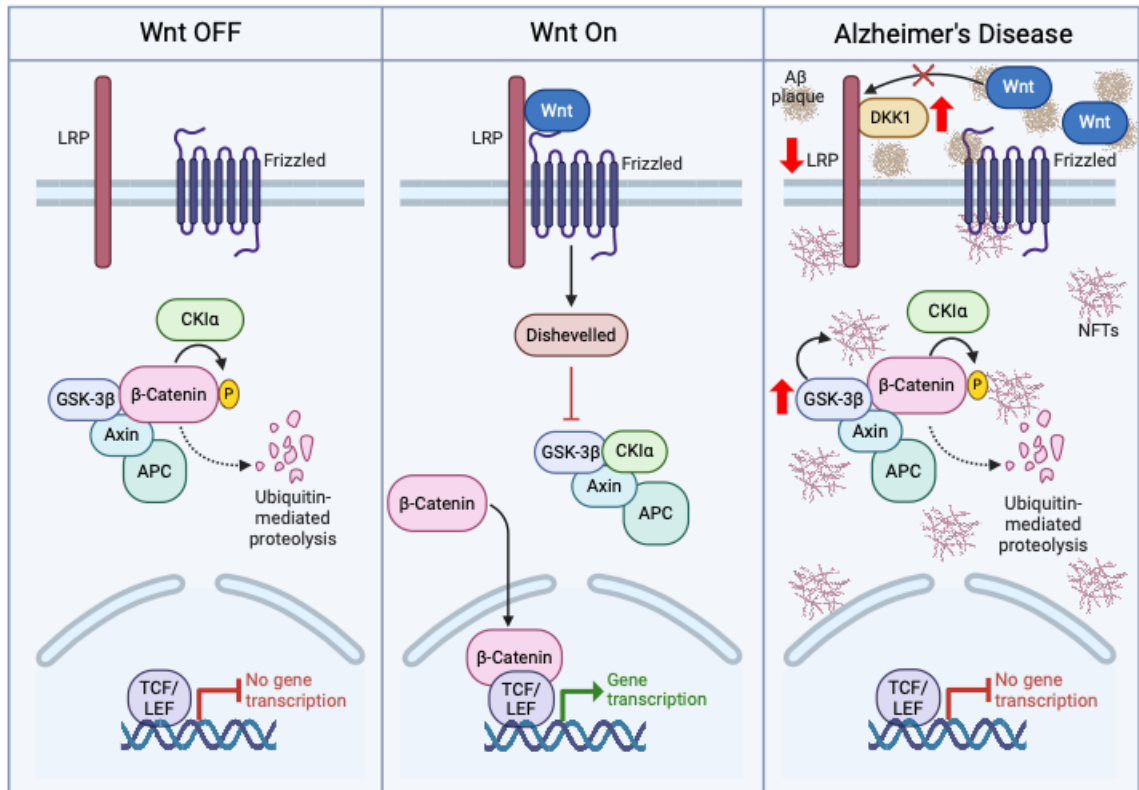


Figure 1. Dysregulation of Wnt/β-catenin Signaling in AD

This figure illustrates the Wnt/β-catenin signaling pathway in its inactive state (left), active state (center), and dysregulated state in AD (right). The left panel depicts the pathway in its inactive state, where the absence of Wnt ligands allows the destruction complex consisting of Axin, APC, CK1a, and GSK-3β, to target β-catenin for ubiquitin-mediated proteolysis. This prevents β-catenin from accumulating in the cytoplasm and translocating into the nucleus, thereby inhibiting gene transcription. The center panel demonstrates the active Wnt/β-catenin signaling pathway. Wnt ligands bind to Frizzled and LRP co-receptors, leading to the recruitment of Dishevelled, which inhibits the destruction complex. This inhibition stabilizes β-catenin, allowing it to accumulate in the cytoplasm, translocate into the nucleus, and interact with TCF/LEF transcription factors to drive the expression of Wnt target genes involved in neurogenesis and synaptic plasticity (Nusse & Clevers, 2017). The right panel depicts the dysregulation of the Wnt/β-catenin signaling pathway in AD. Pathological mechanisms in AD, such as Aβ plaques and NFTs, lead to the upregulation of DKK1, a Wnt antagonist that inhibits LRP6 co-receptor activation. Concurrently, GSK-3β becomes hyperactivated, contributing to excessive β-catenin degradation. This results in lack of β-catenin stabilization and nuclear translocation, silencing TCF/LEF-mediated gene transcription. The dysregulated pathway impairs neurogenesis, synaptic recovery, and neuronal survival, further exacerbating AD pathology. This illustration was created using BioRender.

Neural Stem Cell Secretome: Neurogenesis and Wnt/ β -Catenin Signaling Pathway in Alzheimer's Disease

A recent study demonstrated that the injection of NSC-derived secretome enhanced neurogenesis and stimulated neuronal differentiation of endogenous NSCs in a rodent model of AD by activating the Wnt/ β -catenin signaling pathway. In their recent study, Hijroudi et al. isolated and expanded NSCs from the ganglionic eminences of 14-day-old rodent embryos to harvest NSC-derived conditioned medium (NSC-CM) (Hijroudi et al., 2022). A rodent model of AD induced by A β 1-42 injection, which causes AD-like symptoms and pathology with A β plaque formation, received bilateral ventricular injections of 5 μ l NSC-CM. The researchers then conducted double-immunofluorescence staining for BrdU/Nestin and BrdU/NeuN at two different time points, day 1 and day 35, following the last BrdU administration, to assess effects of NSC-CM injection on the proliferation and differentiation of endogenous NSCs. The results revealed a significant decrease in the number of BrdU/Nestin and BrdU/NeuN double-labeled cells in the untreated NSC-CM AD-induced rodent group compared with the non-AD-induced control group, which indicates that A β plaque formation results in the inhibition of neurogenesis. Conversely, treatment with NSC-CM in AD rodents significantly increased the number of BrdU/Nestin and BrdU/NeuN double-labeled cells, suggesting that the NSC secretome can restore neurogenesis impaired by A β formation in AD via a paracrine mechanism (Figure 2A, 2B). This enhanced neurogenesis was accompanied by improved cognitive performance in learning and memory task in the AD rodents. Furthermore, real-time polymerase chain reaction (RT-PCR) analysis revealed that NSC-CM treatment in AD rodents significantly increased the expression levels of

Wnt/ β -catenin signaling pathway effectors such as Wnt3a, MAPK, extracellular signal-regulated kinases (ERK), phosphoinositide 3-kinases (PI3K), AKT serine/threonine kinase (Akt), and β -catenin genes, which had been downregulated by A β formation, to almost the normal levels observed in non-AD-induced control rodents. Additionally, the administration of NSC-CM decreased the A β -induced overactivation of GSK-3 β when compared with the control rodent groups, resulting in activation of Wnt/ β -catenin signaling pathway (Hijroudi et al., 2022, Figure 2C). Additionally, the administration of NSC-CM decreased the A β -induced overactivation of GSK-3 β when compared with the control rodent groups, resulting in activation of Wnt/ β -catenin signaling pathway (Hijroudi et al., 2022). These results indicate that NSC secretome can effectively counteract the changes induced by A β formation in the Wnt/ β -catenin signaling pathway in AD, which plays a significant role in the pathogenesis of AD. The findings from this study revealed that NSC secretome is capable of regulating adult neurogenesis by activating Wnt/ β -catenin signaling pathway, thereby enhancing the cognitive functions in the AD rodents. However, the precise mechanism through which the NSC secretome activates the Wnt/ β -catenin signaling pathway is unknown. The researchers of this study only hypothesized that these effects are, at least partially, associated with the presence of neurotrophic factors NGF and BDNF in the NSC-CM (Adachi et al., 2007; Hijroudi et al., 2022).

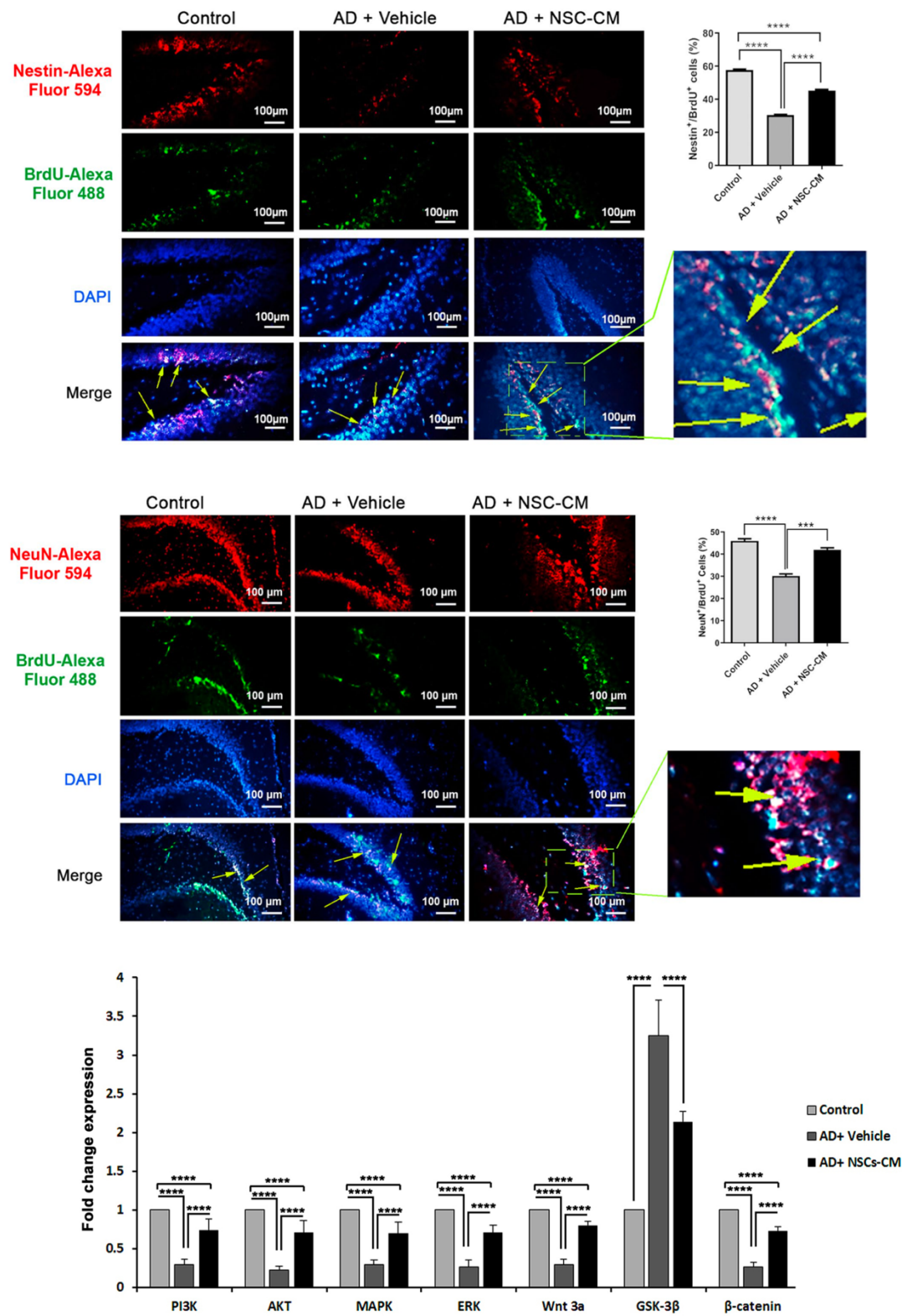


Figure 2. NSCs-CM Injection Enhances Neurogenesis and Modulates Wnt/β-catenin

Signaling in AD Mice: Findings from Hijroudi (2022)

Immunofluorescence imaging and quantitative analysis of double-labeled BrdU/Nestin and BrdU/NeuN cells in the dentate gyrus of hippocampus in AD mice following NSCs-CM injection reveals a significant increase in the percentage of BrdU/Nestin and BrdU/NeuN-positive cells. This demonstrates enhanced proliferation and neurogenesis observed at 1 and 35 days after the injection of NSCs-CM (A, B). This real-time RT-PCR analysis presents the changes in gene expression associated with the Wnt/ β -catenin signaling pathway highlights a restoration of downregulated PI3K Akt, MAPK, ERK, Wnt3a, and β -catenin genes, and a reduction in the overactivity of GSK-3 β , suggesting the role of NSCs-CM in modulating the Wnt/ β -catenin signaling pathway and mitigating AD-associated molecular changes (C). Figures adapted from Hijroudi (2022).

A study conducted by Bahlakeh et al. replicated findings similar to Hijroudi et al. by demonstrating the ability of neurotrophic factor-secreting cells (NTF-SCs), which produce and secrete neurotrophic factors like BDNF and NGF, to enhance neurogenesis and neuronal differentiation in a mouse model of AD via the activation of the Wnt/ β -catenin signaling pathway (Bahlakeh et al., 2022). The study utilized both NTF-SCs and their conditioned medium (NTF-SCs-CM) in AD mouse models induced by A β 1-42 injection, assessing their effects on hippocampal neurogenesis and cognitive function. Double immunofluorescence staining revealed that, much like in the Hijroudi et al. study, there was a significant increase in the number of BrdU/Nestin and BrdU/NeuN double-labeled cells in the SGZ of the hippocampus in AD mice treated with NTF-SCs or NTF-SCs-CM (Figure. 3A, 3B). These results suggest that both approaches can restore neurogenesis impaired by A β plaque formation and other associated pathological mechanisms in AD. The study also demonstrated improved cognitive function in AD mice treated with NTF-SCs or NTF-SCs-CM. The passive avoidance task revealed significantly enhanced memory retention, while the Morris water maze (MWM) test showed reduced escape latency and increase time spent in the target quadrant, indicating

improved spatial learning and memory. Furthermore, real-time RT-PCR analysis corroborated these findings by showing upregulated expression of Wnt/ β -catenin pathway effectors, including PI3K, Akt, MAPK, ERK, Wnt3a, and β -catenin, along with the suppression of GSK-3 β (Bahlakeh et al., 2022, Figure 3C).

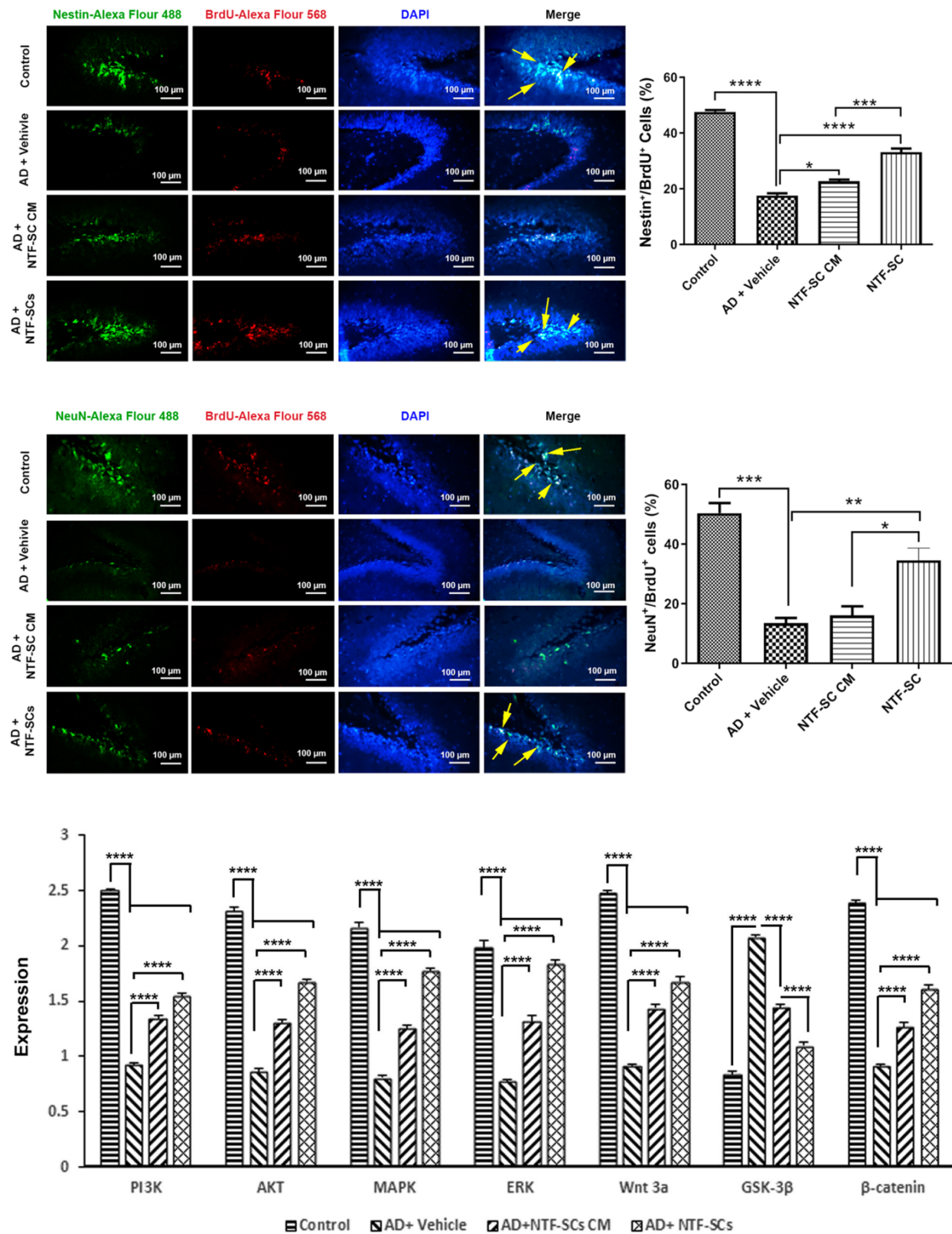


Figure 3. NTF-SCs and NTF-SCs-CM Treatment Promote Neurogenesis and Wnt/ β -catenin Signaling Activation in AD Mice: Findings from Bahlakeh (2022)

Immunofluorescence imaging and quantitative analysis showing a BrdU/Nestin-positive cells increase in AD mice treated with NTF-SCs or NTF-SCs-CM, suggesting enhanced proliferation of endogenous NSCs (A). Increase in BrdU/NeuN-positive cells 35 days post-treatment with NTF-SCs or NTF-SCs-CM, indicating improved neuronal maturation in the treated groups compared to controls (B). Real-time RT-PCR analysis showing that NTF-SCs or NTF-SCs-CM transplantation upregulated the expression of PI3K, Akt, MAPK, ERK, Wnt3a, and β -catenin, while suppressing GSK-3 β in the hippocampus of AD model of mice. These results indicate the activation of the Wnt/ β -catenin signaling pathway, promoting neurogenesis and neuronal survival in AD-related neurotoxic environments (C). Figures adapted from Bahlakeh (2022).

