Technische Universität Dresden
Medizinische Fakultät Carl Gustav Carus

Modeling of Alzheimer’s disease in adult zebrafish brain and characterization of pathology-induced neural stem cell plasticity

DISSERTATIONSSCHRIFT

zur Erlangung des akademischen Grades

Doctor of Philosophy (Ph.D.)

Vorgelegt

der Medizinischen Fakultät Carl Gustav Carus
der Technischen Universität Dresden

von

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geboren am 07.01.1984 in Hazro (Türkei)

Dresden 2020

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Tag der mündlichen Prüfung : 14.09.2020
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ZUSAMMENFASSUNG

Die Alzheimer-Krankheit ist eine gewaltige Bedrohung für eine alternde Gesellschaft. Millionen von Menschen leben weltweit mit der Alzheimer-Krankheit, für die es keine aktuelle Behandlung gibt. Die Amyloidkaskaden-Hypothese (AKH) ist die aktuell am meisten akzeptierte Hypothese zur Ursache der Alzheimer-Krankheit. Die AKH bietet eine mechanistische Sicht auf die pathologische Kaskade, ausgehend von der Amyloid-Aggregation über die chronische Entzündung bis hin zur TAU-Pathologie. Die Medikamente, die auf der Grundlage der AKH entwickelt wurden, konnten Amyloid-Plaques bei Alzheimer-Patienten entfernen, brachten aber keine Verbesserung der kognitiven Fähigkeiten. Diese Misserfolge legen nahe, dass die Alzheimer-Krankheit nicht nur theoretisch im Rahmen der AKH betrachtet werden kann. Neuere Hypothesen kulminieren die Auswirkungen verschiedener Zelltypen (z.B. neurale Stammzellen, Astrozyten, Oligodendrozyten) auf den Ausbruch der Alzheimer-Erkrankung. Komplexe Rückkopplungs- und Feed-Forward-Mechanismen sind in der Pathophysiologie der Alzheimer-Demenz wahrscheinlich. Das Zusammenspiel zwischen der Pathologie und der Beteiligung anderer Zelltypen macht diese Krankheit multifaktoriell und komplex. Kürzlich zeigten zwei Studien (Moreno-Jimenez et al., 2019; Tobin et al., 2019), dass die Produktion neuer Neuronen im menschlichen Gehirn bei der Alzheimer-Erkrankung dramatisch abnimmt. Eine interessante Hypothese wurde durch diese Studien gestützt: Die pathologisch induzierte Erzeugung neuer Neuronen (regenerative Neurogenese) bei Alzheimer-Patienten könnte helfen, die Pathologie der Alzheimer-Erkrankung rückgängig zu machen. Da die Regenerationsfähigkeit bei Säugetieren entwicklungsmäßig wenig ausgeprägt ist (Tanaka und Ferretti, 2009), kann uns die Untersuchung der Neurodegeneration in einem Modellorganismus mit Regenerationsfähigkeit daher lehren, wie man die Proliferation und Neurogenese neuraler Stammzellen unter pathologischen Bedingungen induzieren kann.

Für diese spezielle Frage können uns Modellorganismen mit natürlicher Regenerationsfähigkeit zeigen, wie man Proliferation und Neurogenese unter den

pathologischen Bedingungen der Alzheimer-Erkrankung induzieren kann. Der Zebrafisch bietet eine beispiellose Möglichkeit, die Neurodegeneration und Regeneration zu modellieren, um die molekularen Mechanismen zu untersuchen, wie anhand der Neurogenese in Wirbeltiergehirnen die Alzheimer-Krankheit verbessert werden kann. Dies wurde in unserem Labor bereits in mehreren Publikationen gezeigt. Aus diesem Grund habe ich in meiner Doktorarbeit Zebrafische verwendet, um die Plastizität neuraler Stammzellen (NSZ) zu untersuchen. Besonders interessierte mich die Heterogenität von NSZ-Populationen in Bezug auf ihre molekularen Programme und die molekulare Grundlage der regenerativen Neurogenese von NSZ auf das Amyloid-β-42 (Aβ42) und TAU-Pathologien.

Wir haben zuvor ein Aβ42 Toxizitätsmodell erstellt, das verschiedene pathologische Merkmale der menschlichen Alzheimer-Erkrankung zeigt, wie Entzündung, Zelltod, synaptische Degeneration und kognitive Abnahme. Ich habe eine transgene Zebrafischlinie erzeugt, die die menschliche Version des TAU-Proteins mit einer Mutation exprimiert, die das Protein krankheitsanfälliger macht (TAU-P301L). Diese Linie wurde verwendet, um Aβ42 und Tau zu kombinieren, um ein kombiniertes pathologisches Modell wie bei der Alzheimer-Krankheit beim Menschen zu erzeugen, da diese beiden Proteine während der Krankheit koexistieren. Die Zebrafisch-Linie exprimierte das menschliche Tau-P301L von der Embryonalentwicklung bis zum Erwachsenenalter, und TAU wurde ab dem Embryonalstadium hyperphosphoryliert. Des Weiteren habe ich die Tau-Pathologie untersucht, indem ich auf Entzündung und Zelltod getestet habe. Die Expression von TAU im Zebrafischhirn zeigte keine pathologische Aggregation und keinen Zelltod. Die TAU-Hyperphosphorylierung führte weder in Abwesenheit noch in Anwesenheit von Aβ42 zur Bildung von (neurofibrillären) Verwirrungen. Darüber hinaus änderte die Tau-Expression in Neuralen Stamm-/Vorläuferzellen (NSVZs) und Neuronen die NSVZ-Proliferation nicht. Diese negativen Ergebnisse sind keine technischen Artefakte, denn in anderen Regionen des Zentralnervensystems wie dem Rückenmark verursachte die TAU-Expression eine Pathologie. Unsere Ergebnisse legten eine interessante Hypothese nahe, nämlich, dass das Gehirn des Zebrafischs Schutzmechanismen zur Verhinderung der TAU-Verwirrung haben könnte. Diese Annahme könnte uns das Wissen liefern, um mögliche Interventionen zur Verhinderung von TAU-Pathien im menschlichen Gehirn zu entwerfen (Cosacak et al., 2017).

Da die Tau-Überexpression sowohl die Toxizität von Aβ als auch die Bildung (neurofibrillären) Verwirrungen nicht verschlimmerte, setzte ich meine Arbeit mit dem

Aβ Toxizitätsmodell fort (Bhattarai et al., 2016). In diesem Modell beginnen NSVZs die Proliferation und erzeugen neue Neuronen über einen spezifischen Signalweg (IL4/STAT6-Signalisierung). Dieses neurogene Ergebnis im Zebrafischhirn nach einer Alzheimer-Erkrankung steht in starkem Kontrast zu den Gehirnen von Patienten mit einer Alzheimer-Erkrankung, bei denen mehrere Studien auf einen starken Rückgang des neurogenen Ergebnisses hindeuteten (Moreno-Jimenez et al., 2019; Tobin et al., 2019). Um die Heterogenität der NSVZs im Zebrafischhirn zu verstehen und neue Mechanismen in der Amyloid-Pathologie zu finden, habe ich daher die Einzelzell-Sequenzierung verwendet, um den Transkriptomik-Gehalt der Zebrafisch-NSVZs und anderer Zelltypen wie Neuronen, Oligodendrozyten und Immunzellen zu untersuchen. Meine Arbeit stellt die erste Studie dar, in der die Heterogenität der NSVZs im Telencephalon des adulten Zebrafischs so detailliert analysiert wurde. Mit Hilfe von öffentlich zugänglichen In-situ-Hybridisierungsdatenbanken identifizierte ich die ungefähren Standorte der identifizierten Subtypen von NSVZs im Telencephalon. Darüber hinaus habe ich auch die spezifischen Markergene dieser Untertypen auf der Grundlage meiner Transkriptomik-Analysen und maschinellen Lernalgorithmen bestimmt.

Ein wichtiges Merkmal der NSVZs ist, dass sie auf verschiedene Insults im Gehirn unterschiedlich antworten (Bhattarai et al., 2016). In der vorliegenden Dissertation untersuchte ich, ob räumlich getrennte NSVZs eindeutig auf Aβ42 oder IL4 reagieren. Durch die Bestimmung der Anzahl der proliferierenden Zellen nach der Behandlung mit Aβ42 oder IL4, fand ich unterschiedliche Reaktionen in der proliferativen Reaktion der NSVZs. Wir konnten zeigen, dass sterbende Neuronen IL4 sezernierten und ich entwickelte eine Methode zur in silico-Analyse der Zell-Zell-Interaktionen unter Verwendung von kognitiven Liganden-Rezeptor-Paaren. Diese große Ressource wird nun zusammen mit allen Einzelzellanalysen auf einer öffentlich zugänglichen Website dokumentiert, wo Zellcluster-Marker, Karten der zellulären Interaktion, Gen-Ontologie-Begriffe und die Analyse der Signalwege öffentlich zugänglich sind.

Zusammenfassend kann man sagen, dass ich während meiner Doktorarbeit

(1) erfolgreich ein Tau-Zebrafischmodell generierte, welches das menschliche Tau-Protein kontinuierlich exprimiert,

(2) eine transgene Zebrafischlinie vom Embryonalstadium bis zum Erwachsenenalter durch Untersuchung von TAU-Hyperphosphorlyse, Entzündung, Zelltod, Proliferation, Verwirbelung und Kreuzwirkung von TAU auf die Amyloid-Toxizität charakterisierte,

(3) das ersten Repositorium und den ersten Datensatz zur zellulären Heterogenität von Zebrafisch-NSVZs unter homöostatischen und AD-Bedingungen unter Verwendung von Einzelzell-Transkriptomik-Ansätzen erstellte,
(4) die zellulären Mechanismen mit denen NSVZs auf verschiedene Pathologien reagieren könnten (z.B.: Aβ42 Toxizität), identifizierte,
(5) eine frei zugängliche Website erstellte, damit die Gesellschaft von unseren Ergebnissen und Analysen profitieren kann,

(6) neue Forschungsansätze für weitere Studien basierend auf den vorliegenden Daten erzeugt habe.

SUMMARY

Alzheimer's disease (AD) is a daunting threat for aging societies. Millions of people worldwide live with AD, which has no current treatment. Amyloid cascade hypothesis (ACH) is the one of the current and the most accepted hypotheses regarding the cause of AD. ACH provides a mechanistic view of the pathological cascade starting from amyloid aggregation to chronic inflammation and TAU pathology. The drugs that were developed based on ACH were able to clear amyloid plaques from AD patients but failed to provide improvement in cognitive ability. These failures suggest that AD cannot only be considered within the theoretical framework of ACH. Recent hypotheses culminate the effects of various cell types (e.g., neural stem cells, astrocytes, oligodendrocytes) in the onset of AD. Complex feedback and feed-forward mechanisms are likely in AD pathophysiology, and the interplay between pathology and the involvement of other cell types make this disease multifactorial and perplexing. Recently, two studies (Moreno-Jimenez, et al., 2019; Tobin, et al., 2019) showed that the production of new neurons in in human brains dramatically decreases in AD. An interesting hypothesis was supported by these studies: pathology-induced generation of new neurons (regenerative neurogenesis) in AD patients may help reverting the AD pathology. Therefore, since regenerative ability in mammals is evolutionary poor (Tanaka and Ferretti, 2009), studying neurodegeneration in a model organism with regenerative ability can teach us how to induce neural stem cells proliferation and neurogenesis in pathological conditions.

For this particular question, model organisms with natural regenerative ability can teach us how to induce proliferation and neurogenesis during the pathological conditions of AD. Zebrafish offers an unparalleled opportunity to model neurodegeneration and regeneration to investigate the molecular mechanisms how neurogenesis can be enhanced in vertebrate brains upon AD as exemplified in our laboratory with several publications. For this reason, in my PhD thesis, I used zebrafish to investigate the neural stem cell (NSC) plasticity, in particular the heterogeneity of NSC populations in respect to their molecular programs and the

molecular basis of the regenerative neurogenesis from NSCs upon Aβ42 and TAU pathologies.

We previously established an Aβ42 toxicity model, which shows several pathological features of human AD, such as inflammation, cell death, synaptic degeneration and cognitive decline. I generated a transgenic zebrafish line that expresses the human version of TAU protein with a mutation that renders the protein more disease-prone (TAU-P301L). This line would be used to combine Aβ42 and Tau to generate a combined pathological model as in AD in humans, as these two proteins co-exist during the disease. The zebrafish line expressed the human Tau-P301L from embryonic development to adulthood and TAU was hyper-phosphorylated from embryonic stages. I further checked for the Tau pathology by assaying for inflammation, cell death. TAU expression in zebrafish brain did not show the pathological aggregation and cell death. TAU hyper-phosphorylation did not lead to tangle formation in the absence or presence of Aβ42. Moreover, Tau expression in neural stem/progenitor cells (NSPCs) and neurons did not alter NSPC proliferation. These negative results are not technical artifacts because in other regions of the central nervous system such as the spinal cord, TAU expression causes pathology. Our findings suggested an interesting hypothesis that zebrafish brain might have protective mechanisms to prevent TAU tangle formation and this can provide us the essential knowledge to design potential interventions to prevent TAUopathies in human brain (Cosacak et al., 2017).

As Tau overexpression did not exacerbate Aβ toxicity as well as tangle formation, I continued my thesis with Aβ toxicity model (Bhattarai et al., 2016). In this model, NSPCs start proliferation and generate new neurons via a specific signaling pathway (IL4/STAT6 signaling). This neurogenic outcome in zebrafish brain after AD is in strong contrast to the AD brains in patients where several studies suggested the existence of a sharp decline in neurogenic outcome (Moreno-Jimenez, et al., 2019; Tobin, et al., 2019). As a result in order to understand the heterogeneity in NSPCs in zebrafish brain and finding new mechanisms in amyloid pathology, I used single-cell sequencing to investigate the transcriptomics content of zebrafish NSPCs and other cell types such as neurons, oligodendrocytes and immune cells. My work constituted the first study ever analyzing the NSPC heterogeneity in adult zebrafish telencephalon in such detail. Using publicly available in situ hybridization databases, I identified the approximate locations of identified subtypes of NSPCs in the telencephalon.

Moreover, I also determined the specific marker genes of these subtypes based on my transcriptomics analyses and machine learning algorithms.

An important feature of NSCPs is that they respond differently to different insults in the brain (Bhattarai et al., 2016). In my thesis, I asked whether spatially-distinct NSPCs respond unequivocally to Aβ42 or IL4. By counting the number of proliferating cells after Aβ42 or IL4 treatment, I have found different responses in the proliferative response of the NSPCs. As we showed that dying neurons secreted IL4, I developed a method for in silico analyses of the cell-cell interactions by using cognate ligand-receptor pairs. This large resource together with all single cell analysis is now documented on an open-access website, where cell cluster markers, cellular interaction maps, gene ontology terms and pathway analysis can be publicly accessed.

In summary, during my PhD thesis, I,

(1) Successfully generated a zebrafish model of Tau, which chronically expresses human Tau,

(2) Characterized transgenic zebrafish line from embryonic stages to adulthood by investigating TAU hyperphosphorlyation, inflammation, cell death, proliferation, tangle formation and cross effects of TAU on amyloid toxicity

(3) Generated the first repository and dataset on cellular heterogeneity of zebrafish NSPCs in homeostatic and AD conditions using single cell transcriptomics approaches

(4) Identified the cellular mechanisms by which NSPCs might be responding to various pathologies (e.g.: Aβ42 toxicity)

(5) Generated an open-access website for the community to benefit from our results and analyses

(6) Proposed further research avenues for further publications that used our data for their studies.

ABBREVIATIONS

3R	3-Repeat
4R	4-Repeat
ACH	Amyloid Cascade Hypothesis
AD	Alzheimer Disease
APP	Amyloid Precursor Protein
Aβ	Amyloid-β
Aβ42	Amyloid-β-42
BDNF	Brain Derived Growth Factor
CAA	Cerebral Amyloid Angiopathy
CVMI	Cerebroventricular microinjection
DS	Down Syndrome
EOAD	Early Onset Alzheimer Disease
FAD	Familial Alzheimer Disease
FGF	Fibroblast Growth Factor
FTD	Frontotemporal Dementia
IL4	Interleukin-4
KYNA	Kynurenic Acid
L	Leucin
LOAD	Late Onset Alzheimer Disease
MAPT	Microtubule Associated Protein TAU
MTBD	Microtubule Binding Domain
NFT	Neurofibrillary Tangle
NSC	Neural Stem Cell
NSPC	Neural Stem/Progenitor Cell
P	Proline
PBS	Phosphate Buffer Saline
PS1	Presenilin-1
PS2	Presenilin-2
S	Serine
SAD	Sporadic Alzheimer Disease
SP	Senile Plaque
SVZ	Subventricular Zone
T	Threonine
UAS	Upstream Activating Sequence
WT	Wild Type

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1 INTRODUCTION

1.1. A Brief History and Current State of Alzheimer Disease Research

Dementia is an appalling threat in aging society, and the longer people live the higher is the risk of developing dementia. The leading type of dementia is Alzheimer Disease (AD) (Mayeux and Stern, 2012; Reitz, et al., 2011; Sosa-Ortiz, et al., 2012) that is clinically characterized by deterioration of cognitive abilities (Dolan, et al., 2010a; Dolan, et al., 2010b; Schneider, et al., 2007). Currently, there is no treatment for AD and as the average human age is increasing in societies, it is predicted that the number of AD patients worldwide will increase to 150 million by 2050 (Brookmeyer, et al., 2007), suggesting finding treatments for this devastating disease is a matter of utmost importance.

AD is first characterized by Alois Alzheimer in 1907 with his patient with memory impairment. Post-mortem analysis of the patient's brain showed two obvious pathological phenotypes that are called senile plaques (SPs) and neurofibrillary tangles (NFTs) (Alzheimer, et al., 1995). However, the importance of studying AD got public attention and convinced the scientific community with a seminal work that show correlation between the amount of neurotic SPs (now known as Amyloid-β plaques) and dementia in the brains of elderly patients (Blessed, et al., 1968). The major breakthrough in AD research came by identification of amyloid-β (Aβ) as the component of congophilic angiopathy (or Cerebral Amyloid Angiopathy) (Glenner, 1983; Glenner and Wong, 1984a; Glenner and Wong, 1984b) and in SPs (Masters, et al., 1985) and microtubule associated protein Tau (MAPT) in NFT (Grundke-Iqbal, et al., 1986). Amyloid-β, mostly 40-42 amino acids, is a peptide derived from longer precursor called Amyloid Precursor Protein (APP). Identification of Aβ in Down Syndrome (DS) with trisomy 21 that has extra copy of APP and some mutations found

on APP in AD patients, led to Amyloid Cascade Hypothesis (ACH) which postulated that Aβ42 is the initiator of AD pathology, while subsequent inflammation, aggregation of Tau and neuronal loss follow as a result of Aβ deposition (Hardy and Selkoe, 2002; Hardy and Higgins, 1992; Selkoe and Hardy, 2016). This hypothesis led to several clinical campaigns designing drug by aiming either decreasing the production or increasing the clearance of Aβ (Ji, et al., 2001; Schneider, et al., 2014; Tanzi, et al., 2004; Wisniewski and Goni, 2015). While some of the drugs or immunization protocols were able to clear Aβ, the clearance of Aβ alone did not improve cognitive ability in AD patients (Cummings, et al., 2016; Mehta, et al., 2017; Nicoll, et al., 2019). These failures indicate that AD should not be considered only as a cascade of events, instead as soon as AD is initiated by Aβ deposition, the more proximal Tau pathology, inflammation and contribution of other cell types (e.g., neural stem cells, astrocytes, oligodendrocytes) exacerbates AD pathology by feedback and feed-forward mechanisms that cause the actual AD-onset (De Strooper and Karran, 2016; Dzamba, et al., 2016; Heneka, et al., 2015; Henstridge, et al., 2019; Tincer, et al., 2016). This feedback and feed-forward mechanisms and interplay between pathology and involvement of other cell types make AD a multifactorial and complex disease.

The two key proteins - Aβ (processed from APP) and Tau - are the components of two hallmarks of AD: extracellular plaques and intracellular NFTs, respectively. There are no Tau mutations that are associated with AD. The cascade of the events initiated by Aβ is considered to be leading to Tau hyperphosphorylation, which subsequently causes mislocalization of Tau, oligomerization, fibrillation and tangle formation (Selkoe and Hardy, 2016). Thus, Aβ is the key protein for initiation of AD pathology but Tau is likely to be the proximal cause of the actual neuronal pathology. Studies have shown that generation and accumulation of Aβ start several decades before the first clinical symptoms are observed (Jack and Holtzman, 2013; Jack, et al., 2013; Jack, et al., 2010). While Aβ accumulation starts earlier than Tau accumulation, the cognitive impairment is correlated with Tau pathology. As a result, Aβ is an important factor in initiating AD, while Tau is another key factor involving in neuronal loss followed by deterioration in cognitive ability and memory.

1.2. Hypotheses on the causes of AD and NSC hypothesis

Chronologically, the first AD hypothesis proposed was cholinergic hypothesis. Among several key enzymes in neurotransmitter synthesis, choline acetyltransferase is the

main enzyme whose activity decreases in several brain areas including hippocampus (Davies and Maloney, 1976). Currently, there are a few drugs inhibiting activity of Acetylcholinesterase. These drugs do not cure or prevent AD however decreases the effects of AD and relieving AD patients (Knight, et al., 2018; L, 2000). The second hypothesis was introduced in 1991, thus amyloid-β is the initiator of AD followed by Aβ deposition, tau hyperphosphorylation, NFT formation and finally neuronal death (Hardy and Allsop, 1991; Hardy and Selkoe, 2002; Hardy and Higgins, 1992; Selkoe, 1991; Selkoe and Hardy, 2016). This is called the amyloid cascade hypothesis (ACH) and it still holds true and explain the mechanistic perspective of AD. As aforementioned, Aβ is the initiator of AD, however some other factors involve later in the disease progression. One of these factors is Tau hyperphosphorylation, fibrillation and tangle formation. Alzheimer Disease first starts in entorhinal cortex by Tau accumulation (Sipos, et al., 2007; Van Hoesen, et al., 1991; von Gunten, et al., 2006; Yasuda, et al., 1995) and then spreads to hippocampus and other brain areas. This spreading of tau is called Tau propagation in which Tau behaves like an endopathogen or a prion-like behavior. As a result, tau propagation spreads throughout the brain (Clavaguera, et al., 2009; Frost, et al., 2009).

Aging is the main cause of neurodegenerative disorders and mitochondrial abnormality play role in aging or aging phenotypes. Calcium is an important molecule in cells and Aβ has been shown to increase the intracellular calcium level, making neurons more vulnerable to other factors (Mattson, et al., 1992). Mitochondria dysfunction may affect the expression and processing of Aβ in LOAD and change the chronology of AD (Swerdlow and Khan, 2004). Alzheimer Disease is predicted to be decreased by 25% without any treatment, but just increasing the quality of life, especially preventing neurovascular diseases (Kempermann, 2019). As a result any problem with neurovascular system may affect the substrate delivery and the drainage of the waste from the brain through the blood. Neurovascular or cerebrovascular diseases is associated or cause of many neurodegenerative diseases (Iadecola, 2004). Lymphatic system also plays role similar to neurovascular system and abnormality in this system play role in neurodegenerative disease as well (Da Mesquita, et al., 2018). Moreover, excess level of metals (e.g., Zinc, Copper or Iron) play role in Aβ generation and plaque formation resulting in cognitive decline (Bush, et al., 1994; Clements, et al., 1996; Duce and Bush, 2010; Lovell, et al., 1998; Spinello, et al., 2016). Amyloid-β induces inflammation and inflammation is another hypothesis in AD (Bagyinszky, et al., 2017; Latta, et al., 2015; McGeer and McGeer, 2010). In

addition of all these hypotheses, recently the contribution of the different cell types is also getting attention (De Strooper and Karran, 2016). The role of neural stem cells (NSC) and neurogenesis in AD has been recently discussed (Choi and Tanzi, 2019; Kizil and Bhattarai, 2018). This hypothesis relatively looks simple and naïve, however in theory it has several challenges. As discussed above, once advanced AD becomes a complex neurodegeneration, as a result all possible aspect of AD must be considered and investigated in order to find treatments for AD. One of these is the contribution and the malfunction of various cell types that exacerbate AD symptoms. The role of NSCs in regenerating the brain is an important aspect to be considered for AD treatment (Gage and Temple, 2013; Kizil and Bhattarai, 2018; Tincer, et al., 2016). However, there are challenges to study NSC and their neurogenic outcome in mammals whose regenerative ability is evolutionarily defined as poor (Tanaka and Ferretti, 2009). As a result, studying neurodegeneration in a model organism with natural regenerative ability can teach us new mechanisms that may be transferred to mammals to induce NSC proliferation in pathological conditions. While this strategy looks simple, it has several challenges on the road. These challenges include; (1) activating NSC in pathological conditions, (2) survival and generation of new neurons from these cells (3) integrating new cells in to the existing neural circuit and (4) preventing tumor formation from proliferating NSC. For my thesis, the first step is the main step to focus on, thus modelling AD like pathologies in zebrafish brain and finding new mechanisms to induce NSC proliferation in these contexts in a model organism with a regenerative ability.

1.3. Adult Neurogenesis and Alzheimer Disease

Adult neurogenesis in mammalian brain first identified by H3-Thymidine incorporation in rats (Altman and Das, 1965). In humans, adult neurogenesis has been shown by BrdU-incorporation (Eriksson, et al., 1998) and Carbon-14 in nuclear DNA of human brain (Spalding, et al., 2013). However, the adult neurogenesis has been challenged recently (Arellano, et al., 2018; Paredes, et al., 2018; Sorrells, et al., 2018), while several studies propose the existence of adult neurogenesis in human hippocampus (Boldrini, et al., 2018; Kempermann, et al., 2018; Lima and Gomes-Leal, 2019; Moreno-Jimenez, et al., 2019; Tobin, et al., 2019). Regardless of this profound debate, the decrease of adult neurogenesis in AD patient has been shown recently (Moreno-Jimenez, et al., 2019; Tobin, et al., 2019). These studies show the importance of Adult neurogenesis or generation of new neurons in pathological

conditions and put forward the hypothesis “can AD be tackled by increasing neurogenic outcome?”. This is a similar approach to that of our lab, where we are trying to understand whether zebrafish can teach us how to enhance neurogenesis in AD conditions. This seems to be a fertile approach as for instance IL4 signaling, which is essential for zebrafish brain to respond to AD by neurogenic outcome (Bhattarai et al., 2016, 2017a, 2017b), is also able to enhance neurogenesis in human astrocytes (including neural stem cells) based on the studies in a novel 3D culture system developed in our lab (Celikkaya, et al., 2019; Papadimitriou, et al., 2018). These findings are supported by the fact that mammalian brains can also be coaxed to become more neurogenic and this helps to ameliorate the cognitive decline in disease (Choi, et al., 2018; Choi and Tanzi, 2019; Kizil and Bhattarai, 2018).

1.4. The role of Tau in AD and Tauopathies

Aβ accumulation is the main cause of initiating AD, while another key protein Tau mislocalization has detrimental effect on neuronal function and loss. Tau is a microtubule associated protein and identified as component of NFT (Grundke-Iqbal, et al., 1986). So far no mutations have been identified on Tau in AD cases, so it is the wild type that play role in AD pathology. However, mutations have been identified on tau that causes some other neurodegenerative disease; e.g. Fronto-temporal Dementia (FTD) linked to Chromosome 17 (Hutton, et al., 1998; Poorkaj, et al., 1998; Spillantini, et al., 1998). Either with mutations or with no mutations, Tau plays role in pathology of several Neurodegenerative Diseases that are collectively called Tauopathy, including AD as well. However, in AD Tau is a secondary cause of AD, while in FTD Tau is the main causing protein.

