T.C.
DOKUZ EYLÜL ÜNIVERSITESI
IZMIR INTERNATIONAL
BIOMEDICINE AND GENOME
INSTITUTE

INVESTIGATION OF THE ROLE OF P75NTR HOMOLOGUE NRADD IN FORMATION OF REGENERATION OR DEGENERATION RESPONSES IN ALZHEIMER'S DISEASE IN ZEBRAFISH AMYLOID β42 TOXICITY MODEL

DOĞAÇ İPEKGİL

MOLECULAR BIOLOGY AND GENETICS

MASTER THESIS

IZMIR-2024

THESIS CODE: DEU.HSI.MSc/2021850001

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Supervisor: Prof. Dr. Güneş ÖZHAN

This study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) 1001 Project: 121Z900

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ABBREVIATIONS

AD : Alzheimer's Disease

CNS : Central Nervous System

CRISPR : Clustered Regularly Interspaced Short Palindromic Repeats

Cas9 : CRISPR-Associated 9

HRMA : High Resolution Melting Analysis

IF : Immunofluorescence

Nradd : Neurothrophin Receptor Associated Death Domain

P75NTR : Low-affinity nerve growth factor receptor

CVMI : Cerebroventricular Microinjection

dpi : days post-injection

APP : Amyloid Precursor Protein

Aβ42 : Amyloid beta 42

GOF : Gain of function

LOF : Loss of function

hs : heat shock

dpf : days post-fertilization

DD : Death Domain

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Prof. Dr. Güneş ÖZHAN, for her mentoring and support, for listening to and discussing every question I have had, for giving me the freedom to try out my ideas, and for her invaluable guidance.

I would also like to thank Assoc. Prof. Dr. Evin İŞCAN for sharing her knowledge and guidance. She always listened to me when I felt overwhelmed, helped me, and offered her never-ending support.

I have learned what it is like to work and study in a remarkable environment. The past three years have flown by, leaving me with unforgettable memories. I am grateful to my lab-mates—Esra KATKAT, Umut ÇAĞIRAL, Yiğit KOCAGÖZ, Yusuf Kaan POYRAZ, Sevcan HACOĞLU, Doğa KARAGÜLLE, Ayşe ŞAHİN, Beril ÇATAK, Selin HELVACIOĞLU, İpek AYDOĞDU, Miray SEVİNİN, Dilek NAZLI, Ebru TURHANLAR ŞAHİN, Şevval ÖZDİNÇ, Uğur BORA, Özge ÇARK, and Yağmur AZBAZDAR— and to all my friends at IBG for making our lab an enjoyable and fun place to be. Their support has been a constant presence throughout these years, and I deeply appreciate it.

I would like to thank IBG core facilities Emine GELİNCİ and Meryem ÖZAYDIN from IBG Zebrafish facility Aslı SEREN from IBG Optical Imaging facility and Ece UZUN from IBG Histopathology unit, who addressed every problem in such a timely manner that we did not even notice. Their effort and support are much appreciated.

I would like to thank my parents, Emel and Alpay İPEKGİL, for their unwavering support and for making this journey possible. Without their encouragement and belief in me, I could not have pursued my dreams.

I would like to thank my best friends from our undergraduate years at IZTECH, Arda CEYLAN and Şüheda YAŞARBAŞ. They were always there when I needed them most. We supported each other like family and consistently had each other's backs.

Finally, I would like to acknowledge TÜBİTAK for their support through the 2210-C National MSc/MA Scholarship Program in Priority Fields of Science and Technology.

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ABSTRACT

Neurodegenerative diseases, particularly Alzheimer's Disease (AD) are devastating conditions affecting millions of people, leading to a decline in their cognitive and social abilities. Such effect is a result of low regeneration capacity of the human brain. Current treatment options are limited to symptomatic relief and can neither halt the disease progression nor provide healing. On the other hand, vertebrates such as zebrafish can regenerate their brains even in adulthood. Gaining insights about this mechanism could provide novel treatment options beyond symptomatic relief and could increase the life quality of millions suffering from these diseases. One of the potential candidates that could have play a role in formation of regenerative or degenerative mechanisms is neurotrophin receptor associated death domain (*nradd*). Previous work done in our lab revealed that *nradd* can trigger apoptosis like its homologue p75NTR and can modulate Wnt/β-Catenin signaling pathway by interacting with Wnt receptor complex. In the crossroads of two pathways playing role in both AD and regeneration, revealing the role of zebrafish *nradd* in Aβ42 toxicity can give insights about regenerative mechanism and can provide novel targets for drug development.

To assess the role, two zebrafish lines, one Loss of function (LOF) and one Gain of function (GOF) were generated. These lines were used to restore the *nradd* expression in where *nradd* is downregulated and where its expression gets close to control levels. This setup enabled us to understand that *nradd* is playing a role in inducing apoptosis during Aβ42 toxicity, as in zebrafish development, and it is doing so with a different mechanism than its mouse homologue, without γ-secretase cleavage.

Keywords: Zebrafish, CRISPR, Neurodegeneration, Regeneration, AD, Apoptosis

ZEBRA BALIĞI AMİLOİD β42 TOKSİSİTE MODELİNDE ALZHEİMER HASTALIĞINDA REJENERASYON VEYA DEJENERASYON TEPKİLERİNİN OLUŞUMUNDA P75NTR HOMOLOGU NRADD'IN ROLÜNÜN ARAŞTIRILMASI

Doğaç İPEKGİL, Dokuz Eylül Üniversitesi, İzmir Uluslararası Biyotıp ve Genom Enstitüsü, Sağlık Kampüsü Balçova 35340 İzmir/TÜRKİYE

ÖZET

Nörodejeneratif hastalıklar, özellikle Alzheimer Hastalığı (AD), milyonlarca insanı etkileyen ve bilişsel ve sosyal yeteneklerinin azalmasına yol açan yıkıcı durumlardır. Bu etkiler, insan beyninin düşük yenilenme kapasitesinden kaynaklanmaktadır. Mevcut tedavi seçenekleri yalnızca semptomatik rahatlama sağlamanın yanında ne hastalığın ilerlemesini durdurabilir ne de iyileşme sağlayabilir. Öte yandan, zebrabalığı gibi omurgalılar yetişkinlikte bile beyinlerini yenileyebilirler. Bu mekanizma hakkında bilgi edinmek, semptomatik tedavinin ötesinde yeni tedavi seçenekleri sunabilir ve bu hastalıklardan muzdarip milyonlarca insanın yaşam kalitesini artırabilir. Rejeneratif veya dejeneratif mekanizmaların oluşumunda rol oynayabilecek potansiyel adaylardan biri nörotrofin reseptörüne bağlı ölüm alanı (*nradd*) olabilir. Laboratuvarımızda yapılan önceki çalışmalar, *nradd'nin* homoloğu p75NTR gibi apoptozu tetikleyebileceğini ve Wnt reseptör kompleksi ile etkileşime girerek Wnt/β-Katenin sinyal yolunu modüle edebileceğini ortaya koymuştur. Hem AD'de hem de rejenerasyonda rol oynayan iki yolun kesişim noktasında, zebrafish *nradd'nin* Aβ42 toksisitesindeki rolünü ortaya çıkarmak, rejeneratif mekanizmalar hakkında fikir verebilir ve ilaç geliştirme için yeni hedefler belirleyebilir.

Bu rolü değerlendirmek için biri İşlev Kaybı (LOF) ve diğeri İşlev Kazanımı (GOF) olan iki zebrafish hattı oluşturuldu. Bu hatlar, nradd ifadesinin azaldığı ve ifadesinin kontrol seviyelerine yaklaştığı durumlarda bu durumu tersine çevirmek için kullanıldı. Bu düzenleme, *nradd'nin* Aβ42 toksisitesi sırasında, zebrafish gelişiminde olduğu gibi, apoptozu indüklemede rol oynadığını ve bunu fare homologundan farklı bir mekanizma ile, γ-sekretaz kesimi olmaksızın gerçekleştirdiğini anlamamızı sağladı.

Anahtar kelimeler: Zebrabalığı, CRISPR, Nörodejenerasyon, Rejenerasyon, AH, Apoptoz

1. INTRODUCTION AND AIM

1.1. Statement and Importance of the Problem

Dementia is a broad term defined by the deterioration of learning, memory, orientation, language, and mental functions. Most dementia patients are diagnosed with Alzheimer's Disease (AD), a neurodegenerative disease that progressively affects daily activities. Current treatments for AD target only symptoms but do not halt the progression of the disease. One of the main hypotheses of AD, is the Aβ42 hypothesis, which suggests that the accumulation of neurotoxic Aβ42 plaques causes neurodegeneration. However, the mechanisms that make the brain vulnerable to the neuronal degeneration seen in this pathology are largely unknown. Zebrafish possess a tremendous capacity for regeneration compared to mammals, even in adulthood. This organism's ability to regenerate its central nervous system (CNS) in adulthood makes zebrafish a key model organism for understanding how it overcomes toxicity and regenerates damage, in this case caused by Aβ42 toxicity. Understanding these mechanisms using a regenerative model is crucial for developing therapies that go beyond symptomatic relief.

1.2. Aim of Study

This study aims to utilize the highly conserved signaling pathways in zebrafish related to neurodegeneration and their regenerative capacity. By investigating the mechanisms involved in the formation of neurodegenerative and regenerative responses during Aβ42 toxicity, the study seeks to understand the disease's mechanisms. Additionally, it aims to identify the molecular cues involved in regeneration, with the goal of targeting or applying the differences between zebrafish and mammals to develop new therapeutic approaches in the future.

1.3. Hypothesis of Study

In this study, it is hypothesized that p75NTR homologue neurothrophin receptor associated death domain (*nradd*) may play a role in the formation of neurodegenerative or regenerative responses in zebrafish brain during Aβ42 toxicity.

1. GENERAL INFORMATION

2.1. Adult Neurogenesis and Brain Regeneration

2.1.1. Adult Neurogenesis

Neurogenesis comes from the combination of words "neuro" meaning relating to nerves and "genesis" which means formation of something, and neurogenesis defines the birth of functional new neurons and their successful assembly into new or already formed neural network. During development, most neurogenesis takes place from progenitor cell pools located in neural tube and neurogenesis is completed at the end of the developmental period. Though neurogenesis period is completed in higher vertebrates during development, various animals keep neurogenesis active throughout their lifespan by keeping their progenitor cell pools active in their nervous system and can generate new functional neurons that are mature and capable of integrating into existing circuits.

First studies performed on neurogenesis revealed the presence of mitotic cells located in adult nervous system without telling the fate of proliferating cells. With the help of thymidine labeling cell tracing technologies, understanding the fate of the proliferating cells became possible (Gage, 2000). Research done by Joseph Altman revealed the presence of newborn glia or neuron-like cells in adult injured rat brain upon regeneration (Altman, 1962). The work done revealed that higher vertebrate models are capable of neurogenesis in adulthood as well. Later studies showed that, generated cells possess a neuronal identity and integrated into the neural circuit. And later work on neurogenesis showed other organisms such as songbirds, teleost and human are capable of varying degree of neurogenesis in adulthood as well (Anderson & Waxman, 1983; Eriksson et al., 1998). Studies done so far revealed that adult neurogenesis is evolutionarily conserved across species with differing rate and number of neurogenic zones across species (Kaslin et al., 2007).

There are two main type of neurogenesis called constitutive and regenerative neurogenesis. As it can be understood by name if new neurons are generated for plasticity or memory in intact brain, this is called constitutive neurogenesis. In constitutive neurogenesis, the aim is to generate and integrate new neurons to existing

circuits which helps learning and memory. In the other hand, regenerative neurogenesis is the process of generating new neurons for replacing lost neurons in a circuit upon injury. Both models of neurogenesis generate new neurons, but the trigger is the driving factor for determination of which mode will be favored.

Constitutive neurogenesis requires active neurogenic niches which are tightly controlled by the surrounding environment and molecular cues. Number of these regions are limited in higher vertebrate models and these proliferative zones are called, ventricular-subventricular zone (V-SVZ) and sub-granular zone (SGZ) (Fiorelli et al., 2015; Ming & Song, 2011). Constitutive neurogenesis is restrained by limited zones available and newborn neurons must migrate to their target. For instance, newborn neuroblasts in V-SVZ migrates to olfactory bulb to integrate and maturate. However newborn neurons in SGZ have a limited migration capability and can only migrate towards apical layers of dendrite gyrus (Hayashi et al., 2015).

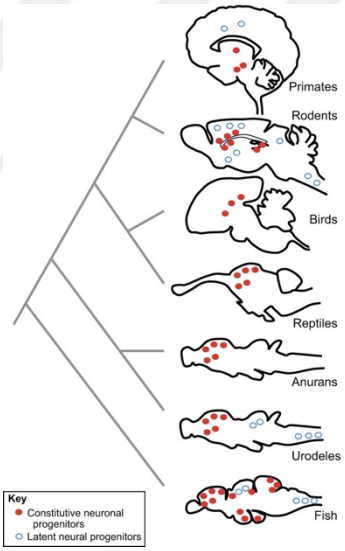

Figure 1: comparative view of regenerative neurogenesis in vertebrates (Alunni & Bally-Cuif, 2016)

As mentioned above, regenerative neurogenesis is not a life-long event, but it requires a trigger, which is most of the time injury or neurodegeneration hence the process is tightly controlled by absence of injury specific proliferative responses by its microenvironment. Regenerative neurogenesis is most of the time controlled by external stimuli after injury and requires activation of the quiescent progenitor cell

pools residing in central nervous system (CNS) for recovery. In the presence of proliferatively permissive environment after damage, progenitor cells undergo rapid and transient proliferation. Regenerative capacity of an organism lays on the number of available progenitor cell pool residing in CNS, though constitutive proliferating zones can also contribute to regenerative neurogenesis. Injury specific ques that are required for the initiation of neurogenesis has recently associated with the injury accompanied acute inflammation which is essential for quiescent progenitor cells to get activated (Ming & Song, 2011). However, other extrinsic factors driving this mechanism remains unknown. Even though the mechanism of neurogenesis is conserved across species, the capability of neurogenesis decreases over evolution (Hayashi et al., 2015). Higher vertebrates such as mammals have very limited capacity of adult neurogenesis than lower vertebrates because of higher vertebrates bearing lower numbers of neurogenic niches than lower vertebrates (Kaslin et al., 2007).

Adult neurogenesis is not limited to fewer zones in non-mammalian vertebrates but happens in more widespread fashion depending on the number of neural progenitor zones available (Figure 1). For example, in birds, neurogenesis occurs frequently in the lateral walls of lateral ventricles and large sets of neuronal precursors are present in wide regions of telencephalon (Alvarez-Buylla et al., 1990). Newborn neurons migrate through radial fibers and reach their destination to be integrated (Alvarez-Buylla & Nottebohm, 1988). Research in frogs reveal similarly high numbers of neurogenic zones in brain areas included but not limited to telencephalon, though there are limited numbers of article (D'Amico et al., 2011). The number of the neurogenic areas are even higher in teleost species. These neurogenic areas are active throughout the fish` lifespan and are present in the entire brain axis (Zupanc, 2008; Zupanc & Horschke, 1995). When the studies coming from multiple species from different evolutionary origin are compared, it is obvious that some of the aspects of neurogenesis is conserved evolutionarily, however others may be species specific.

2.1.2. Zebrafish as a Model Organism in Regeneration and Neurodegeneration Studies

Neuroregeneration model in vertebrates is informative yet hard to achieve since not all the organisms have the regeneration ability equally since this ability often decrease with the increasing phylogenic position of an organism. For instance, in adult mammal tissue healing results generally with scar tissue formation. Though, complete tissue renewal is also possible in mammal, it is often limited to the developmental period. In contrary to mammal, teleost and amphibians in this context bear incredible ability of self-renewal of damaged tissue, organs and even limbs in the adulthood without formation of the scar tissue. *Danio rerio* as known as zebrafish is a teleost bearing high regenerative capacity in adulthood and is a versatile model organism in this context depending on several organ regeneration studies including CNS (Porrello et al., 2011; Seifert & Maden, 2014).

Besides aforementioned regenerative capacity of zebrafish, its genome is approximately 70% similar with human which also creates a platform to generate disease models and investigate how zebrafish recovers from a disease that is detrimental to human. There is multiple well-established injury and disease models in zebrafish which allows us to study the regenerative response in a multifaceted approached such as stem cell biology, behavioral analysis, and drug screening (Cigliola et al., 2020) (Kizil, Kaslin, et al., 2012). Small body size, short life cycle and external fertilization and transparent development of zebrafish also makes this organism an easy to study platform. Emerging gene editing and transgenesis technologies allow researchers to generate cell type specific genetic systems for cellular fate mapping, and to generate knock out or knock in models to perform the functional analysis on candidate proteins (Li et al., 2021). With multiple advantages, research done on zebrafish can contribute to field of regeneration. Discoveries on the mechanisms governing tissue renewal using zebrafish model may be employed in other organisms as a therapy, including humans.

2.1.3. Adult Zebrafish Telencephalon: Neurogenesis and Regeneration

Zebrafish telencephalon is an analogous brain structure to cerebral cortex responsible for higher order brain functions such as learning, memory and spatial navigation. Zebrafish telencephalon includes similar anatomical regions comparable to mammalian brain hence telencephalon is the most studied brain region of zebrafish with respect to regeneration and neurodegeneration studies (Grandel et al., 2006; Lam et al., 2009) .

Zebrafish telencephalic region consists of three sub-regions called olfactory bulb, pallium (dorsal telencephalon) and sub-pallium (ventral telencephalon). In sub-pallium, there are two nuclei identified as ventral nucleus of the ventral telencephalon (Vv) and dorsal nucleus of the ventral telencephalon (Vd) (Wulliman et al., 2012). Pallium region is a more complex region consisting of multiple nuclei which are the central zone (Dc), the dorsomedial zone (Dm), the lateral zone (Dl) and the posterior zone (Dp) of the dorsal telencephalon (Wulliman et al., 2012). Approximately 16 neurogenic niches have been characterized to reside in the telencephalon (Grandel et al., 2006; Kishimoto et al., 2013). Among these, Vv is sharing a homology with SVZ and Dl or Dp share homology with SGZ of dentate gyrus of tetrapod. Previous study conducted by Ganz et al. (2014) aimed to find and compare any homologue telencephalon region of zebrafish by the help comparing expression patterns of conserved genes with mammals. They suggested that Dm corresponds to the pallial amygdala in mammals, Dc corresponds to cortex, Dl to hippocampus and there is no corresponding region for Dp in tetrapods. Diotel et al. (2015) revealed that the adult zebrafish telencephalon has similarities in terms of transcription regulator (TR) gene expression clusters with the mouse brain. Additionally, other compartments of the telencephalon, such as neurotransmitters, their synthesizing enzymes, and the distribution of markers for GABAergic and serotonergic innervation, are expressed in a similar way to those in rodents. These similarities suggest homology between zebrafish and tetrapods in adulthood.

2.1.3.1. Molecular Mechanisms Governing Adult Zebrafish Telencephalon Regeneration

To date, many injury models have been generated in zebrafish to study neural regeneration. Among them, telencephalic stab injuries (Baumgart et al., 2012a; Kishimoto et al., 2012; Kroehne et al., 2011; März et al., 2011; Schmidt et al., 2014a) and zebrafish model of Aβ42 toxicity (Bhattarai et al., 2016, 2020; Cosacak et al., 2019) are the ones characterized the best at cellular and molecular level.

By applying in situ hybridization and transcriptomic techniques to stab injured zebrafish telencephalon, several hundred genes with a potential role in adult zebrafish neurogenesis have been identified. The study performed by Kizil, Kyritsis, et al. (2012) showed increase in expression of a zinc finger transcription factor *gata3* in RGCs, upon stab injury. Morpholino injection to injured zebrafish against *gata3* decreased the numbers proliferative induced RGCs and ceased neurogenesis. This molecular mechanism has been correlated with inflammation and further confirmed by observing higher expression of *gata3* at the ventricular zone followed by increased neurogenesis via Zymosan A induced inflammation. These findings indicate that brain inflammation is enough to trigger regenerative mechanisms for reactive neurogenesis in the zebrafish telencephalon.

Additionally, expression of the transcriptional regulator *id1* is also upregulated upon injury with delay and independent of inflammation but on BMP signaling pathway. BMP signaling factor is crucial for maintaining quiescent NSCs in neurogenic niches. Functional studies suggest that *id1* upregulation upon injury is crucial for the maintenance of NSC pools for later use (Viales et al., 2015).

The molecular mechanisms work in harmony to regenerate the telencephalon upon injury. The field of regeneration in this context is relatively unexplored, and many other pathways contributing to neurogenesis and regeneration have yet to be revealed.

2.1.4. Adult Zebrafish Brain Injury Models

There is multiple acute injury models employed on different organisms like mammals, amphibians, and fish. Employed injury models range from resection of part of an organ to specifically target and kill subset of cells.