These results closely align with Hijroudi et al.'s findings, as depicted in the similarities between Figure 2 and Figure 3, collectively suggesting the involvement of NSC secretome-derived factors, such as NGF and BDNF, in modulating the Wnt/ β -catenin signaling pathway to restore neurogenesis and cognitive function in AD models. These two recent studies offer important insights into how NSC secretome could be utilized to treat AD. However, while both studies emphasized the importance of activating the Wnt/ β -catenin signaling pathway in AD, they also highlight the need to identify the specific NSC secretome components responsible for this activation and to further investigate the mechanisms by which it occurs. As mentioned above, in AD, pathological factors such as A β plaques increase the expression of DKK1, a potent Wnt antagonist, and decrease the expression of LRP6, a Wnt co-receptor, thereby impairing the typical ligand-receptor binding essential for Wnt/ β -catenin pathway activation. This suggests that NSC secretome components may activate the Wnt/ β -catenin pathway through alternative mechanisms, compensating for the blockade caused by AD pathology.

Importantly, both studies not only performed real-time RT-PCR analysis on the canonical markers of the Wnt/ β -catenin signaling pathway, such as Wnt3a and GSK-3 β , but also included analyses of PI3K, Akt, MAPK, and ERK (Figure 2C, 3C). The

reasoning behind the inclusion of these additional factors in both papers was their roles as downstream mediators of Wnt/ β -catenin signaling, as changes in their expression can indicate the activation of the Wnt/ β -catenin signaling pathway in AD. However, these findings raise the intriguing possibility that PI3K/Akt and MAPK/ERK signaling pathways may themselves be directly activated by components of the NSC secretome and could internally cross-talk with Wnt/ β -catenin signaling, indirectly facilitating its activation even under conditions where typical external Wnt ligand-receptor binding is disrupted by pathological mechanisms in AD.

This hypothesis is supported by evidence that both PI3K/Akt and MAPK/ERK pathways are critical regulators of cellular responses in neurodegeneration, including cell survival, synaptic plasticity, and neurogenesis, all of which are impaired in AD (Li et al., 2023; Liu et al., 2020). Studies have shown that PI3K/Akt signaling can modulate GSK-3 β activity, inhibiting its phosphorylation of β -catenin and thereby stabilizing β -catenin levels to promote nuclear translocation and transcriptional activity (Jere et al., 2023; Kitagishi et al., 2012; Liu et al., 2020). Similarly, MAPK/ERK signaling has been linked to β -catenin activation by regulating transcriptional co-activators and facilitating downstream gene expression required for neuronal differentiation and repair (Zeller et al., 2013). The possibility that the NSC secretome simultaneously activates these pathways, creating a feedback loop or synergy with Wnt/ β -catenin signaling pathway, provides a compelling avenue for further investigation.

Given the therapeutic potential of the NSC secretome in AD, and considering the existing gaps in the literature, the present study aims to create a comprehensive NSC secretome library focusing exclusively on neurotrophic factors and growth factors. These

components are known to play pivotal roles in neurogenesis, neuronal survival, and synaptic plasticity, making them key candidates for therapeutic exploration. This approach will involve detailed categorization and functional annotation of NSC secretome components to uncover their roles in signaling pathway activation.

Additionally, advanced bioinformatics analyses with literature review will be used to map potential interactions and regulatory mechanisms within the pathways, enabling the identification of specific NSC secretome components that may facilitate the crosstalk. By elucidating these mechanisms, the study seeks to provide deeper insights into how NSC secretome components mediate neurogenesis and repair in AD, paving the way for the development of targeted, cell-free therapeutic strategies that restore cognitive function by activating Wnt/ β -catenin signaling pathway through pathway crosstalk.

Problem Statement

AD, the most common neurodegenerative disorder and the seventh leading cause of death in the United States, is characterized by progressive neuronal loss and cognitive decline that eventually disrupt behavior, speech, spatial navigation, and motor functions (Alzheimer's Disease Fact Sheet, 2023; DeTure & Dickson, 2019). The hallmark pathologies of AD include the extracellular accumulation of A β into senile plaques and the intracellular deposition of hyperphosphorylated tau as NFTs, leading to widespread neuronal loss and impaired synaptic plasticity (Liu et al., 2024). Current treatment options include seven FDA-approved medications designed to manage cognitive and behavioral symptoms, along with two FDA-approved immunotherapy drugs approved for treating mild cognitive impairment. However, there is no cure for AD; these treatment strategies, including cholinesterase inhibitors, NMDA antagonists, and immunotherapy

targeting A β pathology, remain limited to symptom management and have yielded little clinical success in halting or reversing disease progression (How Is Alzheimer's Disease Treated?, 2023; Kumar et al., 2023). This limitation underscores the critical need for innovative therapeutic approaches that address the root causes of neurodegeneration, capable of regenerating damaged neural networks and restoring lost neural functions.

Neurogenesis, the process by which new neurons are generated from NSCs, plays a vital role in maintaining brain plasticity, synaptic repair, and cognitive function (Faigle & Song, 2012). In AD, hippocampal neurogenesis is significantly impaired due to multiple pathological changes in AD brain. The Wnt/ β -catenin signaling pathway, a key regulator of neurogenesis and synaptic recovery, is particularly vulnerable to pathophysiology of AD (Arredondo et al., 2020). Among these, compelling evidence suggests that A β peptides and metabolites of amyloid precursor proteins (APP) significantly interfering with the Wnt/ β -catenin signaling pathway by increasing Dickkopf-1 (DKK1) expression, a potent antagonist that inhibits Wnt ligand binding, decreasing lipoprotein receptor-related protein 6 (LRP6) expression, a co-receptor essential for Wnt/ β -catenin signaling activation, and enhancing glycogen synthase kinase-3 β (GSK-3 β) activity, which leads to β -catenin degradation (Jia et al., 2019; Oh et al., 2015). These pathological mechanisms of AD create a significant barrier to initiating neurogenesis and synaptic repair, which are necessary for cognitive recovery (Jia et al., 2019).

NSC transplantation has been explored as a potential therapy for regenerating lost neurons and enhancing neurogenesis to restore cognitive function in AD. However, the clinical translation of NSC transplantation faces significant challenges, including

tumorigenicity, immune rejection, ethical concerns, and the complexities of integrating transplanted cells into the existing neural circuitry (Madhavan & Collier, 2010; Tran & Damaser, 2015; Willis et al., 2020; Zhang et al., 2020). In contrast, the NSC secretome, a collection of bioactive molecules secreted by NSCs, offers a promising cell-free alternative, mitigating the risks associated with introducing live cells into patients. The NSC secretome, which consists of growth factors, neurotrophic factors, and other bioactive molecules secreted by NSCs, has demonstrated therapeutic potential in preclinical studies by promoting neurogenesis, synaptic repair, and neuronal survival (Willis et al., 2020). NSC secretome therapy leverages the paracrine effects of bioactive molecules secreted by NSCs, providing targeted neuroprotection and regeneration without the complexities and risks of live cell transplantation, such as immune rejection or uncontrolled cell proliferation leading to tumor formation (González-González et al., 2020; Tran & Damaser, 2015). Compared to NSC transplantation, NSC secretome therapy circumvents many associated risks while harnessing the regenerative properties of NSCs.

Recent studies by Hijroudi et al. (2022) and Bahlakeh et al. (2022) demonstrated the therapeutic potential of NSC secretome in AD. Hijroudi et al. showed that NSC-conditioned medium (NSC-CM) restored impaired neurogenesis and cognitive function in AD-induced rodent models by activating Wnt/ β -catenin signaling pathway (Hijroudi et al., 2022). Similarly, Bahlakeh et al. highlighted the role of neurotrophic factor-secreting cells (NTF-SCs) and their conditioned medium (NTF-SCs-CM) in enhancing hippocampal neurogenesis and cognitive outcomes, with similar activation of the Wnt/ β -catenin signaling pathway (Bahlakeh et al., 2022). These findings highlight the

therapeutic potential of cell-free, NSC secretome-based strategies in addressing the neurodegenerative and cognitive deficits of AD.

Despite these findings, critical knowledge gaps remain. It is unclear which specific growth factors and neurotrophic factors within the NSC secretome are responsible for activating the Wnt/ β -catenin pathway and through what precise mechanisms these components exert their effects. While previous studies suggest that these secretome components may restore Wnt/ β -catenin signaling by bypassing extracellular inhibition caused by DKK1 and other pathological factors, the molecular details of these interactions remain poorly characterized. Understanding the identity and function of these bioactive molecules is essential for translating NSC secretome therapy into effective, targeted treatments for AD. Furthermore, while the current study focuses on the potential of growth factors and neurotrophic factors in directly activating Wnt/ β -catenin signaling, these interactions may also involve intracellular crosstalk with pathways such as PI3K/Akt and MAPK/ERK. These pathways, though not directly addressed here, are known to intersect with Wnt/ β -catenin signaling and may provide additional therapeutic insights. By narrowing the focus to growth factors and neurotrophic factors within the NSC secretome, this study seeks to address these critical knowledge gaps and establish a molecular framework for advancing cell-free, secretome-based therapies as a novel strategy for treating AD.

Hypothesis

This study hypothesizes that specific growth factors and neurotrophic factors within the NSC secretome activate the Wnt/ β -catenin signaling pathway in AD through crosstalk with intracellular signaling pathways such as PI3K/Akt and MAPK/ERK. This

crosstalk enables the indirect activation of Wnt/ β -catenin signaling, bypassing extracellular inhibition caused by pathological mechanisms, including the upregulation of DKK1 and the downregulation of LRP6. These interactions enhance neurogenesis, synaptic recovery, and cognitive improvement in AD, providing a foundation for developing cell-free, NSC secretome-based therapies as a novel therapeutic strategy for AD.

Objectives

The primary objective of this thesis is to investigate the therapeutic potential of NSC secretome components in modulating the Wnt/ β -catenin signaling pathway and its crosstalk with PI3K/Akt and MAPK/ERK pathways in AD through systematic literature review and bioinformatics analysis. To achieve this, the research aims to:

1. To identify growth factors and neurotrophic factors within the NSC secretome:
Develop a comprehensive library of NSC secretome components, systematically categorizing growth factors and neurotrophic factors involved in key intracellular signaling pathways involved in neurogenesis.
2. To analyze the molecular and functional roles of NSC secretome components: Utilize Gene Ontology (GO) enrichment analysis to examine the cellular, molecular, and biological processes associated with NSC secretome proteins.
3. To investigate signaling pathway activation by NSC secretome components: Assess the involvement of NSC secretome components in activating PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin signaling pathways.
4. To analyze the crosstalk between PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin signaling pathways: Elucidate how NSC secretome components facilitate crosstalk

- among these pathways to stabilize β -catenin, bypass extracellular inhibition, and promote neurogenesis in AD.
5. To validate bioinformatics findings through systematic literature review: Cross-reference bioinformatics predictions with experimental evidence to confirm the activation and crosstalk of key signaling pathways.
 6. To visualize and map the molecular mechanisms influenced by NSC secretome components: Construct pathway interaction maps and network diagrams to illustrate the complex interplay between signaling pathways modulated by NSC secretome components.

These objectives aim to advance the understanding of NSC secretome-mediated signaling mechanisms and establish a molecular foundation for future therapeutic strategies targeting neurodegeneration in AD.

Organization of the Thesis

This thesis is organized into five chapters, each designed to address the research objectives and findings. Chapter 1, Introduction, includes a comprehensive literature review, presenting an in-depth analysis of existing research on NSCs, their therapeutic applications, and the mechanisms of their secretome. It also examines the Wnt/ β -catenin signaling pathway's role in neurogenesis and its dysregulation in AD, highlighting critical knowledge gaps addressed in this study. Additionally, this chapter outlines the problem statement, hypothesis, objectives, and provides overview of the thesis structure. Chapter II, Research Methods, explains the systematic review and bioinformatics methodologies used in the study. It describes the processes for identifying and

categorizing NSC secretome components, performing GO enrichment analysis, signaling pathway analysis, PPI network analysis, and validating bioinformatics findings through targeted literature review. Chapter III, Results, presents key findings from the bioinformatics analyses and literature review, detailing the molecular and functional roles of NSC secretome components, their involvement in signaling pathway activation, and the crosstalk between Wnt/ β -catenin, PI3K/Akt, and MAPK/ERK pathways. Finally, Chapter IV, Discussion, interprets the findings, emphasizing the therapeutic implications of NSC secretome-based modulation of these pathways for AD. It also addresses the challenges, limitations, and gaps in current knowledge, along with recommendations for future research.

Chapter II.

Research Methods

This chapter details the research methods employed to identify and analyze growth factors and neurotrophic factors within the NSC secretome that activate the Wnt/ β -catenin signaling pathway in AD, with a focus on its crosstalk with PI3K/Akt and MAPK/ERK signaling pathway. The primary approach used in this research is a systematic literature review combined with bioinformatic analysis. The aim is to identify growth factors and neurotrophic factors within the NSC secretome, to perform bioinformatics analyses, and to validate findings and visualize mechanisms. This methodological approach facilitates the creation of a comprehensive NSC secretome library of growth factors and neurotrophic factors and pathway interaction maps, providing a detailed understanding of the molecular mechanisms involved in the activation of Wnt/ β -catenin signaling pathways in AD. All data, software tools, and bioinformatic analyses used in this study are sourced from publicly available and open-access resources. Additionally, any custom datasets generated during the course of this research are documented in detail to ensure reproducibility and accessibility for future research.

Systematic Literature Review

The primary objective of this systematic literature review was to construct a preliminary list of NSC secretome components, with an emphasis on those with documented roles in neurogenesis and interactions with signaling pathways relevant to AD, particularly the Wnt/ β -catenin signaling pathway. This review identified and

characterized NSC secretome factors present in NSC-conditioned media (NSC-CM) or NSC-extracellular vesicles (NSC-EVs) based on studies that employed either biased or unbiased methodologies. Only growth factors and neurotrophic factors were analyzed, as the hypothesis suggests that these factors may be involved in the intracellular activation of the Wnt/ β -catenin signaling pathway in AD. This preliminary list served as the foundation for subsequent analyses, including categorization, functional annotation, and bioinformatic analysis.

Systematic Literature Search

A systematic literature review was conducted to explore the diverse compositions and functions of the NSC secretome, with a focus on growth factors and neurotrophic factors involved in signaling pathways relevant to neurogenesis and AD pathology. It aimed to establish a comprehensive library of NSC secretome components, specifically growth factors and neurotrophic factors, that could activate the Wnt/ β -catenin signaling pathway in relation to AD. The literature search was conducted using several key databases, including PubMed, Web of Science, Google Scholar, ScienceDirect, SpringerLink, Frontiers, and HOLLIS, covering a broad range of research across neuroscience, cellular biology, and disease pathology. To refine and focus the search results, Boolean operators such as AND, OR, and NOT were employed, allowing for precise combination and exclusion of search terms. The search terms were grouped into three main categories to target specific areas of interest.

To identify NSC secretome components, the search included terms such as “neural stem cell,” “NSC secretome,” “NSC-derived secretory factors,” “NSC secreted proteins,” “NSC-conditioned medium,” “NSC extracellular vesicles,” “NSC exosomes,” and “proteomics of NSC secretome.” Additional terms focused on NSC-derived growth factors and neurotrophic factors, including “growth factors,” “neurotrophic factors,” and specific examples such as “IGF-1,” “VEGF,” “GDNF,” “NGF,” and “BDNF.” These keywords were essential for identifying growth factors and neurotrophic factors within the NSC secretome.

In examining the Wnt/ β -catenin signaling pathway, search terms such as “Wnt/ β -catenin signaling pathway,” “canonical Wnt signaling pathway,” “Wnt target genes,” “Wnt/ β -catenin signaling in NSCs,” “Wnt,” “GSK-3 β ,” “LPR5/6,” and “DKK1” were used to locate studies detailing the components, mechanisms, and modulation of this pathway. By targeting keywords associated with Wnt/ β -catenin signaling pathway, the search aimed to capture both fundamental studies on canonical signaling mechanisms as well as those specifically exploring pathway activation within NSCs. This focus was critical for identifying potential crosstalk mechanisms by which the NSC secretome could influence Wnt/ β -catenin signaling, particularly under conditions that mimic the pathophysiology of AD.

To address AD and the therapeutic potential of NSC secretome in neurodegeneration, search terms included “neurogenesis,” “neurodegenerative disease,” “Alzheimer’s disease,” “A β plaques,” “NFTs,” “NSC transplantation,” “NSC therapy,” “NSC secretome therapy,” “cell-based therapy,” and “cell-free therapy.” Additionally, terms focusing on signaling pathway interactions in AD, such as “Wnt/ β -catenin signaling in AD,” “PI3K/Akt signaling pathway in AD,” and “MAPK/ERK signaling pathway in AD,” were used to capture studies connecting these molecular pathways with AD pathology. This portion of the search aimed to gather studies that link the molecular roles of growth factors and neurotrophic factors within the NSC secretome and Wnt/ β -catenin signaling to the underlying mechanisms of AD. Emphasis was placed on exploring how AD-related factors, such as A β plaques, NFTs, and DKK1, interact with these pathways, establishing a context for potential therapeutic applications. The integration of findings from these searches provided foundation for identifying NSC

secretome components with promising therapeutic implications for AD, particularly those capable of inducing neurogenesis through activation of the Wnt/ β -catenin signaling pathway.

Inclusion and Exclusion Criteria

This systematic literature review applied specific inclusion and exclusion criteria to ensure the relevance and quality of the selected studies. Inclusion criteria focused on studies involving human NSC secretome, either *in vitro* or *in vivo* models, or comparative analyses that offer insights into human application. NSCs derived from healthy human sources were prioritized to ensure an unaltered secretome composition, maintaining conditions that closely reflect a natural cellular environment. The selection emphasized studies investigating growth factors and neurotrophic factors, aligning with the study's hypothesis that these components activate intracellular signaling pathways (e.g., PI3K/Akt, and MAPK/ERK), leading to Wnt/ β -catenin signaling pathway activation. This activation is believed to promote neurogenesis, neuroprotection, and synaptic plasticity in AD and other neurodegenerative diseases, even when extracellular Wnt/ β -catenin activation is impaired. Only studies investigating the relationship between the NSC secretome and signaling pathways in AD or other neurodegenerative diseases were included, ensuring a specific focus on the neurogenic effects of the NSC secretome in AD. Additionally, studies published between 2000 and 2025 were prioritized to capture the latest advancements, except when historical context or foundational insights were necessary.