Tau, natively, is an unfolded protein with several domains including microtubule-binding domains (MTBD). It has six isoforms with either 3-repeat (3R) or 4-repeat (4R) of MTBD and 0N, 1N or 2N combinations following alternative splicing in human brain (Goedert, et al., 1989). The MTBDs or 3R/4R repeats are the tau domains that bind to microtubules; stabilizing and promoting self-assembly from tubulin subunits. Mutations, phosphorylation in these domains may have dramatic effect on the binding capacity of Tau to microtubules, thus releasing Tau from the microtubules (Barghorn, et al., 2000; Biernat, et al., 1993; Hong, et al., 1998). Free soluble Tau is prone to

aggregate mainly by the beta-sheet aggregation, while binding microtubule prevent tau aggregation (reviewed in; Mandelkow and Mandelkow, 2012).

Currently, 44 different FTD associated mutations have been found in Tau (www.molgen.ua.ac.be/FTDMutations), some of which are pathogenic. The majority of these mutations are enriched in the microtubule binding repeats. One of these mutations occur at amino acid 301 converting Proline (P) to Leucine (L), Serine (S) or Threonine (T) at exon 10 or repeat 2.These mutations destabilize binding of Tau to microtubule, as a result accumulation and phosphorylation of Tau cause neurodegeneration such as axonal and synaptic loss via cell death. In the past decades, several mouse models have been generated by expressing FTD related genes (reviewed in Ahmed, et al., 2017). The 2 mutations on Tau (P301L and P301S) are commonly used and known to cause neurodegeneration and pathology such as inflammation, NFT, tau aggregation or transmission impairment in the synapse before the synapse loss (Hoover, et al., 2010; Lewis, et al., 2000; Oddo, et al., 2003; Yoshiyama, et al., 2007). Several studies has been done on these mouse models to evaluate other potential therapeutic strategies, such as Kinase inhibitors (Le Corre, et al., 2006; Noble, et al., 2005), microtubule stabilizers (Brunden, et al., 2010), immunization with a phospho-tau peptide (Asuni, et al., 2007), passive immunotherapy with tau antibodies (Chai, et al., 2011) and the immunosuppressant FK506 (Yoshiyama, et al., 2007), which have some progress to prevent tau pathology but not in the progression or restoration of lost neurons. As a result, clearance of Tau by itself is not a treatment for AD or Tauopathies, additionally generating new neurons to replace the lost ones is also important for treatment of AD. However, the response of neural stem cells and their neurogenic outcome are not well-addressed during Tau pathology, because of limited neurogenesis in pathological conditions in mammals.

1.5. Modelling AD in Mouse

The mechanism to understand neurodegenerative diseases started with the discovery of molecules that accumulate neuropathologically and the ones that cause diseases in familial form of AD. While the majority of AD are sporadic (SAD or LOAD) thus a combination of several genetic and environmental factors involve, a part of AD are caused by mutations on APP or Presenilins (Ertekin-Taner, 2007). The majority of AD is called Sporadic AD (SAD) or commonly used Late-Onset AD (LOAD), that there is no strong genetic cause of it with Mendelian inheritance (reviewed in; Karch and

Goate, 2015). However, mutations in APP, Presenilin-1 (PS1) and Presenilin-2 (PS2) proteins are the main genes causing AD, which leads to Early-Onset of AD (EOAD) or Familial AD (FAD). In EOAD cases, ~81% percentage of cases are caused by mutations on PS1 followed by ~14% of APP and 6% of PS2 (Ertekin-Taner, 2007). In addition to these genes, there are several others genes that have been identified or predicted as AD risk genes (Jansen, et al., 2019; Kunkle, et al., 2019; Lambert, et al., 2013). Among these, ApoE4 allele is the gene with higher risk and identified first (Tsai, et al., 1994). Any mutations that can favor amyloidogenic processing of APP increase the Aβ generation, concomitantly decreasing the onset of AD.

As a result, in order to model AD in mouse model and other model organisms the familial form of APP (Games, et al., 1995; Hsiao, et al., 1996), PS1 (Guo, et al., 1999) and WT or Tau with P301L/S (Lewis, et al., 2000; Yoshiyama, et al., 2007) mutations are commonly used to generate transgenic mouse. Moreover, the combinations of these 3 genes have been used to generate transgenic mice; APP and PS1 (Citron, et al., 1997; Duff, et al., 1996; Flood, et al., 2002; Holcomb, et al., 1998), APP/PS1/TauP301L (3xTg) (Oddo, et al., 2003), or APP and PS1 with several fAD mutations (5XFAD) (Oakley, et al., 2006). Depending on the promoter, there are differences in the production of Aβ and the onset of AD phenotypes in these models, however almost all of them recapitulate some or many features of AD (Gotz and Ittner, 2008). Especially, the mouse models replicated particular symptoms of AD, helped understanding the mechanisms of AD and several drug developments and preclinical studies (Ahmed, et al., 2017; Gotz and Ittner, 2008). To control the expression level of APP, an APP knock-in line was also generated (Saito, et al., 2014). However, one of the challenges in mice is the limited adult neurogenesis. Thus, while mice offer several similarities to human, its limited regenerative ability restricts the studies on regeneration. As a result, modeling neurodegeneration and studying stem cell response in different pathological conditions in an organism with regenerative ability may teach us how to control the proliferative and neurogenic outcome of NSCs. Therefore, in my thesis, I have chosen zebrafish for generating chronic neurodegeneration and for studying the neural stem cell response.

1.6. Modelling Neurodegeneration in zebrafish

The regenerative ability in vertebrates varies widely and especially mammals have limited regenerative ability that is restricted to a few organs. However, some

vertebrates like zebrafish have a higher regenerative ability widespread throughout their body. Zebrafish has been used to study developmental biology (Haffter, et al., 1996; Streisinger, et al., 1981) and regeneration (Bernhardt, et al., 1996; Johnson and Weston, 1995; Kizil, et al., 2012b; Kizil, et al., 2009; Poss, et al., 2002; Raya, et al., 2003; Rowlerson, et al., 1997; White, et al., 1994). Several labs focused on different organs in fish for regenerative studies; including regeneration in caudal fin (Nechiporuk and Keating, 2002; White, et al., 1994), liver (Burkhardt-Holm, et al., 1999), kidney (Reimschuessel, 2001), hair cells (Harris, et al., 2003) and heart (Poss, et al., 2002; Raya, et al., 2003). Additionally, zebrafish nervous system is able to regenerate including spinal cord (Becker, et al., 1998), retina (Vihtelic and Hyde, 2000), cerebellum (Liu, et al., 2004). The regeneration in brain, mainly telencephalon, has been shown by stab injury models (Baumgart, et al., 2012; Kishimoto, et al., 2012; Kroehne, et al., 2011; Marz, et al., 2011). The stab injury models showed that zebrafish brain could regenerate by using the Neural Stem/Progenitor covering the ventricle of fish telencephalon marked by astroglial markers such as s100beta and her4. Interestingly, these cells start to proliferate, migrate and repair/regenerate the injury sites. Further studies have shown that injury dependent molecular programs and inflammation are playing role in the activation and plasticity of NSPC (Kizil, et al., 2012a; Kizil, et al., 2012c; Kyritsis, et al., 2012; Rodriguez Viales, et al., 2015).

The ability of NSPC responding to injury by using specific molecular programs shows the opportunity of using zebrafish to model neurodegeneration and understanding the NSPC response in the pathological conditions. To address this question in our lab, we recently generated an amyloid toxicity model (Bhattarai, et al., 2016; Bhattarai, et al., 2017a; Bhattarai, et al., 2017b) in zebrafish by injection of Aβ-42 peptide using cerebroventricular microinjection (CVMI) by improving this previously established technique (Kizil and Brand, 2011; Kizil, et al., 2015). This model recapitulates many features of AD (inflammation, cell death, synaptic degeneration and cognitive decline) but no tauopathy/tangle formation. Additionally, the NSPCs start proliferation and generate new neurons. This proliferation is induced by IL4 secretion from dying neurons and activated microglia (Bhattarai, et al., 2016; Bhattarai, et al., 2017a; Bhattarai, et al., 2017b). Moreover, the finding from fish has been tested in 3D culture of human fetal Astrocytes (Papadimitriou, et al., 2018). It has been shown that the mechanism from fish has similar effects on NSC proliferation in 3D culture and this may help to prevent AD. These studies show that zebrafish can be used as a model

organism to study human neurodegenerative diseases and can help to specifically address neurogenesis in AD conditions.

Another key gene in many neurodegenerative diseases, collectively called Tauopathy, is Tau gene. Previously, several zebrafish lines have been generated to establish neurodegeneration in zebrafish by over-expressing WT or FTD associated mutations on Tau. The first transgenic zebrafish model was generated to over express human wild type (WT) Tau under the control of enolase promoter (Bai, et al., 2007). However, this line was used only to characterize enolase promoter, but not to study the tauopathy, no further study followed this line, even though the line survived until 14 months old. The second stable line generated was to use Gal4/UAS system under HuC/D promoter (Paquet, et al., 2009). In this line, tau hyperphosphorylation, cell death, motor neuron degeneration observed at early stages (5 days old) and Gallyas silver positive cells observed after 5 weeks. However, the expression was limited to spinal cord and construct expression could not be studied in adulthood and in the brain. Mosaic expression of Tau in zebrafish embryos induces hyperphosphorylation and cell death (Tomasiewicz, et al., 2002; Wu, et al., 2016). Except the amyloid toxicity model, the NSPC response in these models has not been further characterized.

The NSPC response in amyloid toxicity model (Bhattarai, et al., 2016) and stab injury models (Kizil, et al., 2012a; Kizil, et al., 2012c; Kyritsis, et al., 2012; Rodriguez Viales, et al., 2015) show that NSPC use different molecular mechanisms in different contexts to induce NSPC proliferation. Thus, zebrafish NSPC response can be used to understand how NSPC response to different pathological conditions. This can help us to understand and to find out new strategies how to activate NSPC in human neurodegenerative diseases.

1.7. Single-cell sequencing to Study Alzheimer Disease

Single-cell sequencing is new next-generation sequencing that can be used even with sparse sample size, where bulk RNA-sequencing cannot be used (Liang, et al., 2014). Moreover, single-cell sequencing can be used to identify heterogeneity in cell populations that look very homogenous by common markers. The technique has been

developed to sequence a large number of cells in a high-throughput manner (Zheng, et al., 2016). There are several single-cell methods, each of them has its own advantages and disadvantages (Ziegenhain, et al., 2017). For instance, SMART-seq can be used to sequence full-length sequence in a few cells with millions of reads, in-drop sequencing cannot be used to sequence full-length and many read numbers. On other hand, in-drop sequencing is useful for sequencing thousands of cells at the same time (Ziegenhain, et al., 2017).

SMART-Seq and in drop-seq have been used to study nervous system in mice (Artegiani, et al., 2017; Dulken, et al., 2017; Harris, et al., 2018; Hochgerner, et al., 2018; Llorens-Bobadilla, et al., 2015; Luo, et al., 2015; Shin, et al., 2015). In-drop sequencing has been further used in the context of AD-like conditions in mouse cortex and hippocampus to study the heterogeneity in microglia (Keren-Shaul, et al., 2017; Mathys, et al., 2017). Several zebrafish studies employed in-drop sequencing for instance in juvenile brain and whole brain during embryonic development (Raj, et al., 2018a; Raj, et al., 2018b) and in habenula (Pandey, et al., 2018). However, single-cell sequencing has not been used to study the response of different cell types in the context of AD in mouse, zebrafish or human.

1.8. Aims of the thesis

In this thesis, my aim was to generate AD-related neurodegeneration models in adult zebrafish brain and to understand the neural stem/progenitor plasticity in the context of AD like pathologies. In the first paper, I will present successful chronic expression of human Tau P301L in adult zebrafish brain and its phenotypic outcomes in conjunction with Amyloid toxicity. In the second paper, I will present the cellular heterogeneity of zebrafish NSPC and their response in an amyloid toxicity model as determined by our single cell sequencing method and novel analyses tools.

2 PUBLICATIONS

This thesis is based on the following original publications presented in this chapter:

1. **Cosacak, M.I.**, Bhattarai, P., Bocova, L., Dzewas, T., Mashkaryan, V., Papadimitriou, C., Brandt, K., Hollak, H., Antos, C.L., and Kizil, C. (2017). Human TAU(P301L) overexpression results in TAU hyperphosphorylation without neurofibrillary tangles in adult zebrafish brain. Sci Rep 7, 12959.

2. **Cosacak, M.I.**[§], Bhattarai, P., Reinhardt, S., Petzold, A., Dahl, A., Zhang, Y., and Kizil, C.[§] (2019). Single-Cell Transcriptomics Analyses of Neural Stem Cell Heterogeneity and Contextual Plasticity in a Zebrafish Brain Model of Amyloid Toxicity. Cell Rep 27, 1307-1318 e1303.

Other papers published during my PhD thesis:

Bhattarai, P., **Cosacak, M.I.**, Mashkaryan, V., Demir, S., Popova, S.D., Govindarajan, N., Brandt, K., Zhang, Y., Chang, W., Ampatzis, K., et al. (2020). Neuron-glia interaction through Serotonin-BDNF-NGFR axis enables regenerative neurogenesis in Alzheimer's model of adult zebrafish brain. PLoS Biol 18, e3000585.

Bhattarai, P., Thomas, A.K., **Cosacak, M.I.**, Papadimitriou, C., Mashkaryan, V., Froc, C., Reinhardt, S., Kurth, T., Dahl, A., Zhang, Y., et al. (2016). IL4/STAT6 Signaling Activates Neural Stem Cell Proliferation and Neurogenesis upon Amyloid-beta42 Aggregation in Adult Zebrafish Brain. Cell Rep 17, 941-948.

Bhattarai, P., Thomas, A.K., **Cosacak, M.I.**, Papadimitriou, C., Mashkaryan, V., Zhang, Y., and Kizil, C. (2017). Modeling Amyloid-beta42 Toxicity and Neurodegeneration in Adult Zebrafish Brain. J Vis Exp.

Celikkaya, H., **Cosacak, M.I.**, Papadimitriou, C., Popova, S., Bhattarai, P., Biswas, S.N., Siddiqui, T., Wistorf, S., Nevado-Alcalde, I., Naumann, L., et al. (2019). GATA3 Promotes the Neural Progenitor State but Not Neurogenesis in 3D Traumatic Injury Model of Primary Human Cortical Astrocytes. Front Cell Neurosci 13, 23.

Cosacak, M.I., Bhattarai, P., and Kizil, C. (2020). Alzheimer's disease, neural stem cells and neurogenesis: cellular phase at single-cell level. Neural Regen Res 15, 824-827.

Cosacak, M.I.*, Papadimitriou, C. *, and Kizil, C. (2015). Regeneration, Plasticity, and Induced Molecular Programs in Adult Zebrafish Brain. Biomed Res Int 2015, 769763.

Diaz Verdugo, C., Myren-Svelstad, S., Aydin, E., Van Hoeymissen, E., Deneubourg, C., Vanderhaeghe, S., Vancraeynest, J., Pelgrims, R., **Cosacak, M.I.**, Muto, A., et al. (2019). Glia-neuron interactions underlie state transitions to generalized seizures. Nat Commun 10, 3830.

Papadimitriou, C., Celikkaya, H. *****, **Cosacak, M.I. ***, Mashkaryan, V. *****, Bray, L., Bhattarai, P., Brandt, K., Hollak, H., Chen, X., He, S., et al. (2018). 3D Culture Method for Alzheimer's Disease Modeling Reveals Interleukin-4 Rescues Abeta42-Induced Loss of Human Neural Stem Cell Plasticity. Dev Cell 46, 85-101 e108.

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www.nature.com/scientificreports

SCIENTIFIC REPORTS

OPEN

Received: 7 June 2017
Accepted: 20 September 2017
Published online: 11 October 2017

Human TAU^{P301L} overexpression results in TAU hyperphosphorylation without neurofibrillary tangles in adult zebrafish brain

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Microtubule-associated TAU protein is a pathological hallmark in Alzheimer's disease (AD), where hyperphosphorylation of TAU generates neurofibrillary tangles. To investigate the effects of TAU in a regenerative adult vertebrate brain system, we generated a cre/lox-based transgenic model of zebrafish that chronically expresses human TAU^{P301L}, which is a variant of human TAU protein that forms neurofibrillary tangles in mouse models and humans. Interestingly, we found that although chronic and abundant expression of TAU^{P301L} starting from early embryonic development led to hyperphosphorylation, TAU^{P301L} did not form oligomers and neurofibrillary tangles, and did not cause elevated apoptosis and microglial activation, which are classical symptoms of tauopathies in mammals. Additionally, TAU^{P301L} neither increased neural stem cell proliferation nor activated the expression of regenerative factor Interleukin-4, indicating that TAU^{P301L} toxicity is prevented in the adult zebrafish brain. By combining TAU^{P301L} expression with our established Aβ42 toxicity model, we found that Aβ42 ceases to initiate neurofibrillary tangle formation by TAU^{P301L}, and TAU^{P301L} does not exacerbate the toxicity of Aβ42. Therefore, our results propose a cellular mechanism that protects the adult zebrafish brain against tauopathies, and our model can be used to understand how TAU toxicity can be prevented in humans.

Accumulation of microtubule-associated TAU protein is a pathological hallmark of Alzheimer's disease (AD) and frontotemporal dementia (FTD)[1–4]. TAU^{P301L} mutation leads to aggressive TAU aggregation and neurofibrillary tangle (NFT) formation in animal models of FTD[3–6], whereas in AD, Aβ42 is believed to cause hyperphosphorylation of TAU, and subsequent aggregation[7–9]. In animal models of Tauopathies, motor deficits and neurodegeneration were observed[10–13]. Since Tauopathies are also seen in AD, transgenic models aiming to recapitulate the main symptoms of AD brains were generated using known familial mutations of AD and FTD, such as 3xTg[14], TauPS2APP[15], Tg2576[16], PLB1-triple[17], and rTg21221[18]. Although these models mimic the pathological features of the human disease, the proposed causative link between Aβ42 and TAU aggregation is still to be clarified[19–21]. Majority of the studies suggested a link between TAU toxicity and Amyloid aggregation[16,22–27]. However, there are also conflicting studies where Amyloid toxicity is not dependent on TAU[28–30], and a recent study suggested that TAU might even ameliorate Amyloid toxicity[31], indicating that the effects of Amyloid toxicity on TAU-dependent tangle formation, and the role of TAU tangles on exacerbating the Amyloid toxicity is still to be clarified. Therefore, new animal models where the effects of Aβ42 and TAU can be addressed independently and in combination to increase our understanding of the relationship between Aβ42 and TAU.

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Neurodegenerative diseases cause a progressive deterioration of neuronal circuits and pronounced hampering of the stem cell proliferation[32]. Therefore, a plausible way of circumventing these diseases is to design regenerative therapy options where neurons would survive the toxicity burden exerted by TAU and Aβ42, but also the neural stem cells would enhance their proliferation to supply more cells that could be replenishing the function of the lost ones[33]. Mammalian models are excellent to recapitulate the pathophysiology of neurodegenerative diseases, however, since mammals are poorly regenerating, basic and translational questions regarding the regenerative capacity cannot be addressed well. Zebrafish, as a vertebrate serves as an excellent tool for questions concerning regeneration because of its high regenerative ability.

In zebrafish, several Tauopathy models were generated before. Transient expression of human TAU fused to GFP under gata2 promoter was shown to cause TAU phosphorylation in zebrafish larvae using Western blot analyses[34]. Another study using transient expression of human TAU-GFP under neuronal HuC/D promoter has also shown that TAU can be hyperphosphorylated[35]. The first stable TAU transgenic was generated by expressing TAU (0N4R) under the control of enolase promoter[36]. TAU was expressed in adult fish brain; however, no phenotypic characterization was documented. The best-characterized zebrafish TAU model so far is Gal4/UAS-mediated expression of human TAU^{P301L} under the control of HuC/D promoter[37]. This line shows motor neuron degeneration, hyperphosphorylation of TAU and tangle formation in the spinal cord. However, studies in this line only focused on the embryonic to juvenile spinal cord but not the brain. A recent study used a new allele of TAU with a mutation causing A152T conversion and showed hyperphosphorylation of TAU, motor neuron degeneration in the spinal cord, and cell death in larval zebrafish retina[38]. Therefore, these zebrafish models expressing human TAU have documented hyperphosphorylation of TAU and tauopathy in the spinal cord and the retina; however, the effects of TAU in the brain are understudied and therefore the effects are still unknown. In our study, we developed two conditional transgenic models of zebrafish, which expressed human TAU^{P301L} chronically in radial glial cells and the neurons derived from these progenitors in early embryonic development (with her4.1 promoter) and directly in neurons (with neural beta tubulin promoter). Expression of human TAU^{P301L} in adult zebrafish brain showed that human TAU^{P301L} can be phosphorylated, but neither forms any aggregates nor causes neurodegeneration on its own. Additionally, by combining the transgenic model of TAU^{P301L} expression with our recent Amyloid toxicity model[39,40], we showed that Amyloid toxicity does not cause TAU aggregation, and TAU^{P301L} expression does not exacerbate the effects of Amyloid toxicity and AD-like neurodegeneration in adult zebrafish brain. Our results indicate that in adult zebrafish brain, human TAU^{P301L} can be phosphorylated but does not form aggregates and does not cause neurodegeneration, and Amyloid toxicity does not elicit Tauopathies.

Results

Conditional transgenic cassettes lead to strong neuronal expression of of TAUP301L in zebrafish brain. To investigate the chronic effects of human TAU^{P301L} expression in zebrafish brain, we generated a cre/lox-based conditional double-transgenic zebrafish that expresses TAU^{P301L}. The spatial control cassette drives the expression of inducible cre recombinase (creERT2) under the control of *her4.1* promoter, which is active in neural stem/progenitor cells with radial glial identity (Fig. 1a, Tg(her4.1:mCherry-T2A-creERT2)). We specifically chose *her4.1* promoter because we hypothesized that if the expression of TAU^{P301L} started from the stem cell stage, all neurons produced by those stem cells after the recombination would express the protein. In contrast to conditional expression systems that use mature neuronal promoters such as HuC/D[41], enolase[36] or neural beta tubulin (NBT)[42], using a stem cell promoter would increase the number of neurons expressing the transgene throughout the life of the fish. Additionally, since in familial forms of neurodegenerative diseases, mutant proteins are expressed in all cells – including the stem cells - of the patients during development, our approach would also help to address the effects of TAU^{P301L} in neural stem cells of zebrafish brain in a way reminiscent of familial forms of Tauopathies in humans.

The effector cassette is responsive to cre and expresses TAU^{P301L} under the ubiquitous promoter after recombination (Fig. 1a, Tg(ubi:loxP-DsRed-STOP-loxP-GFP-T2A-TAU^{P301L})). From here on, Tg(ubi:loxP-DsRed-STOP-loxP-GFP-T2A-TAU^{P301L}) will be named as sTg, double transgenic (Tg(ubi:loxP-DsRed-STOP-loxP-GFP-T2A-TAU^{P301L}) and Tg(her4.1:mCherry-T2A-creERT2) will be denoted as dTg). Since cre activity is induced by addition of Tamoxifen (TAM), we treated sTg and dTg animals with TAM from 16 hours to 48 hours post fertilization in order to recombine all radial glial cells (RGCs), and analyzed the adult animals at 1, 3, and 6 months post fertilization (Fig. 1b). While the recombination does not take place without TAM, after TAM treatment at 48 hours post fertilization dTg animals show GFP expression along the whole rostrocaudal axis of the central nervous system (Fig. 1c).

To determine how widespread TAU^{P301L} is expressed by using *her4.1* promoter, we performed immunostainings for GFP and TAU^{P301L} on sagittal sections of the adult zebrafish brain (Supplementary Fig. 1). We observed a strong and reproducible expression of TAU^{P301L} in adult zebrafish brain from olfactory bulb to the hindbrain (Supplementary Fig. 1 insets 1–9). These results indicate that TAU^{P301L} is successfully expressed throughout the entire axis of zebrafish brain using a stem cell promoter. In order to further investigate whether the use of *her4.1* promoter is appropriate to express TAU in maximum number of neurons, we expressed TAU by using the same recombination paradigm before but using a cre-driver line expressing cre recombinase under the control of neural beta-tubulin promoter (NBT): Tg(nbt:mCherry-T2A-creERT2) (designated hereafter as nTg). By performing recombination after crossing nTg to sTg, we found that nTg is leading to transgene expression along the rostrocaudal axis of the fish brain, and, compared to *her4.1*-driven TAU expression, *nbt* promoter also drives expression of the transgene in a similarly widespread manner (Supplementary Fig. 2), suggesting that *her4.1* promoter can be used to target neurons in the adult zebrafish brain by cre-mediated recombination.