2.1.4.1. Physical Lesions

The most direct injury model employed in model organisms is physical lesions. This injury model lays on introducing a traumatic injury to tissue of interest to study regeneration and employed widely on zebrafish in regeneration studies as well including studies on neurogenesis and regeneration of the CNS (Baumgart et al., 2012b; Demirci et al., 2020; Rastegar et al., 2019; Schmidt et al., 2014b). Physical lesion injury model includes functional studies to assess the missing organ parts` function in a living organism. This injury model may also trigger the regenerative responses and neurogenesis depending on the site and severity of the injury as well. As a physiological response to traumatic injury, the injured brain undergoes complex multicellular ques including apoptosis, inflammation, proliferation of glial cells and increased activity of progenitor cells which may lead to start of neurogenesis depending on the lesion site (Fitch & Silver, 2008; Kaslin et al., 2008). Though physical lesion models are considered great for regeneration studies, the harsh nature of these models can induce cell death through secondary degeneration, persistent inflammation, and cause dysfunction of the blood-brain barrier. These side effects may impact results and lead researchers to misinterpret the findings regarding regenerative events.

2.1.4.2. Chemical and Peptide Injury

Administration of certain chemicals or peptides can lead to injury in zebrafish, either acting as a nonselective neurotoxin or can target cell types like neuronal or glial cells. Chemical and peptide injury methods are employed using different agents to introduce specific injury types. For instance, triethyltin, somatostatin, 6-hydroxydopamine (6-OHDA), methylmercury, 3-acetylpyridine (3-AP), 1,3-dinitrobenzene (DNB), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridinium (MPP+) are some of the chemical agents that has been

reported to be used to evoke neuronal damage depending on the toxicity model desired (Anichtchik et al., 2004; Balaban et al., 1988; Dorman et al., 2000; Lopachin & Aschner, 1993; Mavroudis et al., 2006; O'Callaghan & Miller, 1988; Philbert et al., 2000; Reali et al., 2005; Sallinen et al., 2009).

There are also toxicity models that depends on injection of toxic peptides directly into the space between skull and brain. This kind of injuries depends on the toxic peptide aggregates formed during neurodegenerative diseases such as Alzheimer's disease (AD). Though the cause of AD remains unknown, Aβ42 accumulation is one of the major hypotheses suggesting the toxic nature of Aβ42 accumulation is one of the causes of neurodegeneration (Bhattarai et al., 2020; Cosacak et al., 2019; Kizil, Kyritsis, et al., 2012). Bhattarai et al. (2017) demonstrated that injection of Aβ42 peptide can evoke neurodegenerative responses and can be employed if mimicking AD via Aβ42 toxicity is desired. Modelling neurodegeneration or introducing damage specifically is a key advantage of this method but extend of the damage can be beyond expected. The dosage and injection technique must be properly optimized to avoid global damage caused by toxic compound's concentration or by the injection technique which can also introduce a physical damage besides chemical. Optimal for inducing regeneration in the brain is generating a transgenic zebrafish line that can induce the production of a toxic compound in specific cell types using well-developed transgenesis techniques. However, in some cases (e.g., not having the characterized transgenic fish line), this technique allows researchers, in this case myself, to model and investigate the regenerative and degenerative responses of nradd's using a peptide-induced toxicity model.

2.1.4.3. Transgenic Injury Models

Generating a transgenic zebrafish line is relatively easy due its fully sequenced and annotated genome. There is multiple reporter lines based on cell-type specific promoters to drive expression of reporters, altered gene products, toxic proteins and regulatory RNAs (Balciunas et al., 2006; Jessen et al., 1998; Kawakami, 2007). Also, many reporter constructs have been generated specifically driving expression on certain cell types of zebrafish brain (Asakawa et al., 2008; Baier & Scott, 2009; Chen

et al., 2009; Kaslin et al., 2013; Kleinjan et al., 2008). By using this foundation, researchers have developed transgenic fish lines that allow promoter-specific cell ablations. As an example, Diphtheria toxin A has been used to specifically ablate the lens growth (Kurita et al., 2003) and bacteria toxin was used to kill germ cells (Slanchev et al., 2005) . But with this method, zebrafish starts to express toxins throughout their developmental period and this type of studies are limited if adult experiments are desired.

Lack of certain type of cells might often be fatal to fish embryo and makes the fish line impossible to develop. To overcome this issue, rather than expressing the toxic compound throughout the development, an enzyme nitroreductase that catalyzes the reduction of pro-drug metronidazole into a cytotoxic product. By that, cellular ablation can be temporally controlled by adding metronidazole into fish water at desired timepoints. However, the efficiency of nitroreductase has been shown to change in different cell types (Davison et al., 2007; Hu et al., 2010; Montgomery et al., 2010).

A well-known and used transgenesis technique Cre-LoxP has been implemented into zebrafish as well. In this transgenesis technique, a driver cassette expressing Cre recombinase under tissue specific promoter gives spatial control, and an effector cassette expressing gene of interest (toxic) under inducible promoter which gives a temporal control of cell ablation (Hans et al., 2009, 2011; Hesselson et al., 2009). In some cases, leaky expression of Cre recombinase in organism may lead to unforeseen ablation of different cell types and misinterpretation of the results. To overcome this issue, a more developed Cre-LoxP system in which Cre recombinase is fused to estrogen receptor ligand binding domain, which allows a precise control over nuclear localization of Cre recombinase by addition of Tamoxifen allowing more controlled gene expression or cell ablation in zebrafish (Hans et al., 2009, 2011).

2.2. Neurodegeneration and Alzheimer`s Disease (AD)

Our nervous system is composed of divergent set of differentiated cells which enables us to perform higher cognitive functions, memory, and sensory functions. Gradual loss of different sets of neuronal cells due to either traumatic or non-traumatic injury is called neurodegeneration. Neurodegeneration is known to cause several different conditions depending in the loss of neurons or neurons in particular brain regions such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS) (Bossy-Wetzel et al., 2004).

Some neurodegenerative disorders may influence cognitive functions. Diseases influencing cognitions are collectively called dementia. Dementia is an umbrella term that encompasses symptoms characterized by the impairment of learning, memory, orientation, linguistic, and mental functions, which affect social life. The most common type of dementia is Alzheimer's disease (AD), a progressive neurodegenerative central nervous system (CNS) disorder that affects mental and cognitive abilities, as well as routine daily activities, thereby reducing the patient's quality of life. Current treatments for AD are symptomatic and do not prevent the onset or progression of the disease; hence, there is an urgent need for new therapeutic approaches aimed at preventing or delaying the progression of the disease. According to the amyloid hypothesis, the primary feature in the pathogenesis of AD is the accumulation of amyloid-beta (Aβ) peptides in the brain, leading to the formation of neuritic plaques. However, the mechanisms that render the brain vulnerable to neuronal degeneration observed in this pathology are largely unknown (McKhann et al., 2011).

2.2.1. Alzheimer`s Disease: Aβ42 Hypothesis and Amyloidogenic Pathway

As explained above, dementia is a generic term which encompasses many neurogenerative diseases. However, most of the dementia patients are diagnosed with Alzheimer`s Disease. AD generally is a late-onset disease. Presently, there are no treatments available for AD; hence the progression of the disease is irreversible. The quality of life for those suffering from AD decreases gradually by losing memory and thinking skills and, eventually, the ability to carry out simple tasks (Heckmann et al., 2019; Selkoe & Hardy, 2016). Although the disease-causative factors are yet not fully

known, it has been demonstrated that about 1-2% of AD cases are inherited in an autosomal dominant pattern that causes its earlier onset and more rapid progression (Morris et al., 2012). Characteristic pathologies caused by AD include accumulation of certain proteins, changes in inflammation, loss of synaptic contacts, and neuronal cell death. One of the major hypotheses that can explain the mechanism of neurodegeneration is Aβ42 hypothesis (Heckmann et al., 2019; Selkoe & Hardy, 2016). Aβ42 production is previously correlated with the disease progression. Aβ42 is a 42 amino acid long hydrophobic peptide that can accumulate at the extracellular environment of the CNS produced by consecutive cleavage of amyloid precursor protein by β-secretase and γ-secretase enzymes respectively (Haass et al., 2012; Long & Holtzman, 2019). Aggregation of Aβ42 is highly susceptible and forms the characteristic amyloid plaques for AD. Aβ42 accumulation can drive pathological cascades, resulting in chronic inflammation and oxidative stress that would disrupt cell function and lead to neurodegeneration (Sasidharakurup & Diwakar, 2020).

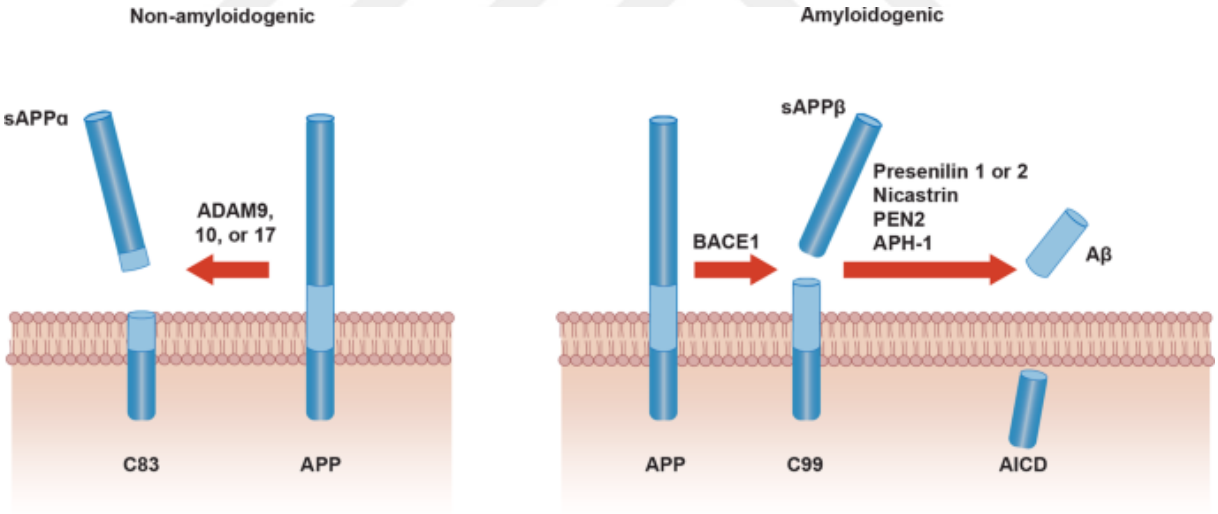

Figure 2: Amyloidogenic pathway versus non-amyloidogenic pathway (Hampel et al., 2021).

Since the discovery that Aβ peptide is a major component of senile plaques, many studies have been conducted to understand its role in AD pathophysiology (Masters et al., 1985). The Aβ peptide is generated from the amyloid precursor protein (APP) through sequential cleavage by the enzymes β-secretase and the γ-secretase protease

complex (Haass et al., 2012; Long & Holtzman, 2019). First, BACE1 cleaves APP at the amino-terminus of the Aβ sequence, resulting in the secreted sAPPβ peptide and a 99-amino-acid-long carboxyl-terminal fragment remaining in the plasma membrane. In the second step, the complex γ-secretase—consisting of four protein subunits (presenilin, presenilin enhancer, APH, and Nicastrin)—cleaves CTF99 at the carboxyl terminus of the Aβ sequence (Vassar et al., 1999). PSEN has two isoforms, PSEN1 and PSEN2, while APH has two isoforms, APHA and APH B/C, therefore, four various γ-secretase complexes may co-exist in the cell (Voytyuk et al., 2018; Xia, 2019). The γ-secretase complex binds the membrane-bound CTF99 and cleaves it at the ε-site to produce the C-terminal fragment of about 10-12 kDa (CTF) and the Aβ48 peptide. After this initial cleavage event, the complex continues to cleave the Aβ peptide at other sites along the C-terminus until it is released, resulting in peptides of generally 38, 40, or 42 amino acids in length. One study has found that in lysates from the mouse brain, the presence of BACE1, γ-secretase complex, and APP protein in a large cellular complex indicates direct translocation of APP from one enzyme to another to produce Aβ (Zhang et al., 2011). Aβ is produced in endosomes and secreted from neurons both pre-synaptically and post-synaptically, whereupon it associates into higher-order oligomers, protofibrils, and fibrils in a β-sheet conformation. Mutations to genes APP, PSEN1, and PSEN2, which act on APP metabolism, lead to the formation of intracerebral amyloid or amyloidogenesis (Lanoiselée et al., 2017).

2.2.2. Current Treatment Approaches Against AD

There are more than 100 compounds that are being tested in various stages of clinical trials for either relieving symptoms or stopping the progression of the disease (Hara et al., 2019). Available treatments for relieving symptoms are the ones targeting the neurotransmitters and their receptors. Cholinesterase inhibitors, such as donepezil, rivastigmine, and galantamine, are used in the symptomatic treatment of Alzheimer's disease (Birks, 2006). They augment synaptic levels of acetylcholine, seeking to counteract the deficiency of this neurotransmitter associated with the cognitive symptoms of Alzheimer's disease. The most common treatment strategy to stop the progression of AD is to target and dissolve Aβ42 tangles via Aβ immunization. One

transgenic mouse model of AD, PDAPP, which overexpresses human APP revealed significant decrease in pre-existing Aβ42 plaques in aged mouse after Aβ42 immunization via injection (Gallardo & Holtzman, 2017; Schenk et al., 1999). However, first human immunotherapy trials depending on passive immunization of patients using AN-1792 which contained synthetic Aβ42 peptide revealed a significant decrease of Aβ42 plaques. However postmortem studies revealed that patients were still showing severe Tau pathology and hallmarks of dementia (Nicoll et al., 2019; Vellas et al., 2009). Other trials aiming to stop the disease progression used monoclonal antibodies against Aβ and most of them failed at phase 3 trials (Doody et al., 2013; Honig et al., 2018; Salloway et al., 2014; Selkoe, 2019). Alternative treatment approaches were also tested targeting and inhibiting the proteins in enzyme complexes involved in amyloidogenic pathway, β- and γ- secretase to prevent production of Aβ42. First studies targeting these enzyme complexes are focused on the rate limiting step of the pathway, BACE1 enzymatic activity. These proposed inhibitors were unable to stop the Aβ42 production and plaque growth in mouse amyloidosis model (Peters et al., 2018). There are multiple β-secretase inhibitors which failed at various stages of trials and there are more candidate inhibitors still on trials (Egan et al., 2019; Henley et al., 2019; Panza et al., 2018). Clinical trials have used gamma-secretase inhibitors, but those studies were aborted because of dose-limiting side effects, low drug efficacy, or worsening of cognitive functions (Coric et al., 2015; Doody et al., 2015).Gamma-secretase modulators are other means to reduce the accumulation of amyloids, acting to modify the processivity of the enzyme without inhibiting gamma-secretase activity, thereby changing the profile of the secreted Aβ peptides (Rynearson et al., 2021; Wagner et al., 2017).

2.3. Role of Neurotrophins and p75NTR Signaling in Neurodegeneration and Neurogenesis

2.3.1. Canonical Neurotrophin Signaling Pathway

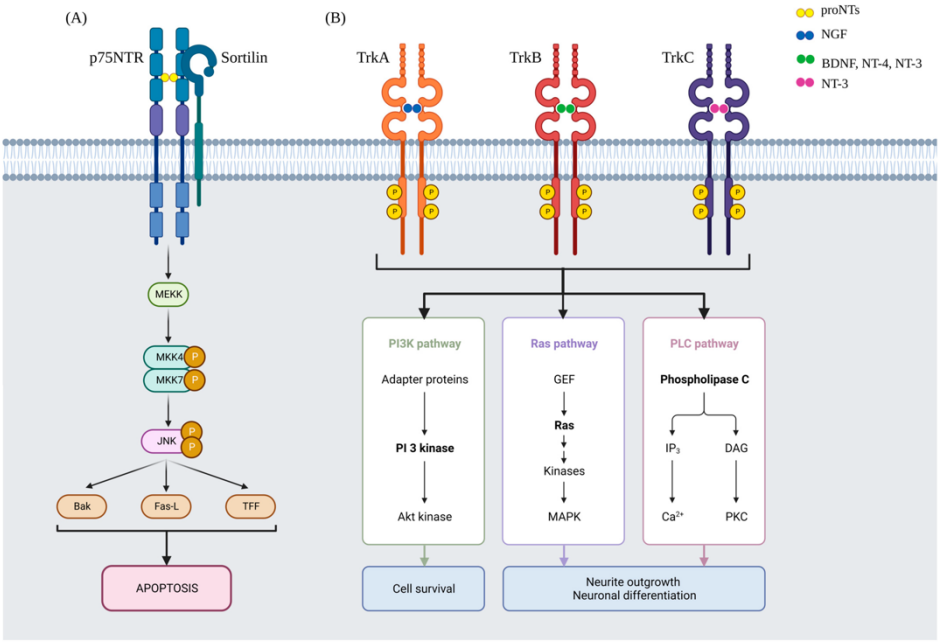

Figure 3: Neurothrophin signaling pathway. Akt—serine/threonine protein kinase; Bak—BCL-2 homologous antagonist/killer; BDNF—brain-derived neurotrophic factor; DAG—diacyloglycerol; Fas-L—tumor necrosis factor ligand superfamily member 6; IP3—inositol trisphosphate; JNK—c-Jun N-terminal kinase; MAPK—mitogen-activated protein kinase; MEKK—mitogen-activated protein kinase kinase kinase 1; MKK4—mitogen-activated protein kinase kinase 4; MKK7—mitogen-activated protein kinase kinase 7; NGF—nerve growth factor; NT-3—neurotrophin 3; NT-4—neurotrophin 4; p75NTR—p75 neurotrophic receptor; PI3K—PI3 -kinase type 3; PKC—protein kinase C; proNTs—premature neurotrophins; Ras—KRAS proto-oncogen; TFF—trefoil factor 1; TrkA/B/C—tropomyosin receptor kinase A/B/C (Gabryelska et al., 2023).

Neurotrophins are a small family of dimeric secreted proteins modulating vertebrate neuron biological properties such as survival, shape, and function (Mitre et al., 2017). The diversity of biological activities regulated by neurotrophins is a consequence of their binding to receptors of the tropomyosin receptor kinase family of tyrosine kinases and to p75 neurotrophin receptor (p75NTR), a member of the tumor necrosis factor receptor superfamily (Barford et al., 2017; Naito et al., 2017;

Wiens & Glenney, 2011). Neurotrophins promote neuronal survival by activating Trk receptors, or depending on conditions, the conditions may be such that, through these receptors, either cell survival or death is obtained (Longo & Massa, 2013). p75NTR is a transmembrane protein bearing the key sequence of cell death "death domain (DD)" on its C-terminus. Induction of this signaling pathway via proNTs promotes α- and γ-secretase mediated proteolysis and induce apoptosis via translocation of released DD into nucleus (Zampieri et al., 2005).

2.3.2. Role of p75NTR in progression of AD

There are multiple studies investigating the expression levels of p75NTR in AD. However, the reports coming out of these studies are rather contradictory. Findings regarding p75NTR expression indicate increasing, decreasing, or unchanged levels (Zeng et al., 2011). These contrasting results may be due to different brain regions analyzed, differences in disease progression levels, and different analyses employed. Aβ peptide can induce apoptosis by interacting with the p75NTR receptor (Yaar et al., 2002). Alongside this information, p75NTR activation induced by Aβ can activate phosphoinositide 3-kinase (PI3K), c-Jun kinase (JNK), and nuclear factor κB (NF κB) and, through these mechanisms, can induce cell death or promote cell survival depending on the interaction partner of p75NTR (Costantini et al., 2005; Coulson, 2006; Hashimoto et al., 2004; Tsukamoto et al., 2003). Also, low concentrations of Aβ can act as a neurotrophic factor and induce neurite growth and neuron survival, while higher concentrations of Aβ, by acting as an NGF antagonist, can decrease GABAergic transduction (Arevalo et al., 2009).

This inconsistency in the promotion of survival or apoptosis by p75NTR can be rooted in the fact that most of the studies characterizing the role of p75NTR in Aβ neurotoxicity have been carried out using p75NTR transfection in tumor cell lines. It might also depend on differences in signal transduction pathway activation between tumor cell lines and primary neurons, cell type- or species-specific effects of Aβ, and its progression through endogenous auxiliary neurotrophic receptors (Zeng et al., 2011). Also, toxicity of Aβ in p75NTR-expressing cells is not associated with threat to survival but rather more promotion of apoptosis (Coulson, 2006). The levels of p75NTR

were shown to be increased in human neuroblastoma SH-SY5Y cells following the stimulation with Aβ42, and the high level of p75NTR in the hippocampus has been observed to be associated with the accumulation of Aβ42 in two transgenic mouse models and in the brains of Alzheimer's disease patients (Chakravarthy et al., 2012). Besides, reports have already been made on reduction of amyloidogenesis by inhibiting β-secretase expression and activity and decrease of Alzheimer's disease-related pathologies at molecular and behavioral levels in the APP/PS1 double transgenic mouse model expressing chimeric mouse/human APP and mutant human PSEN1 by the extracellular domain of p75NTR (Yao et al., 2015). Because most of the previous studies on p75NTR-mediated signaling activation require auxiliary receptors and are restricted to in vitro models, it is necessary to perform further studies in vivo to clarify the mechanisms by which p75NTR may induce Aβ42-mediated neurotoxicity (Coulson, 2006).