Exclusion criteria ruled out studies using NSCs derived from non-human sources unless they provided direct comparisons with human NSCs. Studies employing NSCs derived from cancer cells or disease-exposed environments were excluded, as these could produce an altered secretome profile, affecting reproducibility and therapeutic relevance. Studies were also excluded if they focused on secretome components other than growth factors and neurotrophic factors such as cytokines, chemokines, lipids, miRNA, or extracellular matrix proteins. Secretome studies that lacked relevance to the core focus areas, including neurogenesis, neuroprotection, Wnt/ β -catenin signaling activation, or AD, were not considered. Moreover, studies focused solely on cell replacement therapies or those centered on unrelated mechanisms (e.g., immune regulation or angiogenesis without a direct link to neurogenesis) were excluded. Research with unclear methodologies, inconsistent data, or insufficient validation of secretome components was also omitted to maintain data quality.

Eligible studies included those that utilized unbiased quantitative proteomic approaches, such as mass spectrometry, and RNA sequencing, to analyze NSC secretome derived from hNSC-CM or hNSC-EVs. These unbiased methods were selected to allow for a comprehensive identification of secretome components without prior assumptions, enabling the discovery of a broad range of proteins, including growth factors and neurotrophic factors potentially involved in Wnt/ β -catenin signaling and neurogenesis in AD. Additionally, studies that employed biased methodologies, such as quantitative reverse transcription polymerase chain reaction (qRT-PCR), enzyme-linked immunosorbent assay (ELISA), and Western blotting, were included if they specifically focused on verifying and characterizing growth factors and neurotrophic factors

previously identified through unbiased methods. This integrative approach, consistent with the findings of Willis et al., underscores the value of combining both unbiased and biased techniques for thorough identification and characterization of these secretome components (Willis et al., 2020). This strategy maximizes the identification of diverse growth factors and neurotrophic factors within the NSC secretome while confirming their roles in activating the Wnt/ β -catenin signaling pathway and promoting neurogenesis in AD.

Data Collection and Organization

The NSC secretome components identified through this systematic literature review were compiled and categorized for further analysis. Each component was categorized specifically as growth factors or neurotrophic factors. Other components, such as Wnt ligands, cytokines, and chemokines, were excluded from this categorization to focus specifically on the role of growth factors and neurotrophic factors. This initial categorization was derived directly from the literature search, which served as the preliminary classification for the NSC secretome components. The purpose of this initial categorization is to create a foundational understanding of the NSC secretome's composition, which will be refined later during the bioinformatics analysis phase.

Each NSC secretome component was documented along with its identification technique, whether through unbiased methods (e.g., large-scale proteomic analysis like mass spectrometry) or biased methods (e.g., targeted assays like qRT-PCR). The condition in which each growth factor or neurotrophic factor was identified was also recorded, differentiating between *in vitro* experiments, such as those conducted with NSC-CM, and *in vivo* studies involving animal models of AD or other neurodegenerative

diseases. This distinction provides insights into how these specific components of the NSC secretome behaves in different experimental settings, with potential implications for future translational applications in cell-free or secretome-based therapies for AD. The findings were systematically organized into a preliminary table, which includes the following fields:

- NSC Secretome Component: Lists the name of each identified growth factor or neurotrophic factor within the NSC secretome.
- Category: The specific type of secretome component (e.g., growth factor or neurotrophic factor).
- Technique: The method employed to identify the component, indicating whether it was found through unbiased (e.g., proteomic analysis) or biased (e.g., targeted assays) approaches.
- Condition: Specifies whether the component was detected in *in vitro* (e.g., cell culture experiments) or *in vivo* (e.g., animal models in AD or other neurodegenerative disease) conditions, providing context for its identification.

In addition to the preliminary table, any documented direct or indirect interactions of these growth factors and neurotrophic factors within the NSC secretome with the Wnt/ β -catenin signaling pathway or AD-related pathological markers (e.g., A β plaques and NFTs) were recorded separately. These observations specifically focus on the roles of growth factors and neurotrophic factors in promoting neurogenesis, synaptic recovery, and modulation of AD pathology, providing an additional layer of detail regarding their potential therapeutic impact. Understanding these interactions is key to determining how growth factors and neurotrophic factors within the NSC secretome may activate the

Wnt/ β -catenin pathway, indirectly via crosstalk with other signaling pathways, such as PI3K/Akt or MAPK/ERK, which are involved in intracellular regulation of Wnt/ β -catenin signaling. The initial categorization of growth factors and neurotrophic factors in the preliminary table forms the basis for subsequent bioinformatic analysis, which will further refine the categorization of these components and examine their interactions with intracellular signaling pathways in greater depth. This structured approach ensures that the identified growth factors and neurotrophic factors can be systematically assessed for their role in activating the Wnt/ β -catenin signaling pathway in AD, facilitating a more thorough exploration of pathway crosstalk and potential therapeutic applications.

Bioinformatics Analysis

The primary goal of this phase was to refine and develop the initial NSC secretome list obtained from the systematic literature review using three bioinformatic tools: UniProt, PANTHER, and KEGG. Each tool was used to enhance specific aspects of the table, including accession number, categorization, functional annotation through Gene Ontology (GO) analysis, and signaling pathway analysis, enabling a comprehensive understanding of the NSC secretome components, and their roles in signaling pathways related neurogenesis in AD. Two comprehensive tables were created: the first table includes accession numbers, categories, and functional annotations derived from UniProt and PANTHER, while the second focuses on pathway analysis using KEGG. Additionally, visual representations of the GO analysis results were generated as three pie charts representing the distribution of cellular component, molecular function, and biological process of the NSC secretome components. The resulting comprehensive

datasets highlights NSC secretome components involved in Wnt/ β -catenin signaling pathway activation or its indirect crosstalk with other pathways.

Protein Accession Numbers and Categorization

UniProt, the Universal Protein Resource (<https://www.uniprot.org>), is a comprehensive database that provides detailed information on protein sequences and their functions. It serves as a central repository, integrating data from various sources to offer a unified platform for protein-related information, which includes protein subfamily classifications, subcellular localizations, molecular functions, and biological roles (UniProt Consortium, n.d.). For this study, UniProt served as a primary tool to validate and re-categorize NSC secretome components identified during the systematic literature review and to assign accession numbers for each protein. These steps ensures that the dataset is accurate, standardized, and ready for subsequent bioinformatics analyses.

Accession numbers are unique alphanumeric identifiers assigned to each protein entry in the UniProt database (UniProt Consortium, n.d.). These accession numbers are essential for subsequent bioinformatics analyses, as they serve as standardized references for proteins, enabling researchers to cross-link data across various bioinformatics tools and analyses. To obtain accession numbers, each protein from the preliminary NSC secretome list was manually searched in UniProt using its common name, gene symbol, or protein description derived from the literature review. The search process involved selecting the most relevant UniProt entry based on protein function and associated biological pathways within the *Homo sapiens* species. If multiple entries were found, priority was given to entry that best aligned with the study's focus on Wnt/ β -catenin

signaling pathway activation. The finalized accession numbers were recorded alongside their corresponding protein names in the comprehensive table, providing a standardized reference for all subsequent analyses.

In addition to obtaining accession numbers, UniProt was utilized to refine the categorization of NSC secretome components. Categorization information plays a critical role in understanding the relationships between NSC secretome components and their role in activating the Wnt/ β -catenin signaling pathways. Accurate categorization ensures that each protein is analyzed within the appropriate biological and functional context. For example, Wnt ligand directly activates the Wnt/ β -catenin signaling pathway by binding to Frizzled receptors, while growth factors and neurotrophic factors contribute to neurogenesis and modulate secondary pathways, such as PI3K/Akt or MAPK/ERK signaling pathway, which crosstalk with Wnt/ β -catenin signaling. Categorizing proteins accurately ensures that the functional diversity of the NSC secretome is well-represented, providing foundation for understanding its role in neurogenesis, synaptic recovery, and AD pathology.

Each NSC secretome component was categorized into one of two biologically meaningful groups: growth factors or neurotrophic factors. These categories were chosen for their potential roles in participating in indirect crosstalk with the Wnt/ β -catenin signaling pathway and supporting neurogenesis in AD. For instance, growth factors such as IGF-1 were included for their contributions to intracellular signaling cascades and neuronal survival, while neurotrophic factors like BDNF were grouped for their roles in promoting neurogenesis and synaptic recovery.

In cases where protein could belong to multiple categories, specific criteria were applied to determine the most appropriate classification. Priority was given to the category most relevant to the study's focus on neurogenesis and Wnt/ β -catenin signaling interaction. For example, a protein with dual roles as a cytokine and growth factor was classified based on its predominant function in the central nervous system and its interaction with signaling pathways. Similarly, if a protein demonstrated overlapping characteristics between cytokine and growth factors, it was categorized based on its established role in neurogenesis and its contribution to Wnt/ β -catenin activation. Proteins that could not be confidently categorized or that lacked sufficient annotations were excluded from further analysis.

The accession numbers and categorization obtained through UniProt were systematically recorded in the comprehensive table. This table serves as a foundational resource, providing a standardized and validated reference for the NSC secretome components. It includes columns for accession number and category, which are essential for subsequent analyses, including GO analysis and pathway mapping. By structuring the NSC secretome library around UniProt annotations, this phase ensured that the dataset was both comprehensive and functionally aligned with the study's focus on neurogenesis and Wnt/ β -catenin signaling interactions.

Gene Ontology Enrichment Analysis

Gene Ontology (GO) enrichment analysis is a widely used bioinformatics tool that provides structured annotations for genes or proteins by categorizing them into three primary domains: cellular component, molecular function, and biological process. This

framework helps contextualize gene or protein functions within complex datasets, such as the NSC secretome, identifying their roles in specific cellular locations, biochemical activities, and biological pathways, while also offering insights into their interactions and linking experimental findings to broader biological mechanisms (Tomczak et al., 2018). This study employed GO enrichment analysis to refine and enhance the NSC secretome library by assigning functional and contextual annotations to each component, focusing on their relevance to signaling pathways involved in the activation of Wnt/ β -catenin signaling or its crosstalk with other pathways.

The PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system v.19.0 (<http://pantherdb.org>) is an established bioinformatics tool designed for functional annotation and enrichment analysis of genes and proteins. It integrates GO terms with large-scale protein function and pathway data, enabling researchers to perform comprehensive enrichment analyses. In this study, PANTHER was used to analyze the growth factors and neurotrophic factors identified in the NSC secretome, with a focus on identifying their GO annotations under cellular component, molecular function, and biological process. This step aimed to refine and enhance the dataset, aligning it with the study's emphasis on Wnt/ β -catenin signaling activation and associated pathways that may crosstalk with or influence this signaling pathway.

The three GO domains provide distinct but complementary insights into protein functions. Cellular component refers to the specific subcellular location or structure where a protein is active, such as the extracellular space, cytoplasm, or plasma membrane. Molecular function describes the biochemical activities performed by a protein, such as binding, enzymatic activity, or signal transduction role. Biological

process encompasses the broader physiological or developmental pathways in which a protein participates, such as neurogenesis, signal transduction, or cellular differentiation (Ashburner et al., 2000). Together, these domains provide a multidimensional understanding of each NSC secretome components relevance to signaling and cellular processes.

In this GO enrichment analysis, each protein in the NSC secretome was searched in PANTHER using its UniProt accession number, ensuring consistency and reliability of the data. The organism filter was set to *Homo sapiens* to focus on human proteins, aligning with the study's focus on human NSC secretome and its therapeutic implications for AD. The output consisted of ranked lists of GO terms based on statistical significance, reflecting the relevance of each protein to various cellular components, molecular functions, and biological processes. These lists provided a foundation for interpreting the potential roles of NSC secretome components within signaling pathways.

The selection process for GO annotations followed a structured approach. For the cellular component domain, terms were selected based on the extracellular context of the NSC secretome, as these components are secreted into the extracellular space either in vesicular forms, such as EV, or as soluble factors. Among top three GO hits for cellular component, terms representing the extracellular space were consistently chosen to ensure biological relevance to the function and context of the secretome. For the molecular function and biological process domains, GO terms were chosen based on their direct or indirect relevance to the activation of the Wnt/ β -catenin signaling pathway or other pathways known to crosstalk with canonical Wnt signaling, such as PI3K/Akt or MAPK/ERK. For example, terms associated with receptor binding or kinase activity were

prioritized under molecular function, while biological processes like signal transduction or MAPK signaling pathway were selected if they related to intracellular Wnt/ β -catenin signaling or neurogenesis. In cases where multiple GO terms appeared equally relevant, the term with the lowest P-value (most statistically significant) was chosen. This approach ensured that the most statistically supported and biologically relevant annotations were included in the dataset, enabling accurate determination of which components of the NSC secretome participate in the crosstalk or intracellular activation of the Wnt/ β -catenin signaling pathway. Additionally, for more complex cases where ambiguities arose, UniProt annotations were referenced to resolve these conflicts and verify biological accuracy. This rigorous selection process helped refine and develop the NSC secretome library, allowing for a more precise understanding of the functional roles of its components in activating or influencing the Wnt/ β -catenin signaling pathway. By prioritizing both statistical significance and biological relevance, the study ensured a robust and meaningful dataset to support further analyses of NSC secretome interactions within signaling networks.

Signaling Pathway Analysis

The KEGG, Kyoto Encyclopedia of Genes and Genomes, database (<https://www.genome.jp/kegg/>) is a comprehensive database that integrates genomic, chemical, and systematic functional information to aid in the understanding of complex biological systems and their interactions at the molecular level. Specifically, the KEGG PATHWAY database provides detailed visual representations of molecular interaction networks, including signaling pathways, metabolic processes, and gene regulatory pathways (Kanehisa et al., 2008). Its curated data is especially valuable for mapping

molecular components, such as proteins and genes, to specific biological functions and pathways. For this study, KEGG was utilized to identify the receptors and signaling pathways associated with NSC secretome proteins, with a focus on their role in the PI3K/Akt and MAPK/ERK signaling pathway and their potential to activate the Wnt/ β -catenin signaling pathway via intracellular crosstalk.

To perform pathway analysis, the NSC secretome protein list was first mapped to KEGG gene identifiers (IDs), as KEGG operates using its own gene identification system. This step was conducted using the KEGG Mapper tool, which facilitates the conversion of widely used protein identifiers into KEGG-specific gene IDs. For each protein, the UniProt accession number was entered into KEGG Mapper, which returned the corresponding KEGG gene ID. With the KEGG gene IDs obtained, each protein was then individually queried in the KEGG Pathway database to identify their involvement in signaling pathways. The search process entailed examining signaling pathways associated with each protein, with particular attention to PI3K/Akt signaling pathway and MAPK signaling pathway. When multiple pathways were associated with a single protein, the relevance of each pathway was carefully evaluated based on its alignment with the study's objectives. Priority was given to pathways directly related to Wnt/ β -catenin activation and intracellular signaling pathways known to crosstalk with Wnt/ β -catenin, such as PI3K/Akt and MAPK/ERK. Proteins involved in unrelated pathways were noted but excluded from the primary analysis to maintain focus on the study's objective. As each protein was analyzed, its receptor and associated signaling pathways were identified. Additionally, the NSC secretome proteins were examined within their signaling pathway to map their specific roles in the activation of Wnt/ β -catenin signaling

pathway. This entailed analyzing pathway maps to pinpoint where each protein interacts within the signaling cascade and identifying downstream effectors that influence Wnt/ β -catenin signaling cascade. This mapping provided insights into how NSC secretome proteins may overcome extracellular Wnt inhibition and influence the Wnt/ β -catenin signaling cascade in AD, where antagonists like DKK1 block receptor activation. The data generated through KEGG pathway analysis was compiled into a comprehensive table to summarize the findings. The table includes columns for protein name, KEGG IDs, receptor, and activated signaling pathways. This KEGG pathway analysis was crucial in elucidating the molecular mechanisms by which NSC secretome components produce therapeutic effects in AD.

Protein-Protein Interaction Analysis

The STRING (Search Tool for Retrieval of Interacting Genes/Proteins) database v.12.0 (<https://string-db.org/>) is a comprehensive bioinformatics tool used to explore protein-protein interactions (PPIs). It integrates data from multiple sources, including the scientific literature, computational predictions, experimental interaction databases, and curated pathway resources. STRING provides insights into physical interactions and functional associations between proteins, enabling researchers to visualize complex networks and pathways (Szklarczyk et al., 2023). PPI analysis is a key approach to understanding how proteins within a cellular system interact to carry out biological functions. This analysis helps identify relationships between proteins, map pathways, and uncover mechanisms underlying specific signaling cascades (Farooq et al., 2021). In this study, STRING was utilized to investigate the PPIs between NSC secretome components, their receptors, and key proteins in the PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin

pathways, focusing on how these interactions converge to activate the Wnt/ β -catenin pathway in AD.

The first step involved creating a comprehensive table of proteins associated with the PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin signaling pathways. Both the KEGG database and STRING pathway database were used to identify these proteins. KEGG map from previous section was used to retrieve comprehensive list of proteins involved in each pathway. STRING's pathway enrichment tool further confirmed the relevance of these proteins within their respective signaling networks. Since KEGG identified a large number of proteins for each pathway, for example, 349 proteins were associated with the PI3K/Akt signaling pathway, a careful selection process was conducted to focus on the most important and central proteins. Criteria for selection included functional relevance, known roles in pathway regulation, and potential interactions with Wnt/ β -catenin cascade components. The key proteins were then organized into separate tables for each pathway (Table 1, Table 2, Table 3).