Based on the known pathological locations of TAU in human brains, we focused on the forebrain for the rest of our analyses. By performing immunostainings for GFP and TAU^{P301L} on coronal sections of the zebrafish forebrain, we determined that compared to control animals (sTg), dTg animals show a specific pattern of

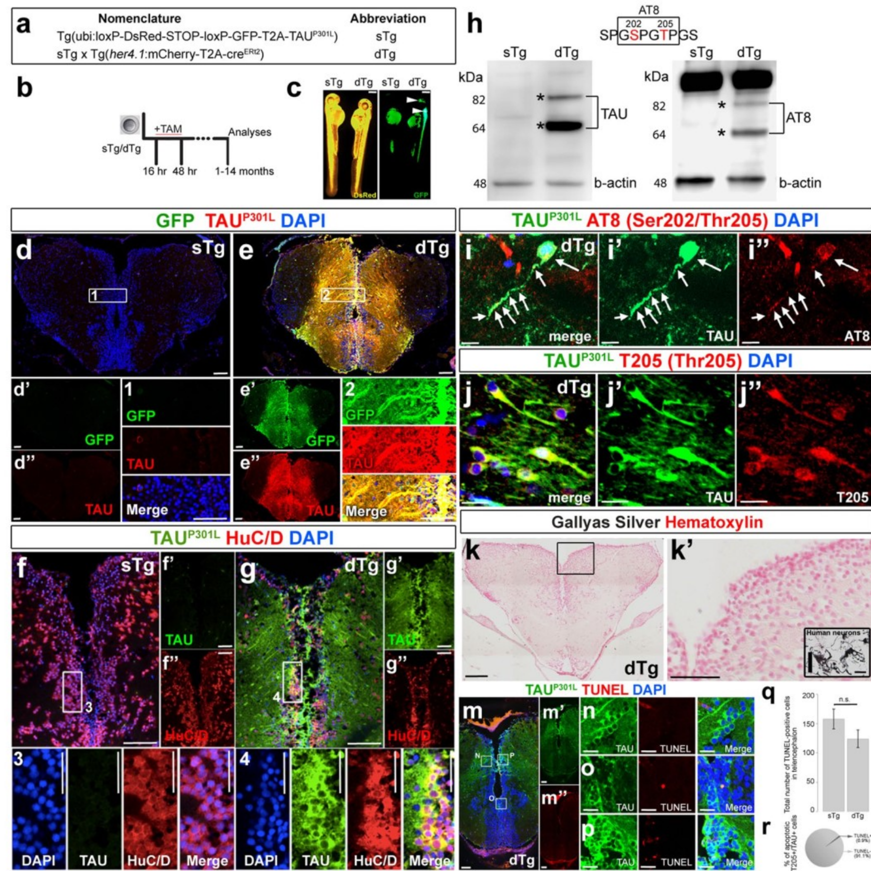

Figure 1. (**a**) Naming of transgenic constructs: sTg – effector transgenic, dTg – double transgenic. (**b**) Scheme for timing of recombination and analyses. (**c**) sTg and dTg animals (5 days post fertilization) treated with tamoxifen. DsRed expression indicates the presence of the effector cassette. Note the recombination in the central nervous system by GFP expression. (**d**) Immunohistochemistry (IHC) for GFP and TAU^{P301L} on coronal sections of telencephalon of a 6 month-old sTg animal. Single channel images of the whole section for GFP (d') and TAU (d"). (1) is the enlarged view of the inset in d. (**e**) IHC for GFP and TAU^{P301L} on coronal sections of telencephalon of a 6-month old dTg animal. Single channel images of the whole section for GFP (e') and TAU (e"). (2) is the enlarged view of the inset in f. (**f**) IHC for HuC/D and TAU^{P301L} on coronal sections of telencephalon of an sTg animal. Single channel images of the whole section for TAU (f') and HuC/D (f"). (3) is the enlarged view of the inset in f. (**g**) IHC for HuC/D and TAU^{P301L} on coronal sections of telencephalon of a dTg animal. Single channel images of the whole section for TAU (g') and HuC/D (g"). (4) is the enlarged view of the inset in g. (**h**) Western blot analyses for expression of TAU^{P301L} (left) and hyperphosphorylated TAU^{P301L} (right) in telencephalon. Beta actin is used as a loading control. (**i**) IHC for GFP and AT8 in a dTg animal. Individual channels are shown for GFP (i') and AT8 (i"). Arrows represent the cytoplasmis signal. (**j**) IHC for TAU^{P301L} and T205 in a dTg animal. Individual channels are shown for TAU^{P301L} (j') and T205 (j"). (**k**) Gallyas silver (black) and Hematoxylin (pink) staining in telencephalon of a dTg animal. (k') Enlarged region in k'. Note the absence of Gallyas silver-positive cells. (**l**) Positive control for Gallyas silver staining in human neurons treated with Amyloid, showing neurofibrillary tangles. (**m**) Immunohistochemistry for TAU^{P301L} (green) combined with TUNEL detection of apoptotic cells (red) in recombined 6 months old dTg animals. Individual fluorescent channels are shown in m' and m". (**n**–**p**) Higher magnification images from the insets in m. (**q**) Quantification of TUNEL-positive cells in the telencephalon in sTg and dTg animals. (**r**) Quantification of apoptotic cells containing hyperphosphorylated TAU^{P301L} (T205-positive). Values represent mean ± s.e.m. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. Scale bars equal 10 μm (**i**–j') and 50 μm elsewhere. n = 6 fish and > 30 histological sections for every staining. Larvae are 5 days old, and adult animals are 6 months old.

recombination, which colocalizes with TAU^{P301L} expression in the telencephalon (Fig. 1d–e"; Supplementary Fig. 3). To investigate whether TAU^{P301L} is expressed in neurons, we performed immunostainings for TAU^{P301L} and neuronal marker HuC/D in sTg (Fig. 1f–f') and dTg animals (Fig. 1g–g"). We observed that neurons express TAU^{P301L} (Fig. 1g4). To determine the percentage of neurons in the pallium that express TAU^{P301L}, we quantified the number of cells that co-localize HuC/D and TAU^{P301L} (Supplementary Fig. 4). We found that TAU^{P301L} is expressed in 91.8% % of the neurons (Supplementary Fig. 4), indicating that in our model, majority of the neurons in the forebrain express TAU^{P301L}.

TAU^{P301L} is hyperphosphorylated in adult zebrafish brain. One of the hallmarks of Tauopathies in human brains is hyperphosphorylation of TAU and formation of neurofibrillary tangles, which can be detected by histological stainings. To analyze whether chronic expression of human TAU^{P301L} in adult zebrafish brain leads to hyperphosphorylation and tangle formation, we analyzed the immunoreactivity of the adult fish brain to AT8, an indicator of late stage Tau hyperphosphorylation at the Serine-202 and Threonine-205 (Fig. 1h). Western blot analyses showed that human TAU^{P301L} is specifically expressed only in dTg but not sTg animals, and when compared to the beta-actin loading control, the expression is strong and abundant (Fig. 1h). We also found that TAU^{P301L} leads to AT8 immunoreactivity (~70 kDa expected size), which is suggestive of TAU^{P301L} hyperphoshorylation (Fig. 1h). The band around 82 kDa is the fusion of GFP and TAU in the cre-effector cassette, and results from inefficient cleavage of t2a peptide in approximately 8% of the cases as documented before[43]. The strong band above 82 kDa is a non-specific recognition of AT8 in zebrafish in the nucleus (Supplementary Fig. 5). By performing quantification of the relative intensities of detected bands, we found that $3.8 \pm 1.4\%$ of the total TAU is AT8-positive in adult zebrafish brain. To confirm the results from western blot analyses, we performed immunohistochemical stainings for AT8 and TAU^{P301L} (Fig. 1i–i"). We observed that a small portion of the TAU^{P301L}-positive cells display AT8 immunoreactivity in the cytoplasm (Fig. 1i–i"). However, since AT8 staining leads to a nuclear background staining in adult zebrafish brain and therefore determining the cytoplasmic staining in adult brain is difficult (Supplementary Fig. 5). To further verify the phosphorylation status of TAU, we performed immunohistochemical staining with an antibody that specifically recognizes phosphorylation of Threonine-205 (T205) (Fig. 1j–j'"). We found that this staining does not cause background signals (Fig. 1j–j"), and approximately 76.1% of the TAU^{P301L}-positive cells are also positive for T205 phosphorylation (Supplementary Fig. 6). These results indicate that chronic expression of TAU^{P301L} can cause hyperphosyphorylation in adult zebrafish brain. We also analyzed the early phosphorylation of TAU by immunohistochemistry and western blot for AT180 (Threonine-231 and Serine-235) (Supplementary Fig. 7) and AT270 (Threonine-181) (Supplementary Fig. 7). We found that AT180 and AT270 give strong non-specific cross-reactivity in zebrafish in immunohistochemistry (Supplementary Fig. 7a,b'", Supplementary Fig. 8a,b'"), and AT270 additionally show non-specific signal in western blots (Supplementary Fig. 8c), indicating that these antibodies cannot be reliably used. AT180, however, shows a specific band of phosphorylated TAU in dTg animals (Supplementary Fig. 7c), confirming the phosphorylation determined by AT8 and T205 antibodies. In overall, these results indicate that TAU^{P301L} is hyperphosphorylated in adult zebrafish brain.

Hyperphosphorylation of TAU^{P301L} is known to cause neurofibrillary tangle (NFT) formation[1,2], and to test whether chronic expression of human TAU^{P301L} would lead to NFTs, we histologically stained coronal sections of adult zebrafish telencephalon with Gallyas silver (Fig. 1k,k'). We did not observe any tangle formation, whereas a positive control from human neurons treated with Amyloid-β42 gave positive signal for Gallyas silver (Fig. 1l), indicating that despite its hyperphosphorylation, human TAU^{P301L} does not form NFTs in adult zebrafish brain.

We hypothesized that if NFTs – as the toxic outcome of TAU – do not form in adult zebrafish brain, the pathological outcomes of neurodegeneration would not manifest in dTg animals. To test this hypothesis, we investigated the levels of apoptosis (Fig. 1m–q), microglia/macrophage activation as an indication of chronic inflammation (Fig. 2a–e) and proliferation of radial glial cells (RGCs) as a standard readout after neuronal loss in adult zebrafish brain[39,44,45] (Fig. 2f–j).

Phosphorylated TAU^{P301L} does not cause cell death, inflammation and stem cell activation in adult zebrafish brain. To determine whether the cells where TAU is hyperphosphorylated undergo apoptosis, we performed TUNEL staining and found that the overall levels of cell death in dTg animals is not significantly different than sTg animals (Fig. 1q), while our previously established AD model of adult zebrafish brain shows extensive cell death (Supplementary Fig. 9), which is also confirmed by unchanged relative expression levels of apoptotic regulator Caspase 3 (Supplementary Fig. 9i). We also observed that only a minor fraction of T205-positive cells containing phosphorylated-TAU are TUNEL positive (Fig. 1r). These results indicate that TAU^{P301L} expression does not lead to cell death in adult zebrafish brain.

Tauopathies cause chronic inflammation by significantly inducing the numbers of macrophages/microglia as well as favoring for their activation state[46]. To test if chronic expression of human TAU^{P301L} in adult zebrafish brain would alter the microglial dynamics, we performed immunostaining for L-Plastin, a marker for microglia and macrophages in adult zebrafish brain (Fig. 2a–d). Compared to sTg, dTg animals did not show a significant difference in the overall number and activation state of the microglia, as the number of ramified and round L-Plastin-positive cells remains unchanged (Fig. 2d). This result indicates that chronic expression of human TAU^{P301L} does not induce chronic activation of microglia in adult zebrafish brain, and this suggests that chronic inflammation does not take place.

Zebrafish has an extensive regenerative ability in its brain, and loss of neurons causes radial glial cells to respond by increasing their proliferation[39,44,45,47–53]. To test if chronic expression of TAU^{P301L} would induce the proliferation of neuronal progenitors, we performed immunostaining for S100β (a progenitor cell marker) and PCNA (proliferation marker) in sTg and dTg animals (Fig. 2f–i). Compared to sTg animals, chronic expression of TAU^{P301L} did not alter the level of proliferation of radial glial cells (Fig. 2j). Additionally, similar to *her4.1*,

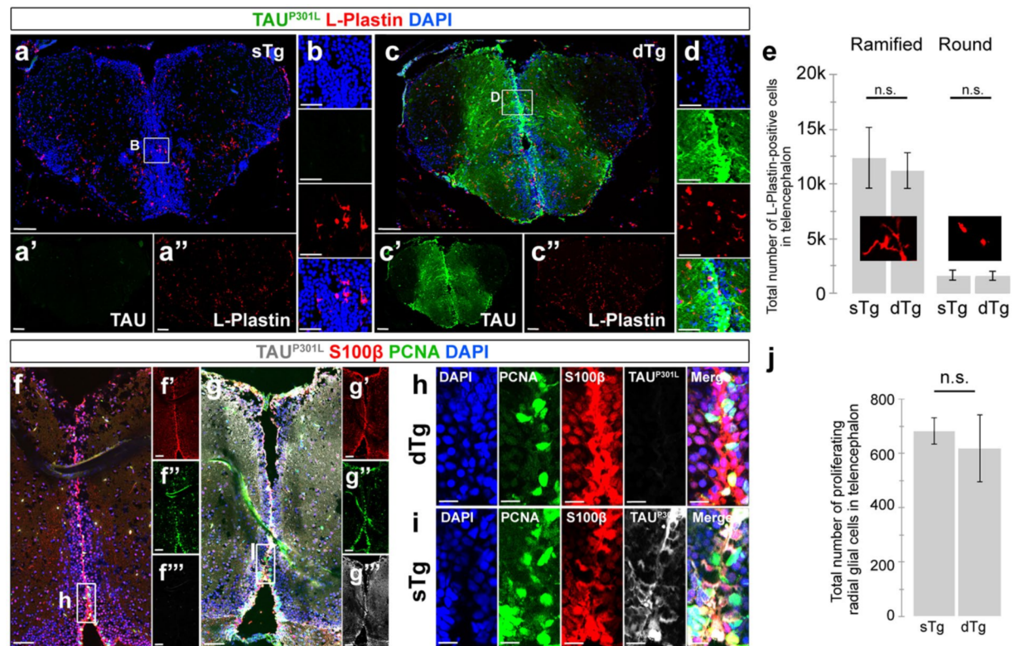

Figure 2. (**a**) Immunohistochemistry (IHC) for L-Plastin (red) and TAU^{P301L} (green) on coronal sections of telencephalon of a 6 month-old sTg animal. Single channel images of the whole section for TAU^{P301L} (a') and L-Plastin (a"). (**b**) The enlarged view of the inset in a. (**c**) IHC for L-Plastin (red) and TAU^{P301L} (green) on coronal sections of telencephalon of a 6-month old dTg animal. Single channel images of the whole section for TAU^{P301L} (c') and L-Plastin (c"). (**d**) The enlarged view of the inset in c. (**e**) Quantification of round and ramified L-Plastin-positive cells in the telencephalon in sTg and dTg animals. (**f**) Immunohistochemistry (IHC) for S100β (red), PCNA (green) and TAU^{P301L} (white) on coronal sections of telencephalon of a 6-month old sTg animal. Single channel images of the whole section for S100β (F'), PCNA (f"), and TAU^{P301L} (f'"). (**g**) IHC for S100β (red), PCNA (green) and TAU^{P301L} (white) on coronal sections of telencephalon of a 6-month old dTg animal. Single channel images of the whole section for S100β (g'), PCNA (g"), and TAU^{P301L} (g'"). (**h**) The enlarged view of the inset in f. (**i**) The enlarged view of the inset in g. (**j**) Quantification of the total number of proliferating glial cells in the telencephalon of sTg and dTg animals. Values represent mean ± s.e.m. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. Scale bars equal 50 μm (**a**–g'") and 20 μm (**h**,**i**). n = 7 fish and > 30 histological sections for every staining. All animals are 6 months old.

nbt-driven TAU did not alter neural stem cell proliferation (S100β-PCNA stainings) or microglial activation in adult zebrafish brain (L-Plastin stainings) (Supplementary Fig. 2). These results indicate that despite the presence and hyperphosphorylation of human TAU^{P301L}, zebrafish brain does not show any pathological hallmarks of Tauopathies, and major pathological hallmarks - cell death, inflammation and cell proliferation - are not affected. We also confirmed these findings by detecting the relative mRNA expression levels of pro-inflammatory cytokines *tnfa*, *ifng*, *il1b*, *il6*, and *il12a* (Supplementary Fig. 10), and observed that these genes do not change their expression levels.

Phosphorylated TAU^{P301L} neither exacerbates the toxicity of Aβ42 nor initiates regeneration programs in adult zebrafish brain. In Alzheimer's mouse models and cultures of human neurons, Amyloidβ42 (Aβ42) deposition leads to hyperphoyphorylated TAU and NFT formation[54–56], and Tauopathies are suggested to mediate the Amyloid toxicity[9,18,56–58]. We have recently generated an Aβ42 toxicity model in adult zebrafish brain, where Aβ42 aggregation leads to cell death, inflammation and induction of radial glial cell proliferation and neurogenesis[39]. We hypothesized that by combining the AD model of adult zebrafish brain to the transgenic expression of human TAU^{P301L} would help determining if chronic expression of TAU^{P301L} would exacerbate the effects of Aβ42. Therefore, we analyzed the radial glial cell proliferation in sTg and dTg animals injected with PBS or Aβ42 as readout of pathology and regenerative response (Fig. 3a–e). In both sTg and dTg animals, injection of Aβ42 increased the cell proliferation to the same degree (Fig. 3e), and Aβ42 aggregation did not cause NFT formation (data not shown), indicating that TAU^{P301L} does not exacerbate the pathological burden of Aβ42 in zebrafish.

A typical symptom of Amyloid toxicity is inflammation and activation of macrophages/microglia[59–61]. To determine whether TAU^{P301L} expression would alter the inflammatory outcome exerted by Aβ42, we performed

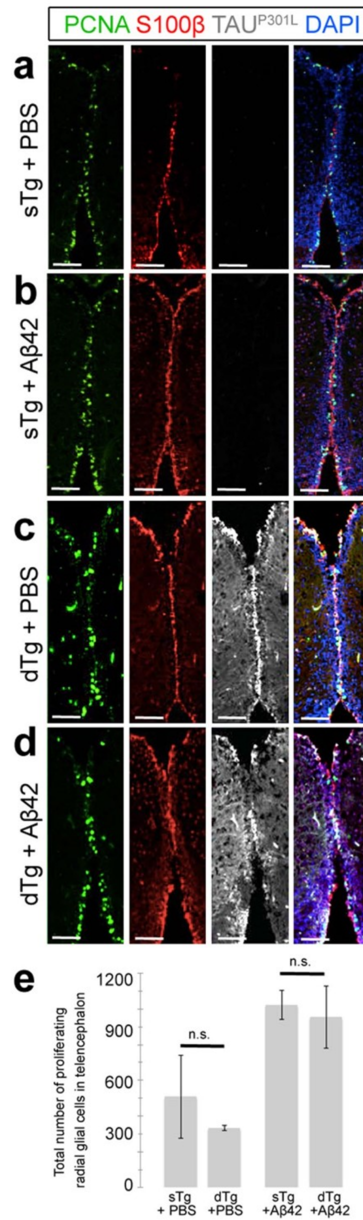

Figure 3. Immunohistochemistry for PCNA (green), S100β (red), and TAU^{P301L} (white) on coronal sections of telencephalon of a 6 month-old sTg animal injected with PBS (**a**), sTg animal injected with Aβ42 (**b**), dTg animal injected with PBS (**c**), and dTg animal injected with Aβ42 (**d**). (**e**) Quantification of the total number of proliferating radial glial cells in the telencephalon of sTg and dTg animals injected with PBS or Aβ42. Values represent mean ± s.e.m. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. Scale bars equal 100 μm. n = 5 fish and > 20 histological sections for every staining. All animals are 6 months old.

immunostaining for L-Plastin – a marker for macrophages/microglia – in sTg and dTg TAU^{P301L} lines (Fig. 4a–d). We found that Aβ42 increases both the ramified and round macrophage/microglia in sTg animals as we have documented before[39], and this increase is statistically not different in TAU^{P301L}-expressing dTg animals (Fig. 4e).

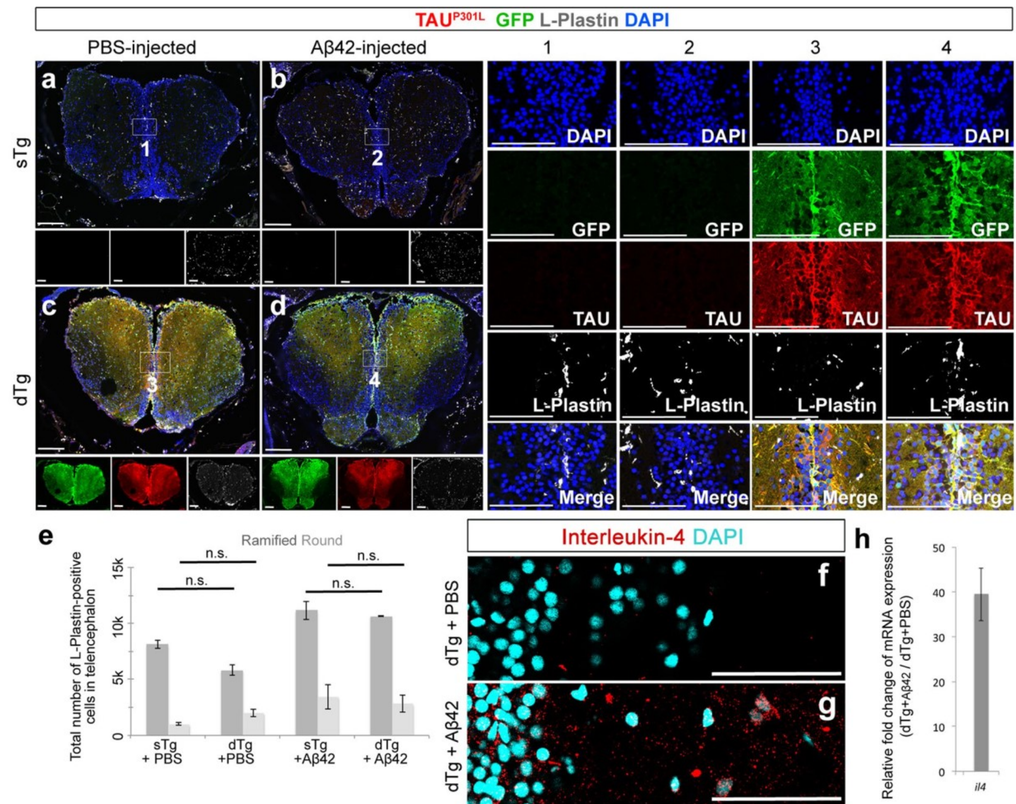

Figure 4. (**a**) Immunohistochemistry (IHC) for TAU^{P301L} (red), GFP (green) and L-Plastin (white) on coronal sections of telencephalon of a 6 month-old sTg animal injected with PBS (**a**), sTg animal injected with Aβ42 (**b**), dTg animal injected with PBS (**c**), and dTg animal injected with Aβ42 (**d**). Insets below the panels show individual fluorescent channels for TAU^{P301L} (red), GFP (green) and L-Plastin (white). Columns 1–4 are single and merged images of the regions indicated in (**a–d**), respectively. (**e**) Quantification of the total number of ramified and round L-Plastin-positive cells in the telencephalon of sTg and dTg animals injected with PBS or Aβ42. (**f–g**) Immunohistochemistry for Interleukin-4 (red) on coronal sections of telencephalon of a 6-month old dTg animal injected with PBS (**f**) or with Aβ42 (**g**). (**h**) Relative change in the expression levels of *il4* after Aβ42 injection compared to control injection. Medial ventricular regions are shown. DAPI (cyan) marks the nuclei. Values represent mean ± s.e.m. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. Scale bars equal 50 μm. n = 6 fish and > 30 histological sections for every staining. All animals are 6 months old.

Additionally, sTg and dTg animals that are not injected with Aβ42 do not differ in the number and activation state of macrophages/microglia (Fig. 4e). These results suggest that chronic expression human of TAU^{P301L} does not change the number and activation state of the microglia/macrophages as determined by L-Plastin staining, and does not exacerbate the effects of Aβ42 in adult zebrafish brain, and interestingly despite the widespread expression of TAU in adult zebrafish brain (Supplementary Figs 1 and 2), Aβ42 does not cause NFT formation from the TAU expressed thereof.

In adult zebrafish brain, Aβ42 was shown to cause a specific neurodegeneration-induced regeneration response by activating the expression of Interleukin-4 (IL4) – a biomarker of neurodegeneration-induced regenerative response in adult zebrafish brain[39]. Since TAU^{P301L} expression and its hyperphosphorylation did not lead to changes in cell death and stem cell proliferation, we hypothesized that in our TAU model, we would not see the neurodegeneration induced IL4 expression. To test this hypothesis, we performed immunohistochemical staining for IL4 in dTg animals injected with PBS or Aβ42 (Fig. 4f,g). We found that IL4 is not expressed in dTg animals with PBS injection (Fig. 4f), whereas Aβ42 injection leads to induction of IL4 expression that is detected by both immunostaining (Fig. 4g) and relative mRNA expression levels (upregulation by 39.5 ± 5.9 folds) (Fig. 4g,h). These results support our previous findings that TAU^{P301L} hyperphosphorylation does not lead to neurodegeneration and subsequent regeneration response in adult zebrafish brain.

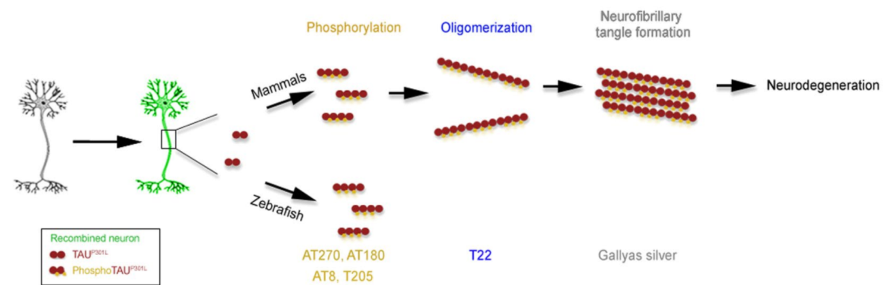

Figure 5. Schematic comparison of the effects of TAU in mammalian and zebrafish brain.

In several mouse models of TAU^{P301L}, the phenotypes manifest in aged animals[62]. We have recently shown that aging exacerbates the neurodegeneration phenotypes in adult zebrafish brain[40]. Therefore, we hypothesized that chronic expression of TAU^{P301L} in older zebrafish could manifest Tauopathies that we did not observe in younger fish (1–6 months of age). To test this hypothesis, we analyzed TAU expression (Supplementary Fig. 11a–f) and microglia activation that is indicative of chronic inflammation (Supplementary Fig. 11g–l) in 14 month-old adult zebrafish brains. We observed that although TAU is expressed strongly at 14 month-old brains of dTg animals (Supplementary Fig. 11d–f), this does not cause macrophage/microglia activation (Supplementary Fig. 11g–m), indicating that the lack of Tauopathies in adult zebrafish brain is not due to the late manifestation as seen in mouse models.

TAU^{P301L} is phosphorylated in zebrafish larvae yet causing no overt behavioral phenotypes. Our TAU model of adult zebrafish brain does not form neurofibrillary tangles despite phosphorylation of the TAU protein. Previously, phosphorylated TAU was documented in zebrafish spinal cord and these fish showed defective escape response at 2 days of development, yet they can regain all behavioral functions at 4 days of development and afterwards[37] suggesting that zebrafish can effectively overcome TAU-mediated pathology. To investigate whether in our zebrafish lines TAU is expressed and phosphorylated in larval stages, we performed immunohistochemical staining for TAU and western blot analysis for phosphorylated TAU markers AT8, AT180, and T205 (Supplementary Fig. 12). We found that TAU is widely expressed in zebrafish larvae along the whole rostrocaudal axis of the central nervous system, and it is phosphorylated (Supplementary Fig. 12). By band intensity quantifications, we found that 91.4 ± 4.7% of total TAU is AT180-positive and 70.3 ± 4.7% of total TAU is AT8-positive in the whole larvae.

In order to examine whether the larval fish shows escape response deficits, we performed a behavioral assay for 2- and 4 day-old larvae as described before[37] (Supplementary Videos 1 and 2). We observed that dTg larvae show normal escape behavior as in their non-transgenic siblings and sTg counterparts (Supplementary Videos 1 and 2), indicating that the Tauopathy in the spinal cord of the larvae is causing the behavioral deficits. These results recapitulate what has been documented before[37,63].

TAU^{P301L} does not form oligomers in adult zebrafish brain. Our results showed that while TAU^{P301L} is phosphorylated, neurofibrillary tangles do not form. We hypothesized that this could be because TAU^{P301L} might not be forming oligomers that are the precursors of tangles. In order to test this hypothesis, we performed immmunohistochemical staining with T22 antibody that detects TAU oligomers. We found that TAU^{P301L} does not form oligomers in adult zebrafish brain in the presence or absence of Amyloid-β42 (Supplementary Fig. 13). These results indicate that in adult zebrafish brain, despite being phosphorylated, TAU^{P301L} does not form oligomeric forms of TAU, and this could be the reason for absence of neurofibrillary tangles.

Discussion

In this study, we generated two cre-lox-based conditional expression lines for adult zebrafish brain to study the effects of chronic expression of human TAU^{P301L} and its relation to Aβ42 toxicity. We found that although chronically expressed TAU is hyperphosphorylated, it does not form oligomers and neurofibrillary tangles, and hence does not cause neurodegeneration in the adult zebrafish brain (Fig. 5).

Previous reports of TAU in zebrafish used larval and juvenile animals[34,37,63] or did not thoroughly investigate the adult phenotypes[36]. Our study is therefore the first study investigating TAU in a chronic expression model of adult zebrafish brain, where we found that human TAU^{P301L} was hyperphosphorylated as documented before in zebrafish[36,64]. However, hyperphosphorylation of TAU did not cause formation of oligomers and neurofibrillary tangles in adult zebrafish brain. We believe that this is not due to the inability of phosphorylated TAU to form neurofibrillary tangles *per se*, but because of a specific blockage of tangle formation in adult zebrafish brain.