2.4. Role of Wnt/β-Catenin Signaling in Neurodegeneration and Neurogenesis

2.4.1. Canonical Wnt/β-Catenin pathway

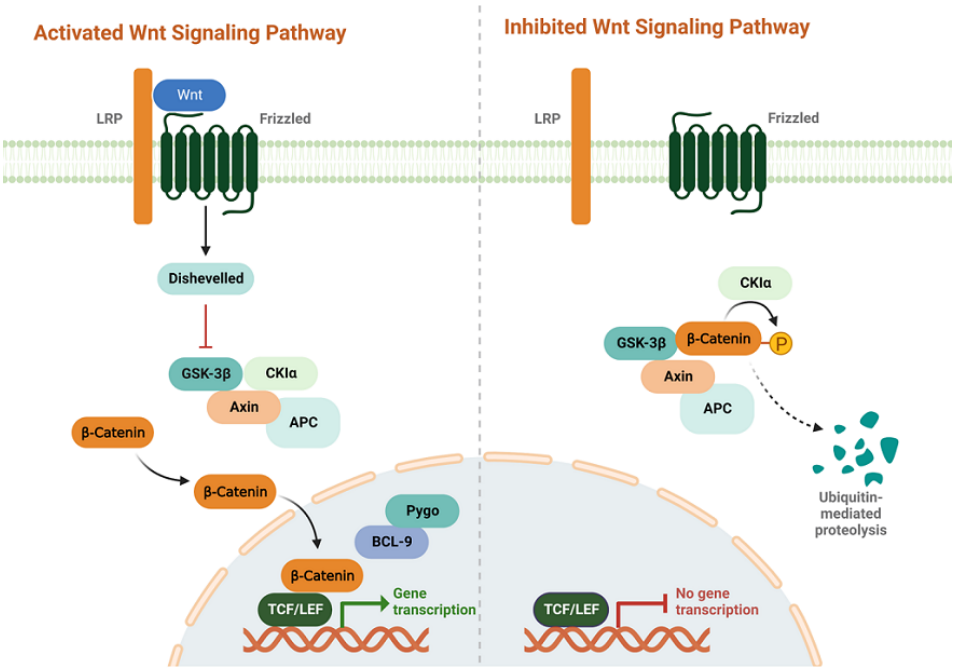

Figure 4: Activated and inhibited canonical Wnt/β-catenin pathway. (Liu et al., 2022)

Wnt signaling, one of the oldest and most conserved signaling pathways from invertebrates to complex mammals, manages numerous physiological activities such as growth, differentiation, migration, and tissue regeneration during all stages of development and in maintaining homeostasis in adult organisms (Nusse, 2005; Aman et al., 2018; Steinhart and Angers, 2018). Due to these vital functions, malfunctions in Wnt signaling have been associated with many pathological processes, including cancer and neurodegenerative diseases (MacDonald et al., 2009; Libro et al., 2016; Azbazdar et al., 2021). The critical roles of Wnt signaling in synaptic transmission and the disruptions occurring in the Wnt signaling pathway during aging suggest that proper functioning of Wnt signaling could enhance synaptic function during aging and improve synaptic pathology associated with Alzheimer's disease (Orellana et al., 2015; Marzo et al., 2016; García-Velázquez and Arias, 2017; McLeod et al., 2018; Folke et al., 2019).

The Wnt signaling pathway, which operates through β-catenin (canonical Wnt pathway), is referred to as Wnt/β-catenin signaling, and its function with the core components is thought to proceed as follows: In the 'Wnt-off' state, when active Wnt ligands are not present in the environment, a cytoplasmic complex consisting of proteins such as Axin, Apc, and Gsk3 phosphorylates β-catenin, leading to its proteasomal degradation (Logan and Nusse, 2004; Angers and Moon, 2009; MacDonald et al., 2009). The 'Wnt-on' state occurs when the Wnt ligand triggers the formation of a complex including the Frizzled (Fz) receptor and co-receptors Low-Density Lipoprotein Receptor-Related Proteins 5/6 (Lrp5/6). This receptor clustering is thought to cause the recruitment of cytoplasmic Dishevelled and Axin proteins to the receptors and phosphorylation of Lrp6. Lrp6 phosphorylation and endocytosis of the receptor complex into membrane vesicles are necessary steps for the activation of the Wnt/β-catenin pathway (Yamamoto et al., 2008). During this process, removal of Axin from the cytoplasmic β-catenin degradation complex, direct inhibition of Gsk3 kinase activity by the phosphorylated cytoplasmic portion of Lrp6, and sequestration of Gsk3 in multivesicular bodies, which keeps it separated from the cytoplasm, are thought to collectively prevent the degradation of β-catenin (Angers and Moon, 2009; MacDonald

et al., 2009; Taelman et al., 2010). β-catenin that enters the nucleus regulates the transcriptional expression of target genes together with T Cell Factor (Tcf)/Lymphoid Enhancer Factor (Lef) family transcription factors.

2.4.2. Role of Wnt/β-Catenin pathway in progression of AD

The Wnt/β-catenin signaling pathway is tightly controlled by numerous positive and negative regulators. Some well-characterized Wnt pathway components and regulators, such as the Wnt antagonist Dkk1 and the β-catenin destruction complex component Axin2, are also target genes and are under the transcriptional control of the pathway (Jho et al., 2002; Gonzalez-Sancho et al., 2005). Dkk1 inhibits canonical Wnt signaling by interacting with the Lrp5/6 coreceptor (Niehrs, 2006). It has been found that Dkk1 is highly expressed in the brains of Alzheimer's patients and mouse models of AD (Caricasole et al., 2004; Rosi et al., 2010). Correspondingly, increased Gsk3ß activity, Lrp6 polymorphism, and decreased Wnt signal activity and cytoplasmic ß-catenin levels due to a cleavage variant have been observed in the brains of patients (De Ferrari et al., 2007; Alarcón et al., 2013). Mass spectrometric analysis of samples obtained from the brains of patients also supports the decrease and disruption of canonical Wnt signaling (Elliott et al., 2018; Xu et al., 2019; Bai et al., 2020). Aß in hippocampal neurons has been shown to increase Dkk1 expression, leading to a decrease in canonical Wnt activity and synapse loss, and that blocking Dkk1 through neutralizing antibodies completely abolishes the effect of Aß on synapses and prevents synaptic loss (Purro et al., 2012; Sellers et al., 2018). In a transgenic mouse model where inducible expression of Dkk1 in the brain cause synapse and memory loss in the striatum and hippocampus, reduce long-term potentiation (LTP), and increase long-term depression (LTD) without affecting cell viability (Galli et al., 2014; Marzo et al., 2016). Additionally, in a mouse model of AD, postnatal deletion of Wnt and Dkk1 receptor Lrp6 from neurons in the forebrain triggered APP amyloidogenesis, leading to synaptic loss and exacerbating AD pathology (Liu et al., 2014). Moreover, Wnt pathway components such as ß-catenin, Tcf4, Gsk3ß, and Dvl1 have been associated with Aß production from APP (Mudher et al., 2001; Parr et al., 2015; Tapia-Rojas et al., 2016; Palomer et al., 2019). Consequently, reactivation of Wnt signaling through inhibition

of Dkk1 and Gsk3ß or increased expression of Wnt ligands has been found to prevent Aß-induced synaptic damage in cells and mouse models of AD (De Ferrari et al., 2003; Alvarez et al., 2004; Quintanilla et al., 2005; Chacón et al., 2008; Cerpa et al., 2010; Vargas et al., 2015; Licht-Murava et al., 2016; Marzo et al., 2016; Ross et al., 2018). Besides these effects on neurons, Wnt pathway is known to have a crucial role in neurogenesis both in development and adulthood. In adulthood NSCs can induce proliferation and neurogenesis via activation of Wnt signaling in autocrine level by WNT ligands and it has been shown that Wnt antagonist Dkk1 decrease the NSC proliferation and neuronal fate determination.

2.5. Neurotrophin Receptor Associated Death Domain (nradd)

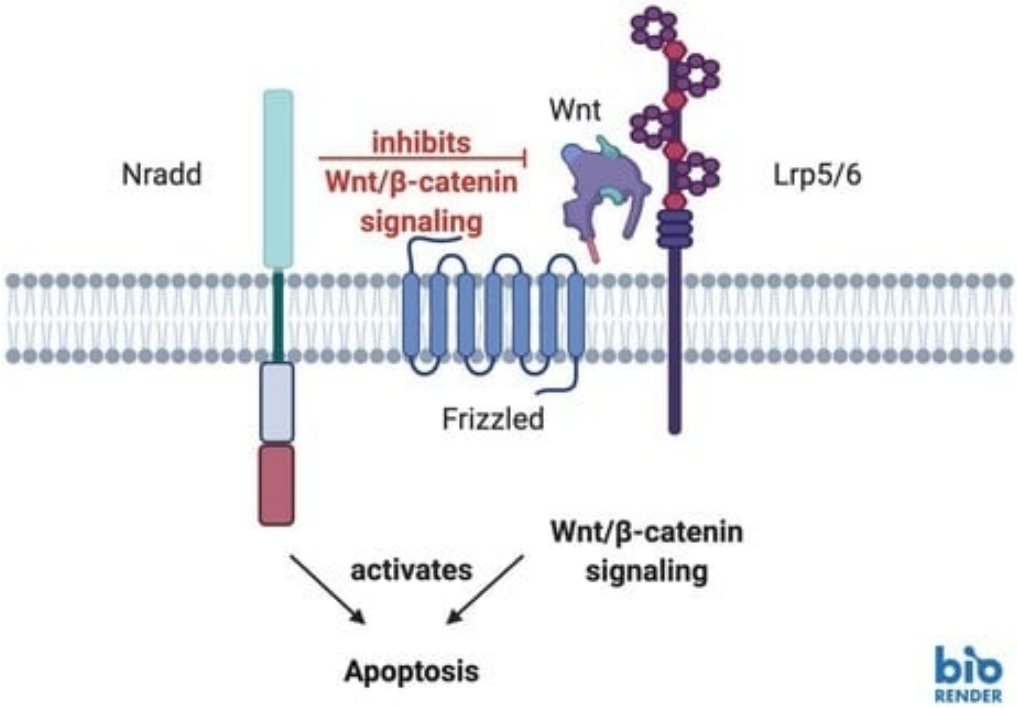

Figure 5: Nradd is a negative modulator of Wnt signaling via interacting with Wnt receptor complex and induces apoptosis working together with Wnt pathway. (Ozalp et al., 2021)

Neurothrophin receptor associated death domain (*nradd*) is in the crossroad of two detrimental signaling pathways playing role in formation of AD. Previously, *nradd* is characterized as homologue of and co-expressed with p75NTR in the spinal canal, neonatal retina and dorsal root ganglion and to bear an intracellular DD like p75NTR

(Kanning et al., 2003; Murray et al., 2004). It has been shown that *nradd* can interact with the receptors of neurotrophin signaling including p75NTR, bind ligands and can induce apoptosis (Kim & Hempstead, 2009; Murray et al., 2004). Alongside, zebrafish *nradd* has been characterized as a Wnt/β-catenin target gene, suppresses Wnt/β-catenin signaling both during development and in mammalian cells, controls Wnt-mediated mesoderm and neuroectoderm formation during development, localizes to the plasma membrane where it physically interacts with the Wnt-receptor complex, induces apoptosis in conjunction with Wnt/β-catenin signaling, and can also trigger apoptosis in mammalian cells (Ozalp et al., 2021).

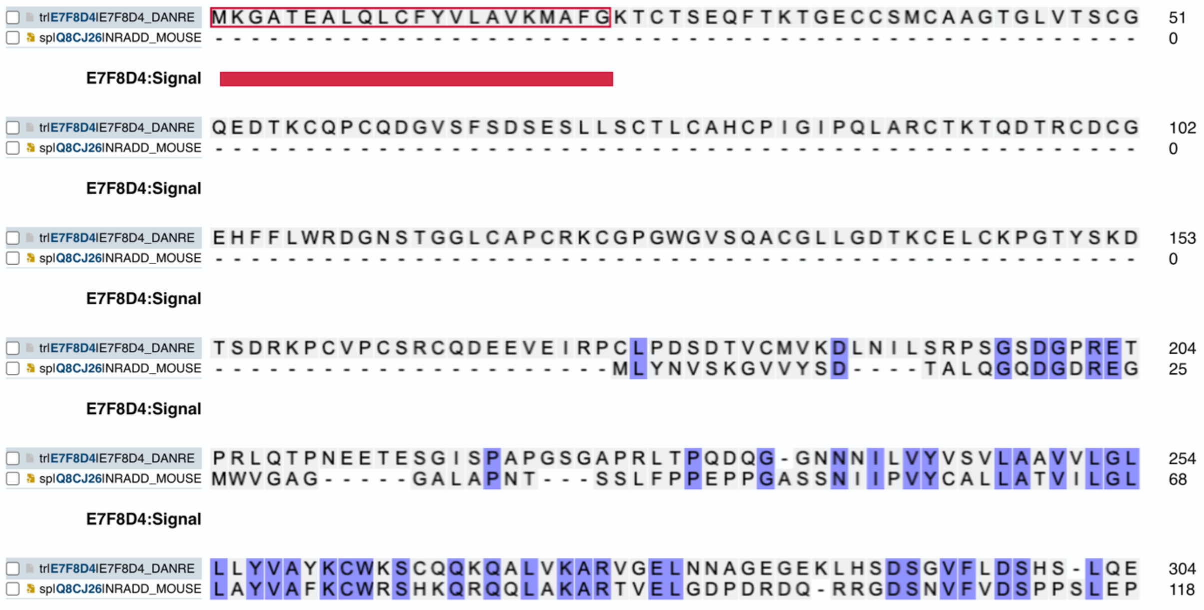

Figure 6: Sequence alignment of the mouse (Mus musculus) NRADD protein (UniProtKB - Q8CJ26, Death domain-containing membrane protein NRADD) and the zebrafish (Danio rerio) Nradd protein (UniProtKB - E7F8D4, Neurotrophin receptor-associated death domain) shows that the zebrafish nradd protein has a much longer extracellular region (approximately 190 amino acids) compared to the mouse Nradd.

Neurotrophin receptor associated death domain is a pseudogene in humans and a functional transmembrane protein lacking N- terminus extracellular domain in mouse and rat. However, zebrafish possess *nradd* as a functional protein with long N-terminus extracellular domain which indicates that it can be contributing to signaling pathways during neurodegeneration or regeneration. When regenerative capacity of the CNS is

compared increased regeneration capacity among human, mouse and rat and zebrafish is observed in adulthood respectively. Also, extensive studies revealed mouse Nradd as γ-secretase substrate and localization of cleaved Nradd into nucleus suggesting alternative functions as well (Gowrishankar et al., 2004; Kanning et al., 2003).

Considering that the zebrafish *nradd* protein's unique long extracellular region, being homologue of p75NTR, its capacity to induce apoptosis during development and homeostasis, and its role as a negative feedback regulator of Wnt/β-catenin signaling, it is very intriguing to elucidate the molecular mechanism of its potential relationship with Aß-induced neurotoxicity. For this purpose, a zebrafish AD model based on Aß42 toxicity will provide a highly suitable platform for evaluating the mechanistic roles of *nradd* protein in the onset of the disease, i.e., the neuronal degeneration process, and the formation of the regenerative response. This approach could reveal the function of *nradd* for AD by uncovering not only the neurodegenerative mechanisms associated with AD but also how the high regenerative capacity of zebrafish overcomes this neurodegeneration.

2. MATERIALS AND METHODS

3.1. Type of Study

This study is used an experimental approach for characterisation of the role of *nradd*'s role in zebrafish model of Aβ42 toxicity.

3.2. Time and Place of Study

This study was conducted at Izmir International Biomedicine and Genome Center from September 2021 and completed in August 2024

3.3. Materials of Study

3.3.1. Zebrafish

The experiments performed in this research used AB wildtype, homozygous nradd mutant line (nradd -/-) and Tg(hsp70:nradd:GFP) transgenic zebrafish line that is a heatschock inducible nradd overexpressing line. All zebrafish lines used in experiments

were between 6 to 12 months old after adulthood is reached. Mutant and transgenic zebrafish lines were produced in house from AB wildtype zebrafish. Zebrafish for this study were provided by Zebrafish Core Unit at Izmir Biomedicine and Genome Center.

3.3.2. Chemicals

A table of chemicals, with their catalog numbers and vendors was given below in Table 1.

Table 1: List of catalog numbers and brands of chemicals used for this study.

Chemical	**Catalog Number**	**Brand**
NaCl	LB.M.106400.5000	Merck
$MgSO_4$	BP213-1	Fisher
$CaCl_2$	1.02382.1000	Merck
KCl	K45117936405	Merck
Methylene Blue	7220-79-3	CARLO ERBA Reagents S.A.S
Tricane Methanesulphonate	886-86-2	Sigma-Aldrich
Qiazol (Trizol)	79306	Qiagen
Borosilicate glass capillary	1B100F-4	World Precision Instruments
Gel and PCR Clean-Up Kit	740609.50	Macherey-Nagel
Digoxigenin (DIG)	11277073910	Roche
Sucrose	CAS 57-50-1	Merck
OCT	10225712	Sakura Finetek USA Inc.
Glass Slide	12-550-17	Fisher Scientific
Proteinase K	25530015	Thermo Scientific
PFA	416780030	Thermo Scientific
Tween 20	P9416-100ML	Sigma-Aldrich
Deionized Formamide	s4117	Merck

Heparin	24590	SERVA
Citric Acid	CIT003.500	Bioshop
Sheep Serum	S2263-100ML	Sigma-Aldrich
BSA	0332-100G	Amresco
Yeast Extract, Bacteriological	J850-500G	Amresco
Tris	0826_1KG	Amresco
$MgCl_2$	0288-500G	Amresco
NBT		
BCIP	0885-500MG	Amresco
MetOH	32213-2.5L	Sigma
Triton-X100	TRX777.500	Bioshop
Sodium Citrate	CIT001	Bioshop
Entellan	1.07961.0100	Merck
Phenol Red	143-74-8	Sigma-Aldrich
Microloader tip	5242956003	Eppendorf
EDTA	0105-1KG	Amresco
Isopropanol	1.09634.2511	Sigma-Aldrich
Sodium Acetate	127-09-3	Merck
EtOH	100983	Merck
96 Well Light Cycler Plate	04729692001	Roche
Nonidet P-40	E109-500ML	Amresco
SDS	SDS001.1	Bioshop
Protease Inhibitor	78429	Thermo Scientific
Phosphatase Inhibitor	04 906 845 001	Roche
6X Loading Dye	B7021S	New England Biolabs (NEB)
Bis-acrylamide	A3574	Sigma-Aldrich
TEMED	T7024	Sigma-Aldrich
APS	7727-54-0	Sigma-Aldrich

Nitrocellulose membrane	10600016	Amersham

3.3.3. Instruments

Table 2: List of instruments used in the experimental procedures during the study.

Device	**Brand**
Basic Power Supply	Biorad
Water Bath and Lid	Nüve
pH Meter	Hanna
Microwave oven	Beko
Balance – Basic	Sartorius
Vortex mixer	Thermo
SZX2 Series stereo microscope	Olympus
SimpliAmp Thermal Cycler	Applied Biosystems
Flaming/Brown Micropipette Puller	Sutter Instrument
PV820/PV830 Pneumatic Picopump MicroCL 17	World Precision Instrument
Applied Biosystems 7500/7500 Fast	Applied Biosystems
MicroCL 17 Microcentrifuge	Thermo
Leica CM 1950	Leica
Light Cycler® 480 Instrument II	Roche
Spectrophotometer Multiskan GO	Thermo
Tissue-Tek® VIP® 6	Sakura Finetek USA, Inc.

Confocal Microscope Zeiss LSM880	Zeiss
ChemiDoc MP Imaging System	Biorad

3.3.4. Kits and Enzymes

Table 3: Kits and enzymes used for this study.

Kits & Enzymes	**Catalog Number**	**Brand**
GoTaq qPCR Master Mix	A6001	Promega
miRNeasy Micro Kit	217084	Qiagen
iScriptTM cDNA Synthesis Kit	1708891	Biorad
NsiI-HF	R3127L	New England Biolabs (NEB)
SbfI-HF	R3642L	New England Biolabs (NEB)
AscI-HF	R0558L	New England Biolabs (NEB)
SphI-HF	R3182L	New England Biolabs (NEB)
In-Fusion® Snap Assembly Master Mix	638947	Takara Bio
Q5 High-Fidelity DNA Polymerase	M0491L	New England Biolabs (NEB)
HiScribe® T7 High Yield RNA Synthesis Kit	E2050S	New England Biolabs (NEB)
RNA Clean & Concentrator-25	R1017	Zymo Research

Guide-it recombinant Cas9 protein	632640	Takara Bio
Fast EvaGreen® qPCR Master Mix	31020	Biotium

3.3.5. Consumables

Table 4: Consumables of the study

Consumables
Needle 30G 0,3x13 mm
Disposable plastic pipette tips
Disposable filter plastic pipette tips
Disposable scalpel
Disposable microtome blades
1,5 mL Eppendorf tubes
2 mL Eppendorf tubes
15 mL centrifuge tubes
50 mL centrifuge tubes
48 well plate
96 well plate
Coplin staining jar
Embedding Molds
Borosilicate glass capillary

3.3.6. Primers

Table 5: Primer sequences of primer pairs used in qPCR experiments.