Table 1. Selected Proteins in the PI3K/Akt Signaling Pathway

Category	Protein Name
Receptor Tyrosine Kinase (RTKs)	EGFR, FGFR, PDGFR, VEGFR, INSR
Adaptor Proteins	IRS1, IRS2
Phosphoinositide 3-Kinase (PI3K)	p110 α , p110 β , p110 δ , p110 γ , p85 α , p85 β , p55 γ
Phosphatidylinositol Molecules	PIP2, PIP3
Akt (Protein Kinase B)	Akt1, Akt2, Akt3
Downstream Target of Akt	mTOR, FOXO, Bad, eNOS
Negative Regulators	PTEN, SHIP2

Table 2. Selected Proteins in the MAPK/ERK Signaling Pathway

Category	Protein Name
Receptor Tyrosine Kinases (RTKs)	EGFR, FGFR, PDGFR, VEGFR, INSR
Adaptor Proteins	Grb2, Shc

Category	Protein Name
Guanine Nucleotide Exchange Factor (GEF)	SOS
Small GTPase	Ras (HRAS, KRAS, NRAS)
MAPK Kinase Kinase (MAP3K)	Raf (Raf-1, B-Raf, A-Raf)
MAPK Kinase (MAP2K)	MEK1, MEK2
Mitogen-Activated Protein Kinase (MAPK)	ERK1, ERK2
Transcription Factors	ELK1, c-Fos, c-Myc, SRF
Dual-Specificity Phosphatase (DUSP)	DUSP1
Kinase Suppressor of Ras (KSR)	KSR1

Table 3. Selected Proteins in the Wnt/ β -catenin Signaling Pathway

Category	Protein Name
Wnt Ligands	Wnt1, Wnt2, Wnt3, Wnt3a, Wnt7a, Wnt8b, Wnt10b
Frizzled Receptors (FZD)	FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, FZD10
Co-Receptors	LRP5, LRP6
Dishevelled Proteins (DVL)	DVL1, DVL2, DVL3
Destruction Complex Components	AXIN1, AXIN2, GSK3B, CSNK1A1, APC
β -Catenin	CTNNB1
Transcription Factors	TCF7, TCF7L1, TCF7L2, LEF1
Wnt Antagonists	DKK1, DKK2, DKK4, SOST, SFRP1, SFRP2, WIF1
R-Spondins	RSPO1, RSPO2, RSPO3
E3 Ubiquitin Ligases	ZNRF3, RNF43

Once the protein tables were finalized, the STRING multiple protein analysis tool was utilized to construct interaction networks. The input for this analysis included: (1) NSC secretome proteins, (2) their respective receptors, (3) PI3K/Akt signaling pathway proteins, (4) MAPK/ERK signaling pathway proteins, and (5) Wnt/ β -catenin signaling pathway proteins. The organism was restricted to *Homo sapiens* to ensure human-specific protein interactions. Interaction confidence levels was set to highest confidence (score $\geq$ 0.9) to focus on high-confidence interactions.

STRING generated an interaction network where proteins were represented as nodes and interactions as edges. For better visualization and interpretation, nodes were color-coded according to their pathway or functional category: NSC secretome proteins were colored orange, their respective receptors were colored purple, PI3K/Akt signaling pathway proteins were colored blue, MAPK/ERK pathway proteins were colored green, and Wnt/ β -catenin signaling pathway proteins were colored yellow. This visual differentiation enabled clear identification of how NSC secretome components interacted with signaling pathway proteins.

The analysis focused on examining the interactions between NSC secretome proteins and proteins in the PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin signaling pathways to identify mechanisms supporting β -catenin stabilization and activation of the Wnt/ β -catenin signaling pathway. Particular attention was paid to identifying patterns where NSC secretome proteins influence PI3K/Akt and MAPK/ERK pathways, supporting β -catenin stabilization and Wnt/ β -catenin signaling activation. Specific proteins with the strongest interactions and crosstalk roles were emphasized, such as Akt's role in phosphorylating GSK-3 β and RAS's direct activation of PI3K. These results contribute to the construction of a detailed molecular framework, demonstrating how NSC secretome proteins influence intracellular signaling cascades to activate Wnt/ β -catenin signaling in AD.

Validation and Interpretation Using Systematic Literature Review

The validation and interpretation of bioinformatics results were conducted through a systematic literature review. This approach aimed to identify existing direct and indirect evidence that supports the bioinformatics analyses or highlight gaps where

further experimental studies are necessary to ensure biological and clinical relevance of identified NSC secretome components and their involvement in Wnt/ β -catenin signaling in AD. By systematically reviewing peer-reviewed studies, the literature review provided a foundation to contextualize bioinformatics predictions, assess their alignment with experimental evidence, and identify potential gaps or novel insights. This comprehensive approach strengthened the interpretation of bioinformatics results and established a framework for understanding the mechanisms by which NSC secretome components could activate Wnt/ β -catenin signaling pathway in AD, thereby establishing a strong scientific basis for potential therapeutic strategies.

Targeted Literature Search

To validate the role of growth factors (EGF, FGF2, FGF8, HGF, IGF-1, PDGF, SCF, VEGF) and neurotrophic factors (BDNF, GDNF, NGF, NT-3, NT-4) in activating the Wnt/ β -catenin signaling pathway through crosstalk with PI3K/Akt and MAPK/ERK pathways, a targeted literature search was conducted across multiple scientific databases, including PubMed, Web of Science, Google Scholar, and Hollis. The primary focus was to identify peer-reviewed studies that provide experimental evidence that directly link these factors to the activation of Wnt/ β -catenin signaling, either independently or via their influence on PI3K/Akt or MAPK/ERK pathways. Additionally, research demonstrating molecular crosstalk between these signaling cascades was prioritized to elucidate the interconnected mechanisms through which these factors might exert their therapeutic effects in AD pathology. The search strategy employed specific keywords to capture relevant studies on pathway activation and crosstalk mechanisms. These keywords included combinations such as “[NSC secretome protein] AND Wnt/ β -catenin

signaling pathway activation,” “[NSC secretome protein] AND PI3K/Akt pathway,” and “[NSC secretome protein] AND MAPK/ERK pathway.” To further refine the results, additional keywords like “Crosstalk Wnt/ β -catenin AND PI3K/Akt and MAPK/ERK” and “[NSC secretome protein] AND neurogenesis AND Alzheimer’s Disease” were incorporated. Boolean operators (e.g., AND, OR) were utilized to optimize the search strategy, ensuring comprehensive coverage of studies across diverse experimental models and biological contexts.

Inclusion of Experimental Studies

The inclusion criteria for studies were carefully defined to prioritize experimental evidence that directly examined the activation of Wnt/ β -catenin signaling and its modulation by PI3K/Akt and MAPK/ERK pathways. Specifically, emphasis was placed on studies employing in vitro models, such as neural cell cultures treated with the listed proteins, or in vivo models of AD, including animal studies with experimentally induced neurodegenerative pathology. In addition to AD and neurodegenerative disease, cancer studies were also observed, as crosstalk between Wnt and growth factor signaling pathway is prevalent in the development and progression of cancer (Thompson et al., 2011). These studies were required to employ quantitative assays to measure pathway activation, such as β -catenin nuclear translocation, GSK-3 β phosphorylation, or transcriptional changes in Wnt target genes. Furthermore, experiments documenting the effects of these factors on PI3K/Akt and MAPK/ERK signaling were included to establish mechanistic links between these pathways and Wnt/ β -catenin activation. Only studies providing quantitative data on pathway activation and modulation were considered to ensure that the findings could be integrated into an evidence-based

framework. This approach ensured that the selected studies not only confirmed pathway activation but also provided mechanistic insights into how these factors modulate signaling interactions to restore neurogenesis in AD.

Growth Factors Validation

The validation process for growth factors included identifying studies that demonstrate their role in activating the Wnt/ β -catenin signaling pathway. Particular attention was given to experimental evidence showing stabilization or nuclear translocation of β -catenin upon growth factor stimulation. The study also focused on studies detailing the upstream activation of PI3K/Akt and MAPK/ERK pathways by growth factors, which subsequently inhibit GSK-3 β activity via Akt, a critical step in β -catenin stabilization. Additionally, findings linking growth factor-induced pathway activation to enhanced neurogenesis, synaptic repair, or cognitive improvement in AD models were systematically summarized to provide a comprehensive understanding of their therapeutic potential.

Neurotrophic Factors Validation

For neurotrophic factors, the review focused on studies demonstrating their interaction with specific Trk receptors (e.g., TrkB for BDNF, TrkA for NGF) and subsequent activation of downstream signaling pathways. Evidence of PI3K/Akt and MAPK/ERK pathway activation following receptor engagement was critically evaluated, as these pathways are known to crosstalk with Wnt/ β -catenin signaling. Experimental findings that highlight β -catenin stabilization or enhanced Wnt target gene transcription, such as the upregulation of Cyclin D1 or c-Myc, were prioritized. Studies were also

examined for their demonstration of neurotrophic effects, including increased neuronal survival, reduced apoptosis, and enhanced differentiation of NSCs in the context of AD pathology. The analysis extended to the therapeutic relevance of these factors, particularly their ability to mitigate AD-related pathology such as A β plaques or tau tangles.

Pathway Crosstalk Validation

The study further validated evidence of pathway crosstalk, specifically the convergence of PI3K/Akt and MAPK/ERK signaling on β -catenin stability and nuclear activity. Studies demonstrating phosphorylation and inhibition of GSK-3 β by Akt, leading to β -catenin stabilization, were analyzed in detail. The impact of MAPK/ERK signaling on Wnt/ β -catenin activation through indirect mechanisms, such as Ras-mediated PI3K activation, was also explored. Emphasis was placed on studies reporting functional outcomes, including reduced neurotoxicity, restored synaptic function, or improved cognitive performance in AD animal models following activation of these pathways. Experimental approaches using AD or other neurodegenerative animal models that measured cognitive improvement provided key evidence supporting the therapeutic potential of these signaling interactions.

Interpretation of Results

The systematic literature review provided insights into the role of growth factors and neurotrophic factors in modulating the Wnt/ β -catenin signaling pathway through crosstalk with PI3K/Akt and MAPK/ERK pathways. The study identified existing experimental evidence that supports individual aspects of these mechanisms, such as the

activation of PI3K/Akt and MAPK/ERK pathways or β -catenin stabilization through GSK-3 β inhibition, but did not find direct studies linking these factors to neurogenesis and therapeutic effects via this specific signaling mechanism in the context of AD, as summarized in the table (Table ??). This highlights important gap in the literature, which will be addressed further in the discussion section. Moreover, during the validation of pathway crosstalk, new mechanism was discovered, revealing an alternate mechanism by which NSC secretome components may influence β -catenin activation independently of the canonical Wnt signaling pathway. This novel finding emphasizes the dynamic and multifaceted regulatory potential of NSC secretome components in bypassing extracellular inhibition and activating downstream signaling pathway.

Pathway Visualization

To consolidate the bioinformatics findings and validate the results from the systematic literature review, a comprehensive pathway map was created using BioRender. This map illustrates the interconnectedness of the Wnt/ β -catenin, PI3K/Akt, and MAPK/ERK signaling pathways, emphasizing the role of NSC secretome components in modulating these pathways. It integrates both established and newly identified mechanisms of β -catenin activation, highlighting how NSC secretome-derived growth factors bypass extracellular Wnt inhibition caused by pathological factors. The illustration provides a clear framework for understanding the therapeutic potential of NSC secretome components in promoting neurogenesis and synaptic recovery in AD.

Chapter III.

Results

This chapter presents a comprehensive analysis of the systematic review and bioinformatics findings, highlighting the composition and functional roles of the NSC secretome in AD. First, the chapter identifies and categorizes growth factors and neurotrophic factors within the NSC secretome, detailing their experimental context and detection methodologies. GO analysis further explores the molecular functions, biological processes, and cellular components of these proteins. Subsequent signaling pathway analysis highlights the role of NSC secretome proteins in activating the PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin signaling pathways, with emphasis on their interconnected regulatory mechanisms. PPI network analysis illustrates the intricate crosstalk among these pathways, identifying GSK-3 β and β -catenin as central nodes mediating their interaction. Finally, the chapter explores evidence of NSC secretome-mediated pathway activation and crosstalk, including the newly identified mechanism. Following this, a complete pathway interaction map was created to visually integrate and summarize these mechanisms. Collectively, these findings underscore the therapeutic potential of NSC secretome components in modulating signaling pathways crucial for neurogenesis and cognitive recovery in AD. By integrating systematic literature review, bioinformatics analyses, and pathway validation, this chapter provides a foundational framework for advancing NSC secretome-based therapeutic strategies.

Identification and Categorization of NSC Secretome Proteins

The systematic literature review identified several growth factors and neurotrophic factors as key components of the NSC secretome, each with potential implications for neurogenesis. A total of 13 growth factors were systematically documented, categorized as growth factors or neurotrophic factors, and analyzed based on their experimental context and identification methods (Table 4). The identified growth factors included EGF, FGF2, FGF8, HGF, IGF-1, PDGF, SCF and VEGF, while neurotrophic factors comprised BDNF, GDNF, NGF, NT-3, NT-4 (Červenka et al., 2021; Gómez-Virgilio et al., 2018; Hicks et al., 2013; Huang et al., 2014; Kondo et al., 2014; Lee et al. 2015; Leonov et al., 2023; Li et al., 2021; McGinley et al., 2016; Mendes-Pinheiro et al., 2018; Romanyuk et al., 2015; Upadhyay et al., 2020; Zhu et al., 2022). These components were detected through a combination of methodologies, including unbiased proteomic analysis and targeted approaches such as WB, ELISA, and qPCR. Experimental contexts varied, with the factors being derived from diverse human NSC sources, including fetal-derived NSCs, iPSC-derived NPCs, and human olfactory epithelium-derived NS/PCs, under both *in vitro* and *in vivo* conditions. These findings highlight the diverse and versatile composition of the NSC secretome, emphasizing its potential in promoting neurogenesis and signaling pathway modulation.

Table 4. Library of Growth Factors and Neurotrophic Factors in the NSC Secretome

NSC Secretome Proteins	Category	Type of NSCs	Technique	Condition	Reference
EGF	Growth factors	Human fetal cortical neuroepithelium-derived NSCs	WB, ELISA	<i>in vitro</i>	Hicks et al., 2013

NSC Secretome Proteins	Category	Type of NSCs	Technique	Condition	Reference
FGF2	Growth factors	Human olfactory epithelium-derived NS/PCs	Proteomic analysis	<i>in vitro</i>	Gómez-Virgilio et al., 2018
		Human fetus-derived NSCs	WB	<i>in vivo</i>	Lee et al., 2015
		Human fetal cortical neuroepithelium-derived NSCs	WB, ELISA	<i>in vitro</i>	Hicks et al., 2013
FGF8	Growth factors	Human iPSCs-derived NPCs	qPCR	<i>in vivo</i>	Romanyuk et al., 2015
		Human iPSCs-derived NPCs	Proteomic analysis	<i>in vitro</i>	Leonov et al., 2023
G-CSF	Growth factors	Human ESCs-derived NSCs	xMAP, cytokine 30-plex kit	<i>in vitro</i>	Červenka et al., 2021
GM-CSF	Growth factors	Human ESCs-derived NSCs	xMAP, cytokine 30-plex kit	<i>in vitro</i>	Červenka et al., 2021
HGF	Growth factors	Human ESCs-derived NSCs	xMAP, cytokine 30-plex kit	<i>in vitro</i>	Červenka et al., 2021
HGFL	Growth factors	Glial-rich Human iPSCs-derived NPCs	qPCR	<i>in vivo</i>	Kondo et al., 2014
		Human iPSCs-derived NSCs	MS, ELISA	<i>in vitro, in vivo</i>	Upadhyaya et al., 2020
IGF-1	Growth factors	Human fetal cortex-derived NPCs	IDA, SWATH MS	<i>in vitro</i>	Mendes-Pinheiro et al., 2018
		Human fetus cortical tissue-derived NSCs	ELISA, WB, ICC	<i>in vitro</i>	McGinley et al., 2016
PDGF	Growth factors	Human iPSCs-derived NPCs	Proteomic analysis	<i>in vitro</i>	Leonov et al., 2023
SCF	Growth factors	Human fetal cortex-derived NPCs	IDA, SWATH MS	<i>in vitro</i>	Mendes-Pinheiro et al., 2018
VEGF	Growth factors	Human ESCs-derived NSCs	xMAP, cytokine 30-plex kit	<i>in vitro</i>	Červenka et al., 2021
		Human fetal cortical	qPCR	<i>in vitro</i>	Hicks et al., 2013

NSC Secretome Proteins	Category	Type of NSCs	Technique	Condition	Reference
BDNF	Neurotrophic factors	neuroepithelium- derived NSCs			
		Glial-rich			
		Human iPSCs- derived NPCs	qPCR	<i>in vivo</i>	Kondo et al., 2014
		Human fetus cortical tissue- derived NSCs	ELISA	<i>in vitro</i>	McGinley et al., 2016
		Human iPSCs- derived NPCs	qPCR	<i>in vivo</i>	Romanyuk et al., 2015
		Human fetus- derived NSCs	WB	<i>in vivo</i>	Lee et al., 2015
		Human iPSCs- derived NPCs	Western blotting, qRT-PCR, ELISA	<i>in vitro</i>	Li et al., 2021
		Human iPSCs- derived NSCs	Western blotting, ELISA	<i>in vivo, in vitro</i>	Zhu et al., 2022
		Human iPSCs- derived NPCs	IF	<i>in vivo</i>	Huang et al., 2014
		Human iPSCs- derived NPCs	ELISA, WB, qPCR, ICC	<i>in vivo</i>	Huang et al., 2014
		Human fetal cortex-derived NPCs	IDA, SWATH MS	<i>in vitro</i>	Mendes- Pinheiro et al., 2018
		Human fetus cortical tissue- derived NSCs	ELISA	<i>in vitro</i>	McGinley et al., 2016
		Human iPSCs- derived NPCs	qPCR	<i>in vivo</i>	Romanyuk et al., 2015
		Glial-rich			
GDNF	Neurotrophic factors	Human iPSCs- derived NPCs	qPCR	<i>in vivo</i>	Kondo et al., 2014
		Human fetus- derived NSCs	WB, IHC	<i>in vivo</i>	Lee et al., 2015
		Human fetal cortex-derived NPCs	qPCR	<i>in vivo</i>	Romanyuk et al., 2015
		Human fetal cortex-derived NPCs	qPCR	<i>in vivo</i>	Kondo et al., 2014

NSC Secretome Proteins	Category	Type of NSCs	Technique	Condition	Reference
NGF	Neurotrophic factors	Human fetus-derived NSCs	WB	<i>in vivo</i>	Lee et al., 2015
		Human iPSCs-derived NPCs	qPCR	<i>in vivo</i>	Romanyuk et al., 2015
		Glial-rich Human iPSCs-derived NPCs	qPCR	<i>in vivo</i>	Kondo et al., 2014
		Human fetal cortex-derived NPCs	IDA, SWATH MS	<i>in vitro</i>	Mendes-Pinheiro et al., 2018
		Human fetus cortical tissue-derived NSCs	ELISA	<i>in vitro</i>	McGinley et al., 2016
		Human fetus-derived NSCs	WB	<i>in vivo</i>	Lee et al., 2015
		Fetal cortical neuroepithelium-derived NSCs	WB, ELISA	<i>in vitro</i>	Hicks et al., 2013
		Human iPSCs-derived NPCs	qPCR	<i>in vivo</i>	Romanyuk et al., 2015
		Glial-rich Human iPSCs-derived NPCs	qPCR	<i>in vivo</i>	Kondo et al., 2014
		Human fetus-derived NSCs	WB	<i>in vivo</i>	Lee et al., 2015
		Glial-rich Human iPSCs-derived NPCs	qPCR	<i>in vivo</i>	Kondo et al., 2014
		Human fetus-derived NSCs	WB	<i>in vivo</i>	Lee et al., 2015
		Human iPSCs-derived NPCs	qPCR	<i>in vivo</i>	Romanyuk et al., 2015
		Human olfactory epithelium-derived NS/PCs	Proteomic analysis	<i>in vitro</i>	Gómez-Virgilio et al., 2018
NT-4	Neurotrophic factors	Human fetus-derived NSCs	WB	<i>in vivo</i>	Lee et al., 2015

This table lists the growth factors and neurotrophic factors identified through the systematic literature review of the NSC secretome. Components are categorized as growth factors or neurotrophic factors, along with details on the sources of NSCs, the identification methods employed, the experimental context (in vitro or in vivo), and relevant references.