The lack of tangle formation despite the presence of hyperphosphorylated form of TAU might be due to several reasons. First, zebrafish brain might not have the circuitries and neuronal subtypes that are susceptible to TAU aggregation in mammals, and therefore modeling Tauopathies in zebrafish might not be appropriate. We believe that this is not the case, because several studies have shown that adult zebrafish brain have analogous regions to the human brains[65,66], and the majority of the neuronal subtypes present in our brains are existent in

adult zebrafish brain[45,67–69]. Additionally, all the constituents of the machinery known to be required for TAU phosphorylation and aggregation is present in zebrafish[37,63] as zebrafish proteome contains more than 80% of the proteins present in humans[70] and the kinases that are implicated in TAU hyperphosphorylation (e.g.: GSK3β, Cdk5, MARK1 and CK1α[71]) are present in zebrafish, and are expressed (Supplementary Fig. 14). Second, the expression level of TAU is known to be a critical determinant of the aggregation dynamics[62]. In our experiments, we used ubiquitin promoter, which drives the expression of the downstream gene efficiently[72]. Also, our western blot results indicate that the levels of TAU are higher than the housekeeping gene beta-actin (Fig. 1h), and immunohistochemical stainings detect strong expression of TAU protein (Figs 1e, g and 2c). Based on these results, we believe that the lack of aggregation of TAU is not due to the levels of TAU protein. Third, our transgenic line may not be expressing TAU in the neurons that are susceptible to Tauopathies in humans. This is a challenging possibility, yet, as we determined by sagittal sections of the adult fish brain (Supplementary Figs 1 and 2), *her4.1* and *nbt* promoters can lead to TAU expression in the majority of the neurons in the adult fish brain, and it is very unlikely that such a widespread expression would not include the neurons of the forebrain that are susceptible to Tauopathies. Therefore, we hypothesize that the lack of neurofibrillary tangles in our model is not due to targeting the right cell types but due to an active protective mechanism that prevents phosphorylated TAU from forming tangles. This is an interesting option suggesting that zebrafish brain could serve as an *in vivo* model for experimentation on how Tauopathies can be prevented by using endogenous cellular programs. Further research on why zebrafish does not form Tauopathies in the brain while it does in the larval spinal cord[37,63] will constitute an exciting basic and pre-clinical research line. For instance, prevention of TAU dephosphorylation by Okadaic acid was shown to increase hyperphosphorylated state of TAU and subsequently leading to neurofibrillary tangles[73]. When we injected Okadaic acid to TAU^{P301L}-expressing adult zebrafish brains, we still did not observe neurofibrillary tangle formation (data not shown), suggesting a currently unknown cellular mechanisms that might be utilized to prevent phosphorylated TAU from forming tangles in human brains. We also found that in the larvae of our zebrafish model, TAU is expressed and hyperphosphorylated (Supplementary Fig. 12) but does not show any behavioral phenotypes such as escape response (Supplementary Videos 1 and 2). Additionally, adult dTg zebrafish do not show any swimming defects or escape response (data not shown) suggesting that TAU hyperphosphorylation does not cause NFT formation in larval and adult stages in our model. Thus, using our zebrafish TAU model as a tool to uncover putative protective mechanisms that prevent phosphorylated TAU from forming tangles will be of great interest to the medical field.

It is still controversial whether neuroinflammatory events lead to TAU phosphorylation or phosphorylated-TAU precedes neuroinflammation[46,57,74–76]. Our results suggest that phosphorylated-TAU is not sufficient to induce microglial activation by itself, and that aggregated forms of TAU might be required for this. We found that in our model, oligomeric TAU is not formed (Supplementary Fig. 13), suggesting that TAU phosphorylation might not be caused by immune cell activity and more toxic forms of TAU (such as oligomers) are required to elicit neuroinflammation by TAU. Our model can help to investigate this interesting hypothesis by analyzing the effects of TAU on various immune cell types, not necessarily confined to microglia, but also other immune cells involved in adaptive immune responses.

We recently established an Amyloid-β42 toxicity model in zebrafish, and observed that Aβ42 aggregation causes cell death, inflammation, synaptic degeneration, and memory deficits, which collectively lead to regenerative response including stem cell activation and neurogenesis[39,62], signifying the role of neuro-immune cross talk for efficient regeneration response in contrast to mammals[77,78]. When we combined amyloid toxicity model with chronic genetic expression of human TAU^{P301L}, we found that TAU expression does not exacerbate the Amyloid phenotypes, and Amyloid toxicity does not cause TAU-mediated neurofibrillary tangle formation. Additionally, unlike Aβ42, TAU^{P301L} does not induce the expression of neurodegeneration-induced factor Interleukin-4[39] (Fig. 4g), providing further evidence that TAU^{P301L} expression fails to elicit a neurodegeneration-regeneration cascade in adult zebrafish brain. Since zebrafish brain is highly regenerative after various type of neuronal loss[33,45,67,79], we believe that the lack of stem cell proliferation response and activation of regenerative programs is an indication that adult zebrafish brain can cope well with the phosphorylated forms of TAU^{P301L}.

Several studies documented Tauopathy-related neurodegeneration phenotypes in the absence of tangle formation[17,18,80–82]. Conflicting with these findings, Tau aggregation was also proposed as the major cause of toxicity in animal models[1,2,4,9,13,56,61,83]. We have to note that in our genetic model, we have used the longest isoform of human TAU protein (2N4R). Different versions of TAU have been shown to have different levels of aggregation potential, and therefore, in future other human TAU versions could be tested in zebrafish to come to a more consolidated conclusion of a protective mechanism preventing tangle formation. Additionally, protein aggregation is temperature dependent, and zebrafish lives at 28 °C while our body temperature is 37 °C. Therefore, a possible link between the temperature and aggregation dynamics of TAU could also account for the observed differences, and this aspect of Tauopathies can also be studied in zebrafish. Given that keeping zebrafish at 37 °C has been used as a means of heat stress, the effects of temperature on folding, phosphorylation and aggregation dynamics and clearance of TAU are therefore interesting aspects of TAU toxicity that can be tested *in vivo* in zebrafish.

In overall, we believe that our new chronic conditional TAU expression model in zebrafish is a useful assay system where various neurodegeneration paradigms could be modularly combined, and the effects of any intervention on the TAU aggregation could be measured. For instance, our model would allow screening for molecules or pathways that could abrogate a putative "protective mechanism" that prevents phosphorylated TAU from getting aggregated. In such a case, adult zebrafish brain may potentially start generating neurofibrillary tangles. Alternatively, possible factors that are related to immune system could have effects on how TAU is phosphorylated and how phosphorylated forms of TAU proceed further to form oligomers and neurofibrillary tangles. Such "reverse" studies could uncover currently unknown mechanisms that may help us to further understand and design therapies for Tauopathies in humans.

Zebrafish brain is highly regenerative and responds to injuries by activating specific molecular programs[33,45,67]. A possible regenerative therapy in human brains could entail mobilizing the endogenous stem cells to form new neurons that replace the lost ones, and regulation of the synaptic plasticity to compensate for the lost circuit connections. Reductionist models of neurodegenerative diseases in zebrafish can therefore help us to understand which types pathogenicity would activate regenerative programs in stem cells. By cataloging the effects of individual pathogenic hallmarks of neurodegenerative diseases in zebrafish, this animal model can help clinical studies to design regenerative therapies or prevention strategies that are naturally occurring in a vertebrate brain. Since previous zebrafish models of Tauopathies did not focus on the regenerative response of the brain, our model is also a useful addition to the toolbox of regeneration research in order to understand how a vertebrate brain responds to toxic protein aggregation or even prevents the progression of disease pathology.

Materials and Methods

Ethics statement. All animal experiments were carried out in accordance with the animal experimentation permits of Referate 24 (Veterinärwesen, Pharmazie, und GMP) of the state administration office of Saxony, Germany (Landesdirektion Sachsen) and the ethical commission of TU Dresden (Kommission für Tierversuche). The approved experimental protocols are licensed under the permit numbers TVV-35/2016 and TVV-52/2015 through the regular official processes.

Generation of transgenic zebrafish lines and recombination. The longest isoform of human TAU (2N4R) with P301L mutation template was a gift from Bettina Schmid and Christian Haass[37]. The template was amplified by PCR using the forward (5′-atggctgagccccgccag-3′) and reverse (5′-tcacaaaccctgcttggccagg-3′) primer pairs that were flanked by *XhoI* and *AscI* restriction enzymes, respectively. The PCR product was subcloned into pGEM-T-Easy vector (Promega) and sequenced by M13 universal primers. The TAU^{P301L} was released from pGEM-T-Easy by *XhoI/AscI* enzymes and cloned into pTol(Red-to-Green) (ubiquitin promoter-driven[72] loxP-DsRed-loxP-GFP cassette-containing Tol2 transgenesis vector[47,84]). For generating neural beta tubulin (nbt) promoter-driven cre line, we amplified the *nbt* promoter using forward (5′-tgggccctctagaccctgtctg-3′) and reverse (5′-ggccggccgattgggttgagtc-3′) primers flanked by *ApaI* and *FseI* restriction enzyme sites. PCR product was subcloned into *her4.1*:mCherry-t2a-Cre^{ERT2} transgenesis plasmid containing Tol2 sites[84,85] by using appropriate restriction enzymes.

For transgenesis, we used a protocol as described[85] with minor modifications. Briefly, 1 nL of 30 ng/μL plasmid and Tol2 transposase mix in RNAse-free water were injected into fertilized zebrafish eggs (F0), the fish were raised to adulthood, and were outcrossed to wild type zebrafish (AB background) to establish the founder fish (F1). 6 founders were identified, and were raised to adulthood for Tg(ubi:loxp-DsRed-loxp-GFP-t2a-hTAUP301L). Tg(nbt:mCherry-t2a-Cre^{ERT2}) founders were identified by outcrossing to wild type animals and screening for mCherry expression. Positive embryos were raised to adulthood and crossed to a control cre-effector line that shows fluorescence after recombination in order to test the effective recombination capacity. To identify a cre-effector TAU line with high efficiency of recombination, all F1 fish for TAU were outcrossed to Tg(her4.1:mCherry-t2a-Cre^{ERT2}) or Tg(nbt:mCherry-t2a-Cre^{ERT2}) cre-driver lines, and the F2 progeny were treated with freshly prepared 5 μM of Tamoxifen (TAM) in E3 medium between 16–32 hpf and 32–48 hpf. At 48hpf, the F2 progeny with high GFP expression were sorted from non-GFP siblings. The outcrosses were repeated to obtain a stably segregating transgene insertion (achieved after F4). The experiments and results given here are from F5 and later offspring.

Tissue preparation and immunohistochemical stainings. The fish were sacrificed, and the heads were fixed in 2% PFA overnight at 4 °C. After washing 3 times 10 min with Phosphate buffer, the heads were decalcified using 20% Sucrose/EDTA. Samples were mounted in 20% Sucrose/7.5% Gelatin as described[39]. Samples were cryosectioned at 12 μM thickness, and were stained with the following antibodies: chicken anti-GFP IgG (1:2000; Abcam), mouse anti-Tau13 IgG1 (IHC 1:1000, WB 1:500; Abcam), mouse anti-phospho-Tau (AT180) IgG1 (IHC 1:500, WB 1:1000; Pierce, Thermo Scientific), mouse anti-phospho-Tau (AT8) IgG1 (IHC 1:500, WB 1:1000; Pierce, Thermo Scientific), mouse anti-phospho-Tau (AT270) IgG1 (IHC 1:500, WB 1:1000; Pierce, Thermo Scientific), mouse anti-PCNA IgG2a (1:500; Dako), rabbit anti-S100β IgG (1:500; Dako), rabbit-anti-L-plastin IgG (1:5000) (gift from Michael Redd), rabbit anti-beta actin (WB 1:1000, Abcam), rabbit anti-synaptophysin (1:500, Abcam), mouse anti-HuC/D IgG2b (1:500, Life Technologies), mouse anti IL4 (1:500, R&D Systems), and rabbit anti-Tau (T22) (1:500, Merck).

Gallyas silver staining. Brain samples were fixed as described above. Heads were embedded in paraffin, sectioned at a thickness of 10 μM. Samples were pretreated with 0.25% potassium permanganate at room temperature for 15 min[86], washed in 2% oxalic acid for 2 min, and incubated in 0.4% lanthanum nitrate/2% sodium acetate/3% H_2O_2 solution at room temperature for 60 min[87]. The following treatments were performed: 5% periodic acid (5 min), alkaline silver iodide solution (1 min), 0.5% acetic acid (10 min), developer solution containing tungsto-silicic acid (5–10 min), 0.5% acetic acid, 0.1% gold chloride (5 min), and 1% sodium thiosulfate (5 min). Samples were counterstained with 0.1% nuclear fast red (2 min), dehydrated using Methanol, and were mounted in DPX.

Western blotting. For western blotting, the telencephalon of 6 month-old fish were dissected or 9 day-old larvae were sacrificed according the local ethical regulations. The telencephalon or whole larvae were used for protein isolation using 200 μL RIPA buffer (Sigma, R0278) with addition of phosphatase (Roche, Catalog Number 04906837001) and protease inhibitors (Roche, Catalog number 04963132001). 5 μl of protein ladder (ThermoFisher, #26634) and 10 μl of total protein was loaded in 4–12% Bis-Tris precast gradient gels (NuPage, Catalog number NP0322BOX) and the gels were run in NuPAGE MES SDS running buffer (Novex, Life

technologies, NP0006-1) at 200 V for 60 min. Blots were transferred to methanol activated PVDF (Novex, Life technologies, LC2002) membrane, and were blocked in 10% milk powder in 0.2% Tween in 1X-PBS for 1h at room temperature. The primary antibodies at appropriate dilutions in 2 ml 0.2% Tween in 1X-PBS were applied overnight at 4 °C in 15 or 50 ml plastic containers. Following the washing steps, secondary antibodies at 1:4000 dilutions (anti-rabbit IgG HRP (Santa Cruz, #sc-2004) or anti-mouse IgG peroxidase (Sigma, #A8924)) were applied at room temperature for 2 h. Gel images were acquired by ImageQuant LAS4000 (GE Healthcare) using Western BLoT Ultra Sensitive HRP Substrate (Takara, #T7104A).

Cerebroventricular microinjection. Zebrafish at the age of 6 months were injected with 20 μM Aβ42 as described previously[39,88–90]. For cerebroventricular microinjection, the fish were first anaesthetized in 0.001% MESAB, and a slit was generated in the skull over the optic tectum without damaging the brain. 1 μl of solution containing monomeric Amyloid-β42[39,40] was injected into the cerebroventricular fluid (for control animals PBS was injected). The fish were returned to their containers and recovery was carefully observed. All the injected fish survived the procedure and did not show any defects that might be due to the injections. 25 μg of Okadaic acid (Sigma #O8010) was dissolved in 500 μl distilled 1x-PBS, and 5 ng/μL was injected into the fish brain as described previously[89,90]. 3 dpi fish were sacrificed and prepared for immunohistochemical stainings as described above.

Detection of mRNA expression levels. mRNA isolation and detection of relative mRNA expression levels were performed as described[39,40].

Imaging, quantification, and statistical analyses. Images were acquired using structured illumination microscope (Zeiss AxioImager Z1) and scanning confocal microscope (Leica SP5 II, DM6000). In order to maintain the comparative intensities of the images and not to introduce bias, we used the same settings for all experimental groups of controls and transgenic animals per experiment. Images were taken using the tiling option of the Apotome microscope with no auto-correction to prevent inconsistencies in fluorescence intensity. Acquired images were stitched together using the Zeiss ZEN software installed to the Apotome configuration. Raw images are used to generate figures using Adobe Photoshop and Adobe Illustrator software. Cell counting was performed manually on the entire sections taken on a slide and stereological calculations were performed as described[91]. The statistical analyses were performed using GraphPad Prism (Version 6.02) for one-way ANOVA followed by a Tukey's post-hoc test and for Student's T-Test. Error bars shown are the s.d, and asterisks indicate significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. $p > 0.05$ is not significant (n.s.). Student's T-test was performed for paired samples, and a T-Test for independent measurements. All sample sizes and analyses powers were calculated using G*Power-Software, (http://www.gpower.hhu.de/en.html). No animals were excluded from the analyses. $n = 5$ fish for every experimental group, and in total more than 20 sections were used for stereological quantification. The ages of the animals used are denoted in the respective figure legends. Quantification of band intensities in western blots were performed as described[92].

Behavioral analyses. Escape response assays were performed as described[37]. Tamoxifen was replaced with fresh E3 medium. The video and imaging taken by changing filter from bright field to DsRED and then to GFP. The escape response was recorded at GFP channel to localize the dTg fish. Escape response was measured by touching the tail using Olympus MVX10 stereo microscope and CellSens Dimension software.

Data Availability. All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Acknowledgements

This work was supported by German Center for Neurodegenerative Diseases (DZNE) Dresden, Helmholtz Association (VH-NG-1021), Center for Regenerative Therapies Dresden (CRTD), TU Dresden, and Deutsche Forschungsgemeinschaft (DFG) (KI 1524/6-1) to C.K. We would like to thank Bettina Schmid and Christian Haass for the plasmids containing human TAU^{P301L}, Stefan Hans and Michael Brand for *her4.1* cre-driver zebrafish

line and the plasmid containing the cre-effector cassette pTol(R2G), Michael Redd for L-Plastin antibody, Alvin Thomas and Yixin Zhang for Amyloid-β42 peptide, and Gerd Kempermann for advice and comments on the results.

Author Contributions

M.I.C. and C.K. conceived and designed the experiments, M.I.C., V.M., H.H. and K.B. generated the Tg(ubi:loxP-DsRed-loxP-GFP-t2a-hTAU^{P301L}) cre-effector TAU expression transgenic line, C.A., M.I.C. and C.K. generated Tg(nbt:mCherry-t2a-cre^{ERT2}) cre-driver line, M.I.C. characterized the transgenic lines, performed tissue preparation, sectioning, stainings and quantifications, L.B. and T.D. helped the stainings and quantifications, P.B. performed Amyloid injections, C.P. prepared human neuronal cultures and Gallyas silver staining. M.I.C. and C.K. analyzed the data and prepared the figures, C.K and M.I.C. wrote and revised the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-13311-5.

Competing Interests: The authors declare that they have no competing interests.

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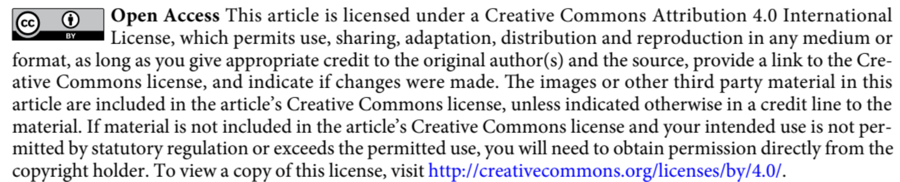

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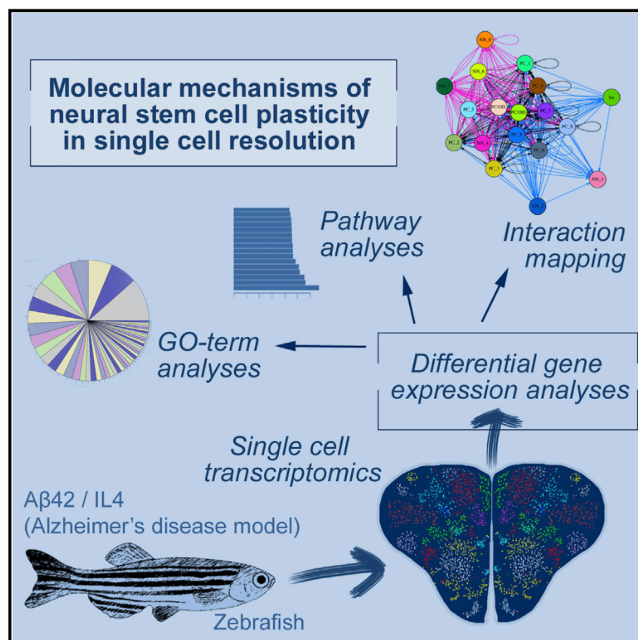
Single-Cell Transcriptomics Analyses of Neural Stem Cell Heterogeneity and Contextual Plasticity in a Zebrafish Brain Model of Amyloid Toxicity

Graphical Abstract



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In Brief

The zebrafish brain combats Alzheimer's disease by producing more neurons, which are derived from neural stem cells. By using single-cell sequencing technology, Cosacak et al. identify distinct stem cell populations that react differently to Alzheimer's disease-like conditions in the adult zebrafish brain and develop tools to investigate their molecular programs.

Highlights

- Single-cell transcriptomics reveals neural stem cell/glia heterogeneity in zebrafish
- Different stem cell populations can be defined spatially and molecularly
- Amyloid-β-42 and interleukin-4 induce plasticity of certain progenitor populations
- Interaction mapping predicts the pathways that regulate neural stem cell plasticity

Cosacak et al., 2019, Cell Reports *27*, 1307–1318
April 23, 2019 © 2019 The Author(s).
https://doi.org/10.1016/j.celrep.2019.03.090

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Single-Cell Transcriptomics Analyses of Neural Stem Cell Heterogeneity and Contextual Plasticity in a Zebrafish Brain Model of Amyloid Toxicity

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https://doi.org/10.1016/j.celrep.2019.03.090

SUMMARY

The neural stem cell (NSC) reservoir can be harnessed for stem cell-based regenerative therapies. Zebrafish remarkably regenerate their brain by inducing NSC plasticity in a Amyloid-β-42 (Aβ42)-induced experimental Alzheimer's disease (AD) model. Interleukin-4 (IL-4) is also critical for AD-induced NSC proliferation. However, the mechanisms of this response have remained unknown. Using single-cell transcriptomics in the adult zebrafish brain, we identify distinct subtypes of NSCs and neurons and differentially regulated pathways and their gene ontologies and investigate how cell-cell communication is altered through ligand-receptor pairs in AD conditions. Our results propose the existence of heterogeneous and spatially organized stem cell populations that react distinctly to amyloid toxicity. This resource article provides an extensive database for the molecular basis of NSC plasticity in the AD model of the adult zebrafish brain. Further analyses of stem cell heterogeneity and neuro-regenerative ability at single-cell resolution could yield drug targets for mobilizing NSCs for endogenous neuro-regeneration in humans.

INTRODUCTION

Zebrafish, with their extensive regenerative ability, have become a key model organism for studies of how tissues heal and regenerate (Alunni and Bally-Cuif, 2016; Kizil et al., 2012b; Zupanc, 2008). Although zebrafish provide valuable opportunities to molecularly dissect the regenerative programs in many tissues, there are still hurdles that render the interpretation of complex cellular interactions and responses difficult, such as the heterogeneity of progenitor cell populations and their differential response to stimuli (März et al., 2010).

Neural progenitor cells can have various identities in vertebrates (Alvarez-Buylla et al., 2002). During development of the nervous system, the neuroectoderm gives rise to neurons through the earliest neural progenitors that have the neuroepithelial character (Klämbt, 2012; Pacary et al., 2012). Later, the neuroepithelium gives rise to radial glial cells, which remain the primary progenitor cell type in lower vertebrates such as zebrafish but are replaced by astrocytes in mammals (Goldman, 2012; Götz, 2012; Kriegstein and Alvarez-Buylla, 2009; Pollen et al., 2015). These two glial cell types are also neurogenic. For instance, in the subventricular zone of the mouse brain, the primary neurogenic progenitors are astrocytes (Doetsch, 2003; Doetsch et al., 1999), whereas, in the zebrafish telencephalon, the analogous region, the pallium, is populated by radial glial cells, which are the neurogenic population (Adolf et al., 2006; Grandel et al., 2006; Kizil et al., 2012b; Than-Trong and Bally-Cuif, 2015; Zupanc, 2008). Therefore, investigation of the heterogeneity of stem cells in the adult zebrafish brain would provide an important understanding of how progenitor subtypes react to diseases, which molecular programs enable the plasticity of stem cells, and whether those programs could be harnessed for regenerative therapies in humans.

CNS regeneration in zebrafish is of particular importance because of the robust neural regeneration ability in zebrafish and potential clinical ramifications, which cannot be elucidated with mammalian models. The zebrafish brain has a widespread constitutive proliferative ability, and radial glial cells (RGCs) constitute the major stem cell population. In the telencephalon, for instance, RGCs form neurons throughout the life of the zebrafish, and they also respond to neuronal loss by reactively proliferating. This proliferation requires induced molecular programs that are required for the special regenerative ability of these stem cells. Different insults have been shown to affect the stem cell response differently, suggesting that distinct progenitor cell populations might differentially react upon stimuli. For instance, we generated an amyloid toxicity model in the adult zebrafish brain and identified that zebrafish can effectively enhance neural progenitor cell proliferation and neurogenesis by inducing interleukin-4 (IL-4), which mediates the crosstalk

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between disease pathology in neurons to initiate the regenerative output in stem cells (Bhattarai et al., 2016, 2017a, 2017b; Kizil, 2018). We also found that IL-4 can directly affect human neural stem cells in a similar fashion to induce proliferative and neurogenic ability (Papadimitriou et al., 2018). However, in traumatic injuries, IL-4 is not induced in the zebrafish brain, although stem cell proliferation increases in both situations. Therefore, there is a need to define the relationship of stem cell heterogeneity and the regenerative plasticity response of those stem cells in the zebrafish brain. This would provide a context-dependent understanding of the molecular programs underlying the regenerative ability rather than a one-size-fits-all approach.

Because of the heterogeneity of stem cell populations and neuronal subtypes in the zebrafish brain, it has not been possible to clearly delineate cell-type-specific responses. This was also the case for the Alzheimer's disease model, where it remained unknown how Amyloid-β-42 (Aβ42) and IL-4 lead to enhanced stem cell plasticity and neurogenesis, which individual subtypes of stem cells and neurons respond to Aβ42 and IL-4, and how individual the responses were. Therefore, in our study, we performed single-cell sequencing in control, Aβ42-treated, and IL-4-treated adult zebrafish telencephalon and categorized the cell type identities and molecular programs of individual cell types, how Aβ42 and IL4 altered those programs, and how different cell types interacted with each other. Our results add further elaboration to our previous findings that Aβ42 and IL-4 affect neural stem cells to enhance their neurogenic capacity by providing detailed analyses of heterogeneous cell populations. We believe that the extensive datasets will not only provide a useful resource for detailed examination of the adult zebrafish brain and its remarkable regenerative ability in a homeostatic and neurodegenerative context but also serve as an improvable database for further research regarding the regenerative ability of vertebrate brains.

RESULTS AND DISCUSSION

Sequencing and Clustering of Cells to Identify Main Cell Types

Previously, we have shown that Aβ42 toxicity in zebrafish induces the expression of IL-4 and that the IL-4-STAT6 pathway induces neural stem- progenitor cell (NSPC) proliferation (Bhattarai et al., 2016, 2017a, 2017b). We also found that IL-4 can directly affect human neural stem cells in a similar fashion to induce proliferative and neurogenic ability (Papadimitriou et al., 2018). To investigate which cell types respond to Aβ42 and IL-4 in the adult zebrafish brain, we injected PBS, Aβ42, and IL-4 into a 6-month-old adult zebrafish brain as described previously (Bhattarai et al., 2016) and designed an analysis pipeline (Figure 1A). We dissected the telencephalon region of the adult brain of a transgenic zebrafish that expressed GFP under the *her4.1* promoter, which marks glial cells (Yeo et al., 2007). Using flow cytometry-assisted cell sorting, we removed cell debris and dead cells (Figures S1A–S1C) and enriched the GFP+ and GFP− cells. To perform single-cell sequencing, we mixed viable GFP+ and GFP− cells in a 1:1 ratio. After single-cell sequencing, we mapped the resulting reads to the zebrafish genome and performed unbiased clustering (Figures 1B–1D) using Seurat software (Butler et al., 2018). After removing cells with minimum and maximum thresholds for nUMI, nGene, and mitochondrial RNA genes, we obtained 609, 737, and 450 cells from PBS-, Aβ42-, and IL-4-treated fish telencephalons, respectively. The read numbers per cell (nUMI), number of genes detected per cell, and mitochondrial mRNA percentages (Figures S1D–S1I) were comparable with previously published datasets (Farrell et al., 2018; Pandey et al., 2018; Raj et al., 2018; Satija et al., 2015), supporting the quality of our datasets. Furthermore, by comparing nUMI, nGene, percent.gfp (percentage of gfp transcripts), and percent.mito (percentage of mitochondrial genes), our datasets were comparable with each other (Figures S1D–S1I). By using the Seurat feature of integrating data analyses from different conditions, we classified our cell types for all samples together (Figures 1B and 1C; Figures S1J and S1K). By integrating and combining all cells, we were able to classify cell types that have fewer cells in one sample; otherwise, these cell types are classified with the closest cell types based on the variable genes (data not shown). By using some marker genes (e.g., *lcp1*, *pfn1*, *her4.1*, *s100b*, *id1*, *sv2a*, *nrgna*, *olig1*, and *olig2*; Figure S1L), we identified 4 major cell clusters as Im (immune) cells, PCs (progenitor cells), NNs (neurons), and OPCs/ODs (oligodendrocyte progenitor cells and oligodendrocytes). Then, we identified the top 10 markers for each of these 4 clusters (OPCs/ODs not combined, kept as OPC/OD_1 and OPC/OD_2) (Figure 1D) and top 10 marker genes (Figure 1E; Data S1).