Gene	**Forward**	**Reverse**
rpl	CCCTCCACCTTATGACAAGAGA	CGTCCAAGCAGGGCAAATTT
pcna	CAAGGAGGATGAAGCGGTAACA	CTGCGGACATGCTAAGTGTG
gfap	ACCCGTGACGGAGAGATCAT	GCCAGTGTCTGAGCCTCATT

ngfra	ACTGATACTCCTTGCGTTGGG	TCCTGTAGCCTACTGTCCTGT
ngfrb	GAGTGCTGCAAACAATGCCA	CCGAGAACGTCTCACTGTCA
nradd	CAGGAAGGCTCCACATTGGG	ACTGATACTCCTTGCGTTGGG

3.3.7. Buffers

Table 6: Composition of buffers used in this study.

Buffer	**Content**
10X Phosphate Buffered Saline (PBS)	80.0 g NaCl 2.0 g KCl 14.4 g Na_2HPO_4 ddH_2O added to final volume of 1000 ml and pH adjusted to 7.2 with HCl.
1X PBSTx	0.1% TritonX-100 in 1X PBS.
4% PFA in PBS	8 g PFA 0.5 ml 2M NaOH 20 ml 10X PBS ddH_2O until 200 ml.
Sodium Citrate Buffer	2.94 g $Na_3C_6H_5O_7$ in 1L ddH_2O and pH adjusted to 6.0 with HCl.
20X SSC Buffer	3M NaCl, 0.3M $Na_3C_6H_5O_7$, set pH to 7.
Hybridization Buffer	50% deionized formamide, 5X SSC, 500μg/ml Yeast RNA, %0.1 Tween 20, %0.1 Heparin and 0.1M Citric acid
Wash Buffer 1 (WB1)	50% deionized formamide, 1X SSC, %0.1 Tween 20.
Wash Buffer 2 (WB2)	2X SSC, %0.1 Tween 20.
Wash Buffer 3 (WB3)	0.2X SSC, %0.1 Tween 20.
Wash Buffer 4 (WB4)	0.1X SSC, %0.1 Tween 20.
NTMT	100mM NaCl, 100mM Tris-HCl, 50mM $MgCl_2$ and 1% Tween 20

3.3.8. Antibodies

Table 7: Antibodies used for this study.

Antibody	**Catalog number**	**Brand**
Anti-mouse NRADD antibody	ABIN908949	AntibodiesOnline
Donkey Anti-Rabbit IgG Antibody (TRITC (Tetramethylrhodamine Isothiocyanate))	711-025-152	Jackson ImmunoResearch
Anti-DIG AP antibody	11093274910	Roche
Cy5-conjugated AffiniPure Donkey Anti-Mouse IgG (H+L) (min X Bov,Ck,Gt,GP,Sy Hms,Hrs,Hu,Rb,Shp Sr Prot)	715-175-150	Jackson ImmunoResearch
Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb #9664	9664	Cell Signaling Technology
GFP (D5.1) XP® Rabbit mAb	2956S	Cell Signaling Technology
Goat anti-Rabbit IgG (H+L) Secondary Antibody, DyLight™ 800 4X PEG	5151S	Cell Signaling Technology
PCNA	MABE288	Merck
Anti S100 beta antibody [EP1576Y] (ab52642)	ab52642	Abcam

Anti-6xHis Mouse Monoclonal Antibody [clone: 1B7G5]	66005-1-IG	Proteintech
Huc/hud Monoclonal Antibody (16A11), Unconjugated, Species Reactivity: Avian, Chicken, Human, Zebrafish, Host: Mouse / IgG2b, kappa	A-21271	A-21271
SOX10 Monoclonal antibody	66786-1-Ig	Proteintech

3.3.9. Aβ42 peptide

The Aβ42 DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA amino acid sequence was purchased from Peptiteam Company, Ankara. The peptide was dissolved in a 1:1:1 ratio of acetonitrile:DMF:ddH2O at a concentration of 400 mM (Bhattarai, Thomas, Cosacak, et al., 2017). It was diluted using 1X PBS half an hour before use.

3.4. Methods Utilized for Study

3.4.1. Zebrafish Handling and Maintenance

All zebrafish in this study were used with approval from the Izmir International Biomedicine and Genome Institute – Local Ethics Comity for Animal Experiments (IBG-AELEC). Zebrafish were maintained in the aquarium systems located in the Zebrafish Core Unit and they were exposed to 12 hours' light and 12 hours' dark per day at 28°C. Embryos of AB wild type fish were used to generate the necessary mutant and transgenic lines. Injected embryos were maintained in E3 medium until 5dpf then transferred into the aquarium system. For the later stages of the study, mainly adult zebrafish were used. Zebrafish were anesthetized using 50mg/L Tricaine (MS-222, Sigma Aldrich) before invasive procedures.

This study is conducted in the scope of 3R principle.

3.4.2. Generation of hs:nradd construct

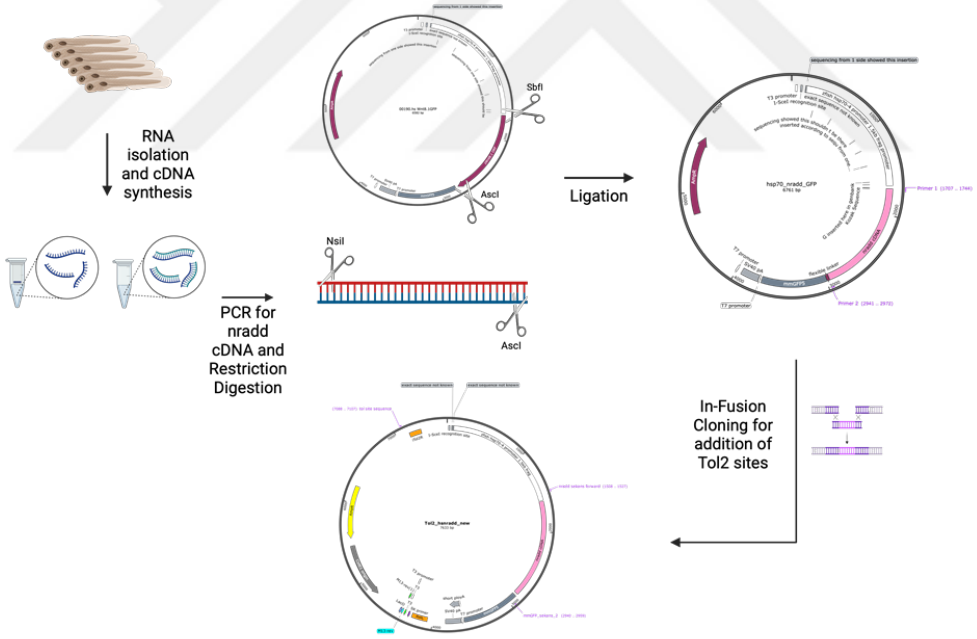

Figure 7: Overall workflow for generation of heat shock inducible nradd overexpressing contruct that have been used to generate Tg(hsp70:nradd:GFP) with Tol2 transgenesis. (Created with BioRender.com)

To generate the heat shock inducible nradd overexpressing transgenic zebrafish line, Tg(hsp70:nradd:GFP), two consecutive sub-cloning were performed. First, for obtaining the hsp70:nradd:GFP cassette and second to transfer this cassette in the middle of Tol2 sites. To obtain the coding sequence of nradd gene, RNA extraction from 3 days post fertilization wilt type AB larvae was conducted using the miRNeasy® Micro Handbook from Qiagen (Germany, 217084) along with QIAzol lysis reagent. The process followed the guidelines provided by the manufacturer. The tissue was homogenized in QIAzol Lysis Reagent using RNase-free disposable pestles and a 20G insulin needle. To avoid genomic DNA contamination, DNAse I from Qiagen, Germany, was used for on-column treatment. The total RNA concentration was measured using a NanoDrop™ 2000/C spectrophotometer from Thermo Fisher Scientific in Massachusetts, United States. By using 1 μg of the product, first strand cDNA was synthesized by using iScript™ cDNA synthesis kit Bio-Rad (CA, USA, 1708890). Produced cDNA was diluted 1:10 and 1μl is used from diluted cDNA in the following step. Then a Polymerase Chain Reaction (PCR) with NEB Q5® High-Fidelity DNA Polymerase (MA, USA, M0491L) was performed with primers flanking the coding sequence of the *nradd* gene with the absence of stop codon and addition of the appropriate single cutter restriction sites added to the Forward and Reverse primers. The restriction sites added were, NsiI (5' ATGCAT 3') to Forward and AscI (5' GGCGCGCC 3') to Reverse primers. Kozak sequence was added to Forward primer to increase the transcription efficiency and triplicate of non-matching bases were also included at 5' of both primers to increase the efficiency of the restriction reaction of the PCR product. The primer sequences used for the PCR reaction are Forward, 5' TCCTATGCATGCAAACATGAAAGGAGCAACTGAAGCACTT 3' and Reverse, 5' TACAAGGCGCGCCGACCACTGATACTCCTTGCGTTG 3'. PCR reactions conditions were adjusted by using the gene specific annealing temperature of primers and is calculated using NEBioCalculator as 63°C. The reaction was set according to the manufacturer's instructions and conditions are as follows; 30 seconds of initial denaturation at 98°C followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 63°C for 30 seconds and elongation step at 72°C followed by final extension at 72°C for 2 minutes. PCR product was controlled by running in 1%

Agarose Gel and visualized using BioRad Gel Doc XR Imaging Systems (CA, USA). After confirming the specificity of the PCR reaction, the product is purified by using Mancherey- Nagel (M&N) NucleoSpin Gel and PCR Clean-up Mini kit (Germany, 740609.50) by following the manufacturer's instructions.

After obtaining the restriction sites added nradd CDS, 1μg of the PCR product was double digested using NEB NsiI-HF & AscI enzymes (MA, USA, R3127S; R0558S) for 2 hours at 37°C. Digested product was run in 1% Agarose Gel and purified by using Mancherey- Nagel (M&N) NucleoSpin Gel and PCR Clean-up Mini kit (Germany, 740609.50) by following the manufacturer's instructions.

For the first sub cloning, backbone was available in our lab with heat-shock inducible promoter (hsp70), Wnt3a gene, 10 amino acids of flexible linker region followed by mmGFP5 fluorescent protein and Ampicillin resistance gene (Amp^R). Cut sites were determined by using SnapGene software and determined as SbfI and AscI (NEB, R3642S; R0558S) and performed at 37°C for 2 hours. SbfI was selected because of its compatible sequence with NsiI after restriction digestion reaction. The product was separated by running in 1% Agarose gel and the backbone was recovered from gel by using Mancherey- Nagel (M&N) NucleoSpin Gel and PCR Clean-up Mini kit (Germany, 740609.50) by following the manufacturer's instructions.

Ligation reaction was performed using obtained nradd insert and backbone by using T4 DNA Ligase (NEB, M0202S) by a 1:3 ratio of backbone to insert. Necessary amounts of both products were calculated by NEbioCalculator and ligation reaction was performed at room temperature for 2 hours. Ligated product was directly transformed to DH-5α *E. Coli* strain and incubated at 37°C overnight. Next day, ten grown colonies were selected and transferred into a liquid culture and grown overnight at 37°C. Then manual plasmid isolation was performed from those cultures and subjected to control digestion using SbfI (NEB, R3642S), a restriction enzyme that cuts from inside of the insert, and EcoRV (NEB, R0195S) that recognizes the promoter region to confirm that *nradd* is successfully ligated in to the hsp70 backbone. Positive colonies were identified by running the digested products on 1%

Agarose gel and plasmids showing double bands in gel was identified as positive colonies.

To further confirm the plasmids, three of them were Sanger Sequenced in order to confirm that there are no mutations. To prepare the plasmids for Sanger Sequencing, a PCR reaction was set with NEB Q5® High-Fidelity DNA Polymerase (MA, USA, M0491L) and with primers specific to hsp70 promoter region and from the mmGFP5 sequence to fully sequence the insert. Primes sequences are as follows, Forward; 5' GAATTTCACTTGTTGACTAG 3' and Reverse; 5' TACCCATTAACATCACCATC 3'. Small amount of the PCR product was run on 1% Agarose gel to control the specificity of the reaction, and the rest was sent to Eurofins, GATC, Germany for Sanger Sequencing.

Second sub cloning was performed using In-Fusion® HD Cloning Kit (TakaraBio, 102518) due lack of necessary restriction sites on backbone containing Tol2 sequences. There was a plasmid containing Tol2 sites and and Amp^R gene in our lab available. As the first step, the generated plasmid on previous step was double digested using NsiI and SacI enzymes (NEB, R3127S; R3156S) at 37°C for 2 hours. The product was run on 1% Agarose gel and the cassette was purified from agarose gel by using Mancherey- Nagel (M&N) NucleoSpin Gel and PCR Clean-up Mini kit (Germany, 740609.50) by following the manufacturer's instructions. The backbone containing Tol2 sites was amplified with primers specifically designed to amplify the region of Tol2 sites and Amp^R with an extra fifteen bases homologue to 5' ends of both strands of the insert cassette by using TakaraBio's primer designing tool from https://www.takarabio.com/learning-centers/cloning/primer-design-and-other-tools. Primer sequences are as follows, Forward; 5' CGGCCGCGGCGCCAAGTTATAATTTCCCTAATTTCCAGGT 3', Reverse: 5' CCGCCACCGCGGTGGTCTCTATCAAGACTAATACACCTCT 3'. Product from this reaction was run on 1% Agarose gel for confirmation and purified by Mancherey- Nagel (M&N) NucleoSpin Gel and PCR Clean-up Mini kit (Germany, 740609.50) by following the manufacturer's instructions.

After obtaining both the insert and the backbone, recombinase reaction was set by using In-Fusion® HD Cloning Kit (TakaraBio, 102518). Manufacturer's instructions were followed. Ligated product was directly transformed to DH-5α *E. Coli* strain and incubated at 37°C overnight. Next day, ten grown colonies were selected and transferred into a liquid culture and grown overnight at 37°C. Then manual plasmid isolation was performed from those cultures and sent directly to Sanger Sequencing for confirmation of the addition of Tol2 sites. Primers for sequencing are as follows; nradd + ; 5' GAATTTCACTTGTTGACTAG 3', nradd - ; 5' TACCCATTAACATCACCATC 3', Tol2 site; 5' TGAATATCTGTTCAGACACC 3'.

3.4.3. Design and Preparation of the Guide RNA (gRNA)

For the generation of nradd homozygous knock out zebrafish line, the guide RNA (gRNA) was designed using the CrisprScan database to target the exon1-intron1 junction with high specificity, aiming to introduce a frameshift mutation and disrupt splicing by targeting the intron1 donor site (García-Tuñón et al., 2019). The gRNA sequence, incorporating a T7 promoter for in vitro transcription, was synthesized and dissolved in nuclease free water to a final concentration of 100 µM. For the annealing PCR reaction, 10 µl each of the nradd gRNA primer and spacer sequence (both at 100 µM concentration) were combined in 180 µl of nuclease-free water. Annealing reaction was set using NEB Q5® High-Fidelity DNA Polymerase (MA, USA, M0491L). Different from a standard PCR reaction, without adding any templates instead, adding 5µl of primer mix prepared before at a final volume of 50µl. Following annealing with spacer sequence, the product was validated for correct size and purity through gel electrophoresis. gRNA and Spacer sequences are as follows; nradd gRNA; 5' TAATACGACTCACTATAGGCGGAAAACCTCACCTTTAGTTTTAGAGCTAGAATAGC 3', Spacer Sequence;5' AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGC TATTTCTAGCTCTAAAAC 3'

Annealed product was purified after confirmation by Mancherey- Nagel (M&N) NucleoSpin Gel and PCR Clean-up Mini kit (Germany, 740609.50) by following the manufacturer's instructions. By using 1µg of the purified product, in vitro transcription

reaction was performed using HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB, E2050L) according to manufacturer's instructions. Produced gRNA was purified using RNA Clean and Concentrator-25 (Zymo Research, R1017).

gRNA was aliquoted to avoid freeze thaw cycles and stored at -80°C freezer until use.

3.4.4. In Vitro Capped Tol2 mRNA Synthesis

Tol2 transgenesis system is well characterized and functioning tool used in gene transfer vectors in vertebrates (Kawakami, 2007) and Tol2 plasmid (pTol2) required for the synthesis of the Tol2 mRNA was available in our laboratory. To obtain the capped Tol2 mRNA, pTol2 was first linearized with restriction enzyme NotI (NEB, R0189S) at 37°C for 2 hours. Linearized plasmid was run on 1% Agarose gel to confirm linearization and purified using Mancherey- Nagel (M&N) NucleoSpin Gel and PCR Clean-up Mini kit (Germany, 740609.50) by following the manufacturer's instructions. 1μg of purified product was used to synthesize capped Tol2 mRNA using mMESSAGE mMACHINE™ SP6 Transcription Kit (Invitrogen, AM1340) following manufacturer's instructions. Produced capped Tol2 mRNA was purified using RNA Clean and Concentrator-25 (Zymo Research, R1017) and stored at -80℃ freezer until use.

3.4.5. Genomic DNA Isolation from 3 Days Post Fertilization Zebrafish Larvae and Adult Zebrafish Caudal Fin

Isolation of pure genomic DNA from 3 days post fertilization (dpf) crispants and adult fish coming from the same progeny is crucial to identify the mutant zebrafish. To achieved that, each sample was put separately in a 50 μl DNA Extraction buffer and incubated at 98°C for 10 minutes. 5μl of proteinase K (5 mg/ml) was added to each tube and incubated at 55°C overnight. Next day proteinase K was inactivated by incubating samples at 98°C for 10 minutes. Samples were centrifuged at 14,000 rpm for 4 minutes to get rid of cell debris and supernatants were transferred in new tubes separately. Then, into each tube, 50μl of nuclease free water, 10μl of 3M Sodium Acetate and 180 μl of isopropanol was added and samples were vortexed. Then samples were incubated on ice for 10 minutes. To precipitate genomic DNA, samples

were centrifuged at 14,000 rpm for 20 minutes at 4°C. Supernatants were discarded and pellets were washed with ice-cold ethanol and left to air-dry. Genomic DNAs were suspended in 10 μl of nuclease free water. Genomic DNA concentration was determined in a NanoDrop™ 2000/C spectrophotometer. Each sample is diluted to concentration of 100ng/μl (Thermo Fisher Scientific, MA, United States) for Sanger Sequencing applications or 2ng/μl for High Resolution Melting Analysis.

3.4.6. Identification of Loss of Function Zebrafish Line by High Resolution Melting Analysis (HRMA) & Sanger Sequencing

High Resolution Melting Analysis is a versatile tool used mostly in variant characterization. Another main usage area is to determine the Insertion-Deletion (Indel) mutations in genome and identify them by calculating the melting point differences among samples and control groups. Working principle is similar to q-RTPCR differs through template, which is genomic DNA in this case in HRMA. In this work HRMA was used as an initial and rapid detection method to identify a founder zebrafish. One set of primer which flanks the gRNA recognition site resulting 148 base pairs of amplicon was designed using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Sequence for the primers is as follows, Forward; 5' AGGAAAAGTGTTGAAGATGAAAGGA 3', Reverse; 5' CAGTTAAAACAACAACAACAGCGT 3'. The PCR reactions utilized 10 µL of Forget-Me-Not™ EvaGreen® qPCR Master Mix for HRM analysis, along with 0.5 µL of each primer (10 µM concentration), 20 ng of genomic DNA (obtained from 3dpf crispants or adult caudal fin), and nuclease-free water to reach a final volume of 20 µL. The amplification was carried out on a LightCycler 480 Instrument II (Roche) using white 96-well plates. The PCR protocol involved an initial denaturation at 95 °C for 2 minutes, followed by 45 cycles of denaturation at 95 °C for 10 seconds and annealing/extension at 56 °C for 30 seconds. A final melting curve analysis was performed by heating from 95 °C to 63 °C at a rate of 0.02 °C/s, with subsequent cooling to 40 °C. Data analysis of the resulting curves was conducted using Roche LightCycler 480 software version 1.5.1.62 (Samarut, Lissouba, and Drapeau, 2016).

Melting temperature shifted samples were further subjected to sanger sequencing to understand the exact mutation caused by CrisprCas9 injections. Genomic DNA from zebrafish were amplified with NEB Q5® High-Fidelity DNA Polymerase (MA, USA, M0491L) using primers flanking the target sequence with a larger amplicon size differing from HRMA. PCR products were directly sent to Eurofins, GATC, Germany for Sanger Sequencing with the same primers used in PCR reaction.