Gene Ontology (GO) Protein Classification of NSC Secretome Proteins

Using GO analysis, the NSC secretome proteins were classified into three distinct categories: cellular component, molecular function, and biological process (Table 5). Each protein was assigned a UniProt accession number, allowing standardized cross-referencing across bioinformatics tools and ensuring dataset reliability. The cellular component analysis revealed that all proteins localized to the extracellular space, aligning with the paracrine signaling roles of the NSC secretome. The molecular functions of these proteins predominantly included receptor binding, such as EGF receptor binding, NGF receptor binding, and insulin-like growth factor receptor binding, as well as growth factor activity, highlighting their crucial roles in activating signaling pathways. Biological processes associated with the proteins were mainly related to the regulation of intracellular signaling pathways, including MAPK cascade, PI3K/Akt pathway, Ras protein signal transduction, and tyrosine kinase signaling pathways, all of which crosstalk with Wnt/ β -catenin signaling pathways. The GO analysis results were visualized using three pie charts, which illustrate the distribution of the identified proteins across the cellular component, molecular function, and biological process (Figure 4).

Table 5. Functional Classification of NSC Secretome Proteins Based on GO Analysis

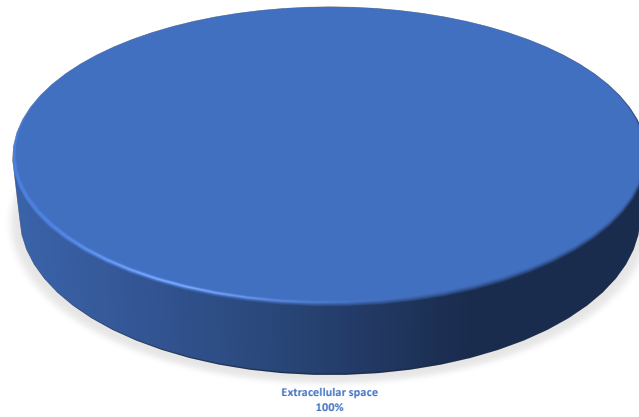
NSC Secretome Proteins	Accession Number	Category	Cellular Component	Molecular Function	Biological Process
EGF	P01133	Growth factor	Extracellular space	Epidermal growth factor receptor binding	Positive regulation of MAPK cascade
FGF2	P09038	Growth factor	Extracellular space	Fibroblast growth factor receptor binding	Positive regulation of MAPK cascade

NSC Secretome Proteins	Accession Number	Category	Cellular Component	Molecular Function	Biological Process
FGF8	P55075	Growth factor	Extracellular space	Fibroblast growth factor receptor binding	Positive regulation of MAPK cascade
HGF	P14210	Growth factor	Extracellular space	Growth factor activity	Positive regulation of PI3K/Akt signal transduction
IGF-1	P05019	Growth factor	Extracellular space	Insulin-like growth factor receptor binding	Positive regulation of MAPK cascade
PDGF	Q9GZP0	Growth factor	Extracellular space	Platelet-derived growth factor receptor binding	Positive regulation of PI3K/Akt signal transduction
SCF	P21583	Growth factor	Extracellular space	Growth factor activity	Positive regulation of Ras protein signal transduction
VEGF	P15692	Growth factor	Extracellular space	Vascular endothelial growth factor receptor binding	Positive regulation of PI3K/Akt signal transduction
BDNF	P23560	Neurotrophic factor	Extracellular space	Nerve growth factor receptor binding	Cell surface receptor protein tyrosine kinase signaling pathway
GDNF	P39905	Neurotrophic factor	Extracellular space	Glial cell-derived neurotrophic factor receptor binding	Glial cell-derived neurotrophic factor receptor signaling pathway
NGF	P01138	Neurotrophic factor	Extracellular space	Nerve growth factor receptor binding	Cell surface receptor protein tyrosine kinase signaling pathway
NT-3	P20783	Neurotrophic factor	Extracellular space	Nerve growth factor receptor binding	Cell surface receptor protein tyrosine kinase signaling pathway

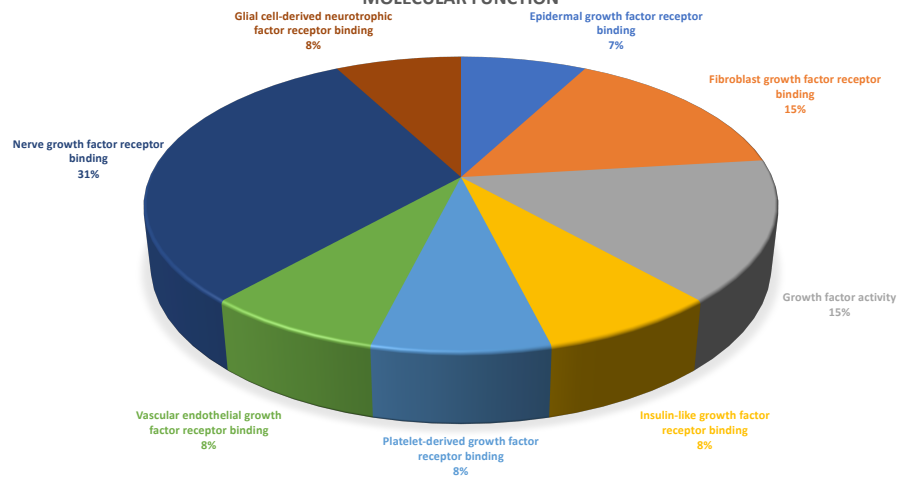
NSC Secretome Proteins	Accession Number	Category	Cellular Component	Molecular Function	Biological Process
NT-4	P34130	Neurotrophic factor	Extracellular space	Nerve growth factor receptor binding	Cell surface receptor protein tyrosine kinase signaling pathway

This table summarizes the functional classification of NSC secretome proteins based on GO analysis. It includes protein names, accession numbers, categories, cellular component, molecular function, and biological process.

CELLULAR COMPONENT



MOLECULAR FUNCTION



BIOLOGICAL PROCESS

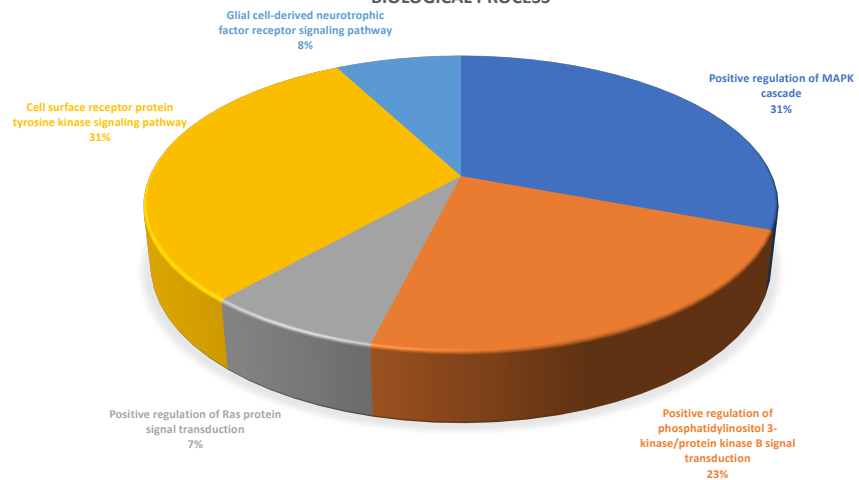


Figure 4. GO Analysis of NSC Secretome Proteins: Cellular Component, Molecular Function, and Biological Process

These pie charts represent the GO Analysis of NSC secretome proteins, categorized into cellular component, molecular function, and biological process. The cellular component chart shows all proteins localized to the extracellular space. The molecular function chart highlights key roles such as receptor binding and growth factor activity, while the biological process chart emphasizes pathways like MAPK cascade regulation and PI3K/Akt signal transduction, illustrating the functional diversity of these proteins.

Signaling Pathway Analysis of NSC Secretome Proteins

The signaling pathway analysis of the NSC secretome proteins revealed that all examined growth factors and neurotrophic factors were involved in the activation of both the PI3K/Akt and MAPK/ERK signaling pathways (Table 6). Specifically, proteins such as EGF, FGF2, FGF8, and HGF, among others, demonstrated activation of these pathways through interactions with their respective receptor tyrosine kinases (RTKs), including EGFR, FGFR, and c-Met. These RTKs served as key mediators, initiating intracellular signaling cascades upon ligand binding. This indicates that RTK-driven pathways are critical to the therapeutic potential of NSC secretome proteins. Additionally, the neurotrophic signaling pathways were also identified as significant contributors to the activation of the PI3K/Akt and MAPK/ERK pathways. Neurotrophic factors such as BDNF and NGF were found to signal through their respected Trk receptors, driving downstream activation of both signaling pathways (Figure 5A). This dual activation underscores the interconnected nature of signaling networks and the potential for neurotrophins to mediate diverse cellular outcomes, including survival, differentiation, and synaptic plasticity.

Further analysis focused on the ability of NSC secretome proteins to influence the Wnt/ β -catenin signaling pathway through intracellular crosstalk. Growth factors and neurotrophic factors activated the PI3K/Akt pathway through their interactions with RTKs (Figure 5B). These interactions triggered the recruitment and activation of PI3K, leading to the phosphorylation of Akt, a key downstream effector. Akt then phosphorylated GSK-3 β , inhibiting its activity. This phosphorylation event was crucial for stabilizing β -catenin, preventing its degradation. The stabilized β -catenin accumulated in the cytoplasm and translocate into the nucleus, where it activated Wnt target gene transcription (Kitagishi et al., 2012). This mechanism highlights how the activation of the PI3K/Akt pathway by growth factors and neurotrophic factors directly supports the Wnt/ β -catenin signaling cascade, effectively overcoming extracellular Wnt inhibitor such as DKK1. This mechanism is particularly relevant in AD, where disruptions in Wnt signaling are common and contribute to disease progression.

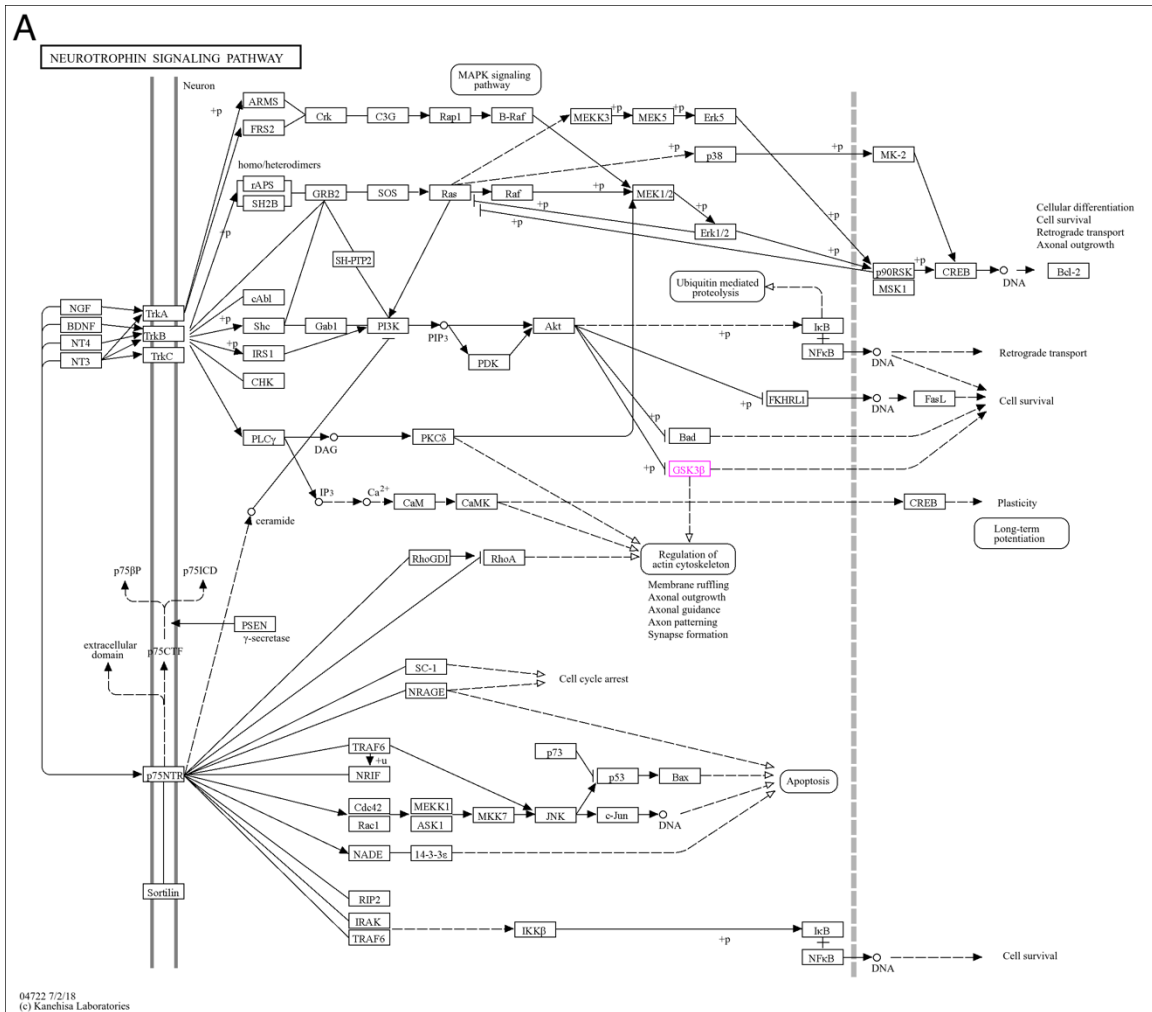
In contrast, the MAPK signaling pathway did not exhibit direct relationship with Wnt/ β -catenin signaling (Figure 5C). Instead, the Ras signaling component of the MAPK pathway exhibited a link to PI3K/Akt activation. Ras activation led to the recruitment and activation of PI3K, which subsequently activated Akt. This indirect connection highlights Ras as an upstream regulator that contributes to the crosstalk between MAPK and Wnt/ β -catenin signaling through its influence on PI3K/Akt activity (Cuesta et al., 2021; Figure 5A, 5B). Collectively, these findings emphasize the prominent role of Akt as a key mediator in the intracellular signaling activation of the Wnt/ β -catenin signaling pathway.

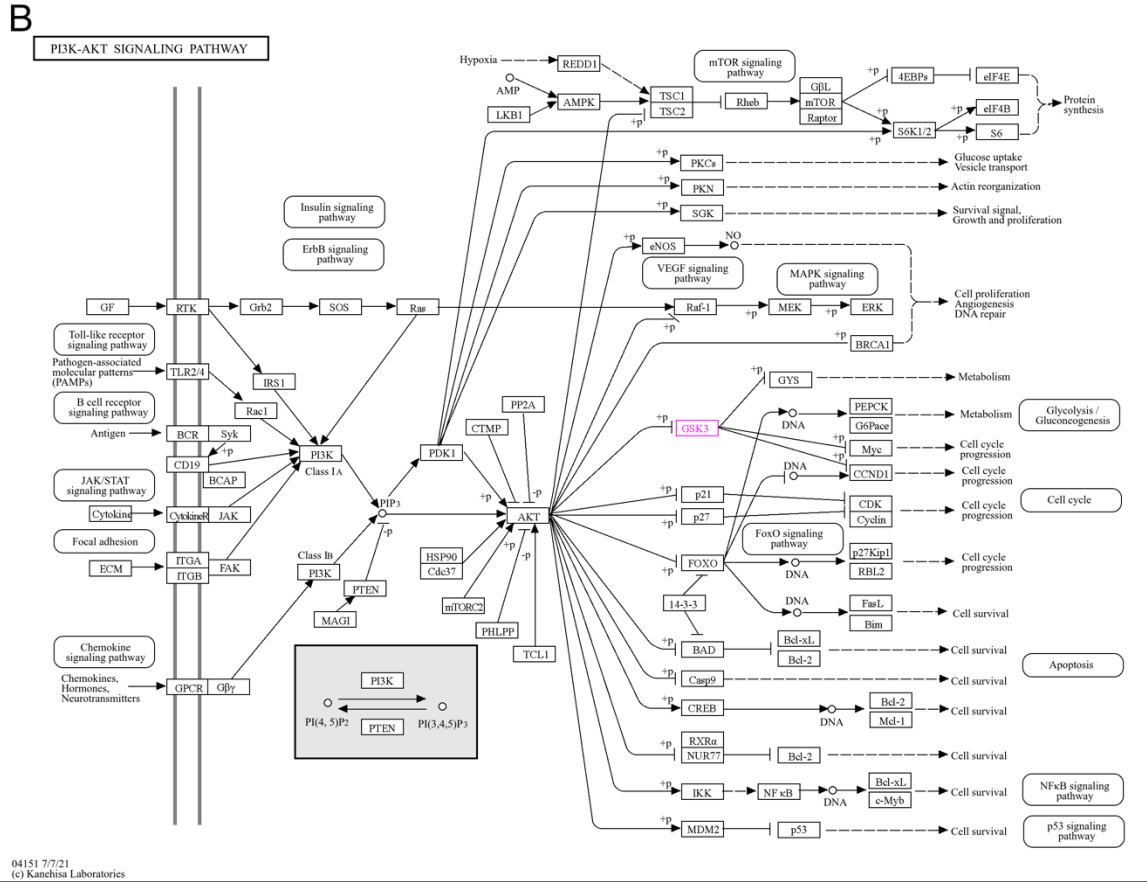
Table 6. Pathway Analysis of NSC Secretome Proteins