Given that Aβ42 and IL-4 have effects on gene expression, we asked whether combining all cells from different conditions would affect cell clustering. Because Seurat can be used to cluster cells from different treatments, technologies, or species (Butler et al., 2018), we observed a clear separation of marker genes in two main cell clusters: NNs and PCs. Interestingly, the top 10 marker genes in NNs are almost completely absent in PCs and vice versa (Figure 1E). Moreover, the majority of neuronal markers are absent in immune cells, suggesting that cluster-based cell separation is successful.

The PCs express some commonly known marker genes such as *fabp7a*, *her4.1*, *her4.2*, and *id1* (Figure 1E). Interestingly, *fabp7a* is found in all PCs, and *her4.1* is not expressed by all PCs, possibly sparing non-glial progenitors such as the neuroepithelium (Figure S1L). Of note, as expected, GFP is expressed in almost all *her4*.1 cells at higher levels (Figure 2C; Figure S2K).

Cell Clustering and Identification of Cell Types for PCs and NNs

After several rounds of analyses, we realized that variable genes in NNs and PCs limit the clear separation of cells. Thus, while finding the top marker genes for PCs by comparing genes from one cell cluster with all other cell clusters (Ims, OPCs, and NNs), we found that marker genes cannot be clearly separated or that one marker genes are shared with 2 or more other PCs. To find subtypes of PCs, we ran clustering using only PCs identified in Figure 1 and using the same analysis steps described above, except for using the "num.dims" value (an indicator of the number of dimensions used for the principal-component analyses) as 20 to increase the stringency for aligning and finding the iterative clusters (Figure 2A; Figures S2A-S2C and S2H). With the same strategy, we further sub-clustered the NNs as

Figure 1. Cell Sorting and Categorization of Cell Types
(A) Overall strategy for cell sorting and single-cell data analyses.
(B–D) tSNE clustering of cells.
(B) Co-existence map of cells from PBS, Aβ42, and IL-4-treated brains on tSNE plots.
(C) Cell clusters identified by resolution 1.
(D) The main cell clusters are color-coded for immune (Im) cells, progenitor cells (PCs), neurons (NNs), and oligodendrocytes/progenitor cells (OPC/OD_1 and OPC/OD_2).
(E) Heatmap of the top 10 markers for the main cell clusters.
One dissected brain was used for every experiment. See also Figures S1 and S2.

well (Figures S2D-S2F and S2I). We then color-coded cell clusters for PCs and NNs (Figures S2C and S2F) and showed the cells on a t-distributed stochastic neighbor embedding (tSNE) plot (Figure 1A) using the new color-coded cells (Figure S2G). With this strategy, we observed better marker enrichment in the PC and NN types through iterative sub-clustering (Figures S2J and S2K; https://www.kizillab.org/singlecell).

Interestingly, after sub-clustering the PCs and neuronal cells, some cell clusters that are mixed by using all cells are clearly separated by iterative sub-clustering. For instance, cell clusters 1 and 4 in Figure 1C can be clustered into 4 neuronal clusters (NN_0, NN_1, NN_4, and NN_7; Figures S2F and S2G). Additionally, by using all cells, we could identify PC 0, PC 1, PC 2, and PC 4 cells and clearly separate them in tSNE plots (Figure 2A; Figure S2C). Therefore, we propose that iterative sub-clustering of PCs is an efficient way to demonstrate the heterogeneity of similar cell types.

In the current study, we focused mainly on PCs and the effect of Aβ42 and IL-4 on those cells; however, wherever required, we provide results from all cell types as well (https://www.kizillab.org/singlecell). After the two-step clustering, we identified 1, 2, 8, and 9 cell clusters for Im cells, OPCs/ODs, NNs, and PCs, respectively (Figure S2G) and focused mainly on PCs. After identifying PC clusters, we aimed to validate our results with biological *in situ* data to predict the regionalization and type of PCs. Therefore, we took the advantage of an unbiased and

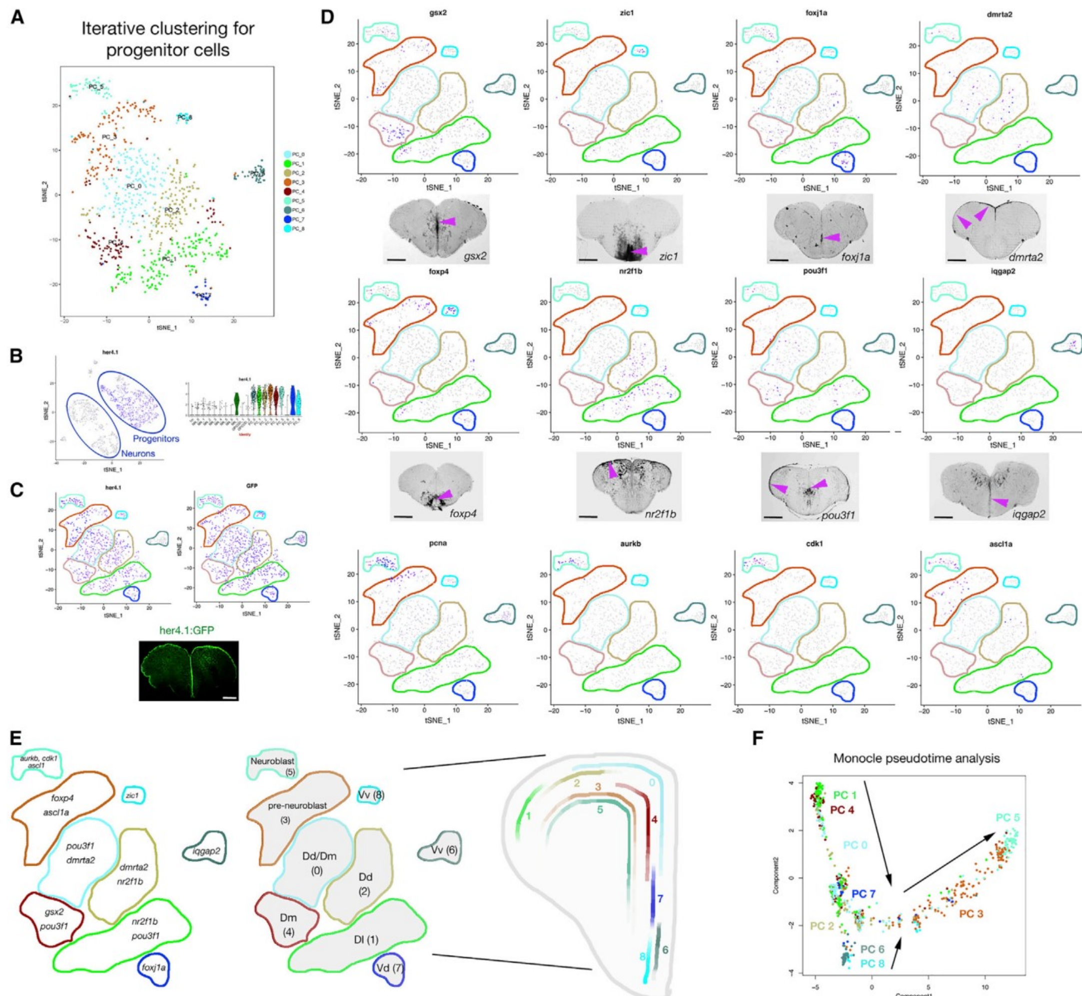

Figure 2. Identification of PC Types

(A) tSNE plots for PC clusters.

(B) *her4.1* expression in a tSNE (left) and violin plot (VLN) of single cell data (right), which contain all cell types.

(C) her4.1 expression (left) and GFP expression (right), shown in tSNE plots for PCs. Also shown is her4.1-driven GFP staining on a telencephalon section (bottom).

(D) tSNE plots for some of the marker genes and relevant *in situ* hybridizations. Pink arrowheads denote expression domains.

(E) Marker genes and localization of cell types on a representative telencephalic scheme.

(F) Cell trajectories on pseudotime generated by monocle2; all colors depict the cell cluster colors in (A).

Scale bars, 200 μm. See also Figures S1–S3.

independent study that is comprehensively documented as a public repository, The Atlas of Gene Expression in the Telencephalon of Adult Zebrafish (AGETAZ) (https://itgmv3.itg.kit.edu/agetaz/index.php; Diotel et al., 2015).

We tested the validity and reliability of our tSNE feature plots by comparing her4.1 and GFP plots with GFP expression in the her4.1-GFP transgenic zebrafish line (Figure 2B) and observed that, indeed, GFP is a general indicator of PC populations (Figure 2C). Later, we selected several regional markers from the *in situ* hybridization database and checked their localizations on feature plots (Figure 2D; Figure S3). As a result, we identified deterministic marker expression for all PC clusters (Figure 2D).

For instance, PC 5 cells express *pcna*, *mki67*, *top2a*, and *ascl1a*, indicating that these cells are actively proliferating cells or neuroblasts. PC 3 cells are the closest cells to PC 5 cells by expressing *pcna* and *ascl1a* but not *mki67* and *top2a* and can be classified as non-proliferating neuroblast precursors or pre-neuroblasts (Figure 2D; Figures S2J). Pre-neuroblasts and neuroblasts share common top marker genes (Figure S2J). Neuroblasts express known markers associated with the neuroblast state (such as *pcna*, *stmn1a*, and *hmgb2*) at higher levels compared with pre-neuroblasts. These two cell types do not show a regional localization but are scattered along the ventricular region in the adult zebrafish telencephalon (Figure 2D).

PC 6 cells do not express *s100b* and *her4.1*, whereas PC 8 cells express *her4.1* but not *s100b*. These two cells are not radial glial cells but, combined with the expression of other markers (e.g., *zic1*, *krt8*, and *clu*; Figure 2D; Figure S3) can be classified as neuroepithelium cells. These cells are mainly localized in the ventral (Vv) telencephalon (based on *zic1* and *iqgap2* expression; Figure 2D). Even though these clusters localize similarly to the ventral telencephalon, they represent two distinct populations.

PC 0 contains cells that express markers of the dorsal (Dd) and dorsomedial (Dm) part of the telencephalon (Figure 2; *pou3f1* and *dmrta2*). Although PC 2 cells express *dmrta2* and *nr2f1b* (Dd markers; Figure 2D), PC 4 cells express *gsx2* and *pou3f1*, which are expressed more in the Dm region (Figure 2D). Interestingly, PC 0 shares common markers either with PC 2 or PC 4. Based on the presence and expression of these marker genes, we classify PC 2 as Dd, PC 4 as Dm and PC 0 as both Dd and Dm. These findings indicate that different regions contain heterogeneous PC populations that differ by marker gene expression.

PC 1 expresses *nr2f1b* and *pou3f1* and, based on the *in situ* hybridization results of these genes, mainly localize on the lateral part of the telencephalon (dorsolateral [Dl]). PC 7 marks an interesting cell type that is characterized by *foxj1a* expression, which is clearly localized to the interstitial region between the ventral and Dm telencephalon (Figures 2D and 2E), and is classified as cells at the ventral part of the dorsal telencephalon (Vd). *foxj1a* is a central regulator of motile ciliogenesis and is co-expressed in PC 7 with *rsph9*, *cfap126*, and *enkur*, which are motile ciliated cell markers (Figure S3) (Lindsey et al., 2012; Ogino et al., 2016). Based on our findings, we propose that PC 7 corresponds to ependymal cells of the adult zebrafish telencephalon. Because ciliated cells are crucial for the development and function of the CNS (Olstad et al., 2019; Sternberg et al., 2018), and because the neurogenic nature of ependymal cells is controversial (Johansson et al., 1999; Spassky et al., 2005), our results provide important molecular information in a normal and Alzheimer's-like state and could contribute to more sophisticated elaboration of the neurogenic capacity and physiological functions of ependymal cells.

Overall, marker genes, *in situ* expression, and differential clustering reveal that the ventricular region of adult zebrafish telencephalon contains distinct progenitor subtypes that can be spatially defined (Figure 2E).

By using iterative sub-clustering of PCs, we identified 7 progenitor and 2 neuroblast types in the telencephalon. To identify how these cell types are related to each other, we constructed a pseudotime using Monocle software (Qiu et al., 2017; Figure 2F). We found that PCs perfectly progressed from progenitor states toward proliferative states (Figure 2F). PC 0, 1, 2, and 4 are distributed on pseudotime but not in a specific location. This indicates that progenitors have a physiological continuum from non-dividing (or quiescent) to potentially dividing states. Pseudotime also predicts the formation of pre-neuroblasts and neuroblasts from other PCs, and, interestingly, regardless from which progenitor state they come, neuroblasts are rather homogeneous cell types in terms of marker gene expression. Based on this observation, we propose that, by using pseudotime, we could also capture the state of PCs and that neurogenic heterogeneity exists at the PC stage rather than at the neuroblast stage. We propose that the adult zebrafish brain contains committed and regionally specified neural progenitors that generate neurogenic diversity through a rather homogeneous neuroblast stage (all gene expression data, gene ontology (GO) term analyses, and tSNE plots for every cell cluster in control, amyloid-treated, and IL-4-treated brains can be found at https://www.kizillab.org/singlecell).

Plasticity Is Affected in a Subset of PCs after Aβ42 and IL-4

Based on the spatially confined PC populations that display distinct marker gene expression, we hypothesized that some of these PC populations would react differentially to amyloid toxicity or IL-4 treatment. A central feature of neural progenitors is their plasticity, which would reflect in a proliferative response. To determine the cell types amyloid and IL-4 affect, we injected amyloid or IL-4 into adult zebrafish brains and analyzed cell proliferation in predefined regions: Dl, Dd, Dm, Vd, and Vv telencephalon (Figure 3A) by performing immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and GFP on the her4.1:GFP transgenic reporter line (Figures 3B–3D'). We observed that amyloid and IL-4 increased cell proliferation in 3 of the 5 regions: the Dd, Dm, and Vd telencephalon (Figure 3E). These results suggest that PC populations react differentially to amyloid toxicity and that heterogeneity of the progenitors underlies neurogenic ability. Because the zebrafish brain can increase neurogenic output after various insults through activation of different molecular mechanisms (Alunni and Bally-Cuif, 2016; Bhattarai et al., 2016; Cosacak et al., 2015; Diotel et al., 2013; Kizil, 2018; Kizil et al., 2012a, 2012b, 2012c; Kyritsis et al., 2012; März et al., 2011; Shimizu et al., 2018; Tincer et al., 2016), understanding the subpopulation of PCs that are responsible for the regenerative ability after particular injuries would have tremendous clinical ramifications. More focused investigations of these special cells could shed light on which programs must be induced in comparative cell types in human brains to boost neurogenic output.

Amyloid Toxicity Changes the Molecular Regulatory Landscape of PCs

Because amyloid toxicity affects PC plasticity in a spatially specific manner, we aimed to determine the molecular changes pertaining to this response using our single-cell analyses. Therefore, we determined differentially expressed genes (DEGs) in every cluster (Data S2) and subsequently identified differentially regulated Kyoto encyclopedia of genes and genomes (KEGG)

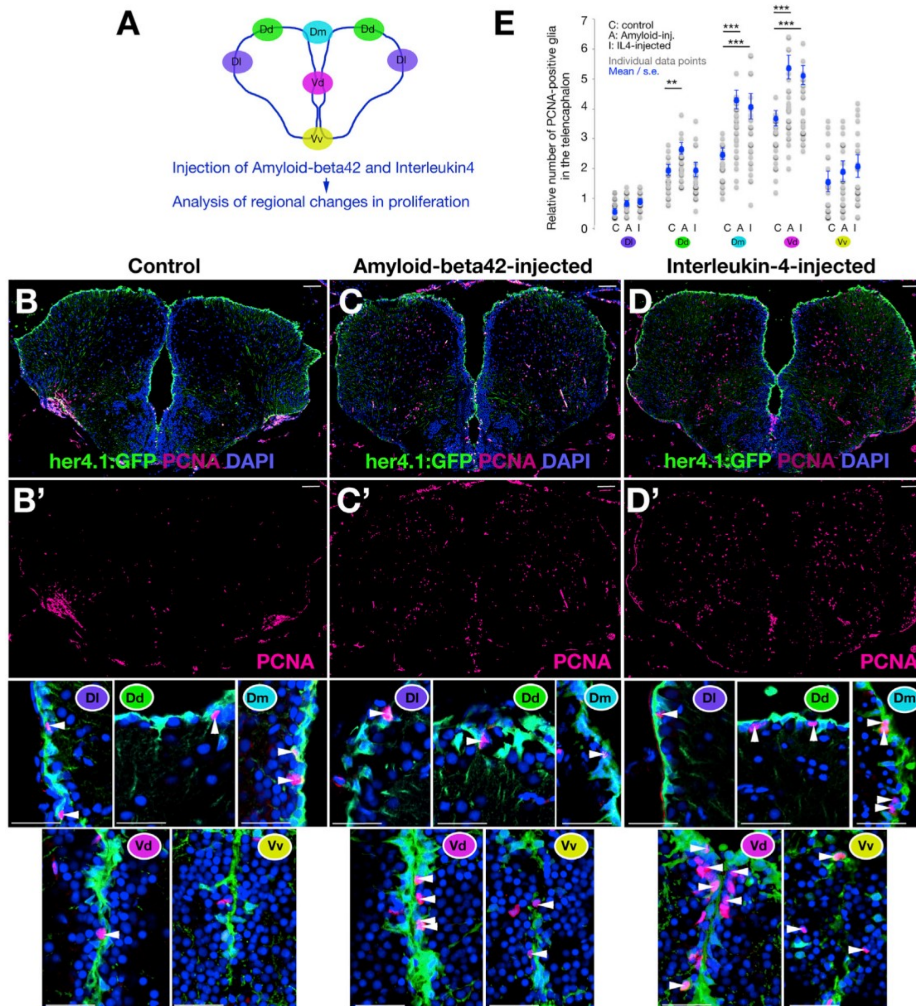

Figure 3. Aβ42 and IL-4 Affect Specific Progenitor Populations in the Adult Zebrafish Brain

(A) Schematic view of regionalization in the adult zebrafish telencephalon.

(B–D') Immunohistochemistry (IHC) staining for her4.1-driven GFP and PCNA on control (B), amyloid-injected (C), and IL-4-injected (D) telencephalic sections. In (B'), (C'), and (D'), PCNA channels are shown. Close-up views of GFP and PCNA double IHC are shown below the single channels. Regions are marked with color-codes as in (A). Scale bars, 50 μm.

(E) Quantification graph for the relative number of proliferating progenitors. Three brains were used for every experimental group. Data are represented as mean ± SEM. The levels of significance are *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

pathways in PCs that are located at the Dd, Dm, and Vd telencephalon (PC 0, 2, 3, 4, and 5; Figures 4A and 4B; Data S3). By comparing the DEGs in every cell cluster within individual treatments (i.e., Aβ42 and IL-4), we found that every cell type has a unique set of DEGs because the percent overlap of DEGs after Aβ42 or IL-4 treatment in a particular cell type is below 30% (percentage of the number of common DEGs in clusters X and Y divided by the number of DEGs in cluster X; Data S4A and S4B). However, when we compared the percent overlap of DEGs that are determined in a given cell type after Aβ42 and IL-4 treatment, we found that, in several cell types, Aβ42 and IL-4 lead to differential expression of more than 30% of the genes in a similar fashion (e.g., PC 0 cells, PC 4 cells, and Im cells; Data S4C). These results suggest that every cell type

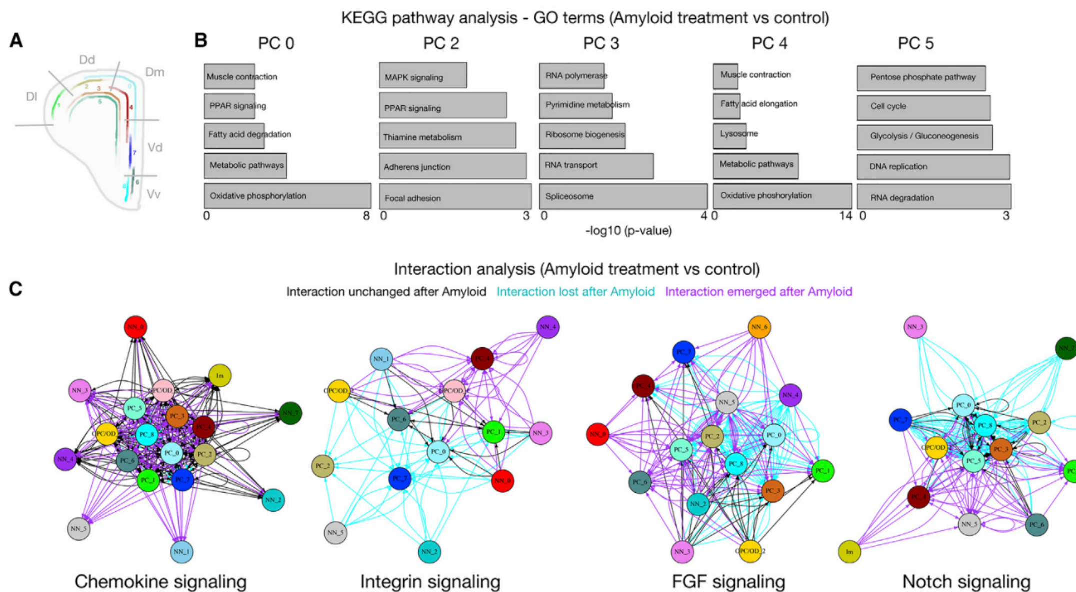

Figure 4. Pathway Analyses and Interaction Maps for PC Populations Affected by Aβ42

(A) Schematic view of the regional distribution of PCs in the adult zebrafish telencephalon.

(B) KEGG pathway analysis, indicating the 5 most affected pathways in PCs 0, 2, 3, 4, and 5.

(C) Representative interaction maps for chemokine, integrin, FGF, and Notch signaling. Black arrows, interactions present before and after amyloid treatment; cyan arrows, interactions lost after amyloid treatment; purple arrows, interactions emerging only after amyloid treatment.

See the Data S1, Data S2, Data S3, Data S4, and Data S5 for extensive datasets of GO terms, DEGs, and other interaction maps.

has a unique molecular signature that is synergistically affected by Aβ42 and IL-4.

With GO term analyses, we also observed that every cell cluster changed distinct molecular pathways after amyloid toxicity, with a few categories overlapping between cell clusters (e.g., oxidative phosphorylation). For instance, cell cycle-related pathways are affected by amyloid only in PC 5 (neuroblasts with proliferative ability), which supports our classification and suggests that, by investigating the differentially regulated pathways, we could determine the signaling landscape that is responsible for induced plasticity in particular cell types after amyloid toxicity. An example could be seen in PC 2, where the mitogen-activated protein kinase (MAPK) signaling pathway is uniquely affected (Figure 4B). Therefore, our single-cell data could be used to determine the signaling pathways that are specifically involved in regulation of neurogenic plasticity of particular PCs.

A prominent way in which cells interact with each other is through secreted molecules and their receptors in target cells. To determine such putative cell-cell communication through secreted molecules, we determined (bioinformatically and based on the literature) 646 ligands interacting with 658 receptors (Data S5). Among the marker genes expressed in clusters (Data S5), we found that 101 ligands and 117 receptors were expressed in identified clusters (Data S5). We generated several interaction maps based on identified genes (https://www.kizillab.org/singlecell; Data S5), portraying complex interaction between cell types.

Because the interaction data we obtained represent a potential interaction landscape based only on ligand-receptor pair interaction, it needed validation. Therefore, we determined the interaction of individual signaling pathways (https://www.kizillab.org/singlecell). Many major signaling pathways, such as fibroblast growth factor (Fgf), Integrin, chemokine, and Notch signaling, are known to be involved in cell-cell communication in the adult zebrafish brain (Alunni et al., 2013; Cacialli et al., 2016; Diotel et al., 2010; Jiao et al., 2011; Kaslin et al., 2009; Kizil et al., 2012a, 2012c; Shimizu et al., 2018). To depict how cell clusters interact using these pathways, we plotted the intercommunication predictions for cell types expressing the receptor or ligand for pathways (Figure 4C). We found that all interaction categories are complex and involve the participation of many cell types, which includes NN-PC and Im-PC interaction. Despite this complexity, such data mining could provide valuable and unprecedented biological information. We also identified other potential interactions with less studied ligands and receptors, such as *agrn*, *appa*, *ctgfa*, *gnai2*, *penkb*, *ptn*, *serpine*, *edil3a*, and *hbegf* (https://www.kizillab.org/singlecell), which might provide still unknown candidates for regulation of NSPC plasticity.

To provide biological verification of our interaction analyses, we selected the fibroblast growth factor (FGF) signaling pathway, which is involved in NCS/PC proliferation in the zebrafish brain

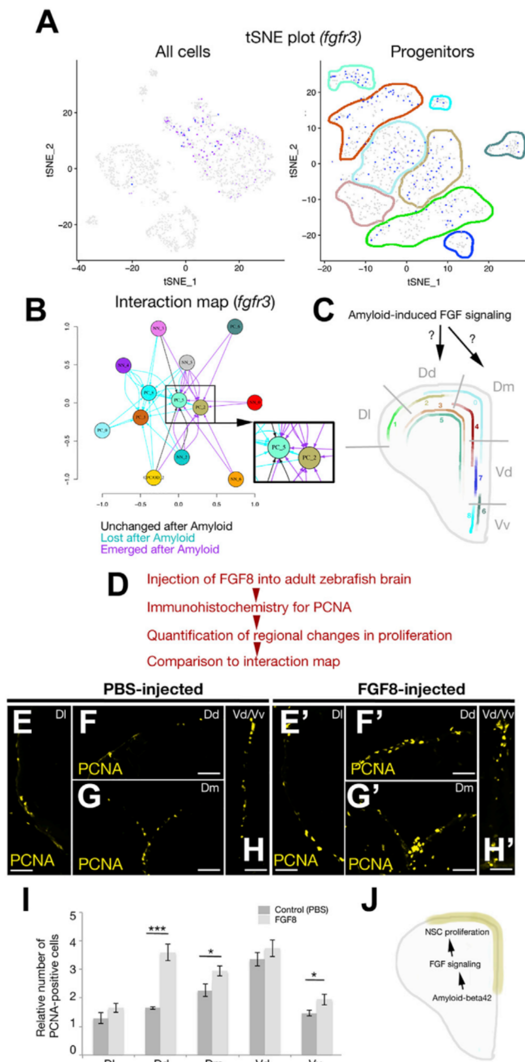

Figure 5. Functional Investigation of Interaction Maps Reveals Fgf Signaling Controlling PC Proliferation

(A) tSNE plot (all cells on the left, progenitors on the right) for *fgfr3*, which is localized specifically to PCs.