3.4.7. Generation of nradd Loss of Function Zebrafish Line

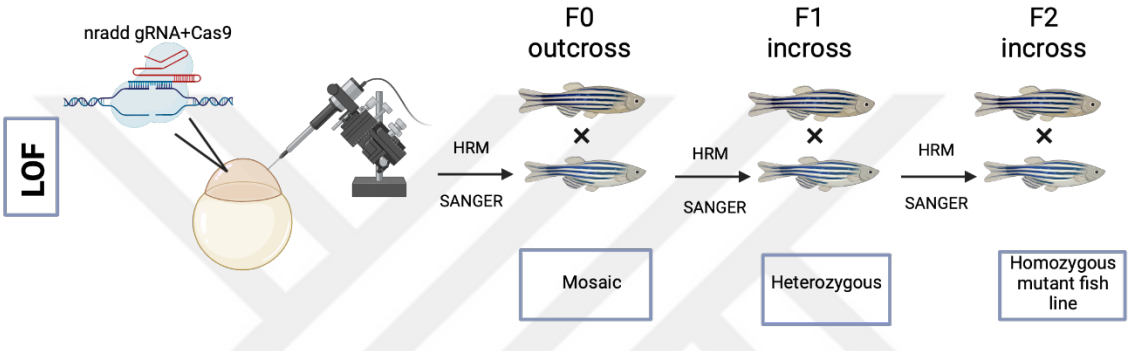

Figure 8: Overall workflow for the generation of the nradd Loss of Function zebrafish line (Created with BioRender.com).

The *nradd* gRNA, along with Cas9 protein at concentrations of 300 ng/μl and 600 ng/μl respectively, were microinjected into zebrafish embryos at the single-cell stage. Rapid validation of resulting *nradd* mutations was performed using high-resolution melting analysis (HRMA) 3 days post-microinjection, providing an early readout (Samarut et al., 2016). Subsequent validation involved sequencing the relevant genomic DNA region. Injected embryos containing *nradd* gRNA and Cas9 generated mosaic F0 fish. Upon maturation, F0 fish were crossed with wild-type AB fish, resulting in an F1 generation containing heterozygous individuals. Heterozygous mutants were identified by HRMA, and genomic DNA isolated from their tail fins underwent Sanger sequencing. These heterozygous mutants were raised and crossed with each other to produce an F2 generation of fish, adhering to Mendelian genetics principles to obtain homozygous mutant individuals.

3.4.8. Generation of nradd Gain of Function Zebrafish Line

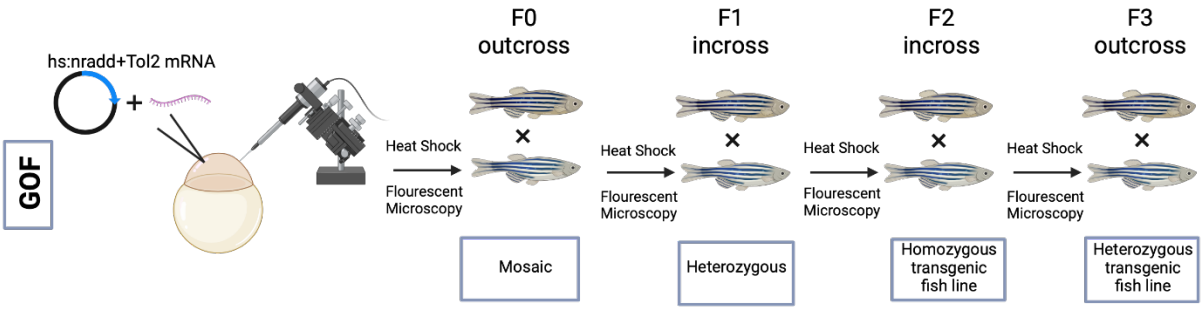

Figure 9: Overall workflow for the generation of the nradd Gain of Function zebrafish line (Created with BioRender.com).

The hs:nradd plasmid, along with capped Tol2 mRNA at concentrations of 25 ng/µl each, was injected into zebrafish embryos at the single-cell stage. Following a one-hour heat shock at 37 °C, larvae were confirmed to harbor the integrated heat shock cassette in their genomes of mosaic F0 fish (Kawakami, 2007). Upon maturation, F0 adults underwent another round of heat shock, and GFP-positive fish were selected one hour later. These fish were crossed with wild-type AB zebrafish to establish a heterozygous F1 line. Through heat shocks during both larval and adult stages, positive individuals were identified and used for incrossing to generate a homozygous *nradd* GOF (Gain-of-Function) line in the F2 generation. While the initial focus was on creating a heterozygous GOF line, the *nradd* GOF zebrafish line was established and maintained as planned by outcrossing the F2 homozygous *nradd* GOF line with wild-type AB zebrafish to obtain a new heterozygous *nradd* GOF line.

3.4.9. Aβ42 toxicity model via Cerebroventricular Micro Injection (CVMI)

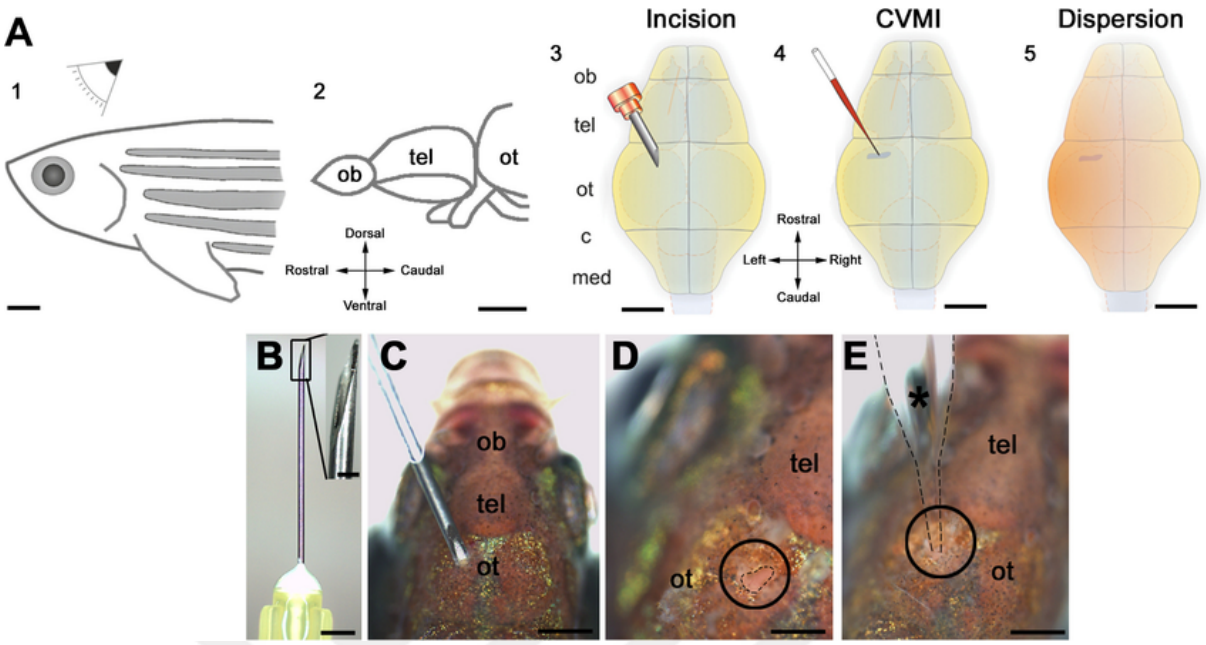

Figure 10: Schematic representation of generation of zebrafish model of Aβ42 using cerebroventricular micro injection (CVMI) (Kizil & Brand et. al., 2011).

Aβ42 toxicity model in zebrafish was generated and characterized by Kizil & Brand (2011) previously. This model is used in this study to mimic the Ab42 toxicity during Alzheimer's Disease (AD), which is one of the main hypotheses in formation of AD. To generate this model for this study, fish were anesthetized in Tricane (Sigma Aldrich, MS-222). After observing the loss of caudal fin reflex, skulls of AB wild type fish, *nradd* LOF and GOF line were punctured using 30G needle towards optic tectum without damaging the brain. 2μl of 40μM Aβ42 was administered from the puncture with micro capillary needles (Figure 11). After the procedure, fish were transferred into fresh system water for recovery.

3.4.10. Tissue Fixation and Cryosectioning

At the end of each experimental procedure, fish were sacrificed by transferring them into system water containing ice and waiting for a few minutes. After the sacrifice procedure, the skulls of zebrafish were removed, and the brains were exposed and transferred into 4% Paraformaldehyde (PFA) solution in PBS and fixed overnight at 4°C with gentle agitation. The next day, tissues were washed three times with

Phosphate Buffer (PB), pH 7.4, to remove PFA. Then, telencephalon tissues were removed from the cranial region and transferred into 30% Sucrose in Phosphate Buffered Saline (PBS) solution until the tissues were completely submerged at 4°C, which took approximately 2 to 3 days. Finally, preserved tissues were embedded in Tissue-Tek O.C.T., inside molds prepared by cutting the upper sides of 1.5 ml tubes and stored at -80 °C until further use.

Embedded tissues were cryosectioned at 12 µm thickness and mounted on Colorfrost Plus™ Microscope Slides Blue Tab (Fisherbrand, 1255017) and slides were dried at 65 °C for 2 hours. Then, stored at -20 °C for Immune Fluorescent and -80 °C for in situ hybridization staining.

3.4.11. Immune Fluorescent Staining and Imaging

Slides coming from section 3.5.10 were taken from the -20°C freezer and left to dry at RT for 30 minutes. Then, they were washed with 0.003% PBSTx twice for 10 minutes, followed by two washes with xPBS for 5 minutes each. The antigen retrieval step was performed using 10 mM Sodium Citrate buffer, pH 6, for 15 minutes at 85°C. Then, the sodium citrate was removed by washing the slides twice with xPBS for 5 minutes, followed by washing with PBSTx twice for 10 minutes. Primary antibodies were diluted in PBSTx to a concentration of 1:300 regardless of the antibody and incubated overnight at 4°C. The next day, excess antibody was cleared with PBSTx wash three times for 10 minutes before secondary antibody incubation. Secondary antibodies were diluted in PBSTx to a concentration of 1:500 and incubated for 2 hours at RT in the dark. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) diluted 1:500 in PBSTx during the last 30 minutes of secondary antibody incubation. Excess antibodies were cleared by washing three times with xPBS for 5 minutes. Slides were mounted in 70% Glycerol in xPBS and stored at 4°C until imaging.

Images were acquired by Zeiss LSM880 confocal microscope and processed using Zen Blue software and ImageJ.

3.4.12. In Situ Hybridization

Slides coming from section 3.5.10 were taken from the -80°C freezer and left to dry at RT for 30 minutes. Slides were rehydrated with xPBS by washing three times for 5 minutes each. The first two washes were performed at RT, and the last was performed at 37°C. Then, slides were incubated in 10 µg/ml Proteinase K in PBST for 5 minutes. Proteinase K was inactivated by incubating the slides in 4% PFA in PBS for 20 minutes at RT. PFA was cleared by washing the slides five times for 5 minutes each in PBST. Then, the slides were incubated in Hybridization buffer for 1 hour at 65°C. The probe against *nradd* was already synthesized previously in our laboratory and used in Ozalp et al. (2021). For this step, the recovered probe solution was used, which contained 0.5 ng/µl *nradd* probe in Hybridization buffer. This solution was pre-heated to 65°C, added to the slides, and incubated overnight at 65°C.

The next day, slides were washed with Hybridization buffer for 20 minutes at 65°C. Then, consecutive washing steps were performed with buffers containing gradually decreasing salt content to protect and stabilize the hybridization of the probe. Each washing step lasted 30 minutes, was repeated twice, and was performed with Wash buffer 1, Wash buffer 2, Wash buffer 3, and Wash buffer 4, respectively. After the washing steps, slides were rinsed with xPBS and blocked for 45 minutes with 10 mg/ml Bovine Serum Albumin (BSA) in PBST at RT. Blocked slides were incubated with anti-digoxigenin-AP (Roche, 11093274910) diluted 1:500 in 2 mg/ml BSA in PBST at 4°C overnight.

On the final day, slides were washed to clear excess antibody with PBST four times for 30 minutes at RT, followed by three washes with Alkaline Phosphatase (AP) assay buffer NTMT for 5 minutes each. Finally, AP substrate containing 3.5 µl each of 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro blue tetrazolium (NBT) in 993 µl NTMT was added to the slides and incubated at 37°C and monitored closely for signal development (Thisse & Thisse, 2008).

3.4.13. RNA Isolation, cDNA synthesis and Quantitative Real Time Polymerase Chain Reaction

After experimental procedures, telencephalon tissues were dissected and immediately homogenized with QIAzol lysis reagent. Total RNA isolation and cDNA synthesis were performed as previously described in section 3.5.2, using the miRNeasy® Micro Handbook from Qiagen (Germany, 217084) and the iScript™ cDNA synthesis kit from Bio-Rad (CA, USA, 1708890). In all processes, manufacturer's instructions were followed.

Quantitative Real-Time PCR (q-RT PCR) reactions were set up with GoTaq® qPCR Master Mix (Promega, A6002) according to the manufacturer's instructions, with a final template concentration of 40 ng/well, with primers in table 4.

3.4.14. Behavioral Assays: Novel Tank, Mirror Biting and Social Interaction Assay

Three different behavioral analyses were employed in this study, each focusing on different behavioral aspects altered in neurodegenerative diseases, specifically in the context of neurodegeneration caused by Alzheimer's Disease.

The Novel Tank Assay aims to measure the anxiety levels of zebrafish introduced to a novel environment. For this assay, the fish were placed individually in a system tank and recorded for 30 minutes with a video camera (Sony, HDR-CX405), without an acclimation period. Zebrafish behavior was traced using Smart 3.0 (Panlab). During the analysis, the tank was equally divided into three 5 cm lateral sections marking the bottom, middle, and top sides of the tank. The software retrieved the following metrics: total distance traveled (cm), mean speed (cm/s), distance traveled in the bottom zone (cm), time spent in the bottom zone (s), and the number of entries into the top zone. Statistical analysis was performed as described in section 3.5.20 (Blaser & Rosemberg, 2012).

The Mirror Biting Assay aims to measure the aggressiveness levels of zebrafish. To assess this, a mirror designed to fit on the narrow facet of the tank cover by an opaque barrier was placed. The behavior of the fish was recorded for 20 minutes with a video camera (Sony, HDR-CX405), following an acclimation period of 5 minutes. After

this period, the opaque barrier was removed, making the mirror visible to the fish. Analysis was conducted using Smart 3.0 (Panlab) software. During the analysis, the tank was segmented into three zones: the attack zone, extending 1 cm from the mirror; the approach zone, extending 3 cm from the mirror; and the mirror far zone, spanning the remainder of the tank. The software retrieved the following metrics: total distance traveled (cm), distance traveled in the attack zone (cm), distance traveled in the approach zone (cm), mean speed (cm/s), time spent in the approach zone (s), entries into the approach zone, time spent in the attack zone (s), and entries into the attack zone. Statistical analysis was performed as described in section 3.5.20 (Audira et al., 2018).

The Social Interaction Assay aims to measure the social behavior of the zebrafish. To assess this, a glass separator designed to divide the tank into two equal parts, enabling the fish to see each other, was used. The experimental group was placed on one side and wild-type Golden zebrafish were placed on the other. The fish were recorded for 20 minutes, with the first 5 minutes being the acclimation period. Analysis was conducted using Smart 3.0 (Panlab) software. During the analysis, the tank was segmented into three zones: the social zone, extending 1 cm from the mirror; the approach zone, extending 3 cm from the mirror; and the barrier far zone, spanning the remainder of the tank. The software retrieved the following metrics: total distance traveled (cm), distance traveled in the social zone (cm), distance traveled in the approach zone (cm), mean speed (cm/s), time spent in the approach zone (s), entries into the approach zone, time spent in the social zone (s), and entries into the social zone. Statistical analysis was performed as described in section 3.5.20 (Audira et al., 2018).

3.4.15. Cell culture

In this study, HEK293T and SH-SY5Y cell lines were utilized. HEK293T cells were chosen for their ease of transfection, while the SH-SY5Y cell line, derived from malignant neuroblastoma, was selected due to its representation of neural cell types. Notably, both cell lines exhibit activity for γ-secretase (Jämsä et al., 2011; Teng et al., 2010). HEK293T cells were cultured in Roswell Park Memorial Institute (RPMI) 1640

Medium (Gibco, 21875-034) supplemented with 10% Fetal Bovine Serum (FBS) (Biological industries, 04-007-1A) and 1% Penicillin Streptomycin (Gibco 15070063). SH-SY5Y cells were cultured in Dulbecco's Modified Eagle Medium F12 (DMEM F12) (Thermo Fisher Scientific, 11-320-033) supplemented with 10% Fetal Bovine Serum (FBS) (Biological industries, 04-007-1A) and 1% Penicillin Streptomycin (Gibco 15070063). Each cell line was subcultured every two days and stored in an incubator at 37°C, 5% CO_2 and 95% humidity.

3.4.16. Generation of Nterm-6xHis-nradd-GFP-Cterm Construct

Nterm-6xHis-nradd-GFP-Cterm construct was generated to be able to detect the extracellular and intracellular domains of the protein in western blot analysis for understanding whether nradd is a substrate of γ-secretase complex. A plasmid which enables nradd expression in cell culture was generated before (Ozalp et al., 2021). Nradd possesses a membrane localization signal which gets truncated after reaching to the plasma membrane. Addition of the 6xHis-tag had to be done after the signal sequence. That sequence was determined using SignalP 5.0 database (https://services.healthtech.dtu.dk/services/SignalP-5.0/) as first 22 amino acids and 6xHisTag was inserted after signal peptide.

To insert 6xHisTag, plasmid was linearized via PCR with CloneAmp™ HiFi PCR Premix (Takara Bio, 639298) using Forward: 5' ACCATCACCACCATCACACGTGCACCAGTGAGCAG 3' and Reverse: 5' GATGGTGGTGATGGTGCTTGCCAAAGGCCATCTTTACGG 3' primer pair. Forward primer contained the 6xHisTag sequence and this sequence was inserted to the plasmid during the PCR reaction and purified using Mancherey- Nagel (M&N) NucleoSpin Gel and PCR Clean-up Mini kit (Germany, 740609.50) by following the manufacturer's instructions. Purified product was circularized using In-Fusion® HD Cloning Kit (TakaraBio, 102518) and transformed into DH5-α *E. Coli* strain and incubated at 37°C overnight. Next day, ten grown colonies were selected and transferred into a liquid culture and grown overnight at 37°C. Plasmids were isolated using NucleoSpin Plasmid Mini kit (Macherey-Nagel, NC0389395) and were sent directly to Eurofins, GATC, Germany for Sanger Sequencing.

3.4.17. Transfection

HEK293T and SH-SY5Y cell lines were subcultured into appropriate flasks and transfected with Nterm-6xHis-nradd-GFP-Cterm construct using Lipofectamine™ 3000 Transfection ReagentG (Invitrogen™ #L3000008) directly after subculturing according to the manufacturer's instruction.

3.4.18. N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) Treatment

N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, commonly known as DAPT, is a well-known inhibitor of the γ-secretase complex. DAPT treatment was initiated 24 hours after transfection, once the GFP signal was observed and all the cells were attached. DAPT was added to the medium to achieve a final concentration of 10 μM and treated for 24 hours (Jang et al., 2022).

3.4.19. Cell Lysate Extraction and Western Blot

Cells obtained from the previous step were scraped from flasks, washed 3 times with PBS and transferred on ice. RIPA buffer with protease and phosphatase inhibitors added was added onto cells, vortexed every 5 minutes for 30 minutes and at the end passed through 30G needle to increase the efficiency. Samples were centrifuged for 45 minutes at 4°C at maximum speed. Supernatants were transferred into fresh tubes and subjected to Bicinchoninic Acid assay (BCA) immediately using Pierce™ BCA® Protein Assay Kit (Thermo Scientific, 23227) according to manufacturer's instructions. After determination of the protein concentration in samples, desired amounts were immediately mixed with Invitrogen novex NuPAGE LDS Sample Buffer (4X) (Fisher Scientific, NP0008) containing 20% 2-Mercaptoethanol (Sigma-Aldrich, M6250) and boiled for 95°C for 5 minutes and stored at -80°C until use.

The samples were separated using a 12% acrylamide-bis-acrylamide gel and transferred to a nitrocellulose membrane (Amersham Protran 0.45 NC, Global Life Sciences Solutions USA LLC, #10600016) at 400mA for 2 hours. The membranes were then blocked with 5% Skim Milk Powder (Bioshop, SKI400.250) for 1 hour at room temperature in TBST. After blocking, the samples were incubated with GFP (D5.1) XP® Rabbit mAb (Cell Signaling Technology, 2956S) and β Actin (D6A8) Rabbit mAb (Cell

Signaling Technology, 8457S) primary antibodies diluted 1:2000 in 5% Skim Milk Powder in TBST overnight at 4°C. For detection, the membranes were treated with Thermo Scientific™ Pierce™ ECL Western Blotting Substrate (Thermo Scientific™ 32106) for HRP-linked staining and imaged using the BIO-RAD ChemiDoc MP Imaging System.