NSC Secretome Proteins	KEGG IDs	Receptor	Pathway Activated
EGF	hsa:1950	EGFR	MAPK signaling pathway, ErbB signaling pathway, Ras signaling pathway, Rap1 signaling pathway, Calcium signaling pathway, HIF-1 signaling pathway, FoxO signaling pathway, Phospholipase D signaling pathway, PI3K/Akt signaling pathway, JAK-STAT signaling pathway
FGF2	hsa:2247	FGFR	MAPK signaling pathway, Ras signaling pathway, Rap1 signaling pathway, Calcium signaling pathway, PI3K/Akt signaling pathway
FGF8	hsa:2253	FGFR	MAPK signaling pathway, Ras signaling pathway, Rap1 signaling pathway, Calcium signaling pathway, PI3K/Akt signaling pathway
HGF	hsa:3082	c-Met	MAPK signaling pathway, Ras signaling pathway, Rap1 signaling pathway, Calcium signaling pathway, PI3K/Akt signaling pathway
IGF-1	hsa:3479	IGF-1R	MAPK signaling pathway, Ras signaling pathway, Rap1 signaling pathway, HIF-1 signaling pathway, FoxO signaling pathway, p53 signaling pathway, mTOR signaling pathway, PI3K/Akt signaling pathway, AMPK signaling pathway
PDGF	hsa:80310	PDGFR- β	MAPK signaling pathway, Ras signaling pathway, Rap1 signaling pathway, Calcium signaling pathway, Phospholipase D signaling pathway, PI3K/Akt signaling pathway
SCF	hsa:4254	c-Kit	MAPK signaling pathway, Ras signaling pathway, Rap1 signaling pathway, Phospholipase D signaling pathway, PI3K/Akt signaling pathway
VEGF	hsa:7422	VEGFR	MAPK signaling pathway, Ras, signaling pathway, Rap1 signaling pathway, Calcium signaling pathway, HIF-1 signaling pathway, PI3K/Akt signaling pathway, VEGF signaling pathway, Relaxin signaling pathway
BDNF	hsa:627	TrkB	MAPK signaling pathway, Ras signaling pathway, cAMP signaling pathway, PI3K/Akt signaling pathway, Neurotrophin signaling pathway
GDNF	hsa:2668	RET	MAPK signaling pathway, Calcium signaling pathway, PI3K/Akt signaling pathway
NGF	hsa:4803	TrkA	MAPK signaling pathway, Ras signaling pathway, Rap1 signaling pathway, Calcium signaling pathway, PI3K/Akt signaling pathway, Neurotrophin signaling pathway

NSC Secretome Proteins	KEGG IDs	Receptor	Pathway Activated
NT-3	hsa:4908	TrkC	MAPK signaling pathway, Ras signaling pathway, PI3K/Akt signaling pathway, Neurotrophin signaling pathway
NT-4	hsa:4909	TrkB	MAPK signaling pathway, Ras signaling pathway, PI3K/Akt signaling pathway, Neurotrophin signaling pathway

This table summarizes the signaling pathway analysis of NSC secretome proteins conducted using the KEGG database. The table includes the identified proteins, their corresponding KEGG IDs, receptors they interact with, and the associated signaling pathways activated, with a focus on PI3K/Akt, MAPK/ERK, and their potential crosstalk with the Wnt/ β -catenin signaling pathway.





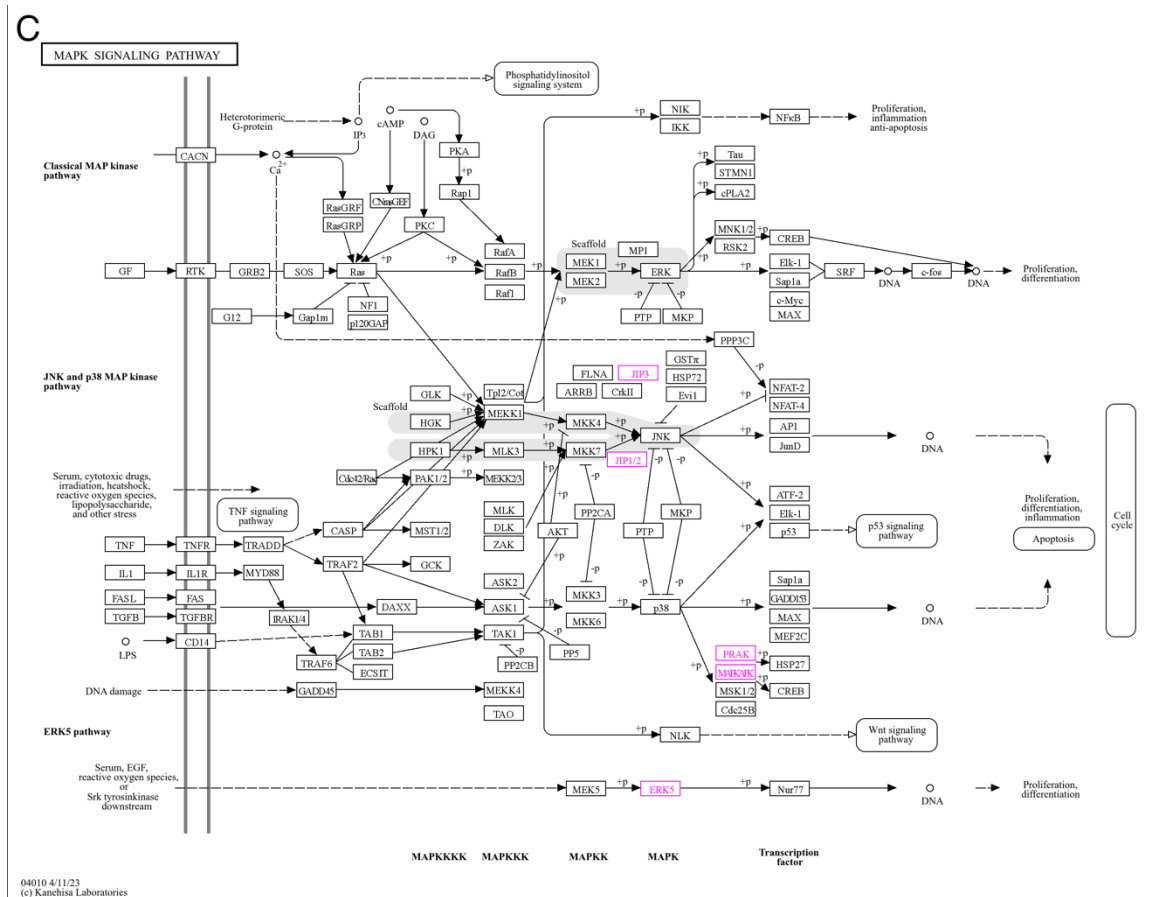


Figure 5. Neurotrophin Signaling Pathway, PI3K/Akt Signaling Pathway, And MAPK Signaling Pathway from KEGG

(A) This figure from KEGG illustrates the neurotrophin signaling pathway, focusing on the activation of the PI3K pathway and its downstream effects. Neurotrophins bind to Trk receptors, leading to the activation of PI3K through adaptor proteins. Activated PI3K generates PIP3, which activates PDK1 and subsequently Akt. Akt plays a crucial role in phosphorylating and inhibiting GSK-3 β , preventing β -catenin degradation and allowing its stabilization and nuclear translocation, where it drives gene transcription (Reichardt, 2006). The figure also depicts the activation of MAPK pathway, which indirectly influences the PI3K/Akt pathway by activating Ras. Ras facilitates PI3K activation, reinforcing the role of Akt in blocking GSK-3 β (Cuesta et al., 2021). (B) This figure from KEGG illustrates the PI3K/Akt signaling pathway, highlighting its central role in cellular survival and intracellular signaling. The pathway is activated when RTK or Ras recruit PI3K to the plasma membrane. PI3K catalyzes the production of PIP3, which serves as a docking site for downstream effectors such as PDK1 and Akt. Activated Akt phosphorylates key targets, including GSK-3 β , inhibiting its activity. This inhibition prevents β -catenin degradation, stabilizing it and allowing its translocation to the

nucleus, where it activates *Wnt/β-catenin* target genes (Kitagishi et al., 2012). The figure also shows how the MAPK pathway, through Ras, contributes to PI3K activation, reinforcing the essential role of Akt in mediating the pathway's downstream effects (Cuesta et al., 2021). (C) This figure from KEGG illustrates the MAPK signaling pathway, which is primarily involved in regulating cell proliferation, differentiation, and stress responses. The pathway is activated by RTKs, leading to the activation of Ras and the subsequent kinase cascade involving Raf, MEK, and ERK (Cuesta et al., 2021). However, there is no direct connection between the MAPK pathway and the *Wnt/β-catenin* signaling pathway. Additionally, while Ras is known to activate PI3K/Akt signaling pathway, this connection is not shown in this figure. Furthermore, the figure highlights the noncanonical MAPK signaling pathway, involving TGF-β-activated kinase 1 (TAK1) and Nemo-like kinase (NLK), which influences the *Wnt* signaling pathway. In this pathway, TAK1 activates NLK, which phosphorylates TCF/LEF transcription factors, preventing their interaction with β-catenin and thus repressing *Wnt/β-catenin* target gene expression. However, this represents an indirect interaction between noncanonical MAPK signaling and the *Wnt* signaling pathway, distinct from the MAPK/ERK signaling pathway (Ishitani et al., 2003).

Protein-Protein Interaction Analysis of NSC Secretome Proteins

The PPI network analysis, conducted at the highest interaction confidence score (0.9) using the STRING database, revealed a complex interaction network comprising of 108 nodes and 633 edges (Figure 6). The network is structured as two distinct but interconnected networks, joined through the key hub proteins GSK-3β and CTNNB1 (β-catenin). The upper-left cluster, predominantly colored yellow, is associated with the *Wnt/β-catenin* signaling pathway, featuring central components such as Wnt ligands, FZD receptors, and DVL proteins. This cluster represents the core regulatory elements of *Wnt* signaling. In contrast, the bottom-right section comprises of blue nodes representing the PI3K/Akt pathway, green nodes representing the MAPK/ERK pathway, orange nodes representing growth factors and neurotrophic factors, and purple nodes representing their receptors. This section illustrates a signaling cascade where five neurotrophic factors, along with their respective receptors, are positioned in the outermost area of the bottom-right section. Additionally, eight growth factors and their receptors are interconnected in

this network. These interactions emphasize the importance of growth and neurotrophic factors in triggering intracellular signaling pathways. Moreover, these growth factors and neurotrophic factors activate both PI3K/Akt and MAPK/ERK pathways, as shown in the figure's tight interaction.

Crosstalk between these pathways is evident, with key nodes such as GSK-3 β , CTNNB1 (β -catenin), and Akt mediating the connection between the PI3K/Akt and Wnt/ β -catenin pathways, and Ras bridging the MAPK/ERK and PI3K/Akt pathways. These interactions suggest a crosstalk of signaling pathways, where Akt phosphorylates and inhibits GSK-3 β , leading to β -catenin stabilization and subsequent activation of the Wnt/ β -catenin pathway.

An unexpected finding in this network is the interaction of MAPK31 (ERK1) and MEK1 (MAP2K1) with GSK-3 β , which was not observed in the previous KEGG pathway analysis. This novel interaction suggests a potential regulation mechanism by which MAPK signaling components influence β -catenin stabilization via GSK-3 β cross-regulation. Overall, this PPI network highlights the intricate interplay among the Wnt/ β -catenin, PI3K/Akt, and MAPK/ERK pathways, offering insights into how these pathways may collectively contribute to cellular signaling dynamics, particularly in the context of AD.



Figure 6. PPI Network Analysis of NSC Secretome at the Highest (0.9) Confidence Score.

The PPI network analysis of the NSC secretome at the highest confidence score (0.9) highlights interactions between signaling pathways. Orange nodes represent growth and neurotrophic factors, purple nodes correspond to their receptors, yellow nodes show Wnt/ β -catenin signaling pathway proteins, blue nodes represent PI3K/Akt signaling pathway proteins, and green nodes indicate MAPK/ERK signaling pathway proteins. The network demonstrates crosstalk between pathways, with growth and neurotrophic factors interacting with their receptors to activate PI3K/Akt and MAPK/ERK pathways, which in turn intersect with Wnt/ β -catenin signaling cascade through key nodes GSK-3 β and CTNNB1. This network highlights the upstream regulatory role of NSC secretome proteins in coordinating intracellular signaling cascades to activate Wnt/ β -catenin signaling pathway.

Pathway Activation by NSC Secretome Proteins

The systematic literature review validated bioinformatics predictions that NSC secretome proteins, including growth factors and neurotrophic factors, can activate key intracellular signaling pathways. As summarized in Table 7, most of the analyzed NSC secretome proteins were capable of activating the Wnt/ β -catenin, PI3K/Akt, and MAPK/ERK pathways (Table 7). Notably, proteins such as EGF, FGF2, FGF8, HGF, IGF-1, PDGF, SCF, VEGF, BDNF, and NGF consistently showed activation across all three pathways. This suggests the potential for crosstalk between these signaling cascades, wherein PI3K/Akt and MAPK/ERK pathways may converge to modulate and stabilize Wnt/ β -catenin signaling.

However, some neurotrophic factors, such as NT-3, and NT-4, no data or studies were found that directly examined their relationship with the Wnt/ β -catenin signaling pathway, despite evidence supporting their role in PI3K/Akt and MAPK/ERK activation. These findings suggest that while these proteins participate in intracellular signaling, their potential influence on Wnt/ β -catenin signaling remains unexplored and may be indirect. Collectively, these results establish a strong foundation for the role of NSC secretome proteins in modulating intracellular signaling pathways critical for neuronal survival and regeneration while also highlighting gaps that warrant further investigation.

Table 7. Pathway Activation by NSC Secretome Proteins

NSC Secretome Proteins	Wnt/ β -catenin Activation	PI3K/Akt Activation	MAPK/ERK Activation
EGF	Yes	Yes	Yes
FGF2	Yes	Yes	Yes
FGF8	Yes	Yes	Yes
HGF	Yes	Yes	Yes
IGF-1	Yes	Yes	Yes
PDGF	Yes	Yes	Yes
SCF	Yes	Yes	Yes

NSC Secretome Proteins	Wnt/ β -catenin Activation	PI3K/Akt Activation	MAPK/ERK Activation
VEGF	Yes	Yes	Yes
BDNF	Yes	Yes	Yes
GDNF	Yes	Yes	Yes
NGF	Yes	Yes	Yes
NT-3	No	Yes	Yes
NT-4	No	Yes	Yes

This table summarizes the ability of NSC secretome components, including growth factors and neurotrophic factors, to activate the Wnt/ β -catenin, PI3K/Akt, and MAPK/ERK signaling pathways. The data is derived from systematic literature review findings that validate bioinformatics predictions of pathway activation. Each protein is assessed for its role in these pathways, independent of specific experimental context. This table establishes the foundational evidence for signaling pathway modulation by NSC secretome proteins.

Crosstalk Between PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin Signaling Pathway

The systematic literature review also explored evidence of crosstalk between PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin signaling pathways, focusing on the capacity of NSC secretome proteins to mediate such interaction as observed in bioinformatics analysis. As shown in Table 8, growth factors and neurotrophic factors such as EGF, FGF2, HGF, IGF-1, BDNF, and NGF demonstrated strong experimental evidence for promoting crosstalk, as highlighted in various models including spinal cord injury, cancer, and healthy conditions (Table 8). For instance, BDNF has been shown to promote human neuronal growth through crosstalk with the Wnt/ β -catenin signaling pathway via GSK-3 β inhibition, stabilizing β -catenin and enhancing neurogenesis (Yang et al., 2015). Similarly, IGF-1 was shown to enhance crosstalk between PI3K/Akt and Wnt/ β -catenin pathways in colorectal cancer models, where simultaneous activation of these pathways through IGF-1 and Wnt3a treatment increased β -catenin transcriptional activity and promoted cell proliferation and migration (Fleming-de-Moraes et al., 2022). This

evidence supports the notion that these proteins facilitate crosstalk by modulating shared molecular targets like GSK-3 β as seen in STRING PPI analysis.

In contrast, growth factors such as PDGF, SCF, and VEGF showed weaker evidence for pathway crosstalk, with studies primarily focusing on their activation of individual pathway but lacking detailed exploration of their interactions with Wnt/ β -catenin signaling pathway. For instance, VEGF has been shown to promote angiogenesis through the PI3K/Akt pathway and β -catenin/Tcf-Lef-dependent transcription; however, this mechanism relies on surviving-mediated amplification rather than direct integration of VEGF into a Wnt-dependent signaling cascade. VEGF-induced β -catenin transcriptional activity appears to depend on PI3K/Akt activation, which stabilizes β -catenin and enhances Tcf-Lef target gene expression, such as VEGF itself and COX-2 (Fernández et al., 2014). This suggests a positive feedback loop amplifying VEGF signaling, but does not establish a direct link between VEGF and canonical Wnt signaling pathways, thus constituting weaker evidence of crosstalk. Furthermore, NT-3, and NT-4 lacked experimental evidence for crosstalk entirely, indicating a significant gap in understanding how these neurotrophic factors may engage with or influence Wnt/ β -catenin signaling pathway.

Importantly, while strong evidence of crosstalk exists in cancer and other models, the review did not identify studies directly examining these crosstalk mechanisms in the context of AD, except for the paper analyzed in this study by Bahlakeh et al. (2022). This absence highlights a critical gap in the literature, particularly regarding the therapeutic implications of these signaling interactions in AD pathology. Further research is needed

to elucidate how NSC secretome proteins modulate these pathways to restore neurogenesis and mitigate neurodegenerative changes in AD.