(B) Predicted interaction map for *fgfr3* and its ligands.

(C) Based on the interaction prediction, PC 2 and PC 5 are exposed to FGF signaling after amyloid treatment.

(D) Methodology for functional validation of the effects of FGF signaling on PC proliferation.

(E–H') Immunohistochemical staining for PCNA in control (E–H) and FGF8-injected (E'–H') adult zebrafish brains. Regional identities are indicated with the names denoted in (C). Panels E–H modified from Kizil and Brand, 2011.

(I) Quantification of regional proliferation response after FGF8-injection.

(J) Working model of the regulatory cascade of NSPC proliferation in the adult zebrafish brain by Aβ42-induced FGF signaling.

Three sections were quantified for every experimental group. Scale bars, 50 μm. Data are represented as mean ± SEM. The levels of significance are *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

(Ganz et al., 2010; Kaslin et al., 2009; Kizil and Brand, 2011; Sleptsova-Friedrich et al., 2001; Topp et al., 2008), and investigated whether it would affect the proliferative ability of the brain regions that correspond to the cell clusters found to be affected by FGF in our interaction analyses (Figure 4C).

We performed tSNE plot analyses for all receptors for FGF (data not shown) and found that *fgfr3* is specifically expressed in PCs (Figure 5A). We then constructed an interaction map by determining the potential interactions through Fgfr3 (Figure 5B). With such a sub-interaction map, all possible communications through *fgfr3* and its ligands are sketched, and we hypothesized that Fgfr3 signaling would positively affect mainly PC 2 (Dd) and PC 5 (neuroblasts) (Figure 5C). Amyloid treatment seemed to cause interactions that were not present in control brains from various cell types toward PC 2 and PC 5 (Figures 5B and 5C). If the interaction map analyses were reliably predictive, then this would suggest that, after activation of Fgfr3 signaling upon Aβ42, the Dd and Dm regions of the zebrafish brain could change their proliferative response. To test this hypothesis, we injected the Fgfr3 ligand FGF8 (Chellaiah et al., 1999; Sleptsova-Friedrich et al., 2001) into the adult zebrafish brain and determined the changes in cell proliferation (Figure 5D) by performing immunohistochemical staining for PCNA (Figures 5E–5F'). We found that, compared with control brains, FGF8 injection significantly increased cell proliferation in the Dd and Dm regions, exactly as predicted by the interaction mapping (Figures 5G and 5H). Overall, our interaction studies provide an extensive high-resolution cell-cell communication map, and these predictions provides a further level of elucidation for the heterogeneity of neural stem cell populations and the molecular mechanisms controlling the plasticity response thereof.

Further investigation of our data and experimental validation of the candidates presented in our resource platform would provide the molecular programs underlying the neural stem cell (NSC) plasticity and regenerative response of the adult zebrafish brain in Alzheimer's disease conditions as well as the downstream regulation of IL-4. Given that we have recently shown that IL-4 is sufficient to enhance the human NSC plasticity and regenerative capacity in a 3D human Alzheimer's model and to circumvent the AD pathology (Papadimitriou et al., 2018), our data will undoubtedly provide more candidates that can be clinically relevant. Such an understanding would provide new therapeutic targets for Alzheimer's disease for clinical and pharmaceutical use, not only for neurogenesis but also for neuronal survival and synaptic integrity, as exemplified recently (Reinhardt et al., 2019).

Conclusion

Heterogeneity of NSC activity and fate decisions mainly rely on activation or suppression of distinct molecular programs in respective cell types. Single-cell sequencing uniquely allows investigation of individual cell types and their response to stimuli. In our analyses, we identified distinct cell types of the adult zebrafish telencephalon, which is a widely used experimental

model for studying neuronal regeneration and NSC plasticity. By focusing on stem cell populations, we also identified how Aβ42, the hallmark of Alzheimer's disease, affects these cell types and which genes and pathways are altered upon Aβ42 toxicity. Because the zebrafish brain can enhance its stem cell activity and neurogenesis in Alzheimer's disease conditions, understanding in more detail how Aβ42 affects individual cells would certainly enhance our understanding of how a vertebrate brain could counteract Alzheimer's pathology and propose previously unidentified targets for clinical use or drug development.

In mammals, radial glial cells give rise to astrocytes during the second wave of neurogenesis (Hansen et al., 2010; Kriegstein and Alvarez-Buylla, 2009; Urbán and Guillemot, 2014). In fish, however, radial glia remain as such morphologically, and, by definition, there is no astrocyte in the telencephalon (Adolf et al., 2006; Ganz et al., 2012; Grandel et al., 2006; März et al., 2010; Mueller et al., 2004). In our study, the PC populations were almost entirely positive for S100beta, a canonical marker for astrocytes in mammals (Doetsch et al., 1999). Therefore, we believe that marker-based interpretation of a cell type in evolutionary terms cannot be performed and that, in zebrafish, radial glial cells take over astrocytic functions. Additionally, in our tSNE analyses, no progenitor cluster generated a branch that could be considered astrocytes. Therefore, we believe that there are no astrocytes in the telencephalon, which supports previous studies (Barbosa et al., 2015; Grupp et al., 2010; Kaslin et al., 2008; Kizil et al., 2012b; Lam et al., 2009; Mueller and Wullimann, 2009; Rothenaigner et al., 2011; Than-Trong and Bally-Cuif, 2015). In mice, astrocytes also co-segregate with NSCs in single-cell analyses (Artegiani et al., 2017), suggesting that, even in species where astrocytes are evident, without specific sorting for these cells, they cannot be distinguished from existing cell types.

Our analyses subdivided the *her4.1*-positive PC population, which had previously been considered a uniform cell type: radial glia (Kroehne et al., 2011; März et al., 2010; Than-Trong and Bally-Cuif, 2015; Yeo et al., 2007). We identified 9 sub-clusters, 2 of which are ventral neuroepithelial cell types that are not radial glia, which supports previous findings (Kaslin et al., 2008). Another two clusters are neuroblasts, which separate according to their proliferative status. Pre-neuroblasts express almost identical markers as proliferating neuroblasts but also share common markers with the remaining five sub-clusters of progenitors, from which they emanate. Whether PCs are lineage-restricted or multipotent has been an intriguing question (Alvarez-Buylla et al., 2002; Laywell et al., 2000; Raj et al., 2018; Temple, 2001). Studies in salamanders have proposed that regenerative masses of progenitors are strictly lineage-restricted and remember their original identities (Antos and Tanaka, 2010; Kragl et al., 2009), whereas, in mammals, where neurogenic competency and potency of the neural progenitors decline with age, progenitors are lineage-restricted in adult stages and multipotent in embryonic development (Costa et al., 2010; Götz, 2012, 2015). Our study in zebrafish suggests that neuroblasts—cells that are proliferative and leading to actual neurogenesis—cluster into a single population. We therefore propose that the mode of neurogenesis and lineage competency of the zebrafish brain involves transition from a single, rather homogeneous cell population (neuroblasts) with common molecular landmarks and represents the embryonic mode of neurogenesis in mammals. This also suggests that zebrafish can be used as a viable comparative tool for mammalian brains to understand the molecular programs that would mimic the early embryonic stages of neurogenesis in adult stages. Because one of the aims in regenerative therapies is to rejuvenate the adult mammalian brain and to impose regenerative ability back to it (Gage et al., 2016; Katsimpardi et al., 2014; Wyss-Coray, 2016), the adult zebrafish brain would serve as an excellent model, with its endogenous life-long neurogenic competency and regenerative ability.

Our results provide complex and multi-layered information that can be retrieved from our accompanying website (http://www.kizillab.org/singlecell). First, it provides refinement of the cell types and progenitor states in the adult zebrafish telencephalon. This will allow investigation of certain regions or cell types at a higher resolution and can allow cell-specific investigation of biological phenomena. Our analyses of the pathways that are active in certain clusters in homeostatic states of the fish brain also provide candidate signaling pathways that can be investigated in detail. Parallels between our results and previous literature as well as our validation studies confirm the fidelity of our analyses and interpretations. In addition to known signaling molecules, our data provide extensive resources for identification of new molecules and cellular interactions in the adult fish brain. Additionally, our analyses with Aβ42 and IL-4 treatment provide unprecedented information regarding the stem cell plasticity and regenerative output of the fish brain in Alzheimer's conditions.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.03.090.

ACKNOWLEDGMENTS

This work was supported by German Center for Neurogenerative Disease (DZNE) and the Helmholtz Association Young Investigator Award (VH-NG-1021 to C.K.), Deutsche Forschungsgemeinschaft (DFG) (KI1524/6, KI1524/10, and KI1524/11 to C.K.), CRTD, TU Dresden (FZ-111 and 043_261518 to C.K.). Neurowind e.V. supported the open access publication of this manuscript. C.K. dedicates this paper to Adnan Kizil.

AUTHOR CONTRIBUTIONS

M.I.C. and C.K. conceived and designed the experiments. P.B. performed cerebroventricular microinjections. Y.Z. provided the amyloid peptide. S.R., A.P., and A.D. performed single-cell sequencing. M.I.C. performed bioinformatics analyses. M.I.C., P.B., and C.K. performed immunohistochemical staining and quantifications. M.I.C. and C.K. generated the supplemental website. C.K. supervised the study, acquired funding, and wrote the manuscript. C.K. and M.I.C. revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: October 8, 2018
Revised: February 21, 2019
Accepted: March 25, 2019
Published: April 23, 2019

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-GFP	Abcam	Cat# ab13970, RRID:AB_300798
Anti-PCNA	Dako	Cat# M0879, RRID:AB_2160651
Anti-S100β	Dako	Cat# Z0311, RRID:AB_10013383
Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 488	Thermo Fischer	Cat# A11039, RRID:AB_142924
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fischer	Cat# A11008; RRID: AB_143165
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fischer	Cat# A-11001, RRID:AB_2534069
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	Thermo Fischer	Cat# A21422; RRID: AB_141822
Chemicals, Peptides, and Recombinant Proteins		
Transportan signal peptide-coupled Amyloid-β42	(Bhattarai et al., 2016)	N/A
Recombinant human Protein IL4	Thermo Fischer	Cat# PHC0044
Recombinant human Protein FGF8	Thermo Fischer	Cat# PHG0184
Bovine Serum Albumin (BSA)	Sigma	Cat# A2153
MESAB	Sigma	Cat# A5040
Critical Commercial Assays		
Neural Tissue Dissociation Kit (P)	Miltenyi	Cat# 130-092-628
10X Chromium Kit	10X Genomics	Cat# 1000092
Deposited Data		
Single cell transcriptome	This paper	GEO: GSE118577
Experimental Models: Organisms/Strains		
Tg(her4.1:GFP) transgenic zebrafish line	(Yeo et al., 2007)	N/A
Software and Algorithms		
Cell Ranger 2.01	(Zheng et al., 2016)	RRID:SCR_016957
GOstats_2.48.0	(Falcon and Gentleman, 2007)	http://gostat.wehi.edu.au; RRID: SCR_008535
monocle_2.10.1	(Qiu et al., 2017)	N/A
Seurat_2.3.4	(Butler et al., 2018)	RRID:SCR_016341
R scripts for data analyses	This paper	https://www.kizillab.org/resources
R version 3.5.2 (2018-12-20)	N/A	RRID:SCR_001905
x86_64-w64-mingw32/x64 (64-bit)	N/A	https://sourceforge.net/, RRID:SCR_004365
Transcriptomics dataset website	This paper	https://www.kizillab.org/singlecell

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Caghan Kizil (caghan.kizil@dzne.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics statement

All animal experiments were performed under the permission of Landesdirektion Dresden with the following permit numbers: TVV-52/2015 with all relevant amendments.

Animals
Transgenic zebrafish expressing GFP under the *her4*.1 promoter (Tg(her4.1:GFP, Yeo et al., 2007) was used for sorting progenitors and the rest of the telencephalon. 6 months post fertilization (mpf) female fish were used for analyses.

METHOD DETAILS

Cerebroventricular microinjection
Tg(her4.1:GFP) zebrafish were injected through a slit generated over the optic tectum using a barbed end 30-gauge needle (Bhattarai et al., 2016; Kizil and Brand, 2011; Kizil et al., 2013). Glass capillary filled with injection liquid was inserted into the cerebrospinal space of the adult brain and 1 μl of the solution was injected. Fish were injected with either with PBS (control), Aβ42 (20 μM) or Interleukin-4 (1 μM), and were kept at 14 hr light/10 hr dark cycle in normal water system for 24 hours. FGF8 was injected at 0.2 mg/ml final concentration. Experimental fish were sacrificed using 0.2% MESAB according to the animal experimentation permits.

Cell Dissociation and sorting
The telencephalon of the fish were dissected in ice-cold PBS and directly dissociated with Neural Tissue Dissociation Kit (Miltenyi) at 28.5°C as described previously (Bhattarai et al., 2016). After dissociation, cells were filtered through 40 μM cell strainer into 10 mL 2% BSA in PBS, centrifuged at 300 g for 10 min and cells resuspended in 4% BSA in PBS. Viability indicator (Propidium iodide) and GFP were used to sort viable GFP(+) or GFP(-) cells by FACS. The resulting single cell suspension was promptly loaded on the 10X Chromium system (Zheng et al., 2016). 10X libraries were prepared as per the manufacturer's instructions. The raw sequencing data was processed by the cell ranger software provided by the 10X genomics with the default options. The reads were aligned to zebrafish reference transcriptome (ENSEMBL Zv10, release 91) and eGFP CDS (with arbitrary ensemble ID; ENSDARG99999999999). The resulting matrices were used as input for downstream data analysis by Seurat (Butler et al., 2018).

Droplet encapsulation sequencing
To prepare the cells for droplet-based sequencing, 2700 GFP-positive and 2700 GFP-negative cells were flow-sorted into a coated tubes containing 2 μl saline solution with 0.04 % Bovine serum albumin (BSA). Subsequently, the single cell suspension was carefully mixed with reverse transcription mix before loading the cells on the 10X Genomics Chromium system. During the encapsulation, the cells were lysed within the droplet and they released polyadenylated RNA bound to the barcoded bead, which was encapsulated with the cell. Following the guidelines of the 10x Genomics user manual, the droplets were directly subjected to reverse transcription, the emulsion was broken and cDNA was purified using Silane beads. After the amplification of cDNA with 10 cycles, purification and quantification was performed.

The 10X Genomics single cell RNA-sequencing library preparation - involving fragmentation, dA-tailing, adapter ligation, and 12-cycle indexing PCR – was performed. After quantification, the libraries were sequenced on an Illumina NextSeq 550 machine using a HighOutput flowcell in paired-end mode (R1: 26 cycles; I1: 8 cycles; R2: 57 cycles), thus generating 80-125 million fragments. The raw sequencing data was then processed with the 'count' command of the Cell Ranger software provided by 10X Genomics. The option of '–expect-cells' was set to 2700 (all other options were used as per default). To build the reference for Cell Ranger, zebrafish genome (GRCz10) as well as gene annotation (Ensembl 91) were downloaded from Ensembl and the annotation was filtered with the 'mkgtf' command of Cell Ranger (options: '–attribute=gene_biotype:protein_coding –attribute=gene_biotype:lincRNA –attribute=gene_biotype:antisense'). Genome sequence and filtered annotation were then used as input to the 'mkref' command of Cell Ranger to build the appropriate CellRanger Reference.

Data Analysis by Seurat
All matrices were read by Read10X function; cell names were re-named as sample names and the column number, to trace back cells if required. First, we removed all ribosomal RNA genes, then we filtered out cells as following; cell with more than 15000 UMI or less than 1000 UMI, cells with less than 500 or more than 2500 unique genes, cells with more than 6% mitochondrial genes. The genes found in less than 5 cells were filtered out. Moreover, we removed cells with potential of mulitplet. The remaining cells and genes were used for downstream analysis for all samples. The data normalized by using "LogNormalize" method, data scaled with "scale.factor = 1e4." For each datasets variable genes found with FindVariableGenes with the following options mean.function = ExpMean, dispersion.function = LogVMR, x.low.cutoff = 0.125, x.high.cutoff = 10, y.cutoff = 0.5. The top1000 most highly variable genes from each sample were merged, mitochondrial genes were removed. Then, the intersection of these genes with all genes in each samples were used for CCA analysis. The 3 Seurat objects and the variable genes found above were used to generate a new Seurat object with RunMultiCCA function, using num.ccs = 30. The canonical correlation strength were calculated using num.dims = 1:30 and the samples were aligned using dims.align = 1:10. The cell clusters were found using aligned CCA and 1:10 dims, with higher resolution 1.0. The cell clusters were shown on 2D using t-SNE (RunTSNE function). By using some general markers, we found 4 major cell types; Progenitor Cells (PCs) [*fabp7a+*], Immune cells (Im) [*lcp1+*, *pfn1+*], Oligodenrocytes / Progenitor Cells (OPC/OD_1, OPC/OD_2), and Neuron (*sv2a+*, *synpr+*). The marker genes for these 5 cell poplations were calculated by using FindAllMarkers function with options min.pct = 0.25, thresh.use = 0.25. The Progenitor cells or Neurons were separated from all other

cells and PCs or Neuronal cell clusters were identified using aforementioned steps with some changes; instead of num.dims = 10, num.dims = 20 were used. The marker genes for PCs or Neuronal cell types were identified as above.

Monocle analysis of progenitor cells

In order to identify cell trajectories on pseudotime, we converte the Progentor cells Seurat object to Monocle (Qiu et al., 2017). The estimated size factor set to 1.0 as the data were already normalized by Seurat. We used the genes used for CCA analysis for ordering in monocle, with max_components = 4. The cells were colored as the colors on Seurat TSNE plots.

Identification of cell types

Feature plots were generated by Seurat software and cell types were determined by the expression of marker genes that define specific cell types.

GO-term analyses

We used the all marker genes with False Detection Rate < 0.1 for Gene Ontology analysis and KEGG pathway analysis using GOStats (1.7.4) (Falcon and Gentleman, 2007) and GSEABase (1.40.1), p value < 0.05 as threshold as described previously (Papadimitriou et al., 2018). To determine the differentially expressed genes (DEGs) after Aβ42 and IL4 treatment, we used FindMarkers function using cell cluster that have at least 3 cells from all samples. Then, we used the p value < 0.05 for significantly expressed genes. These genes were used for GO and pathway analysis using the scripts that are available on www.kizillab.org/resources.

Construction of interaction maps

For the ligand-receptor interaction, we downloaded all Ligand and receptors from a previous publication (Ramilowski et al., 2015) (http://fantom.gsc.riken.jp/5/suppl/Ramilowski_et_al_2015/vis/#/hive), and orthologs for zebrafish were found using BiomaRt of ENSEMBL. The ligand-receptor for zebrafish were used for downstream analysis as following; for cell-cell interaction, all ligands found in 20% of a cell types were chosen, then their receptors were identified in all cell types. If a ligand-receptor pathway was found, then we draw a direction from the cell with the ligand to the cells with the receptor. We used igraph in R to draw interaction map as previously described (Skelly et al., 2018). In order to show the lost and/or new interaction after treatment, we calculated all the interaction for PBS and the treatment (Aβ42 or IL4) and then generated an interaction maps from all interaction of PBS and the treatment. Then, we colored the edges as cyan (lost after treatment), purple (induced after treatment) and black that are not affected by the treatment; but may be differentially expressed.

QUANTIFICATION AND STATISTICAL ANALYSES

Images from histological sections were acquired using Zeiss ZEN software in Zeiss Apotome, Leica SP5 confocal microscope or using AxioScanner slide scanner. Counting was performed on acquired images in a double-blinded manner. The statistical analyses were performed using GraphPad Prism and two-tailed Student's t tests. The levels of significance were $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. In all graphs, means ± standard errors of the means were shown. The data conforms to normal distribution as determined by Pearson's chi-square test.

DATA AND SOFTWARE AVAILABILITY

All feature plots, VLN plots, GO terms and pathway analyses, and interaction maps and the list of genes associated with these analyses can be found on the accompanying website (http://www.kizillab.org/singlecell), which can be accessed freely.

All raw data are deposited to GEO (http://www.ncbi.nlm.nih.gov/geo/) with accession number GEO: GSE118577.

R scripts

All scripts used for the data analysis can be found on https://www.kizillab.org/resources.

3 DISCUSSION

While several hypotheses have been postulated about the causes of AD, amyloid cascade hypothesis explains most the mechanistic aspects of the disease (Hardy, 1992; Hardy and Selkoe, 2002; Selkoe and Hardy, 2016). Based on this hypothesis many drugs were developed and some of those were able to clear amyloid plaques in AD patients without improving the cognitive ability (Cummings, et al., 2016; Nicoll, et al., 2019; Schneider, et al., 2014). The failure in cognitive improvements can be due to many factors and is an intense research area. One reason could be the lack of generation of new neurons in AD patients (Moreno-Jimenez, et al., 2019; Tobin, et al., 2019). While Aβ is the initiator of events that leads to AD, the contribution of Tau, inflammation, synaptic degeneration and neuronal loss collectively lead to the decrease in cognitive ability. The malfunctioning of the functions of other cell types exacerbates AD by feedforward and feedback mechanisms (De Strooper and Karran, 2016). Neural stem cells are one of those cells that are related to the AD pathology (Choi and Tanzi, 2019; Kizil and Bhattarai, 2018; Tincer, et al., 2016). Recently, it has been shown that adult hippocampal neurogenesis drops sharply in Alzheimer disease patients (Moreno-Jimenez, et al., 2019; Tobin, et al., 2019) and the drugs targeting Aβ clearance are able to do so in AD patients but no improvement in cognitive ability was observed after treatment regimens (Nicoll, et al., 2019). This supports the interesting hypothesis that generation of new neurons (regenerative neurogenesis) in AD patients may help to revert the AD pathology as we and others have shown using animal models and 3D culture systems (Bhattarai, et al., 2016; Choi, et al., 2016; Papadimitriou, et al., 2018). While regenerative ability in mammals is evolutionary poor (Tanaka and Ferretti, 2009), studying neurodegeneration in a model organism with regenerative ability can teach us how to induce neural stem cells proliferation and neurogenesis in pathological conditions. Zebrafish can regenerate its nervous system after various insults or disease conditions by using molecular programs pertaining to the regenerative state (Bhattarai, et al., 2016; Cosacak, et al., 2015; Kizil, 2018; Kizil, et al., 2012a; Kizil, et al., 2012b; Kizil, et al., 2012c; Kyritsis, et al., 2012) . In my PhD

thesis, I aimed to generate new transgenic zebrafish models by expressing the AD-associated genes and their variants. Here, I am presenting two studies in zebrafish models (Cosacak, et al., 2017; Cosacak, et al., 2019).

3.1. Zebrafish to study Tau and amyloid pathologies

Tau is a microtubule-associated protein and mutations or hyperphosphorylation on Tau play role in several neurodegenerative diseases that are collectively called Tauopathies (Lee, et al., 2001). In AD, there is no AD associated mutations of Tau. Yet, Tau mutations that are associated with other neurodegenerative diseases are commonly used to generate transgenic animals for addressing Tauopathies (Gotz and Ittner, 2008) and majority of those lines are not expressed in adult brain. To check the effects of Tau in zebrafish telencephalon, I generated a zebrafish transgenic line (Cosacak, et al., 2017) expressing the human Tau with P301L mutations (Dumanchin, et al., 1998; Hutton, et al., 1998). The line expresses human Tau-P301L from early embryonic development to adulthood (18 months), and is among the first lines that can still express the transgene in adult brain by overcoming silencing mechanisms, which in majority of the cases render any transgene inserted into the zebrafish genome inactive in adult animals. One of the modifications on Tau that leads to pathologies is the hyperphosphorylation of Tau and tangle formation (Mandelkow and Mandelkow, 2012). In this line, Tau is hyperphosphorylated from embryonic stages to adulthood and did not induce any overt phenotypes in development of the fish. Moreover, Tau hyperphosphorylation did not induce overt neurodegenerative phenotypes such as cell death and neuroinflammation. This suggests that, despite its hyperphosphorylation, Tau does not form tangles in zebrafish. Additionally, we hypothesized that zebrafish brain might have some protective mechanisms that prevent Tau toxicity. Moreover, we did not observe fibril or tangle formation by electron microscopy as well (Cosacak et al., unpublished data). I have checked the tau localization with electron microscopy, and Tau is mainly localized in bi-layered vesicles in the cytoplasm. This suggests that one of the mechanisms can be autophagy of Tau in this line as well as in zebrafish (Lopez, et al., 2017), in cell lines (Hamano, et al., 2008; Inoue, et al., 2012) and human patients (Piras, et al., 2016). In mouse models, increasing autophagy reduces Tau accumulation (Kruger, et al., 2012; Schaeffer, et al., 2012). Further transcriptomics and functional studies in our Tau line can tell us how zebrafish prevent Tau toxicity in its brain.

Alzheimer disease starts with accumulation of Aβ followed by Tau pathology as has been supported by transgenic expression of APP gene or injection of Aβ42 itself in Tau expressing lines (Gotz, et al., 2001; Gotz, et al., 2004; Oddo, et al., 2003). We tested this hypothesis in our zebrafish line by injecting Aβ42 peptide, which did not lead to exacerbation of amyloid toxicity. I further checked the formation of neurofibrillary tangles in this line after the injection of Aβ42 and did not observe tangle formation either (unpublished data). In overall, studies in this line suggest that zebrafish might have a protective mechanism that prevents Tau toxicity even in the presence of acute Aβ42 or hyperphosphorylated Tau.

3.2. Single-cell sequencing, heterogeneity of NSPCs, and contextual response to amyloid toxicity model

Recently, we have generated an amyloid toxicity model in adult zebrafish brain by injecting Aβ42 peptide (Bhattarai, et al., 2016; Bhattarai, et al., 2017a; Bhattarai, et al., 2017b). I combined this model with my Tau line and did not observe exacerbation in Tau amyloid-toxicity. In the amyloid toxicity model, we have shown an increase in inflammation, synaptic degeneration and cell death, followed by cognitive decline, which are common symptoms in Alzheimer Disease (LaFerla and Oddo, 2005; Reitz and Mayeux, 2014). In contrast to humans (Moreno-Jimenez, et al., 2019; Tobin, et al., 2019), zebrafish NSPCs proliferate and generate new neurons (Bhattarai, et al., 2016). The proliferation of neural stem cells is induced by Interleukin-4 (IL4), which is secreted by dying neurons and activated microglia, through the activation of STAT6 signaling in cells that express the IL4-receptor. Interestingly, the NSPC activation in amyloid toxicity model (Bhattarai, et al., 2016; Bhattarai, et al., 2017b) is molecularly different than the NSPC activation injury zebrafish models (Kizil, et al., 2012a; Kizil, et al., 2012c; Kyritsis, et al., 2012; Rodriguez Viales, et al., 2015), as neurodegeneration-induced proliferation follows distinct molecular players when compared to other injury models (Bhattarai et al., 2016). This suggests that zebrafish brain has context-dependent mechanisms that regulate the activation of NSPC in the zebrafish brain. Moreover, IL4/STAT6 mechanism was further tested in human 3D-cell culture and IL4 was shown to counteract the AD pathology human 3D-gel culture by inducing NSC proliferation (Papadimitriou, et al., 2018). We also showed that Aβ42-induced cellular metabolites such as tryptophan metabolite KYNA, is upregulated during AD in human AD brains (post-mortem tissue analyses) as well as in mouse APP/PS1 model of

Alzheimer's but are suppressed after IL4 treatment (Papadimitriou, et al., 2018). Similarly, we recently showed that IL4 decreases tryptophan metabolism in zebrafish and allows neuroregeneration (Bhattarai, et al., 2020). These results indicate that the mechanisms identified in zebrafish have decent potential to be used for clinical directions in human brains.