3.4.20. Statistical Analysis

All data generated in this study were initially tested for distribution using the Shapiro-Wilk test. Depending on the distribution and group size, either Student's t-test or one-way ANOVA was used if data shows normal distribution, Mann-Whitney U test or The Kruskal-Wallis test was performed if the data had non parametric distribution for statistical analysis.

Datasets with normal distribution was plotted using mean and standard deviation while non-normal data was represented using median and interquartile range.

All statistical analyses were performed using GraphPad Prism 8 software.

4. RESULTS

4.1. Confirmation of *nradd* Expression in Adult Zebrafish Telencephalon Tissue

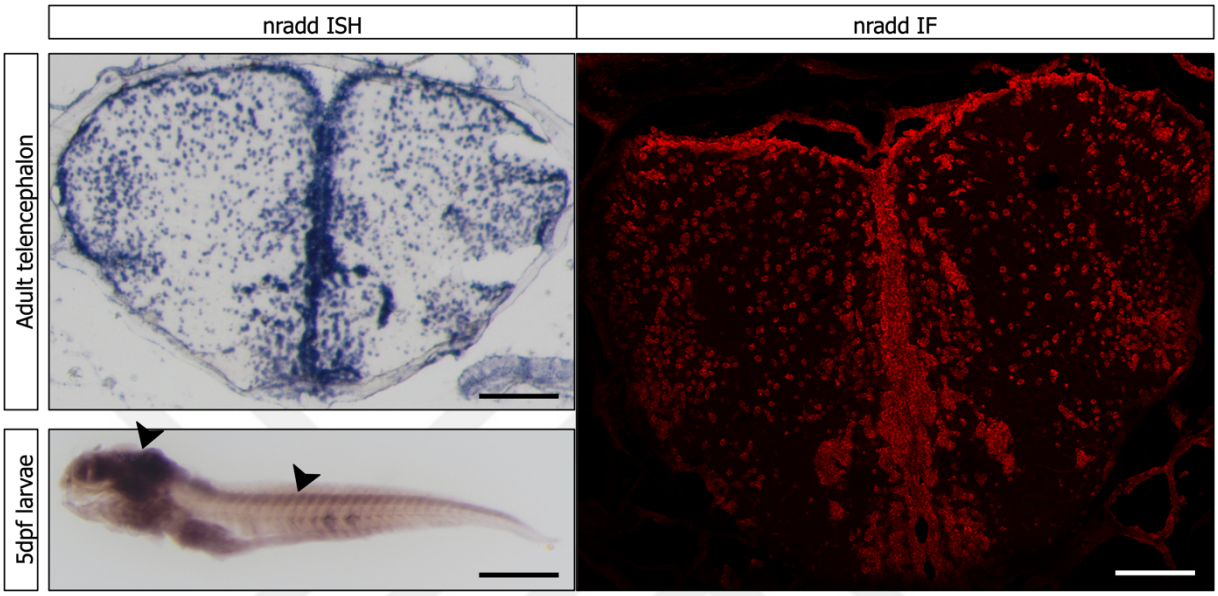

Figure 11: Confirmation of nradd expression in transcript (left) and protein (right) levels in adult zebrafish telencephalon. Error bars represent distance of 100μm. (n=3)

It has been previously shown that nradd is expressed in developing zebrafish nervous system by in situ hybridization (Ozalp et al., 2021). However, there are no knowledge about whether nradd is expressed in adult zebrafish brain. To clarify this question, both mRNA and protein expression was checked via in situ hybridization and immunofluorescence staining and it was confirmed that nradd is expressed and translated into protein in adult zebrafish telencephalon (Figure 11). Expression pattern observed both in ISH and IF staining revealed nradd does not show cell-type or region-specific expression and is globally expressed in adult zebrafish telencephalon.

After confirmation, homozygous nradd knock out and transgenic heat shock inducible nradd expressing fish were generated.

4.2. Generation of nradd Homozygous Knock Out Loss of Function Line

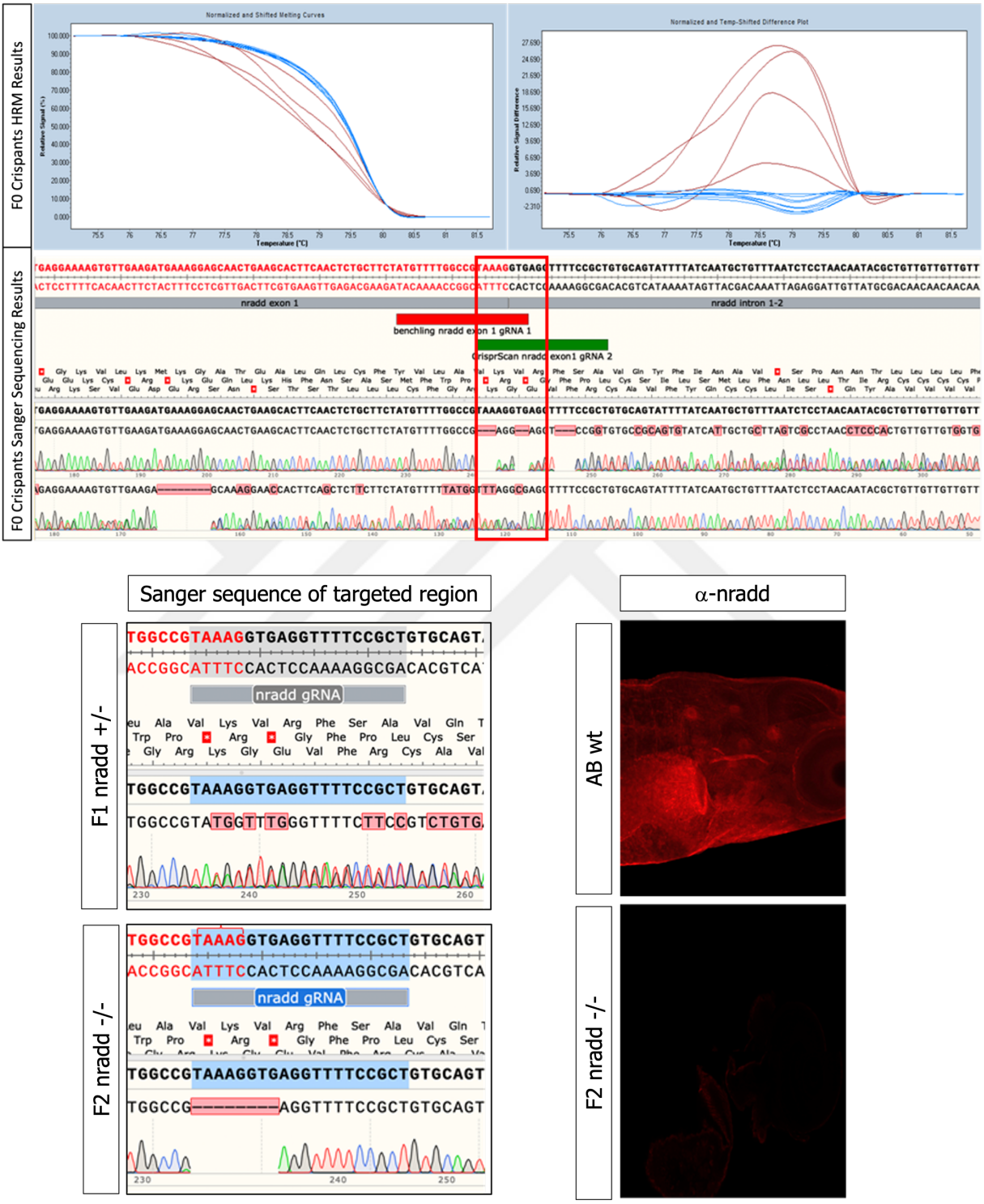

Figure 12: HRMA and Sanger sequencing results of targeted region of nradd reveals a frameshift deletion mutation in F2 homozygous knock out fish and confirmation of the line via whole mounth IF staining with nradd antibody. (n=3)

Nradd homozygous knock out line or Loss of Function (LOF) line was generated using Crsipr/Cas9 gene editing tool. After manipulation of zebrafish embryo at single cell stage, a mutation in that region was identified using genomic DNA coming from caudal fin. In HRMA, amplicons coming from mutant zebrafish line decreased melting points and their melting curves are well separated from wild type zebrafish nradd (Figure 12). After confirming that the gRNA design is working as intendent, F0 fish were outcrossed to obtain heterozygous knockouts. F1 fish were raised to adulthood and targeted region was sent directly to sanger sequencing. Sanger sequence of F1 fish revealed no deletion in the targeted region however reads were observed to have double peaks after targeted pam sequence. Then heterozygous fish were in-crossed to obtain F2 homozygous knock out line. Targeted region coming from F2 was sent to sanger sequencing and the results revealed an 8 bases of deletion, of which 5 of the deleted sequence was on exon 1 and remaining 3 was on intron 1 which confirms there is a frameshift mutation as intended.

As next step, *nradd* expression was checked via whole mouth IF in LOF larvae. As expected, there were no *nradd* staining in LOF line when compared with AB wilt type fish.

4.3. Generation of Heat-shock Inducible *nradd* Overexpressing Gain of Function Line

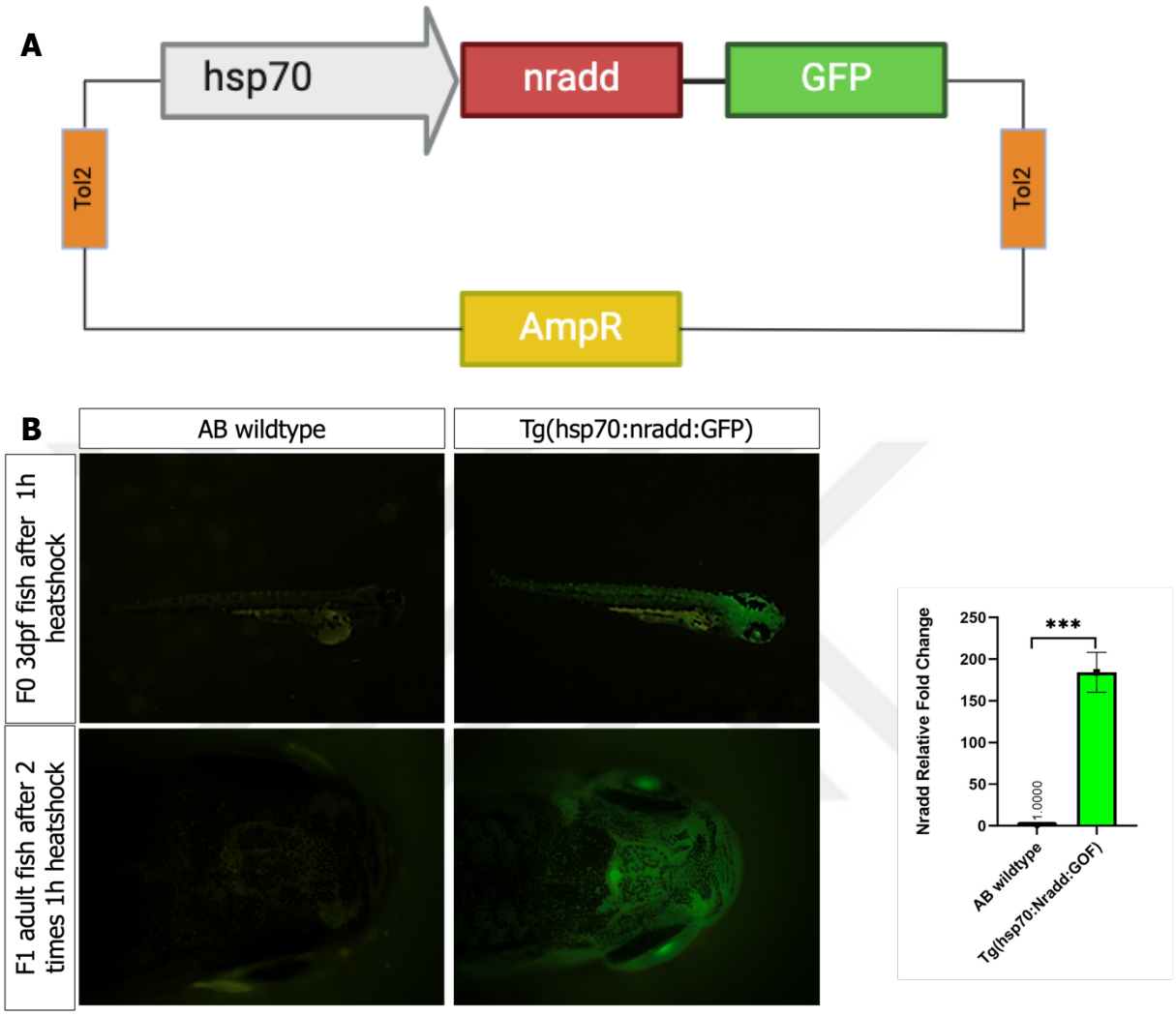

*Figure 13: A) Representative plasmid map generated for GOF line with heat shock inducible cassette and Tol recognition sites. B) Tg(hsp70:nradd:GFP) zebrafish line fluorescing under GFP filter after 2 times 1 hour heat shock and relative nradd expression levels in the adult telencephalon after heat shock. Statistical analyses were performed using student's t-test, *** p<0.001. (n=3)*

To generate transgenic *nradd* line, a new plasmid is cloned containing *nradd* sequence between heat shock inducible promoter (hsp70) and connected to GFP sequence by a flexible linker sequence. This construct was injected alongside Tol2 mRNA into single cell zebrafish embryo and waited for 3 days to zebrafish to develop. 3 dpf larvae were heat shocked and GFP signal was observed under fluorescent

microscope. Positive embryos indicated that transgenesis is successful. Then, after maturation, these fish were outcrossed to obtain heterozygous transgenic fish. Adult fish were heat shocked and GFP expression was confirmed. Same fish revealed to increase nradd expression nearly 200 folds after heat shock in telencephalon tissue in RNA level.

4.4. Characterization of nradd Expression During Amyloid Toxicity

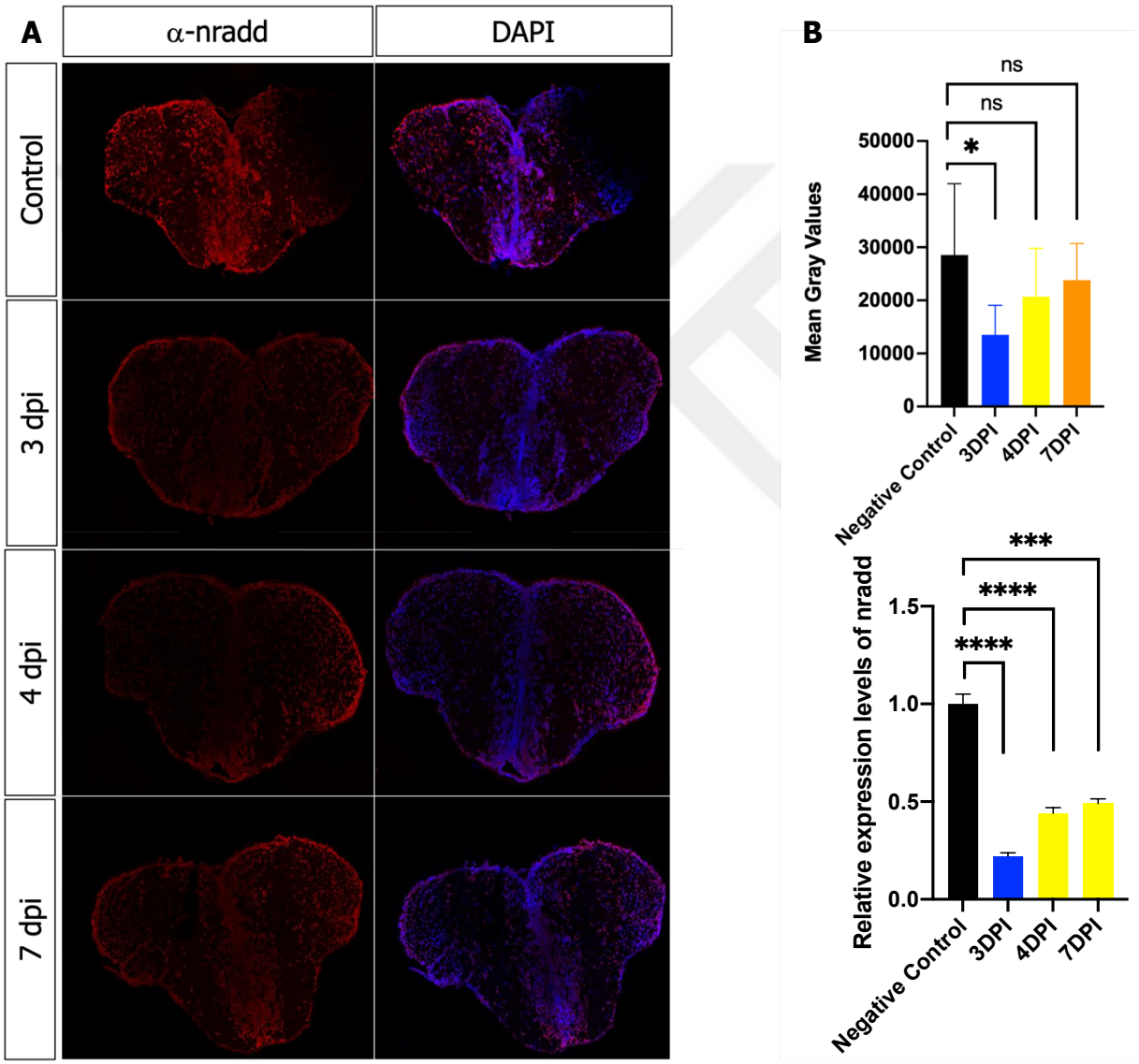

*Figure 14: Immunofluorescence stainings against nradd in wildtype adult zebrafish during Aβ42 toxicity on left. Flourescent intensity measurements and q-RT PCR analysis of nradd expression were represented on the right panel. Student's t-test was used to perform statistical analysis, * p<0.05, ** p<0.01, *** p<0.001 and ns is not significant. (n=3)*

After generating and confirming the necessary lines, *nradd* expression during amyloid toxicity was investigated. For this part it is aimed to understand the expression change of nradd for determining the timepoints for further experiments using LOF and GOF lines (Figure 14). Immunofluorescent staining revealed a significant decrease in 3 dpi of amyloid toxicity, then recovery of expression was observed to control levels in protein level when fluorescent intensity was measured at 4 and 7 dpi. Same pattern was also observed in transcript levels of *nradd* in the telencephalon tissue, a sharp decrease in the expression in 3dpi but trying to recover the expression in transcript level towards 4 and 7 dpi. In transcript level the recovery is not like it is in protein level. Especially in 7 dpi, protein levels show no significant difference while in transcript level 7 dpi seems to be expressed significantly lower than control levels.

By looking at this data, 3 dpi and 7 dpi timepoints were the most suitable for understanding the role of *nradd* in neurodegeneration and regeneration. The 3 dpi was picked because lowest level of expression through timepoints and it is aimed to increase the *nradd* expression using GOF line. LOF line was used in 7 dpi to understand what will happen in the absence of *nradd* when its expression is recovering during amyloid toxicity.