Table 8. Crosstalk Evidence Between PI3K/Akt and MAPK/ERK with Wnt/ β -catenin Signaling Pathway

NSC Secretome Proteins	Experimental Model	Crosstalk Evidence	Crosstalk Evidence Reference
EGF	Spinal Cord Injury	Strong	(Yang et al., 2016)
FGF2	Healthy Model	Strong	(Israsena et al., 2004; Krejci et al., 2012)
FGF8	Healthy Model	Weak	(Kimura et al., 2022)
HGF	Liver Cancer, Head and Neck Cancer	Strong	(Yassin et al., 2022; Raj et al., 2022)
IGF-1	Colorectal Cancer; Healthy Model	Strong	(Fleming-de-Moraes et al., 2022; Jamwal et al., 2018)
PDGF	Healthy Model	Weak	(Yang et al., 2006)
SCF	Ocular Neovascularization	Weak	(Kim et al., 2019)
VEGF	Healthy Model	Weak	(Fernández et al., 2014)
BDNF	Healthy Model, AD	Strong	(Yang et al., 2015; Yang et al., 2016; Bahlakeh et al., 2022)
GDNF	Irritable Bowel Syndrome	Weak	(Lin et al., 2020)
NGF	AD, Healthy Model	Strong	(Bahlakeh et al., 2022; Krejci et al., 2012)
NT-3	N/A	No Evidence	No Reference Found
NT-4	N/A	No Evidence	No Reference Found

This table summarizes the evidence for molecular crosstalk between PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin signaling pathways mediated by NSC secretome components. It includes the experimental models used to study crosstalk, the strength of evidence supporting such interactions, and references to peer-reviewed studies that provide direct validation to these interactions. While the systematic literature review

identified experimental evidence supporting the activation of PI3K/Akt and MAPK/ERK pathways and their role in β -catenin stabilization through GSK-3 β inhibition, no studies have directly examined this crosstalk in the context of AD, except for the paper analyzed in this study by Bahlakeh et al. (2022). This critical gap highlights the need for further research to explore how these mechanisms may contribute to neurogenesis and therapeutic effects specifically within AD pathology.

Summary of Findings

The systematic literature review verified the bioinformatic analysis results, providing evidence for the activation of the Wnt/ β -catenin signaling pathway mediated by growth factors and neurotrophic factors through their interactions with PI3K/Akt and MAPK/ERK pathways. The review confirmed that eight growth factors – EGF, FGF2, FGF8, HGF, IGF-1, PDGF, SCF, VEGF – and three neurotrophic factors – BDNF, GDNF, NGF – activates Akt in PI3K/Akt pathway, which phosphorylates GSK-3 β , inhibiting its activity (Bahlakeh et al., 2022; Fernández et al., 2014; Fleming-de-Moraes et al., 2022; Israsena et al., 2004; Jamwal et al., 2018; Kim et al., 2019; Kimura et al., 2022; Krejci et al., 2012; Raj et al., 2022; Thompson et al., 2011; Yang et al., 2006; Yang et al., 2015; Yang et al., 2016; Yassin et al., 2022). This inhibition stabilizes β -catenin, enabling its accumulation and nuclear translocation to activate Wnt target genes. Concurrently, Ras from MAPK/ERK pathway promotes the phosphorylation of PIP2 to PIP3, establishing a positive feedback loop that further stabilizes β -catenin and amplifies its activity within the Wnt/ β -catenin signaling pathway (Cuesta et al., 2021; Vidal et al., 2022).

In addition to the well-established crosstalk between the PI3K/Akt and RAS/ERK signaling pathways with GSK-3 β through Akt, the literature review identified a new mechanism where EGF binding to the EGFR receptor activates the MAPK/ERK

pathway, leading to ERK phosphorylating CK2, which subsequently phosphorylates α -catenin at Ser641. This phosphorylation disrupts the interaction between α -catenin and β -catenin within the β -catenin-E-cadherin complex, causing the dissociation of α -catenin. This dissociation enables β -catenin to disengage from E-cadherin and translocate to the nucleus, where it binds to TCF/LEF transcription factors to activate Wnt target genes such as c-Myc and Cyclin D1 (Ji et al., 2009; Lu et al., 2003; Thompson et al., 2011). This mechanism demonstrates a novel, Wnt-independent pathway of β -catenin activation triggered by EGF signaling.

The systematic literature review also highlighted how these signaling interactions collectively enhance β -catenin stabilization and transcriptional activity. These interactions between PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin pathways validated the bioinformatics predictions and uncovered intricate crosstalk mechanisms. For clarity, these interactions were visually illustrated using BioRender (Figure 7), providing a detailed representation of how growth factors, such as EGF in this figure, activate the Wnt/ β -catenin signaling pathway intracellularly through two distinct mechanisms under AD conditions. This figure illustrates the complex interplay between signaling pathways influenced by the NSC secretome, emphasizing its role in activating the Wnt/ β -catenin signaling cascade in AD. The diagram integrates key intracellular pathways, including PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin, with a focus on their functional crosstalk and downstream effects on neurogenesis, synaptic plasticity, and neuronal survival. Growth factors and neurotrophic factors from the NSC secretome, such as BDNF, bind to their respective receptors (e.g., TrkB) on the cell surface, initiating downstream signaling cascades. The PI3K/Akt pathway is activated through recruitment of adaptor proteins

such as GRB2 and SOS, leading to the phosphorylation of PIP2 and PIP3 and subsequent activation of Akt via PDK1. Activated Akt phosphorylates and inhibits GSK-3 β , a negative regulator of β -catenin, thereby stabilizing β -catenin and promoting its nuclear translocation. This nuclear translocation of β -catenin facilitates transcription of target genes involved in neurogenesis, synaptic recovery, and cellular survival. Simultaneously, the MAPK/ERK pathway is activated through Ras-GTP binding, which phosphorylates and activates Raf, MEK, and ultimately ERK. While the MAPK/ERK pathway does not directly activate Wnt/ β -catenin signaling, Ras serves as a critical crosstalk node by supporting the PI3K/Akt activation. This interplay reinforces β -catenin stabilization and nuclear translocation. In addition to the well-established crosstalk between the PI3K/Akt and Ras/ERK signaling pathways with GSK-3 β through Akt, the figure highlights an alternate mechanism identified through literature review. EGF binding to the EGFR receptor activates the MAPK/ERK pathway, which leads to ERK phosphorylating CK2 (Thompson et al., 2011). CK2 subsequently phosphorylates α -catenin, disrupting its interaction with β -catenin within the β -catenin–E-cadherin complex. This phosphorylation event causes the dissociation of α -catenin from the complex, allowing β -catenin to disengage from E-cadherin at the cell membrane. Freed β -catenin translocates to the nucleus, where it engages with transcription factors like TCF/LEF to promote the expression of genes involved in neurogenesis, synaptic plasticity, and neuronal survival. This novel pathway provides an alternative mechanism for β -catenin activation, complementing the PI3K/Akt-mediated inhibition of GSK-3 β . The Wnt/ β -catenin signaling pathway is shown under conditions mimicking AD pathology, where A β plaques and neurofibrillary tangles disrupt extracellular Wnt ligand binding by

upregulating DKK1, a known antagonist of LRP receptors (Ji et al., 2009; Lu et al., 2003; Thompson et al., 2011). Despite extracellular inhibition, intracellular activation of β -catenin is achieved via PI3K/Akt-mediated GSK-3 β inhibition and through the newly described mechanism involving ERK-CK2- α -catenin interaction. The diagram also highlights downstream outcomes of mTORC1 and ERK, which support cell proliferation, growth, and survival, complementing β -catenin's role in neurogenesis and synaptic plasticity. This integrated pathway map provides a mechanistic framework for understanding how NSC secretome components bypass extracellular Wnt inhibition in AD, emphasizing their therapeutic potential in promoting neuronal recovery and combating neurodegeneration. Furthermore, this visualization underscores how NSC-secreted factors modulate multiple signaling cascades simultaneously, reinforcing the therapeutic potential of the NSC secretome in restoring neurogenesis in AD.

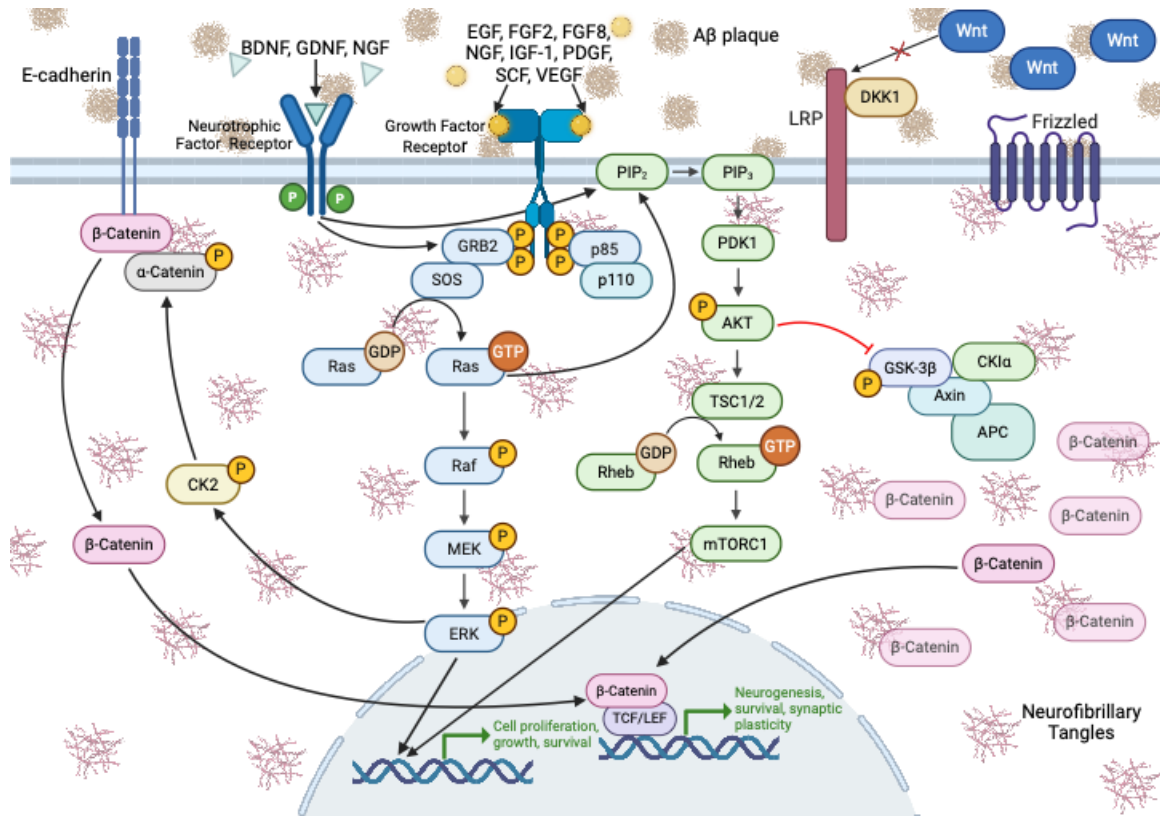


Figure 7. Comprehensive Mechanism of NSC Secretome-Mediated Crosstalk Between PI3K/Akt, MAPK/ERK, and Wnt/β-catenin Pathways in AD

This figure illustrates the complex interplay between signaling pathways influenced by the NSC secretome, with emphasis on its role in activating the Wnt/β-catenin cascade in AD. It integrates key intracellular pathways—PI3K/Akt, MAPK/ERK, and Wnt/β-catenin—highlighting their crosstalk and downstream effects on neurogenesis, synaptic plasticity, and neuronal survival. NSC-secreted growth factors like BDNF bind to receptors (e.g., TrkB), triggering cascades that activate PI3K/Akt and inhibit GSK-3β, stabilizing β-catenin and promoting its nuclear translocation. Concurrently, MAPK/ERK is activated via Ras, indirectly reinforcing β-catenin signaling through PI3K/Akt. The figure also presents an alternate mechanism: EGF activates EGFR, initiating ERK-driven phosphorylation of CK2, which then phosphorylates α-catenin, disrupting the β-catenin–E-cadherin complex and releasing β-catenin for nuclear translocation. Despite extracellular Wnt inhibition in AD via DKK1 upregulation, intracellular activation of β-catenin proceeds via both PI3K/Akt-mediated GSK-3β inhibition and ERK–CK2–α-catenin signaling. Downstream, mTORC1 and ERK promote cell survival and plasticity, reinforcing the therapeutic potential of the NSC secretome in bypassing Wnt blockade and supporting neuronal recovery in AD. This illustration was created using BioRender.

Chapter IV.

Discussion

AD is a progressive neurodegenerative disorder characterized by memory loss, cognitive decline, and behavioral changes. It is estimated that nearly 6.9 million Americans aged 65 and older are living with AD in 2024 (“2024 Alzheimer’s disease facts and figures,” 2024). Decades of research have significantly advanced our understanding of the disease; however, despite this progress, a definitive cure remains elusive, highlighting the urgent need for effective therapies. The brain atrophy of the hippocampus, caused by neuronal loss, and impaired neurogenesis are prominent pathological hallmarks of AD (Niikura et al., 2006). Although the exact mechanisms of altered neurogenesis in AD are not fully understood, numerous studies have identified several key factors and signaling pathways involved in the regulation of neurogenesis (Gonçalves et al., 2016). Among the identified signaling pathways, Wnt/ β -catenin signaling plays a crucial role in adult neurogenesis by regulating the proliferation, survival, and differentiation of NSCs within the adult brain, primarily in the hippocampus. This regulation is achieved by influencing gene expression through the accumulation of β -catenin in the nucleus and activation of target genes (Westphal et al., 2022). Its activation not only plays a crucial role in neurogenesis, but also plays critical roles in regulating processes like synaptic plasticity, blood-brain barrier integrity, and astrocyte maturation, all of which are essential for normal brain function making it a potential therapeutic target for AD (Jia et al., 2019). Recent studies have explored various

approaches to promote neurogenesis and alleviate cognitive and behavioral symptoms in AD. While NSC transplantation has demonstrated potential to replace lost and damaged neurons, with compelling evidence from experimental animal models and early-phase clinical trials demonstrating positive outcomes, its clinical applications have been slow due to stem cell-associated risks, particularly tumorigenicity and immunogenicity (Ghaffari et al., 2016; Willis et al., 2020). As an alternative, cell-free therapy using the NSC secretome, which contain range of bioactive molecules capable of modulating cellular and molecular pathways crucial for neurogenesis, has emerged as a promising approach. Hijroudi et al. (2022) demonstrated that the secretome derived from NSCs enhanced neurogenesis and cognitive performance in a mouse model of AD by activating the Wnt/ β -catenin signaling pathway. These effects are evident through the regulation of key genes and proteins involved in neurogenesis and synaptic repair, as well as the reduction of neurotoxicity and cell death induced by A β plaques (Figure 1). Similarly, a study reported that the transplantation of NTF-SCs restored hippocampal neurogenesis and improved cognition in AD mice model through the activation of the Wnt/ β -catenin signaling pathway (Bahlakeh et al., 2022; Figure 2). Researchers suggest that these effects, at least in part, may be attributed to the presence of specific growth factors within the NSC secretome. However, despite these promising outcomes of NSC secretome, the specific growth factors and neurotrophic factors within the NSC secretome that activates the Wnt/ β -catenin signaling pathway remains unidentified and the underlying molecular mechanisms remain unknown, necessitating further research of the composition of NSC secretome and its precise mechanisms of action. The present study aimed to identify the components of the NSC secretome, specifically growth factors and neurotrophic factors,

that are responsible for activating the Wnt/ β -catenin signaling pathway in AD, thereby enhancing neurogenesis, and to investigate their potential mechanisms of action through a systematic literature review and bioinformatics analysis.

Summary of Key Findings

The study identified eight growth factors – EGF, FGF2, FGF8, HGF, IGF-1, PDGF, SCF, VEGF – and three neurotrophic factors – BDNF, GDNF, NGF – within the NSC secretome that play a crucial role in activating the Wnt/ β -catenin signaling pathway through Wnt-independent mechanisms in AD. Given that Wnt/ β -catenin signaling is significantly suppressed in AD, largely due to A β plaques and NFTs, which upregulate DKK1, downregulate LRP6, and hyperactivate GSK-3 β , alternative intracellular pathways are crucial to bypass extracellular Wnt inhibitory mechanisms (Jia et al., 2019). This study reveals that NSC secretome-derived factors can activate β -catenin stabilization through intracellular crosstalk mechanisms involving PI3K/Akt and MAPK/ERK pathways, providing a potential therapeutic approach to restore neurogenesis in AD.

The research approach began with a systematic literature review, which identified a total of 13 growth and neurotrophic factors secreted by NSCs, specifically eight growth factors – EGF, FGF2, FGF8, HGF, IGF-1, PDGF, SCF, VEGF – and five neurotrophic factors – BDNF, GDNF, NGF, NT-3, NT-4 (Červenka et al., 2021; Gómez-Virgilio et al., 2018; Hicks et al., 2013; Huang et al., 2014; Kondo et al., 2014; Lee et al. 2015; Leonov et al., 2023; Li et al., 2021; McGinley et al., 2016; Mendes-Pinheiro et al., 2018; Romanyuk et al., 2015; Upadhyay et al., 2020; Zhu et al., 2022). These growth and neurotrophic factors contribute to various neurogenesis-related processes, including neuronal proliferation, migration, differentiation, integration, and maturation (Shohayeb

et al., 2018). Given their ability to regulate neurogenesis, they hold significant potential for counteracting neurodegeneration in AD. Following their identification, bioinformatics analysis was performed to categorize these proteins and explore their functional relevance. UniProt was used to assign accession number and classify each factor based on its function and source, while PANTHER GO analysis confirmed that all identified proteins are extracellular, primarily involved in receptor binding and growth factor activity, and functionally regulate PI3K/Akt and MAPK/ERK signaling pathways. KEGG analysis further mapped these proteins to their respective signaling cascades, verifying their involvement in neurogenesis related pathways. Finally, STRING PPI network analysis revealed extensive interactions and crosstalk among these pathways, with GSK-3 β and β -catenin emerging as central nodes linking PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin pathways. Notably, an unexpected interaction between MAPK/ERK components, ERK1 and MEK1, and GSK-3 β was identified, which had not been observed in KEGG pathway analysis, suggesting a previously unexplored regulatory mechanism in β -catenin stabilization.