Several studies in zebrafish considered zebrafish neural stem/progenitor cells as a uniform cell population (Kroehne, et al., 2011; Marz, et al., 2011; Than-Trong and Bally-Cuif, 2015; Yeo, et al., 2007). Recently, two studies focused on juvenile fish brain using single-cell sequencing (Pandey, et al., 2018; Raj, et al., 2018b). One study mainly focused on all cell types in juvenile brain, however did not further identified sub-clusters of these cells (Raj, et al., 2018b), in the second study the neuronal subtypes in juvenile habenula has been investigated. In order to check the heterogeneity of NSPCs in zebrafish telencephalon, I utilized single-cell sequencing (Cosacak, et al., 2019). I identified seven progenitor cell clusters (or neural stem/progenitor cells) and two pre-neuroblast and neuroblast clusters. Moreover, by using markers in each cell clusters and in-situ hybridization from an independent study (Diotel, et al., 2015), I identified the spatial localization of these cell populations in zebrafish telencephalon. Here, I propose that zebrafish brain has a heterogeneous set of neural progenitor cells. The neural stem cell heterogeneity has been postulated in mouse hippocampus (Artegiani, et al., 2017; Harris, et al., 2018; Hochgerner, et al., 2018; Shin, et al., 2015) and sub-ventricular zone (Dulken, et al., 2017; Llorens-Bobadilla, et al., 2015; Mizrak, et al., 2019). When combined with the studies in mouse brains, our results support the evolutionary functionalization of NSPCs in adult vertebrate brains and their potential differential response to various injury types.

I showed that different subtypes of NSPCs respond differently to Aβ42 and IL4 at the transcriptomics level. Dorsal and medial NSPC start proliferation and responds more to Aβ-42 and IL4 when compared to other subtypes. Overall, this shows the importance of studying AD-like conditions to understand the response of each cell type.

In my thesis, I developed new tools to analyze the interaction of cells through ligand-receptor pairs. I aimed to understand which kind of ligand-receptor interaction exist in normal brain environment, and which of those interactions are modified (e.g.: lost or emerged) after Aβ42 or IL4 treatment. Among these interactions, cytokine signaling emerged significantly after amyloid toxicity confirming our previous findings (Bhattarai,

et al., 2016). Additionally, we also confirmed previously published interactions in regenerating zebrafish brain using our in silico analyses tool (Cosacak et al., 2019). Thus, our single-cell data and whole transcriptome analyses correlated with high reliability. In order to further confirm the interaction maps identified by single-cell data, we injected FGF8 using CVMI and found that mainly dorsal progenitor cells increases their proliferation, as these cells expresses Fgf receptor, *fgfr3* (Cosacak et al., 2019; for analyses, gene markers, interaction maps and Gene Ontology analyses public database can be accessed under https://kizillab.org/singlecell/).

Our single-cell data also led to further studies, which benefited from our conclusions and repository (Bhattarai, et al., 2020; Diaz Verdugo, et al., 2019). In one of those studies, we found that IL4 decreased the production of the tryptophan metabolite serotonin, which decreased NSPC proliferation (Bhattarai et al., 2020). Excess of serotonin suppresses NSC proliferation by decreasing BDNF expression in periventricular neurons. In this work, instead of a direct ligand-receptor interaction, we identified a sequential neuron-glia communication to regulate neural stem cell plasticity in AD-like conditions in zebrafish brain. Our results would not be possible without our single cell sequencing approach and datasets (Cosacak et al., 2019).

Our results and studies in mice signify the importance of studying the contribution of different cell types to AD pathology (Bhattarai, et al., 2020; Cosacak, et al., 2019; Cox, et al., 2019; Keren-Shaul, et al., 2017; Mathys, et al., 2017). Recently, single nuclear RNA sequencing has been used to sequence nuclear RNA from postmortem human frontal cortex cells (Mathys, et al., 2019). By comparing AD patients to age and sex matched controls, the cell type-specific changes were observed at early stages of AD, the late stages of which displayed a common global stress response. This data corroborated the cellular phase of AD, and once again underscored the importance of studying individual cell types and their contribution to the disease progression. As hippocampal neurogenesis decreases in AD patients (Moreno-Jimenez, et al., 2019; Tobin, et al., 2019), a comprehensive single-cell sequencing analysis of hippocampal and SVZ cells will open new perspective and approaches to study AD in future.

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5 ACKNOWLEDGMENTS

Firstly, I would like to thank my supervisor Dr. Caghan Kizil, for accepting me as a doctoral researcher; giving freedom and independency in performing research, creating an international working environment, a collaborative group that give me the opportunity to contribute in different projects that resulted in several publications.

I would like express my gratitude to my Thesis Advisory Committee; Prof. Dr. Gerd Kempermann, Prof. Dr. Alexandre Storch and Prof. Dr. Andreas Hermann for their advices and comments during my thesis.

I would like to thank all Kizil Lab members that make it a friendly working environment; Prabesh Bhattarai as an amazing collaborative lab mate that we have several papers tigether, Kerstin Brand and Heike Hollak for their support/help, Dr. Christos Papadimitriou, Dr. Stanislava Popova, Hilal Celikkaya, Dr. Violeta Mashkaryan, my students Tim Dzewas and Ledio Bocova.

I would like to thank: Fish Facility members; Jitka, Markia and Dr. Judith Konantz, CRTD imaging facility members, FACS facility; Dr. Anne Gompf, RNA Seq facility members; Dr. Susanne Reinhardt, Dr. Andreas Petzhold, and Dr. Andreas Dahl.
I would like to friends; Nandini, Vania, Luis, Lisa, Jan.

I would like to thank PhD office members; Anja, Carolyn, Arantxa.

Son olarak da sevgili esim Seher Akbas Cosacak'a; cocuklarimizin büyütülmesinden ve fedekarligindan dolayi cok tesekkur ediyorum.

Tezim sirasinda az zaman gecirdigim cocuklarimiz Cinar Selahattin ve Pinar Havin'i de cok seviyorum.

6 APPENDIX

Supplementary Information

Human TAU^{P301L} overexpression results in TAU hyperphosphorylation without neurofibrillary tangles in adult zebrafish brain

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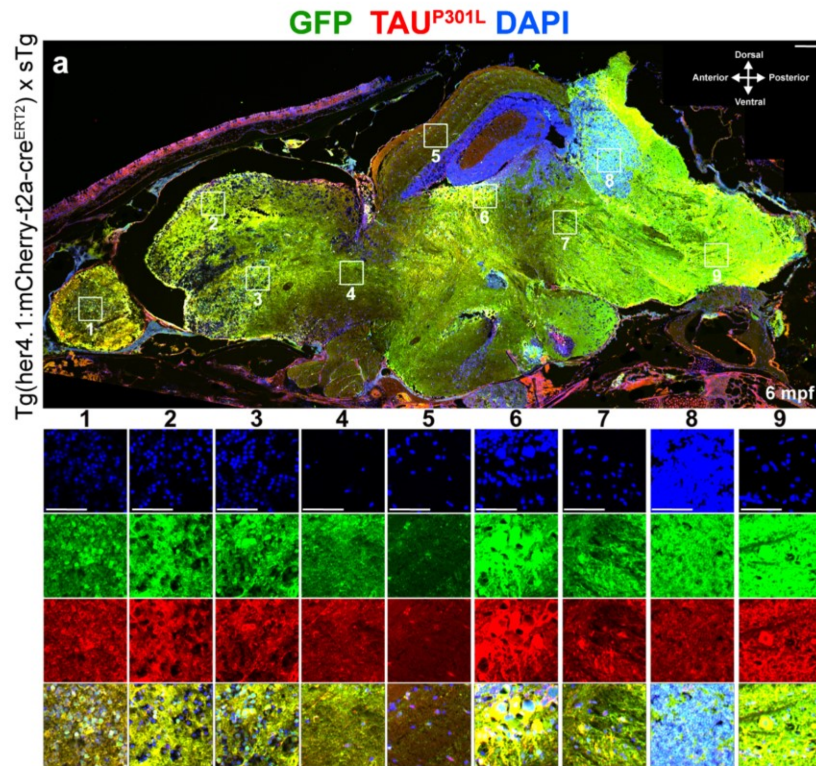
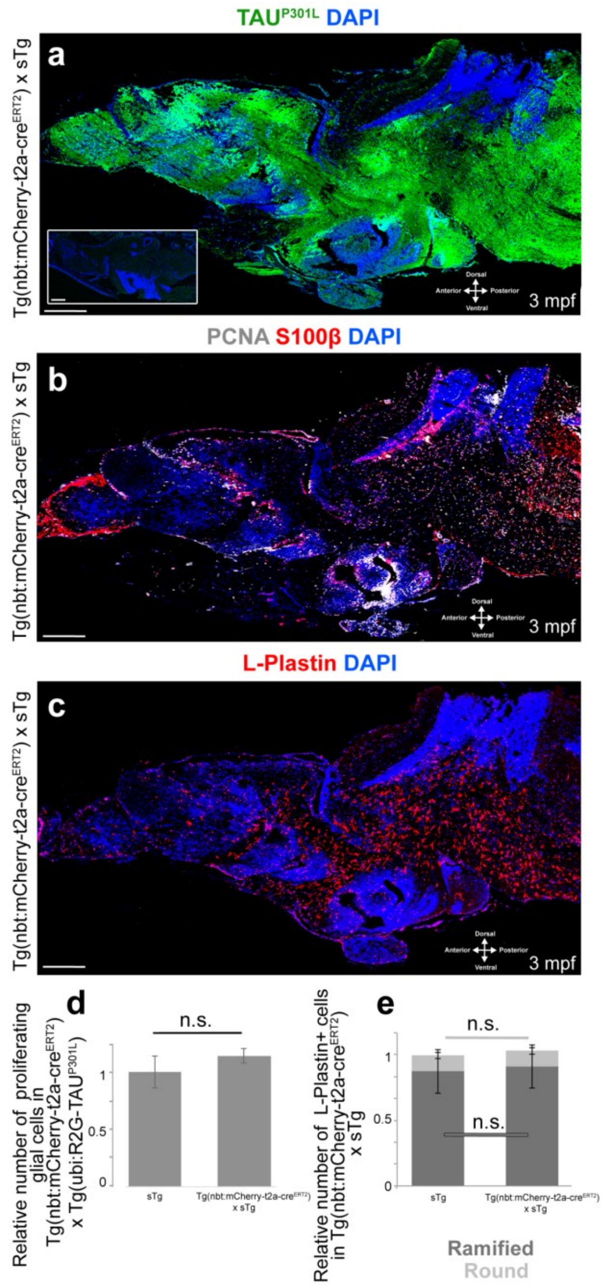
Supplementary Figures and legends

Supplementary Figure 1



Supplementary Fig. 1: (a) Immunohistochemical staining for GFP and TAU^{P301L} on a sagittal section of a 6 month-old dTg animal. Insets from 1 to 9 are DAPI (blue), GFP (green), TAU^{P301L} (red), and merged images from olfactory bulb (1), pallium (2), subpallium (3), midbrain (4), optic tectum (5), medial longitudinal fascicle (6), tegmentum (7), cerebellum (8) and medulla oblongata (9). Scale bars equal 100 µm. n = 8 fish.

Supplementary Figure 2



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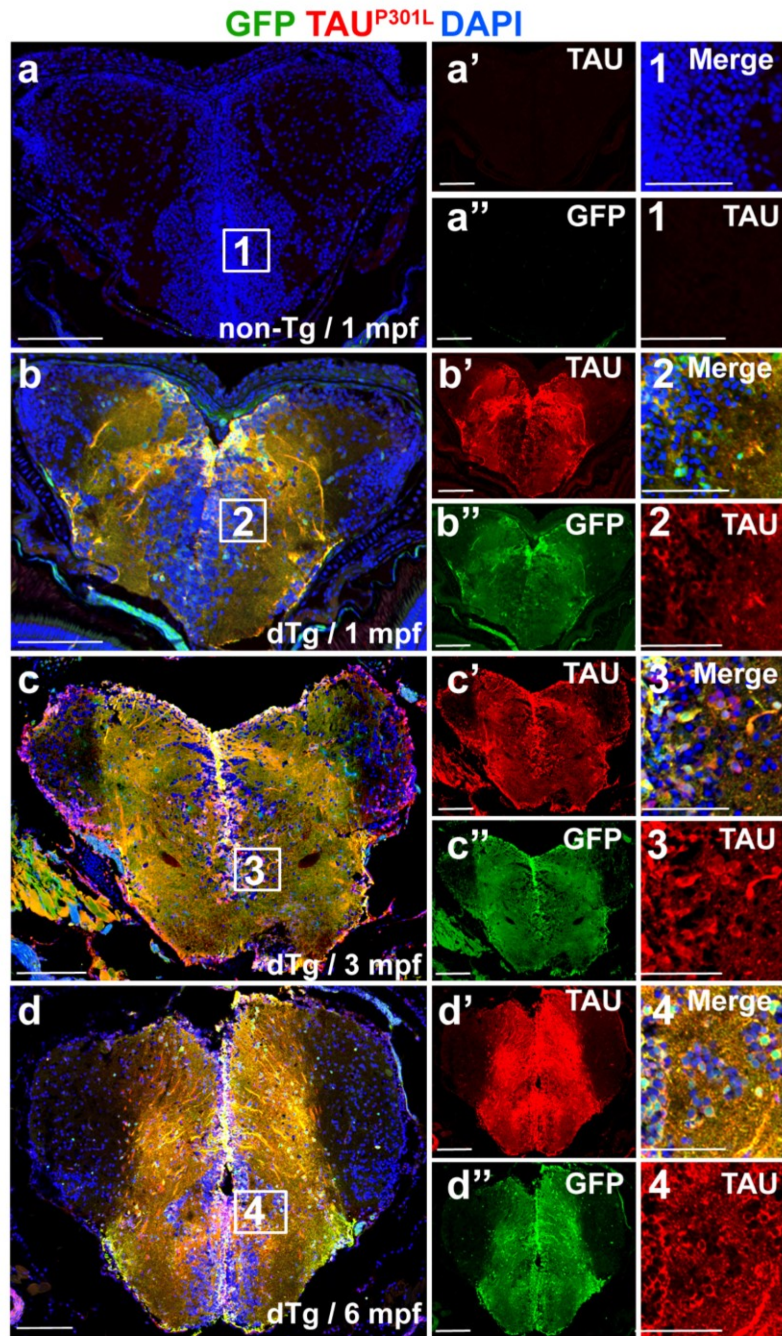
Supplementary Fig. 2. (a) Immunohistochemical staining for TAU^{P301L} on a sagittal section of a 3 month-old Tg(nbt:mCherry-t2a-creERT2) x Tg (ubi:loxP-DsRed-loxP-GFP-t2a-TAU^{P301L}) animal recombined as in Fig. 1. Inset shows the unrecombined animal. (b) Immunohistochemical staining for PCNA and S100β on a serial section of the same animal. (c) Immunohistochemical staining for L-Plastin on a serial section of the same animal. (d) Comparative quantification of the number of proliferating glial cells. (e) Comparative quantification of the relative numbers of L-Plastin-postive cells. Values represent mean ± s.e.m. n.s.: not significant. Scale bars equal 100 μm.

Supplementary Figure 3



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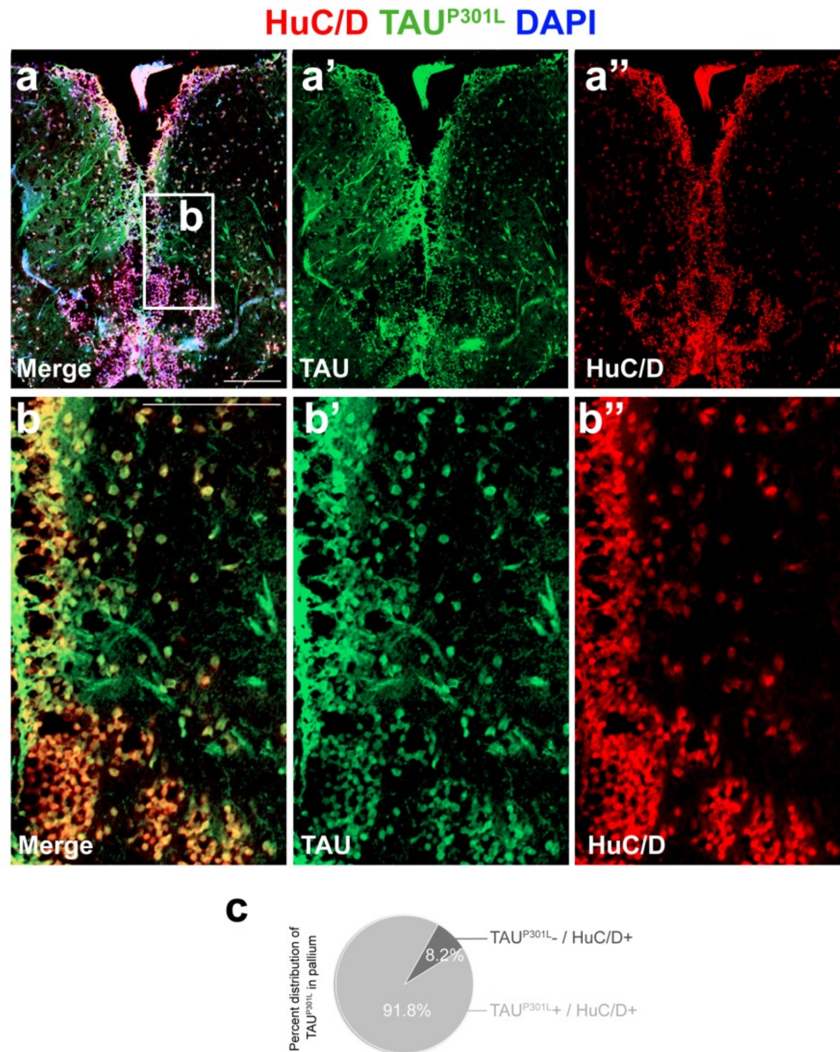
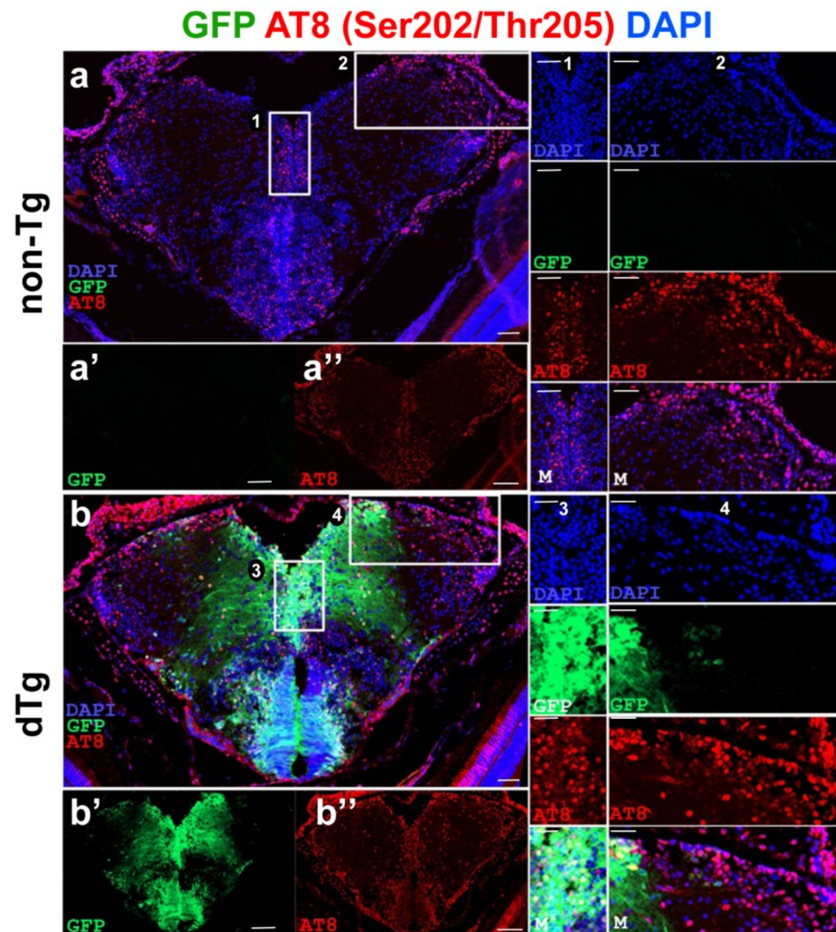
Supplementary Fig. 3: (a) Immunohistochemistry (IHC) for TAU^{P301L} (red) and GFP (green) on coronal sections of telencephalon of a 1 month-old non-transgenic animal. (a', a'') Individual fluorescent channels for TAU^{P301L} (a') and GFP (a''). (1) The enlarged view of the inset in a. (b) IHC for TAU^{P301L} and GFP on coronal sections of telencephalon of a 1-month old dTg animal. (b', b'') Individual fluorescent channels for TAU^{P301L} (b') and GFP (b''). (2) The enlarged view of the inset in b. (c) IHC for TAU^{P301L} and GFP on coronal sections of telencephalon of a 3-month old dTg animal. (c', c'') Individual fluorescent channels for TAU^{P301L} (c') and GFP (c''). (3) The enlarged view of the inset in c. (d) IHC for TAU^{P301L} and GFP on coronal sections of telencephalon of a 6-month old dTg animal. (d', d'') Individual fluorescent channels for TAU^{P301L} (d') and GFP (d''). (4) The enlarged view of the inset in d. Scale bars equal 50 µm. n = 4 fish and >25 histological sections for every staining.

Supplementary Figure 4



Supplementary Fig. 4: Immunohistochemistry for HuC/D (red) and TAU^{P301L} (green) on the telencephalon of a 6 month-old dTg animal. (a', a'') Individual channels for green and red. (b-b'') High-magnification image of the frame in a. (c) Quantification of the percentage of HuC/D-positive neurons expressing TAU in the pallium. Scale bars equal 100 µm. n = 4 fish and >20 histological sections for every staining and quantification.

Supplementary Figure 5

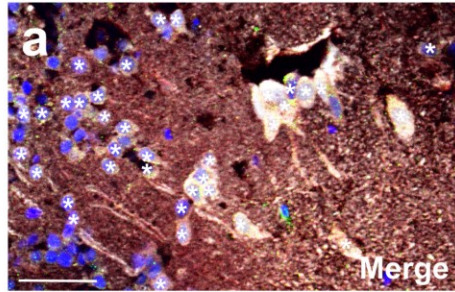


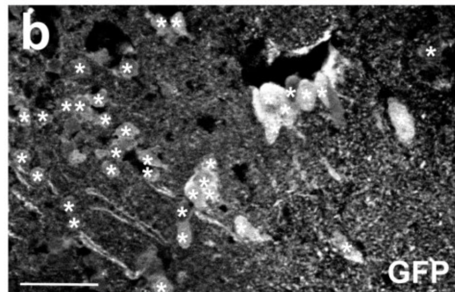
Supplementary Fig. 5: (a) Immunohistochemistry (IHC) for AT8 (red) and GFP (green) on coronal sections of telencephalon of a 6 month-old non-transgenic animal. (a', a'') Individual fluorescent channels for GFP (a') and AT8 (a''). (1 and 2) The enlarged view of the inset in A with individual channels for DAPI, GFP and AT8, and merged image. (b) IHC for AT8 and GFP on coronal sections of telencephalon of a 6-month old dTg animal. (b', b'') Individual fluorescent channels for GFP (b') and AT8 (b''). (3 and 4) The enlarged view of the inset in b with individual channels for DAPI, GFP and AT8, and merged image. Scale bars equal 25 μm. n = 5 fish for every staining.

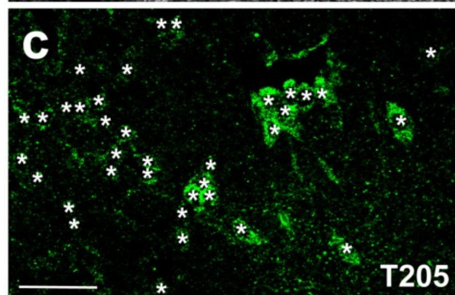
8

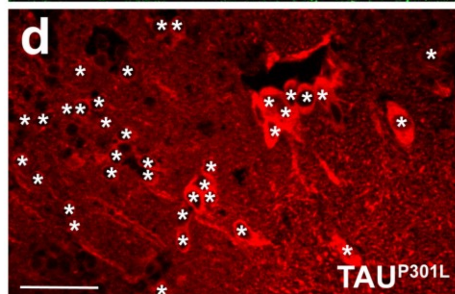
Supplementary Figure 6

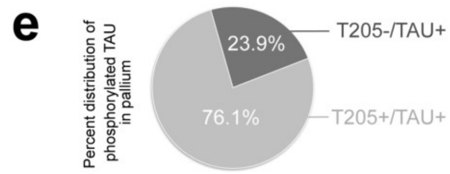
GFP T205 TAU^{P301L} DAPI











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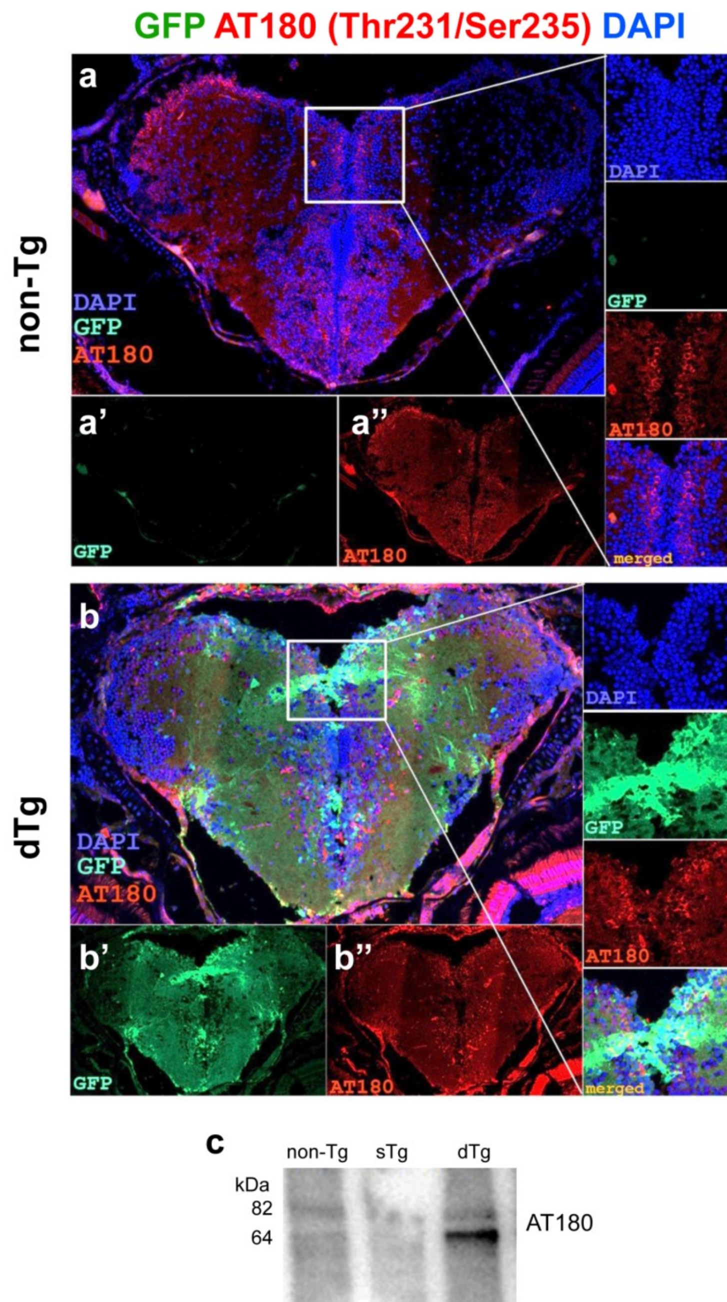
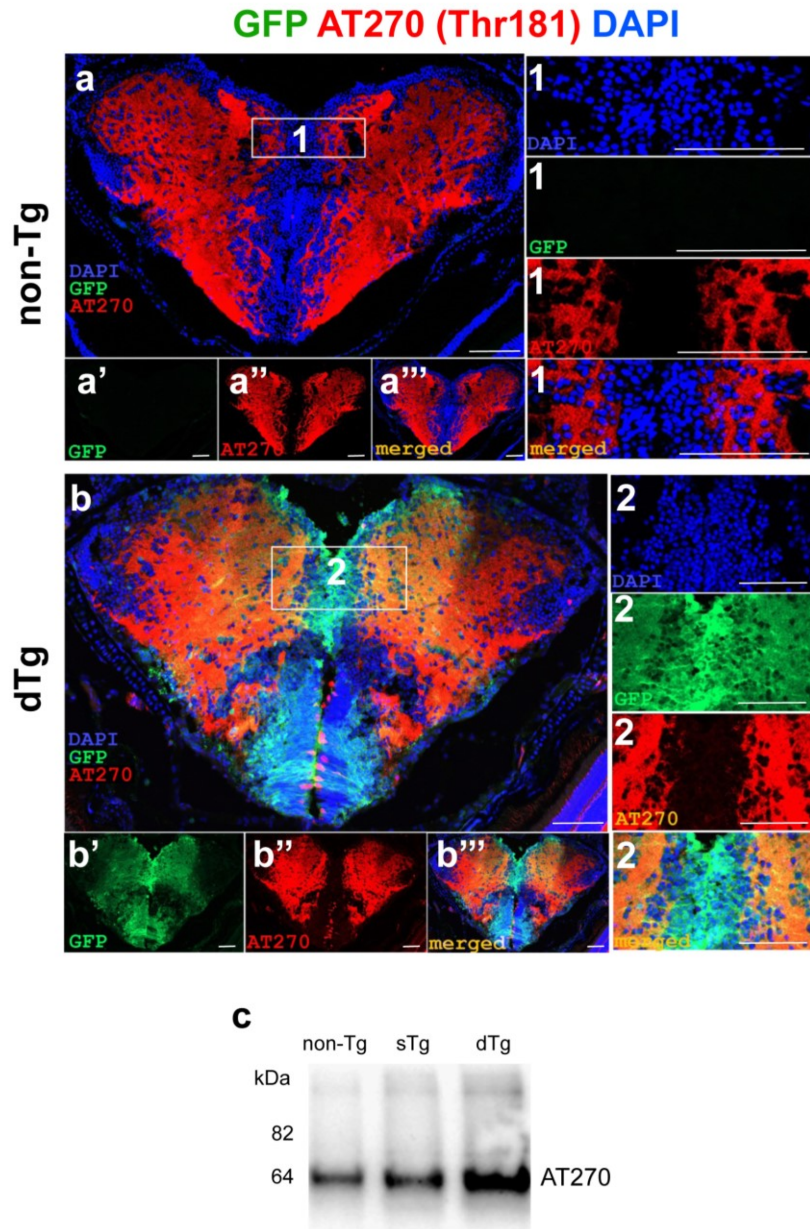
Supplementary Fig. 6: Immunohistochemical staining for GFP (recombined cells, white), T205 (hyperphosphorylated TAU, green), and TAU^{P301L} (red) in 6 month-old dTg animal. (a) merge image. Individual channels for (b) GFP, (c) T205, and (d) TAU^{P301L}. (e) Quantification of the percentage of TAU^{P301L}-positive cells that are T205 positive or negative. Scale bars equal 50 μm. n = 3 fish and >20 histological sections for every staining and quantification.