4.5. Characterization of Neurodegenerative Responses Using LOF And GOF Lines

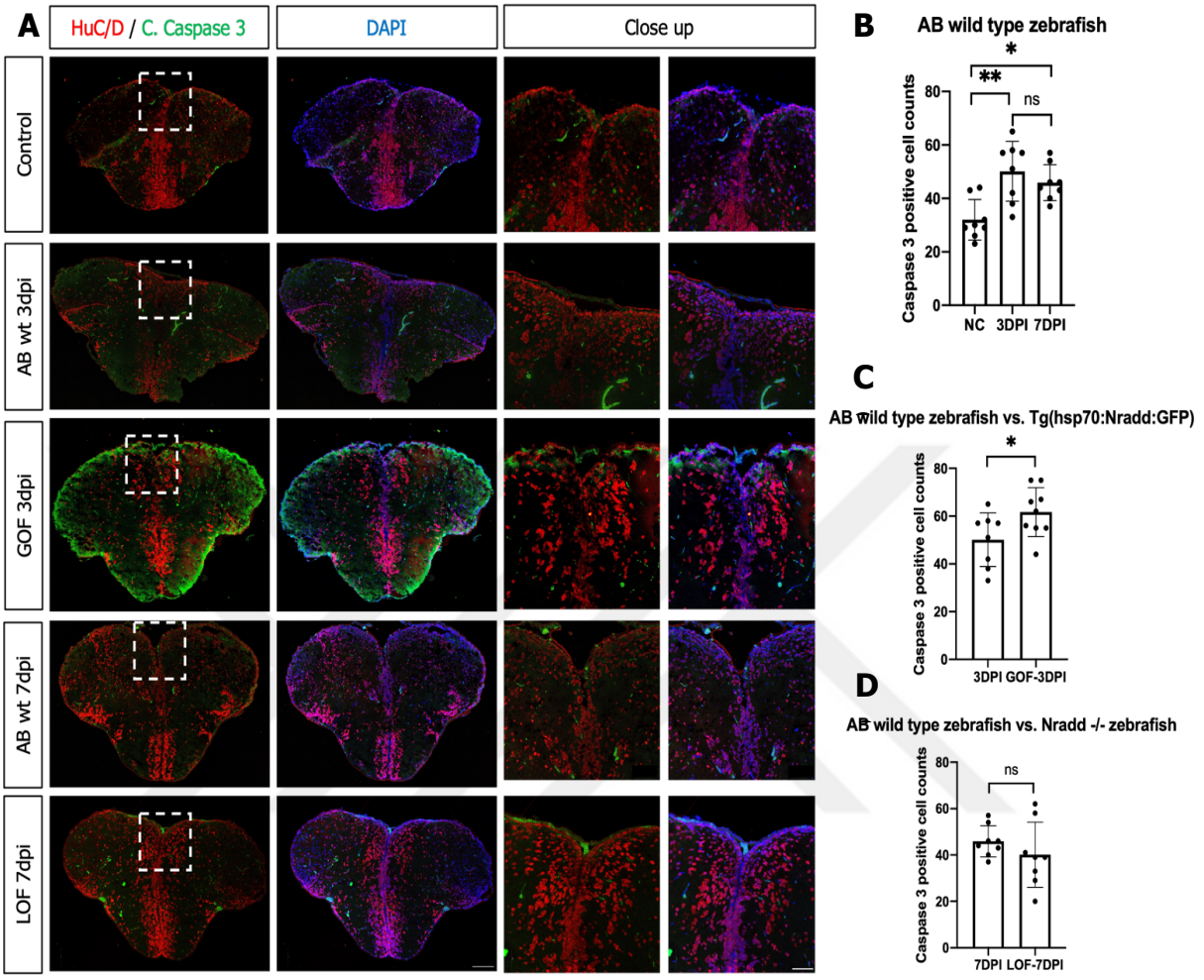

*Figure 15: IF stainings against HuC/D and cleaved caspase 3 reveals neurodegenerative effects of nradd using generated zebrafish lines. Student's t-test and one-way ANOVA were used to perform statistical analysis. * p<0.05, ** p<0.01 and ns is not significant. Scale bars were represented on the final row and corresponds to 100μm in whole telencephalon and 50μm in close up pictures. (n=3)*

For the characterization of neurodegenerative response, a pan-neuronal marker HuC/D and apoptotic marker cleaved caspase 3 were used. It is aimed to quantify and compare the numbers of neurons that undergo apoptosis by counting double positive cells. However, in neither group, apoptotic signal colocalized with pan-neuronal markers as it could be seen in close ups (Figure 15A). Indicating that there is no neurodegeneration in the time points where *nradd* decreased. Regardless of

colocalization, cleaved caspase 3 positive cells were quantified to understand the changing apoptotic responses that may affect other cell types besides mature neurons.

First, it was confirmed that Aβ42 induced toxicity is acting by comparing wildtype control, wildtype 3 dpi and wildtype 7dpi in terms of cleaved caspase counts. This data revealed an increased apoptotic response in 3 dpi and 7 dpi fish when compared with control fish. However apoptotic signal did not decrease towards 7 dpi as well and showed no significant difference between cleaved caspase 3 positive cell counts (Figure 15B).

Then change of apoptotic responses in terms of *nradd* expression is investigated. As previously described, at 3 dpi over expression of *nradd* at the stage where normally expressed the lowest in wildtype fish was achieved by inducing *nradd* expression via heat shock in the day before and in the day of sacrification. In this data, neuronal apoptosis was not seen (Figure 15A), however apoptotic signal is significantly increased in 3dpi GOF line when compared to 3 dpi wt fish (Figure 15C).

Finally, same procedure was done also for 7dpi, using *nradd* LOF fish. For this group, again apoptotic signal did not colocalize with neurons (Figure 15A) and there was no significant difference in cleaved caspase 3 positive cell numbers as well (Figure 15D).

4.6. Characterization Of Degenerative Responses in Other Cell Types

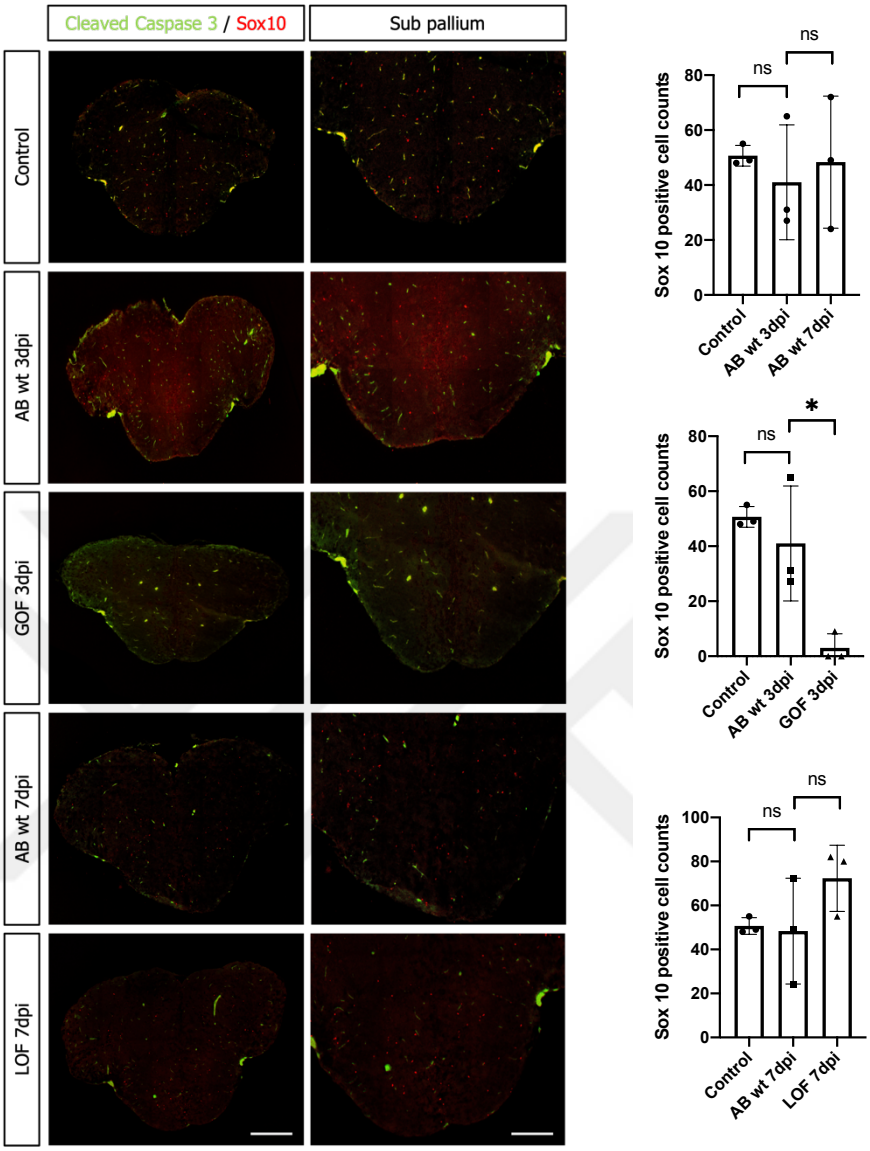

Figure 16: Immunofluorescence images of Sox10 and Cleaved Caspase 3 to reveal other degenerative effects of nradd. Error bars were represented on the final row and corresponds to 100μm in whole brain and 50μm in close up pictures. (n=3)

Since *nradd* is determined to be expressed globally, next changes in oligodendrocyte marker sox10 was investigated. For this section, cleaved caspase 3 staining did not colocalized with sox10 staining indicating, increased cleaved caspase 3 staining observed in GOF line is not affecting oligodendrocytes. Interestingly, there were nearly zero sox10 positive cells in GOF line. In contrary, at 7dpi the cell counts

give increased numbers of sox10 positive cells in LOF 7dpi group however this increase is calculated not significant. Though it is not significant, in this data absence and presence of *nradd* affected a subset of cell population inversely. Indicating *nradd* may influence sox10 positive cells of the zebrafish telencephalon and *nradd* may make certain cell types more vulnerable during Aβ42 toxicity.

4.7. Behavioral Assays

Zebrafish behaviors is well studied and behavioral tests for zebrafish gets more common each day. During amyloid neurotoxicity, some hallmark behaviors have been characterized such as increase in anxiety, alterations in locomotor activity, aggressiveness and decrease in the social behaviors (Audira et al., 2018; Blaser & Rosemberg, 2012). For this panel, zebrafish were subjected to three behavioral tests.

4.7.1. Novel Tank Assay

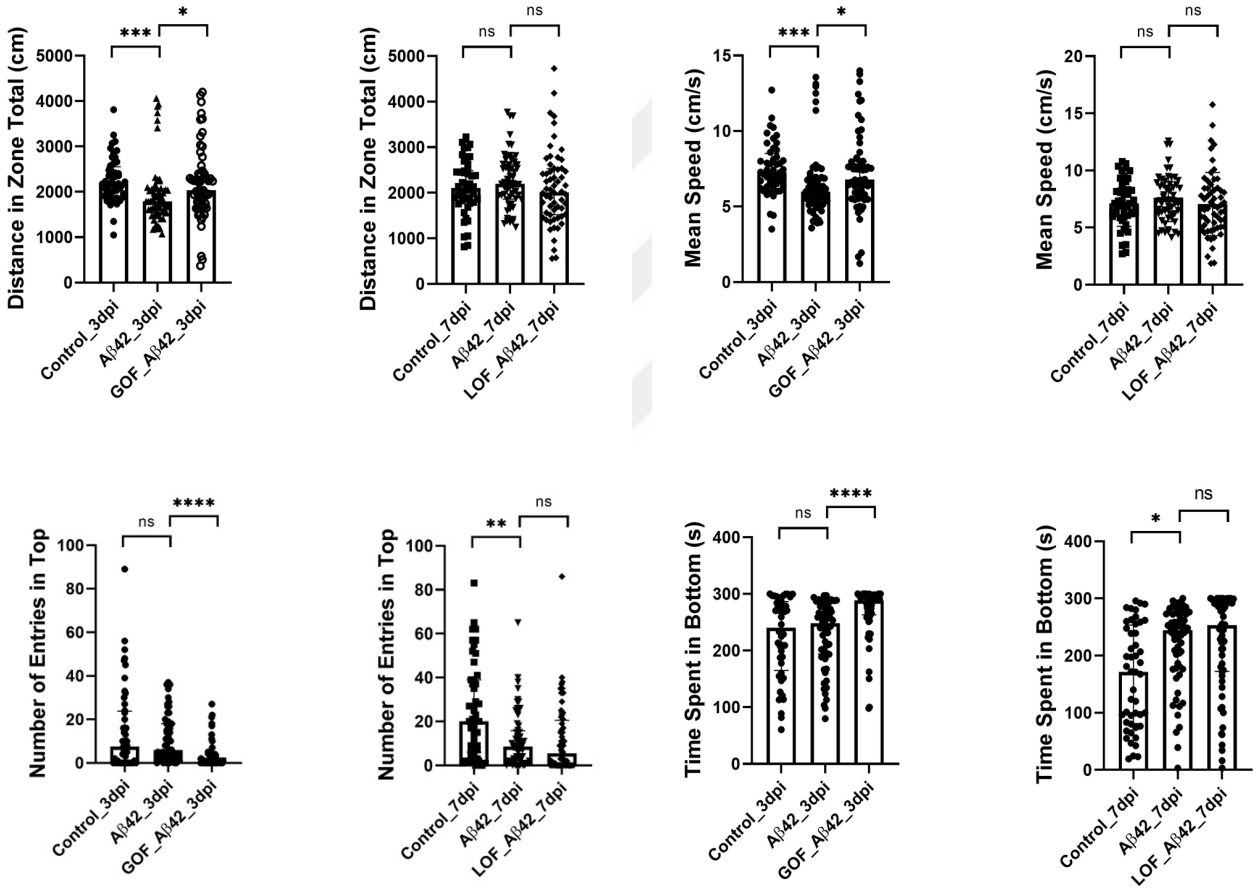

*Figure 17: Analysis of zebrafish behaviors in novel tank assay. 10 zebrafish from each group have been recorded and analyzed. Statistical tests used were one-way ANOVA or Kruskall-Wallis depending on normality of the data. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 and ns is not significant.*

Zebrafish tent to swim at the bottom of the tank when introduced to a new environment. This behavior has been previously associated with anxiety and one can measure the time spent at the bottom of the tank to quantify the anxiety levels. Also, by this method, one can define dimensions of the tank to analyze the mean speed as well for measuring locomotor activity.

Locomotor activities of AB wt 3 dpi fish was decreased when it is compared to AB wt control group as expected. Slight but significant decrease is observed in distance in total zone (cm) and mean speed (cm/s). However, *nradd* GOF line showed increased locomotor activity both in in distance in total zone (cm) and mean speed (cm/s) when compared to AB wt 3dpi.

Locomotor activity of AB wt 7dpi fish revealed no significant difference when compared to AB wt control and *nradd* LOF line revealed no significant difference with AB wt 7 dpi as well.

When number of entries to the upper part of the tank and time spent in bottom of the tank (s) is compared, AB wt control and 3dpi group showed no significant difference indicating that anxiety levels were similar between wildtype groups. However, significant decrease in number of entries to top and an increase in the time spent in bottom was observed when GOF 3dpi is compared with AB wt 3dpi. These results indicate GOF 3dpi group tent to swim more at the bottom and had higher anxiety levels than AB wt 3dpi.

For 7dpi groups, AB wt 7dpi significantly decreased number of entries to the upper zone and significantly increased time spent in bottom meaning at 7dpi Aβ42 injected fish increased anxiety levels. When LOF 7dpi and AB wt 7dpi were compared, there are no significant difference in terms of anxiety, meaning the absence of *nradd* did not affect the anxiety levels 7 days post Aβ42 injection.

4.7.2. Mirror Biting Assay

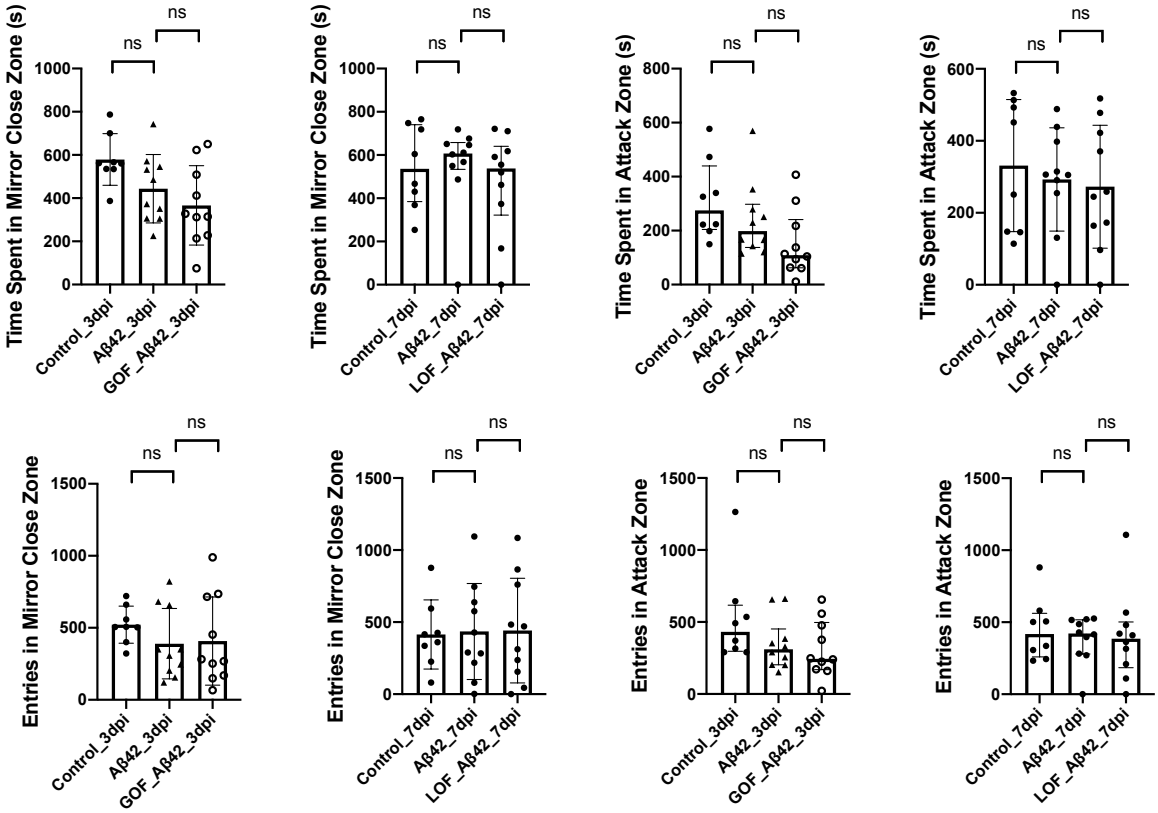

*Figure 18: Analysis of zebrafish behaviors in mirror biting assay. 10 zebrafish from each group have been recorded and analyzed. Statistical tests used were one-way ANOVA or Kruskall-Wallis depending on normality of the data. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 and ns is not significant.*

In the presence of a mirror, zebrafish exhibit a biting and attacking behaviors to their own reflection. For this part, this behavior was measured to quantify the aggressiveness level. To assess aggressiveness, time spent in mirror close zone and attack zone, entries in mirror close and attack zone have been quantified. Observations revealed in 3dpi groups, there are no significant difference in terms of aggressiveness hallmark however, in all graphs a decreasing pattern was observed towards GOF 3dpi. Experimental group where AB wt 7dpi and LOF 7dpi is compared revealed similar data without significance indicating either absence or presence of *nradd* does not affect the aggressive behavior.

4.7.3. Social Interaction Assay

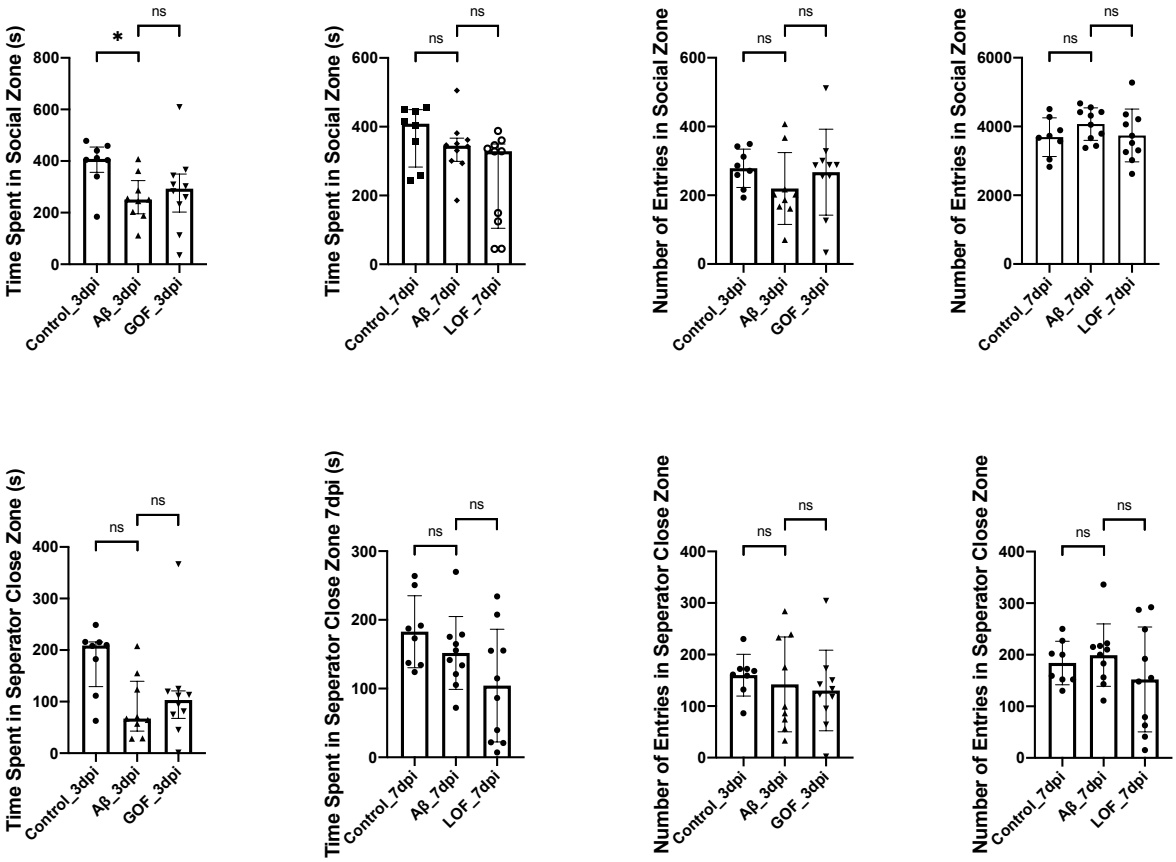

*Figure 19: Analysis of zebrafish behaviors in social interaction assay. 10 zebrafish from each group have been recorded and analyzed. Statistical tests used were one-way ANOVA or Kruskall-Wallis depending on the data distribution. * = p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 and ns stands for not significant.*

In social interaction assay, the tank is divided into half by a clear separator and the time spent and entries in the separator close zone. For this part, analysis was performed accordingly to assess the social behavior of LOF and GOF lines during Aβ42 toxicity. Social zone in our experiment is defined as a more confined space than separator close zone. In 3dpi group, both AB wt 3dpi and GOF 3dpi fish spent less time than control fish however neither number of entries in social zone nor separator close zone differed significantly. For 7dpi group, the situation does not change and neither of the groups showed significant change when compared to their controls. Concluding, nradd does not alter the social behavior, during Ab42 toxicity.