To validate these bioinformatics predictions, a targeted systematic literature review was conducted to identify experimental evidence supporting the role of these growth factors and neurotrophic factors in Wnt-independent β -catenin activation. The review confirmed that eight growth factors – EGF, FGF2, FGF8, HGF, IGF-1, PDGF, SCF, VEGF – and three neurotrophic factors – BDNF, GDNF, NGF – identified in the study activate the Wnt/ β -catenin signaling pathway through PI3K/Akt and MAPK/ERK crosstalk (Bahlakeh et al., 2022; Fernández et al., 2014; Fleming-de-Moraes et al., 2022; Israsena et al., 2004; Jamwal et al., 2018; Kim et al., 2019; Kimura et al., 2022; Krejci et

al., 2012; Raj et al., 2022; Thompson et al., 2011; Yang et al., 2006; Yang et al., 2015; Yang et al., 2016; Yassin et al., 2022). However, no direct evidence was found linking NT-3 and NT-4 to Wnt/ β -catenin signaling, highlighting an area for further research. Experimental studies confirmed that NSC secretome-derived proteins, growth factors and neurotrophic factors, stabilize β -catenin through two primary mechanisms: (1) direct PI3K/Akt inhibition of GSK-3 β , and (2) indirect MAPK/ERK activation of PI3K/Akt via Ras signaling (Cuesta et al., 2021; Vidal et al., 2022). This suggests that both pathways contribute to β -catenin stabilization in an Akt-dependent manner, ultimately leading to Wnt-independent activation of Wnt/ β -catenin target genes. Additionally, another mechanism was identified in which EGF-EGFR activation of MAPK/ERK triggers CK2-mediated phosphorylation of α -catenin, which facilitates β -catenin release from the E-cadherin complex, allowing its nuclear translocation. Together, these findings reveal two direct and one indirect crosstalk mechanism that enable Wnt/ β -catenin activation in the absence of extracellular Wnt ligands in AD:

1. Direct Crosstalk Mechanism: PI3K/Akt-mediated β -catenin Stabilization. The first direct mechanism occurs when growth and neurotrophic factors bind to their respective receptors (RTKs, Trk receptors), activating PI3K/Akt signaling. This leads to Akt phosphorylation of GSK-3 β , inhibiting its activity, thereby preventing β -catenin degradation and promoting its nuclear translocation to activate Wnt target genes (Yang et al., 2015; Thompson et al., 2011).
2. Direct Crosstalk Mechanism: EGF-EGFR-ERK-CK2- α -catenin Pathway. The second direct mechanism follows a separate intracellular route. Here, EGF binding to EGFR activates ERK signaling, which phosphorylates CK2, leading to

CK2-mediated phosphorylation of α -catenin. This disrupts α -catenin's binding to β -catenin, allowing β -catenin to disengage from the E-cadherin complex and translocate to the nucleus, thereby activating Wnt target genes without direct extracellular Wnt activation (Ji et al., 2009; Lu et al., 2003; Thompson et al., 2011).

3. Indirect Crosstalk Mechanism: MAPK/ERK Activation of PI3K/Akt via Ras. The indirect crosstalk mechanism identified in this study involves MAPK/ERK activation of PI3K/Akt via Ras. In this pathway, Ras activation leads to the recruitment of PI3K, which in turn activates Akt. This supports the inhibition of GSK-3 β , further stabilizing β -catenin through an indirect route. Since Ras is an upstream regulator of both PI3K/Akt and MAPK/ERK, this mechanism reinforces β -catenin stabilization and neurogenesis indirectly through pathway convergence (Cao et al., 2019; Thompson et al., 2011).

Overall, these findings provide novel insights into how NSC secretome-derived factors can bypass extracellular Wnt inhibition in AD and activate β -catenin stabilization through intracellular crosstalk mechanisms. The identification of multiple Wnt-independent activation pathways highlights the therapeutic potential of NSC secretome-based interventions for restoring neurogenesis in AD.

Interpretation of Results in Context

The findings of this study build upon previous research that demonstrated the ability of NSC-derived secretome to enhance neurogenesis and cognitive function in AD models, primarily through activation of the Wnt/ β -catenin signaling pathway (Bahlakeh

et al., 2022; Hijroudi et al., 2022). However, these studies did not identify the specific growth factors and neurotrophic factors within the NSC secretome responsible for this effect, leaving a critical gap in understanding the molecular composition driving these neurogenic outcomes. This study addresses this gap by systematically identifying eight growth factors – EGF, FGF2, FGF8, HGF, IGF-1, PDGF, SCF, VEGF – and three neurotrophic factors – BDNF, GDNF, NGF – within the NSC secretome that are implicated in activating Wnt/ β -catenin signaling and promoting neurogenesis in the context of AD. Additionally, while both Hijroudi et al. (2022) and Bahlakeh et al., (2022) reported increased expression of PI3K, Akt, MAPK, and ERK following NSC secretome treatment, neither study provided a detail mechanistic framework explaining how these pathways interact to regulate Wnt/ β -catenin signaling. The present study addresses this limitation by elucidating two direct and one indirect crosstalk between PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin signaling pathways in AD. By elucidating these interactions, this study provides a more comprehensive molecular framework for how NSC secretome components regulate neurogenesis in AD, filling a critical knowledge gap left by previous research and offering new therapeutic target for AD treatment.

Implications for AD Therapy

The identification of specific growth and neurotrophic factors within the NSC secretome that activate Wnt/ β -catenin signaling pathway has significant therapeutic implications for AD. Current FDA-approved AD therapies, such as cholinesterase inhibitors (galantamine, benzgalantamine, rivastigmine, and donepezil) and NMDA antagonist (memantine, memantine and donepezil), primarily target symptomatic relief rather than disease modification. Moreover, recent immunotherapy that help reduce A β

plaques like lecanemab and donanemab have shown only modest clinical benefits, and their long-term efficacy remains uncertain (How Is Alzheimer's Disease Treated?, 2023). The progressive neuronal loss and impaired neurogenesis that characterize AD demand regenerative strategies capable of restoring neurogenesis and synaptic integrity rather than merely slowing cognitive and behavioral symptoms (DeTure & Dickson, 2019). The findings of this study suggest that NSC-secreted factors could serve as potent therapeutic agent by enhancing Wnt/ β -catenin-mediated neurogenesis, thereby counteracting neuronal atrophy in AD-affected regions, particularly the hippocampus. Unlike stem cell transplantation, which faces challenges such as tumorigenicity, immune rejection, and ethical concerns, cell-free therapy using NSC secretome-derived bioactive molecules offers a safer and more feasible alternative for promoting endogenous repair mechanisms like neurogenesis (Madhavan & Collier, 2010; Tran & Damaser, 2015; Willis et al., 2020)

Moreover, the therapeutic potential of NSC secretome-derived factors extend beyond Wnt/ β -catenin activation, as these growth and neurotrophic factors contribute to various neurogenesis-related processes, including neuronal proliferation, migration, differentiation, integration, and maturation. Given their ability to regulate neurogenesis, they hold significant potential for counteracting neurodegeneration in AD. Below, the individual roles and relevance of each growth factor and neurotrophic factor to neurogenesis are discussed.

Growth Factors:

- EGF (Epidermal growth factor): EGF is a potent mitogen that stimulates the proliferation of various cell types, including NSCs. By binding to EGFR, it

promotes the expansion of NPCs, facilitating neurogenesis and contributing to brain development and repair (Ostenfeld & Svendsen, 2004).

- FGF2 (Fibroblast growth factor 2): FGF2 plays a crucial role in the proliferation and differentiation of multipotent NSCs during both development and adulthood. It binds to FGFRs to regulate neurogenesis in the SVZ and the SGZ of the hippocampal dentate gyrus. FGFR1 signaling also modulates inflammatory pathway through the surface glycoprotein CD200, which plays a role in controlling microglial activation. Due to its role in neurogenesis and neuroinflammation, FGF2/FGFR1 signaling has been a focus of therapeutic target for neurodegenerative diseases like AD (Woodbury & Ikezu, 2014).
- FGF8 (Fibroblast growth factor 8): FGF8 plays a crucial role in neural development by promoting neurite outgrowth and axonal extension, particularly in placode-derived sensory ganglia. It binds to FGFR1 and FGFR2, enhancing neuronal connectivity and contributing to the maturation of sensory circuits during neurogenesis (Zhou et al., 2024)
- HGF (Hepatocyte growth factor): HGF is a multifunctional cytokine and growth factor primarily known for its role in organ development and tissue regeneration. In the CNS, HFG binds to its receptor, c-Met, promoting neurogenesis by enhancing proliferation, migration, and differentiation of NPCs (Shang et al., 2011).
- IGF-1 (Insulin-like growth factor): IGF-1 is a key growth factor that regulates neurogenesis and synaptogenesis from embryonic to adult stages of the brain. It influences NSC proliferation, differentiation into neurons and glia, and neuronal

maturation, including synapse formation. Additionally, IGF-1 plays a crucial role in adult neurogenesis by regulating NSC number and differentiation and influencing neuronal positioning and migration, particularly in the subventricular zone-olfactory bulb (SVZ-OB) neurogenesis through IGF-1R signaling (Nieto-Estévez & Vicario-Abejón, 2016).

- PDGF (Platelet-derived growth factor): PDGF is a key regulator of neural progenitor proliferation, differentiation, and survival, particularly influencing oligodendrocyte lineage development. In neurogenesis, PDGF signaling through PDGFR- α and PDGFR- β is essential for the maintenance and expansion of neural progenitors, coordinating with other factors to regulate their differentiation into neurons, astrocytes, and oligodendrocytes (Funa & Sasahara, 2014).
- SCF (Stem cell factor): SCF plays a crucial role in hematopoiesis by promoting the survival, proliferation, and differentiation of hematopoietic stem cells. Its receptor, c-kit, is expressed in various cell types, including NPCs. Research indicates that SCF promotes neurogenesis by stimulating the proliferation and differentiation of neural progenitors, suggesting its potent involvement in brain development and response to injury (Jin et al., 2002).
- VEGF (Vascular endothelial growth factor): VEGF is primarily known for its role in angiogenesis, playing a vital role in vascular development and endothelial cell function. In the CNS, VEGF is expressed by neurons, particularly in the developing brain and during pathological conditions, where it promotes neurogenesis through two mechanisms. It acts as a paracrine factor, stimulating endothelial cells to enhance pro-neurogenic angiogenesis, and as an autocrine

factor, directly supporting the survival, proliferation, and differentiation of neural progenitors through VEGFR signaling (Rosenstein et al., 2010).

Neurotrophic Factors:

- BDNF (Brain-derived neurotrophic factor): BDNF is a central neurotrophic factor expressed in regions like the hippocampus and cerebral cortex. It binds to TrkB receptors and promotes neuronal survival, differentiation, synaptic plasticity, and the formation of new synapses, processes vital for learning and memory (Bathina & Das, 2015; Liu & Nusslock, 2018)
- GDNF (Glial cell-derived neurotrophic factor): GDNF, expressed in various regions of the CNS, supports the survival and maintenance of dopaminergic neurons. It acts through the Ret receptor to promote neuronal proliferation, differentiation, maturation neurite outgrowth and survival (Cortés et al., 2017).
- NGF (Nerve Growth factor): NGF is crucial for the survival and function of cholinergic neurons in the hippocampus and brain cortex. It binds to TrkA receptors and promotes the survival, growth, differentiation, and migration of NSCs and enhances neuronal repair, particularly in regions affected by neurodegenerative diseases (Siegel & Chauhan, 2000). NGF has also been shown to regulate A β peptide production through the amyloidogenic pathway (Matrone et al., 2008).

Additionally, the identification of PI3K/Akt and MAPK/ERK crosstalk mechanisms as key modulators of NSC secretome-mediated neurogenesis presents an opportunity to develop targeted combinatorial therapies for AD. The PI3K/Akt signaling pathway is a vital intracellular signaling cascade that regulates multiple cellular

processes, including cell growth, survival, metabolism, and motility (Deng et al., 2022). It plays a protective role in the brain by inhibiting neurotoxic mechanisms and is responsible for survival, proliferation, and apoptosis of neurons (Guo et al., 2024). In AD, PI3K/Akt signaling is dysregulated, leading to hyperphosphorylation in tau, accumulation of A β , increased neuronal apoptosis, impaired synaptic function, and reduced neurogenesis (Pan et al., 2024). Whereas the MAPK/ERK pathway is involved in regulating cellular biological functions, such as cell proliferation, differentiation, cell cycle regulation, apoptosis, and tissue formation, but is also related to tumor formation (Guo et al., 2020). In brain, MAPK/ERK plays a crucial role in induction and maintenance of synaptic connections and long-term potentiation (LTP), a cellular mechanism underlying learning and memory (Kelleher et al., 2004). However, in AD, MAPK/ERK signaling pathway becomes dysregulated often contributing to excessive tau phosphorylation, neuroinflammation, and synaptic dysfunction (Kim & Choi, 2010). By targeting these interconnected pathways, NSC secretome therapy has the potential to restore neurogenesis, enhance neuronal survival, and mitigate neurodegenerative processes in AD. Given that PI3K/Akt and MAPK/ERK pathways also regulate neuronal survival, synaptic plasticity, and inflammation, therapeutic strategies that enhance their crosstalk with Wnt/ β -catenin could provide multifaceted benefits, addressing both neurodegeneration and cognitive impairment. Future therapeutic strategies should focus on leveraging these molecular interactions to enhance the regenerative potential of NSC secretome, potentially through controlled delivery method or biomaterial scaffolds that optimize their stability and activity in the AD brain environment. Advancing these approaches could transform NSC secretome therapy into viable clinical intervention,

offering a disease-modifying solution that supports neurogenesis and behavioral and cognitive recovery in AD patients.

Limitations of the Study

Several limitations exist in this research that should be acknowledged. First, this study relied on a systematic literature review and bioinformatics analysis to identify the specific growth factors and neurotrophic factors within the NSC secretome that activate Wnt/ β -catenin signaling. While this approach effectively consolidates existing knowledge and provides a computational framework for mechanistic predictions, it lacks experimental validation. The identified factors and their proposed roles in PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin crosstalk must be experimentally tested *in vitro* and *in vivo* to confirm their actual biological effects. Without direct experimental data, the conclusions remain hypothetical. Future studies should employ functional assays, such as NSC-CM treatment on neural cultures or animal models of AD, to validate the efficacy of these secretome components in promoting neurogenesis and cognitive improvement in AD models.

Second, the study focused primarily on growth factors and neurotrophic factors as key drivers of NSC secretome-mediated neurogenesis, but the NSC secretome is highly complex, containing cytokines, chemokines, metabolites, morphogens, bioactive lipids, and miRNAs that also play significant roles in neurogenic signaling (Dause et al., 2022; Foo et al., 2021; Willis et al., 2020; Stevanato et al., 2016). By excluding these components, this study provides an incomplete picture of the full therapeutic potential of the NSC secretome, which suggests that potentially important secretome-derived molecules could be involved in modulating Wnt/ β -catenin signaling. Additionally, the

literature review was limited by the availability of studies examining specific NSC secretome components in the context of AD. Many studies focused on individual growth factors or neurotrophic factors rather than the combined effects of multiple secretome components, which may underestimate the synergistic interactions observed in vivo. Furthermore, while PI3K/Akt and MAPK/ERK signaling pathways were identified as key mediators of Wnt/ β -catenin activation, other intracellular pathways, such as JAK/STAT and Hippo/YAP, were not analyzed in this study, which may also be involved in NSC secretome-mediated neurogenesis (Terry & Kim, 2022; Wang et al., 2015). A more comprehensive approach, integrating proteomics, transcriptomics, and metabolomics, would create a more comprehensive profile of the NSC secretome and its mechanisms of action in AD.

Lastly, this study explores potential crosstalk between key signaling pathways in AD, but the actual behavior of these pathways in AD-relevant conditions may be more complex than the findings suggest. In the context of AD, factors such as age-related changes in neurogenesis, chronic inflammation, and disease progression could all influence how these pathways interact. Additionally, feedback loops, compensatory mechanisms, and interactions with other cellular processes might alter the expected outcomes. Future studies could explore these dynamics in experimental AD models to better understand how the NSC secretome interacts with the AD microenvironment. Despite these uncertainties, the findings of this study provide a foundation for further investigation into the therapeutic potential of NSC secretome-based treatments.

Future Directions

Future research should focus on experimental validation of NSC secretome components to determine their precise role in modulating the Wnt/ β -catenin signaling pathway and their crosstalk with PI3K/Akt and MAPK/ERK pathways in AD. This can be achieved through *in vitro* studies using human-induced pluripotent stem cell-derived NSCs (hiPSC-NSCs) or primary NSCs cultured from rodent brain tissue. Conditioned media from these NSCs should be fractionated to isolate individual growth factors and neurotrophic factors, such as BDNF, NGF, and IGF-1, and their effects on β -catenin stabilization, NSC proliferation, and neuronal differentiations should be analyzed using Western blot, immunocytochemistry, and real-time PCR (Bahlakeh et al., 2022; Hijroudi et al., 2022). Additionally, gene knockdown (siRNA/CRISPER-Cas9) and overexpression studies should be conducted to confirm the involvement of specific secretome components in activating Wnt/ β -catenin signaling pathway. Functional assays, including TOPFlash luciferase reporter assays, can be used to quantify β -catenin transcriptional activity in response to different secretome fractions. To establish casual relationships, pharmacological inhibitors such as XAV939 (a Wnt/ β -catenin pathway inhibitor), LY294002 (a PI3K/Akt inhibitor), and U0126 (a MAPK/ERK inhibitor) should be applied to dissect the contributions of each pathway (Tang et al., 2015).

To investigate pathway crosstalk, co-immunoprecipitation (Co-IP) and proximity ligation assays should be used to examine direct protein-protein interactions between β -catenin and key signaling mediators such as phosphorylated Akt (p-Akt) and ERK1/2 (p-ERK). Additionally, time-course experiments should be performed to determine whether Wnt/ β -catenin activation occurs independently or downstream of PI3K/Akt and MAPK/ERK activation following NSC secretome treatment. Single-cell RNA sequencing

(scRNA-seq) could further elucidate pathway activation at the cellular level, identifying whether distinct subpopulations of NSCs or neurons preferentially respond to secretome-derived factors via specific signaling cascades.

For *in vivo* validation, NSC-derived secretome components should be tested in transgenic AD mouse models (e.g., 5xFAD or APP/PS1 mice) to assess their ability to restore neurogenesis and improve cognitive function (Zhang et al., 2015). Stereotactic injections of NSC-conditioned media, exosomes, or purified neurotrophic factors into the hippocampus should be followed by BrdU/NeuN immunostaining to quantify newly generated neurons. Behavioral tests such as Morris Water Maze, Novel Object Recognition, and Y-maze spontaneous alteration test should be performed to evaluate cognitive recovery. To further confirm pathway crosstalk *in vivo*, phospho-protein arrays and multiplex immunohistochemistry should be employed to assess simultaneous activation of Wnt/ β -catenin, and PI3K/Akt, and MAPK/ERK pathways in brain tissues. Additionally, RNA sequencing and proteomic profiling of treated brain tissue should be conducted to identify downstream molecular changes and confirm interactions between these pathways (Bahlakeh et al., 2022; Hijroudi et al., 2022). By integrating these experimental approaches, future research can provide definitive evidence of the therapeutic potential of NSC-secretome derived factors, establish mechanistic links between Wnt/ β -catenin and its crosstalk with PI3K/Akt and MAPK/ERK pathways, and facilitate the development of targeted secretome-based therapies for AD.

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