4.8. Zebrafish *nradd* Is Not a Substrate for γ-Secretase Complex

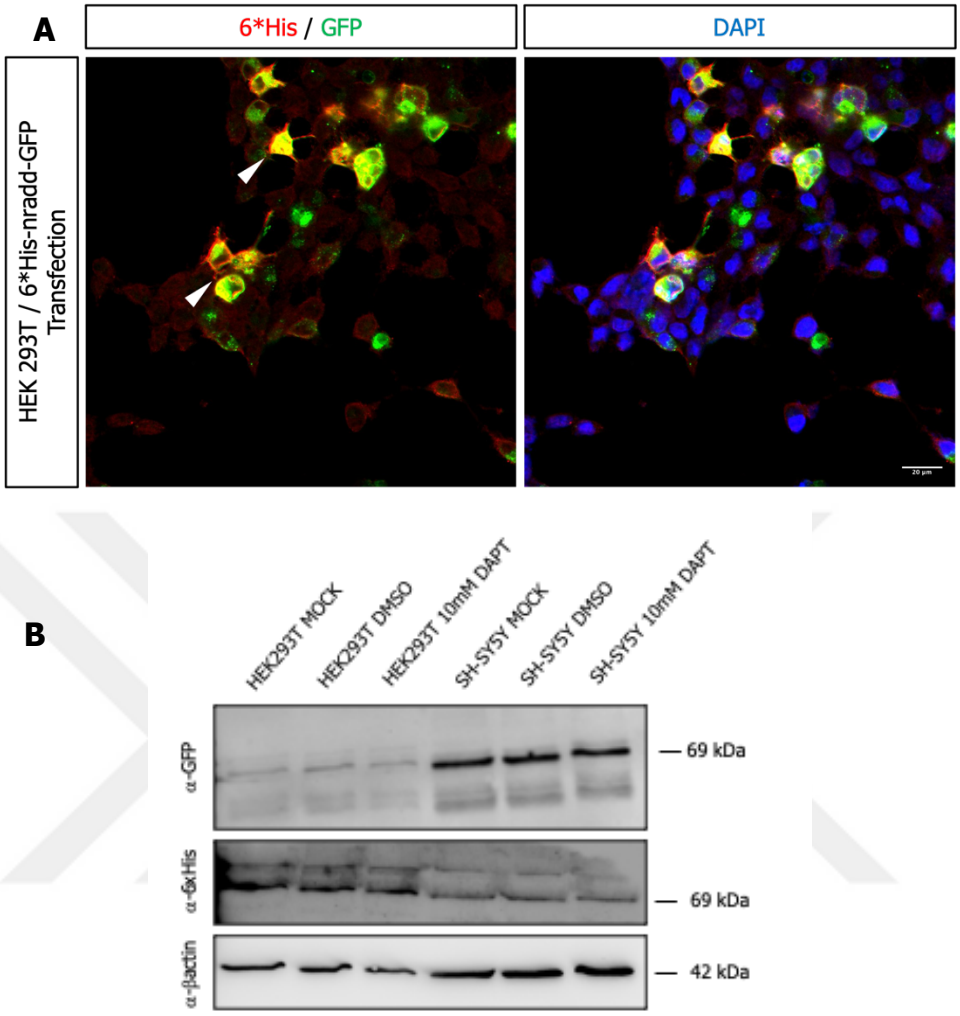

*Figure 20: A) Confirmation of generation of 6*His-nradd-GFP plasmid. Scale bar represents 20μm B) Western Blot image of GFP, 6*His and β-actin proteins in 6*His-nradd-GFP transfected HEK293T and SH-SY5Y cell lines treated with DAPT.*

The other question about zebrafish *nradd* was whether it is a γ-secretase substrate or not. To assess that, 6*His-nradd-GFP construct was generated to be able to detect both ends of the protein. Generated construct was confirmed to bear both tags via IF staining in HEK293T by observing colocalization of two channels (Figure 20A). Confirmed construct was then transfected to γ-secretase active HEK293T and SH-SY5Y cell lines treated with a γ-secretase inhibitor DAPT. Western Blot results reveal no difference in size of the protein in DAPT treated conditions, nor the absence of histidine tag at 69 kDa which is full length *nradd* protein translated from plasmid

(Figure 20B). In contrary to its mouse homologue, zebrafish *nradd* is not a γ-secretase substrate.

5. DISCUSSION

Overall, this study revealed that adult zebrafish globally expresses *nradd* in telencephalon tissue. Global expression of *nradd* could have a serious effect on either neurodegeneration or regeneration. In humans and mice, Aβ42-induced neurotoxicity dysregulates certain signal pathways including p75NTR pathway. Upregulation of p75NTR, which bears a dead domain like *nradd*, may increase the vulnerability of certain cell types to Aβ42 toxicity more than others (Fombonne et al., 2009). To maintain the homeostasis, adult zebrafish must tightly regulate these pathways during neurogenesis. This study reveals the expression pattern of *nradd* in the whole organism; however, a single-transcriptomic analysis can reveal more information on the cell type specificity of the expression pattern. That way, cell type specific mutant and transgenics could have been generated to target only certain cell types, without altering the overall physiology of the model organism.

In wt zebrafish during Aβ42 toxicity *nradd* levels were downregulated at the initial days of toxicity with an increasing trend towards the end of the experimental period; however, nradd levels never reached the control levels within the timepoints tested, including 7dpi. In some cases, as discussed above, p75NTR can get upregulated in the event of toxicity and amyloid fibrils can act as a ligand of p75NTR and trigger apoptotic pathways and cause neurodegeneration (Chakravarthy et al., 2012; Coulson, 2006; Hashimoto et al., 2004; Zeng et al., 2011).Previous studies revealed zebrafish nradd could trigger apoptosis in developing zebrafish (Ozalp et al., 2021). Adult zebrafish may have downregulated nradd expression to favor neuroprotection in the event of endogenous Aβ42 administration. Besides neuroprotection, zebrafish may favor to down regulate nradd expression to protect its neurogenic niches located in the extremities in zebrafish telencephalon. Our toxicity model relays on injecting pre-synthesized Aβ42 peptide directly into cranial cavity. First affected cell populations from this toxicity would be the cells located in outermost layer where neurons and neurogenic niches are located, and these niches would be the most important cell type

during regeneration since neurogenesis capacity is directly correlated with the proliferating cells located at these niches. So, this downregulation may also be due to protection of these niches hence continuum of the regenerative capacity in adult zebrafish telencephalon. At this point, a detailed, cell type-specific analysis of *nradd* expression can give valuable insight into its role in neurogenesis.

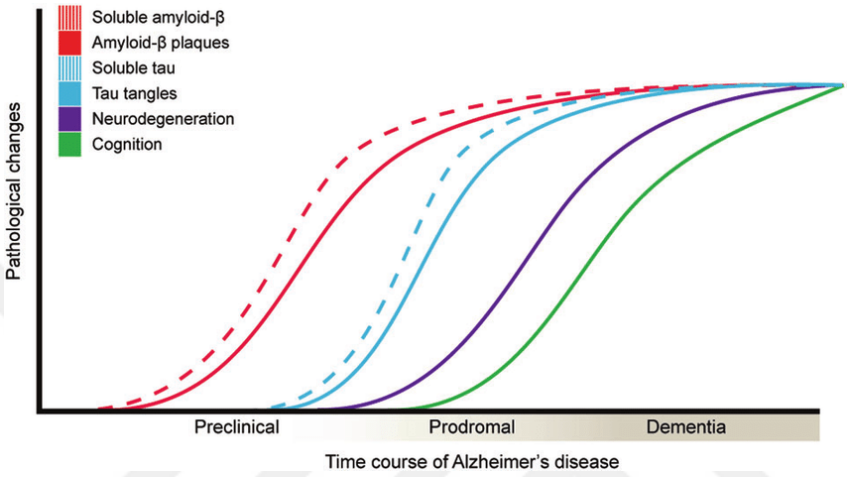

Figure 21: Progression of neurodegenerative events through time course of Alzheimer's disease (Leuzy et al., 2019).

Our findings using LOF and GOF lines also reveal high *nradd* expression also in apoptosis during Aβ42 toxicity. It is observed that zebrafish neurons did not undergo apoptosis in our model of neurodegeneration. Moreover, almost all *sox10* positive cells were ablated upon Aβ42 treatment. This may seem uneusual however, in the clinical progression of disease, neurodegeneration starts at where Aβ42 accumulation peaks (Figure 22) (Leuzy et al., 2019). Our acute model which relies on the accumulation of a single dose of Aβ42 injection in the brain may not be effective enough to induce a robust neurodegenerative response within the timepoints investigated. However, using a chronic transgenic model, like a zebrafish version of mouse PSEN1/APP, for neurodegenerative diseases or daily injections of amyloid plaques would be more informative and mimic the disease pathology more accurately than the acute injury approach. However increased apoptotic responses caused a near ablation of *sox10* positive cells in adult telencephalon during toxicity. This may be caused because during development, *sox10* and *p75NTR* are the key players of neural crest cell differentiation

into Schwann cells and other glial lineages. Sox10 and p75NTR are two molecules thought to be essential for the differentiation of neural crest cells into diverse glial lineages. Sox10 is a transcription factor controlling cell fate through induction of genes concerned with glial identity and function, such as MPZ or S100B (Dechant & Barde, 2002; Drerup et al., 2009; Petratos et al., 2004). The neurotrophins receptor p75NTR controls cell survival and differentiation by modulating the neurotrophin signaling pathways, which impact development and function in these glial cells. Interplay between Sox10 and p75NTR results in a proper balance in the development of glial cells; misregulation of this contributes to peripheral nervous system abnormalities (Dechant & Barde, 2002; Drerup et al., 2009; Petratos et al., 2004). From our results, over expression of *nradd* at 3dpi have cell type specific responses indicating zebrafish *nradd* may be one of the receptors in p75NTR signaling, and its dimerization might result on different outcomes, either survival or cell death, depending on with which receptor *nradd* dimerizes. Absence of *nradd*, did not cause any disruptions in apoptotic mechanism. This phenomenon might be explained by the increased expression of *ngfrb*, a zebrafish homologue of p75NTR, to recover the function of absent *nradd*. This is also observed in homozygous Nradd knock out mouse model where certain types of cells produce more *p75NTR* in the absence of *Nradd* (Unsain et al., 2016). However, it must be considered that, zebrafish *nradd* possess an extracellular domain that can serve as a receptor and might have different functions in zebrafish. Also, *nradd* might be involved in other signaling pathways as well. Zebrafish *nradd's* ability to downregulate Wnt/β-catenin pathway via interacting with Wnt receptor complex may be also valid in the adulthood during amyloid toxicity.

The increased locomotor activity observed in the GOF 3dpi group, compared to Ab wt 3dpi and control, indicates that increased locomotor activity may be due to heightened *nradd* expression. Zebrafish, when introduced to a new environment, exhibit hyperactivity due to their "fight or flight" responses. This hyperactivity may also be caused by heightened neuronal excitability due to amyloid toxicity (Targa Dias Anastacio et al., 2022). Alongside increased locomotor activity in GOF 3dpi, a significant reduction in *sox10*-positive cells was observed in the same group. *sox10*-

positive cells in the adult zebrafish telencephalon are an oligodendrocyte marker and essential for maintenance of oligodendrocytes, which are crucial for proper myelination and neuronal support. Depletion of *sox10*-positive cells indicates that *nradd* overexpression might alter oligodendrocyte precursor cell dynamics, contributing to neuronal dysfunction and increased stress. Moreover, ablation of *sox10* positive cells might cause GOF 3dpi to exacerbate anxiety-like behaviors, like preferring to remain at the bottom of the tank during a novel tank assay.

In contrast, LOF 7dpi showed an insignificant increase in *sox10*-positive cell population, possibly indicating a compensatory response to reduced *nradd* levels. This may suggest that *nradd* may alter the oligodendrocyte susceptibility dealing with neurodegenerative stress, indicating a complex interaction between neuronal and glial cell responses to amyloid toxicity. In 7 dpi groups for behavioral assays, there is no significant difference in locomotor activities and stress levels among AB wt and LOF 7 dpi, indicating there is no change in toxicity progression depending on the reduced levels of *nradd*.

The mirror biting assay and social interaction assay results show that changing the levels of *nradd* does not have a big effect on how aggressive and social adult zebrafish are when amyloid is present. While increased locomotor activity is observed that activity did not translate into aggression and social behavior. These data align with previous findings suggesting zebrafish aggression is often separated from general stress-related hyperactivity. Therefore, *nradd* might only be affecting stress and anxiety responses rather than aggression and social behavior. Moreover, not seeing a significant difference in both GOF and LOF lines on aggression and social behavior might point out that *nradd's* function being more related to cellular stress responses and neurodegeneration rather than social behaviors.

Mouse *Nradd* is also shown to increase apoptosis upon expression in neuronal cells by release of its death domain via γ-secretase cleavage (Gowrishankar et al., 2004). Also, γ-secretase also plays a major role in formation of AD, by intermembrane cleavage of APP to give rise to senile plaques which is a key hallmark of AD (Hampel et al., 2021). After cleavage, intracellular domain of mouse *Nradd,* death domain*,*

translocate in nucleus to induce apoptosis. Given the amino acid sequence of both mouse and zebrafish *nradd* and increment in apoptosis levels during ectopic expression of both *nradds* in mammalian neuronal cells suggests that in zebrafish, *nradd* could trigger apoptosis with the same mechanism and if so, during AD, elevated activity levels of γ-secretase could induce a more severe degeneration in mouse, possibly in zebrafish. Revealing the mode of action can reveal fundamental mechanisms relevant to human neurodegenerative diseases by providing insides into the conserved apoptotic pathways and neurodegenerative mechanisms. To assess whether similar mechanism occurs in zebrafish, 6*His-nradd-GFP plasmid was engineered which bears different tags at opposing termini to detect potential cleavage of *nradd*. The construct was first expressed in HEK293T cells and stained against both tags using fluorophores with different excitation wavelengths. In Figure 20A, white arrowheads indicate the successful generation of the construct. Western blot results revealed no difference in protein size between control and DAPT treated samples revealing *nradd* is not a substrate for γ-secretase. This result suggests that *nradd* may be acting through a pathway distinct from γ-secretase or utilizing alternative proteases in zebrafish.

6. CONCLUSION

In this study, one mutant and one transgenic zebrafish line were produced to characterize the degenerative and regenerative role of *nradd* during Aβ42 toxicity. As synopsis of findings, *nradd* is associated with increased apoptosis during Aβ42 toxicity, specifically acting on oligodendrocytes and affected the anxiety-like behavior but did not affect social behavior and aggressiveness. This mechanism is first thought to act on translocalization of *nradd* DD into the nucleus after γ-secretase cleavage like its homologue *p75NTR* and like mouse *Nradd*. However, mechanistic studies revealed that zebrafish *nradd* is not a substrate of γ-secretase and probably acts on different mechanism or utilizing alternative proteases in zebrafish. To comprehend the mode of action, Co-immunoprecipitation followed by Mass Spectrometry analysis would deepen our understandings by revealing the interaction partners of *nradd,* and could give us a clear picture.

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8. APPENDIX

İZMİR BİYOTIP VE GENOM MERKEZİ
HAYVAN DENEYLERİ YEREL ETİK KURULU (İBG-HADYEK)
KARARI

Toplantı Tarihi	:13/01/2022	**Toplantı Günü**	: Perşembe
Toplantı Sayısı	:1	**Toplantı Saati**	: 11:00

Sayın Doçent Güneş ÖZHAN,

2021-027 Protokol No'lu; yürütücüsü olduğunuz "Alzheimer hastalığında nöronal dejenerasyon ve rejenerasyon yanıtlarının oluşumunda Wnt/β-katenin sinyal iletiminin ve p75NTR homoloğu Nradd'ın rollerinin zebrabalığı amiloid β42 toksisite modelinde araştırılması" başlıklı projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Dr. Gülçin ÇAKAN AKDOĞAN **Başkan**	**Vet. Hek. Kerem ESMEN** **Başkan Yardımcısı**
Prof. Dr. Ensari GÜNELİ **Üye**	(Proje Yürütücüsü) **Doç. Dr. Güneş ÖZHAN** **Üye**
Doç. Dr. Kasım DİRİL **Üye** (Katılmadı)	**Dr. Yavuz DOĞAN** **Üye**
Vet. Hek. Umur KELEŞ **Üye**	**Vet. Hek. Ceren ÜLKER** **Üye**
Vet. Hek. Gizem ARALAN **Üye**	**İrem KARADAŞ** **Üye**

Dogac Ipekgil

Date of birth: 19/07/1999 | **Nationality:** Turkish | **Phone number:** **Email address:** **Email address:** **Website:** | | |

Address:

ABOUT ME

MSc student in Ozhan Development and Regeneration lab. Currently working on investigation of the possible regenerative role of a neurothrophin receptor in zebrafish model of amyloid-beta42 toxicity.

EDUCATION AND TRAINING

01/09/2021 – CURRENT Izmir, Türkiye

MSC IN MOLECULAR BIOLOGY AND GENETICS Izmir Biomedicine and Genome Institute

Supervisor: Prof. Dr. Gunes OZHAN

Generation of mutant and transgenic zebrafish lines via Crispr/Cas9 and Tol2 system.
Zebrafish single cell injection
Molecular biology techniques
Cloning
Immunofluorescence
Confocal Microscope
In-situ hybridisation
High Resolution Melting (HRM) Analysis
Cell culture Techniques, Lentiviral systems
Cerebroventricular Microinjection (CVMI)
Retroorbital Injection
Usage of cryostat device
Western Blot
Zebrafish Behavioral Assays: Novel Tank, Mirror Biting and Social Interaction Assay

Address

Final grade 4.00/4.00

Thesis Investigation of the role of p75NTR homolog nradd in formation of degeneration and regeneration responses in Alzheimer's disease in zebrafish amiloid ß42 toxicity model

12/10/2017 – 15/06/2021 Izmir, Türkiye

BSC IN MOLECULAR BIOLOGY AND GENETICS Izmir Institute of Technology

Address Final grade 3.47/4.00

01/07/2019 – 01/09/2021 Izmir, Türkiye
UNDERGRADUATE RESEARCHER Izmir Institute of Technology

The main subject of the research was an investigation of the effects of the invasion of a protein called Semaphorin 6d in non-metastatic breast cancer cell line MCF7. To achieve this; lentiviral transduction, basic molecular biology and cell culture methods as well as a lab-on-a-chip system were used.

Address G, Türkiye **Field of study** Biology

LANGUAGE SKILLS

Mother tongue(s): **TURKISH**

Other language(s):

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken production	Spoken interaction	
ENGLISH	C1	C1	C1	C1	C1
ITALIAN	A2	A2	A2	A2	A2

Levels: A1 and A2: Basic user; B1 and B2: Independent user; C1 and C2: Proficient user

ADDITIONAL INFORMATION

PUBLICATIONS

Comparative membrane lipidomics of hepatocellular carcinoma cells reveals diacylglycerol and ceramide as key regulators of Wnt/β-catenin signaling and tumor growth

High-fat diet feeding triggers a regenerative response in the adult zebrafish brain – 2023

SEMA6D differentially regulates proliferation, migration, and invasion of breast cell lines – 2022

CONFERENCES AND SEMINARS

09/06/2022 – 12/06/2022 – Acibadem University, Istanbul/Turkey
8th International Congress of the Molecular Biology Association of Turkey (MolBiyoKon'22)

HONOURS AND AWARDS

12/06/2022
Best Poster Prize – FEBS Journal Awarded FEBS Journal best poster prize
Poster Title: Investigation of the role of p75NTR homolog nradd in formation of degeneration and regeneration responses in Alzheimer's disease in zebrafish amiloid ß42 toxicity model

01/05/2022
2210/C National MSc/MA Scholarship Program in the Priority Fields in Science and Technology – THE SCIENTIFIC AND TECHNOLOGICAL RESEARCH COUNCIL OF TÜRKİYE (TUBITAK)

01/04/2020
2209/A Research Project Support Programme for Undergraduate Students – THE SCIENTIFIC AND TECHNOLOGICAL RESEARCH COUNCIL OF TÜRKİYE (TUBITAK)

REFERENCES

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Adress:

Prof. Dr. Ozden Yalcin-Ozuysal

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