Supplementary Figure 7



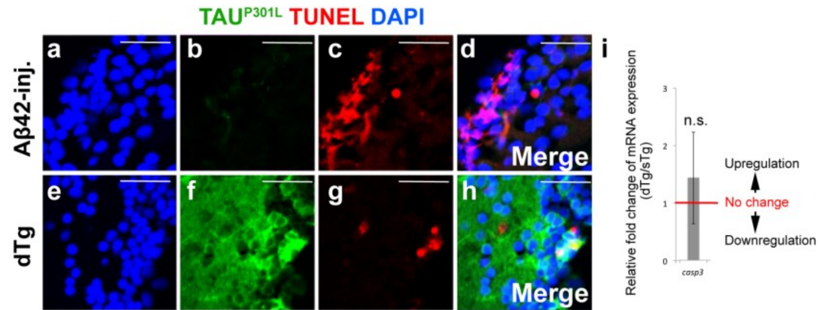
Supplementary Fig. 7: (a) Immunohistochemistry (IHC) for AT180 (red) and GFP (green) on coronal sections of telencephalon of a 6-month old non-transgenic animal. (a', a'') Individual fluorescent channels for GFP (a') and AT180 (a''). Insets show the enlarged view of the frame in A with individual channels for DAPI, GFP and AT180, and merged image. (b) IHC for AT180 and GFP on coronal sections of telencephalon of a 6-month old dTg animal. (b', b'') Individual fluorescent channels for GFP (b') and AT180 (b''). Insets show the enlarged view of the frame in b with individual channels for DAPI, GFP and AT180, and merged image. (c) Western blot for AT180 from brains of non-Tg, sTg and dTg animals. Scale bars equal 25 µm. n = 5 fish for every staining.

Supplementary Figure 8



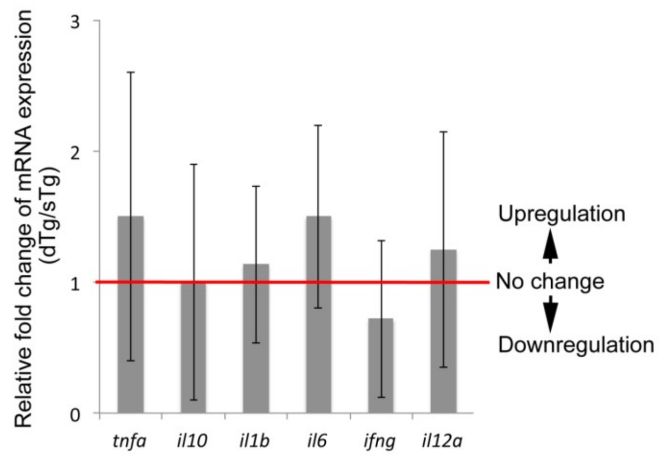
Supplementary Fig. 8: (a) Immunohistochemistry (IHC) for AT270 (red) and GFP (green) on coronal sections of telencephalon of a 6-month old non-transgenic animal. (a’, a’’) Individual fluorescent channels for GFP (a’) and AT270 (a’’). Insets show the enlarged view of the frame in a with individual channels for DAPI, GFP and AT270, and merged image. (b) IHC for AT270 and GFP on coronal sections of telencephalon of a 6-month old dTg animal. (b’, b’’) Individual fluorescent channels for GFP (b’) and AT270 (b’’). Insets show the enlarged view of the frame in b with individual channels for DAPI, GFP and AT270, and merged image. (c) Western blot for AT270 from brains of non-Tg, sTg and dTg animals. Scale bars equal 50 µm. n = 6 fish for every staining.

Supplementary Figure 9



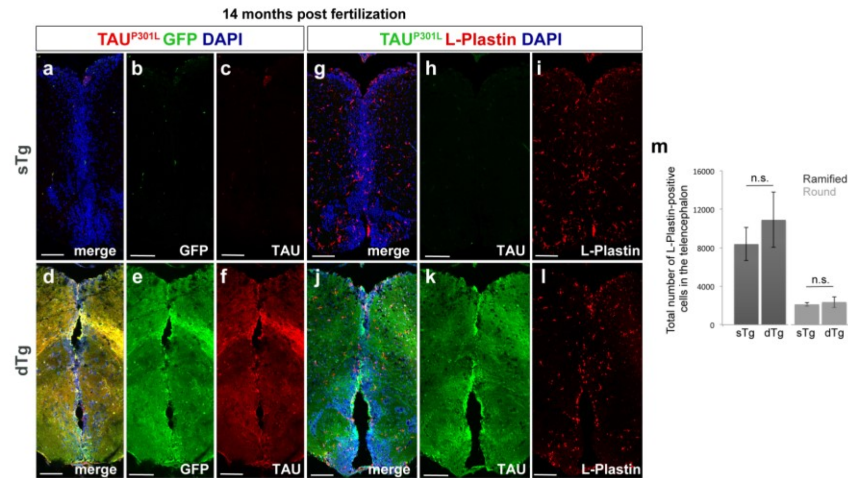
Supplementary Fig. 9: (a-d) Immunohistochemistry (IHC) for TAU^{P301L} (green) and staining for apoptotic cells with TUNEL (green) telencephalon of a 6-month old non-transgenic animal injected with Amyloid-beta42. (a-c) Individual fluorescent channels for DAPI (a), TAU^{P301L} (b), and TUNEL (c). (d) Merged image. (e-h) IHC for TAU^{P301L} (green) and staining for apoptotic cells with TUNEL (green) telencephalon of a 6-month old dTg animal. (a-c) Individual fluorescent channels for DAPI (e), TAU^{P301L} (f), and TUNEL (g). (h) Merged image. (i) Quantification of relative change in the expression levels of *casp3* mRNA in dTg compared to sTg adult zebrafish brains. Scale bars equal 25 µm. n = 5 fish for every staining.

Supplementary Figure 10



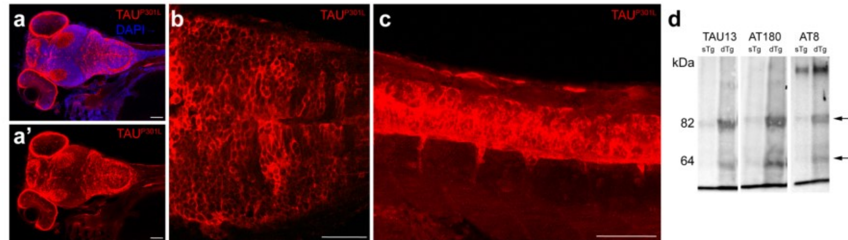
Supplementary Fig. 10: Quantification of the relative change in the expression levels of proinflammatory in dTg compared to sTg adult zebrafish brains.

Supplementary Figure 11



Supplementary Fig. 11: (a) Immunohistochemistry (IHC) for TAU^{P301L} (red) and GFP (green) on coronal sections of telencephalon of a 14-month old sTg animal. (b,c) Individual fluorescent channels for TAU^{P301L} (b) and GFP (c). (d) IHC for TAU^{P301L} and GFP on coronal sections of telencephalon of a 14 month-old dTg animal. (e,f) Individual fluorescent channels for TAU^{P301L} (e) and GFP (f). (g) IHC for L-Plastin and TAU^{P301L} on coronal sections of telencephalon of a 14 month-old sTg animal. (h,i) Individual fluorescent channels for TAU^{P301L} (h) and L-Plastin (i). (j) IHC for L-Plastin and TAU^{P301L} on coronal sections of telencephalon of a 14-month old dTg animal. (k,l) Individual fluorescent channels for TAU^{P301L} (k) and L-Plastin (l). (m) Quantification of round and ramified L-Plastin-positive cells in the telencephalon of 14-month-old sTg and dTg animals. Values represent mean ± s.e.m. *: $p<0.05$, **: $p<0.01$, ***: $p<0.005$. Scale bars equal 50 µm. n = 5 fish and >25 histological sections for every staining.

Supplementary Figure 12



Supplementary Fig. 12: (a) Immunohistochemical staining for TAU^{P301L} in 9 day-old dTg larvae. Anterior view of the brain is shown. (b) Single fluorescence channel for TAU^{P301L}. (c) High-magnification image of the frame in b. (d) TAU^{P301L} expression in the spinal cord. (e) Western blot analyses of TAU^{P301L} (TAU13), and hyperphosphorylated form of TAU with AT180 and AT8. Scale bars equal 50 µm.

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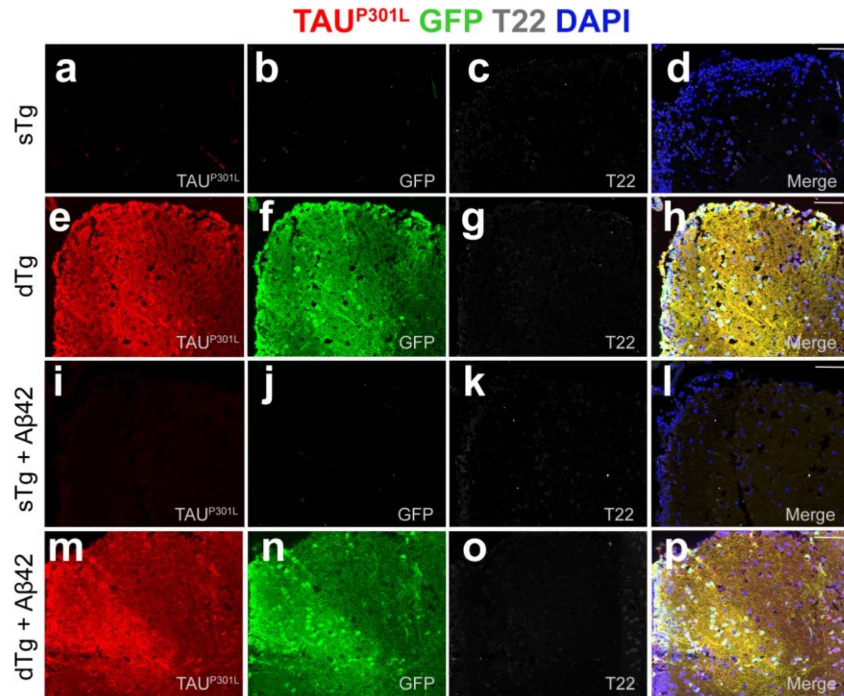
Supplementary Figure 13



Supplementary Fig. 13: Immunohistochemical staining for TAU^{P301L} (red), GFP (green), and T22 (TAU oligomers, gray) in sTg (a-d), dTg (e-h), sTg injected with Aβ42 (i-l), and dTg injected with Aβ42 (m-p). Scale bars equal 50 μm. All animals are 6 month-old.

Supplementary Video Captions

Supplementary Video 1: Escape response assay for 2 day-old dTg animals.

Supplementary Video 2: Escape response assay for 4 day-old non-transgenic, sTg and dTg animals for *her4.1* or *nbt* promoter-driven conditional TAU transgenic lines of zebrafish.

The supplementary video files for Cosacak et al. 2017 can be found in the following links;

https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-017-13311-5/MediaObjects/41598_2017_13311_MOESM2_ESM.mov

https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-017-13311-5/MediaObjects/41598_2017_13311_MOESM3_ESM.mov

Cell Reports, Volume *27*

Supplemental Information

Single-Cell Transcriptomics Analyses of Neural Stem Cell Heterogeneity and Contextual Plasticity in a Zebrafish Brain Model of Amyloid Toxicity

Mehmet Ilyas Cosacak, Prabesh Bhattarai, Susanne Reinhardt, Andreas Petzold, Andreas Dahl, Yixin Zhang, and Caghan Kizil

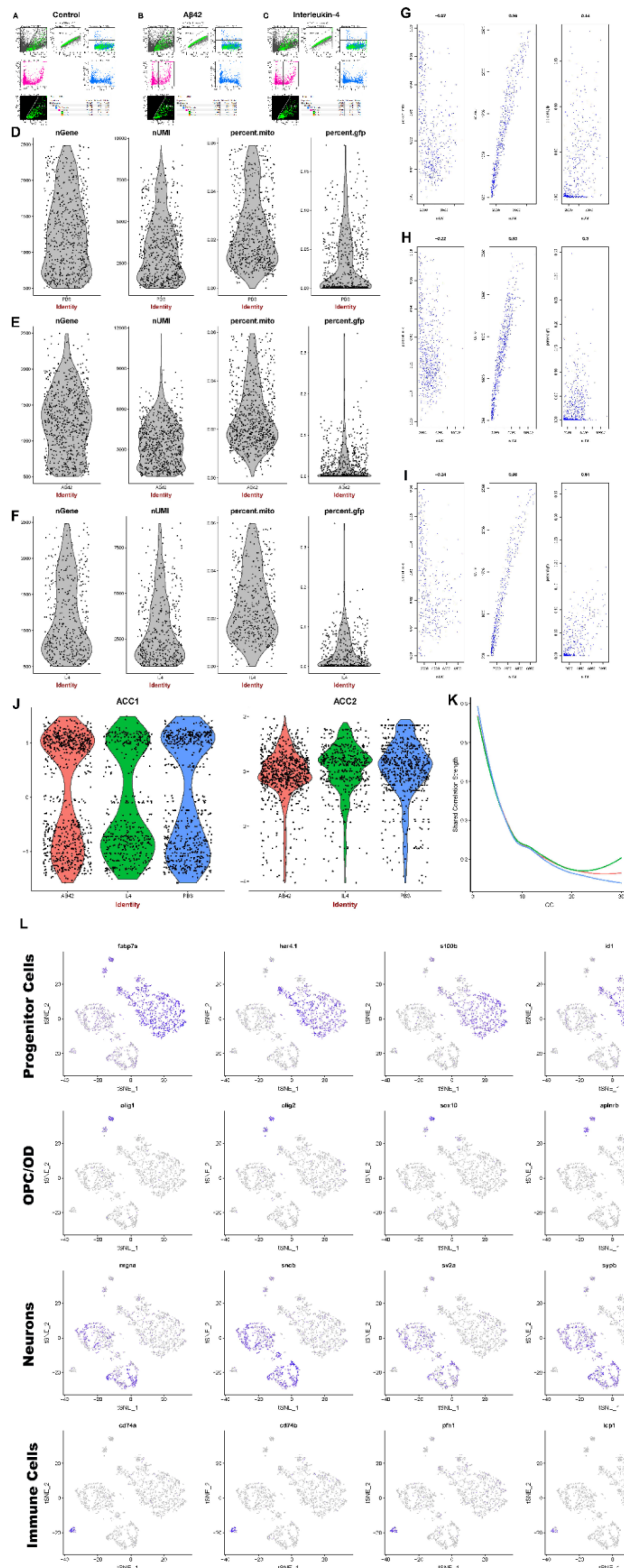

A
Control
B
Aβ42
C
Interleukin-4
D
nGene
nUMI
percent.mito
percent.gfp
PBS
Identity
E
Aβ42
F
IL4
G
H
I
J
ACC1
ACC2
Aβ42
IL4
PBS
K
Shared Correlation Strength
CC
L
Progenitor Cells
OPC/OD
Neurons
Immune Cells
fabp7a
her4.1
s100b
id1
olig1
olig2
sox10
mgna
sncb
sv2a
sypb
cd74a
cd74b
pfn1
tSNE_1
tSNE_2

Figure S1: Cell sorting and categorization of cell types, Related to Figure 1. (A-C) FACS results of PBS, Aβ42 and IL4 fish brain cells. (D-F) VLN plots for the number of gene per cells (nGene), number of reads per cells (nUMI), mitochondrial genes percentages (percent.mito) in nUMI per cell and GFP read percentages in nUMI per cells (percent.gfp) in PBS (D), Aβ42 (E) and IL4 (F). (G-I) scatter plot and correlation between nUMI and nGene, percent.gfp and percent.mito in PBS (G), Aβ42 (H) and IL4 (I). (J) Canonical correlation between unaligned (ACC1) and aligned (ACC2) cells, (K) metagene plot for num.ccs = 30. (L) Feature plots of marker genes that identified four main cell types as progenitor cells (*fabp7a, her4.1, s100b* and *id1*), oligodendrocyte progenitors/oligodendrocytes (OPC/OD) (*olig1, olig2, sox10* and *aplnra*), neurons (*nrgna, scnb, sybp* and *sv2a*), and immune cells (*cd74a/b, pfn1, lcp1*).

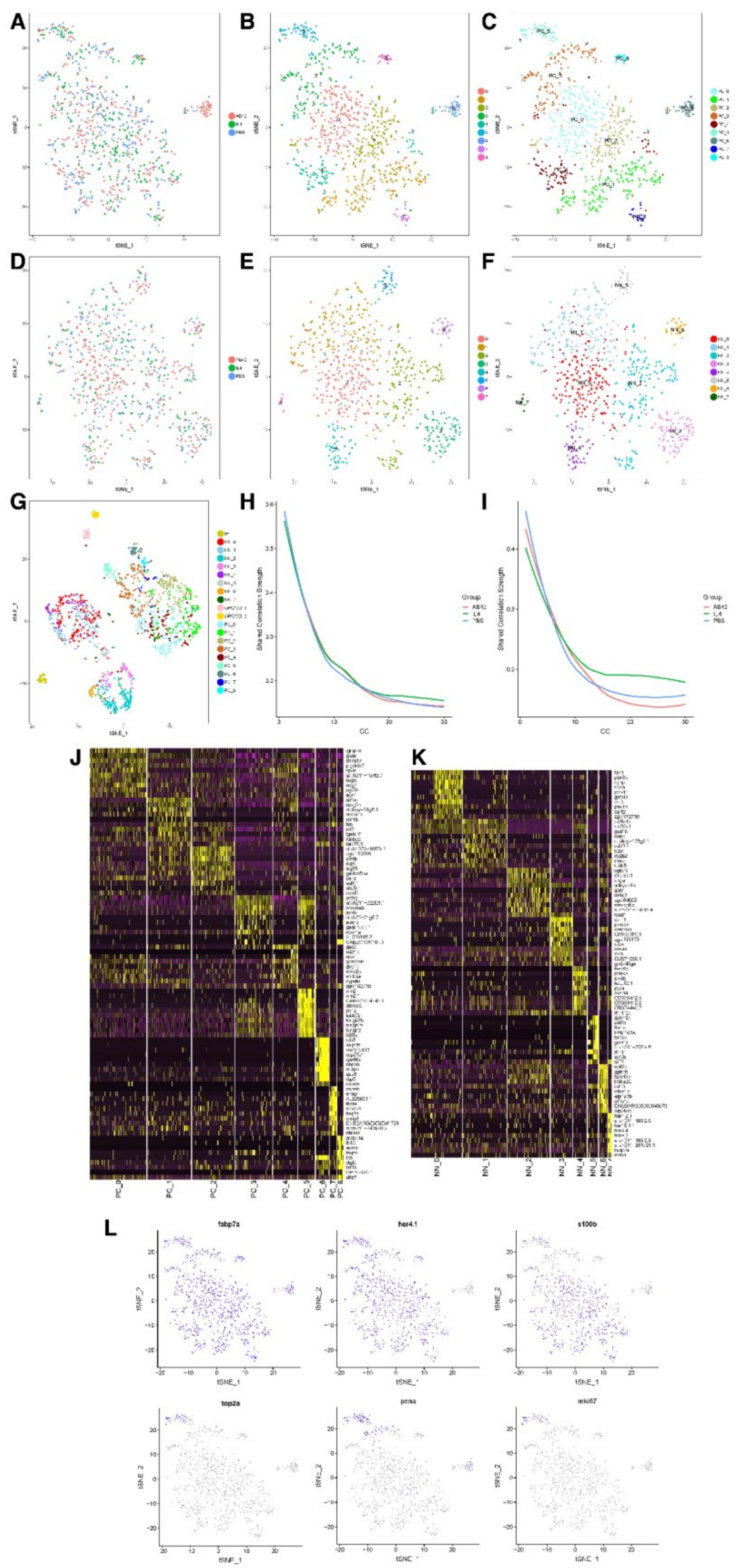
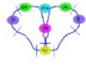
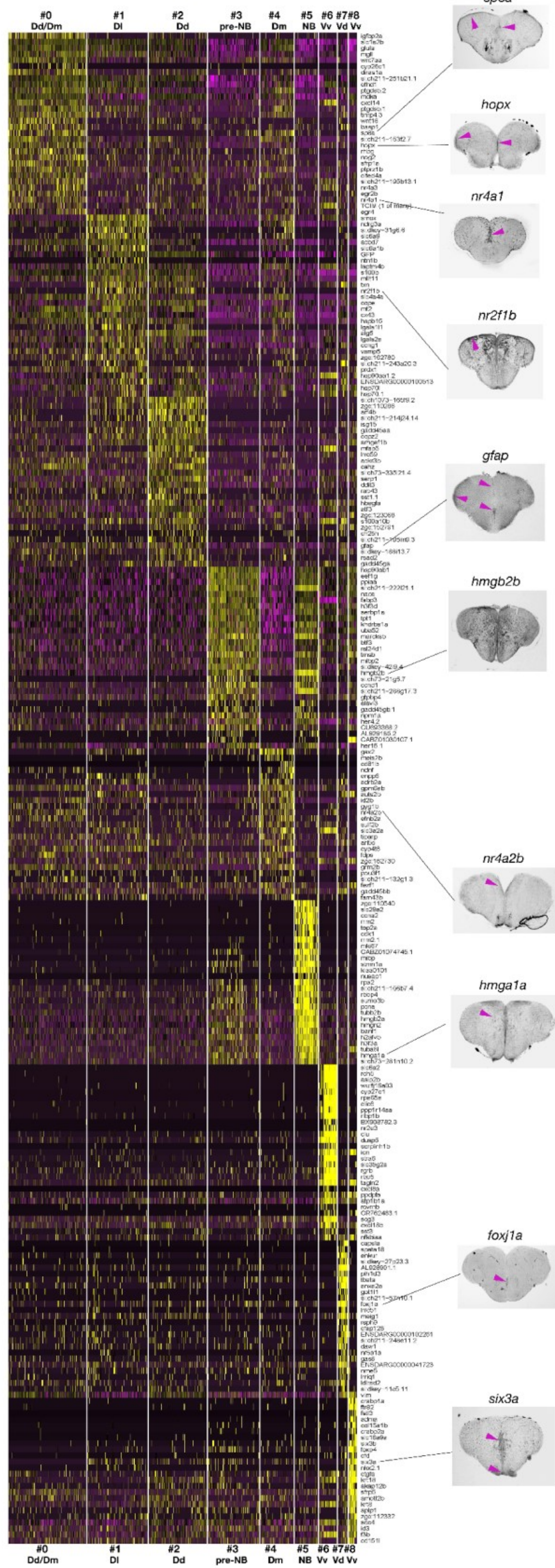


Figure S2: Iterative sub-clustering of progenitor cells and neurons separately, Related to Figure 2. The progenitor cells (PC) and neurons (NN) were re-clustered separately. (A-F) tSNE plots of PC and NN clusters. (A, D) samples are color coded, (B, E) default colors by Seurat, and (C, F) new color codes for cells. (G) All cell clusters are color coded on the tSNE plots in Figure 1. The color codes for PC and NN are from C and F, Im, OPC/OD cells are given new colors. These color codes are used in the remaining figures. (H-I) Metagene plots for PC (I) and neurons (I). (J, K) top 10 marker genes for PC (J) and NN (K). (L) Feature plots of progenitor cell markers (*fabp7a, her4.1, s100b*) and proliferation markers (*top2a, pcna, mki67*).




#0
Dd/Dm
#1
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Dd
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pre-NB
#4
Dm
#5
NB
#6
Vv
#7
Vd
#8
Vv
sp8a
hopx
nr4a1
nr2f1b
gfap
hmgb2b
nr4a2b
hmga1a
foxj1a
six3a

Figure S3: Heat-map of top 30 marker genes for progenitor cell clusters, Related to Figure 2. Purple the lowest, yellow the highest expression level. In situ hybridization for some genes from every cluster are shown on the right. Pink arrowheads denote expression domains.

Anlage 1

Erklärungen zur Eröffnung des Promotionsverfahrens

1. Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

2. Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts habe ich ausschließlich die im Manuskript angegebenen Unterstützungsleistungen erhalten.

3. Weitere Personen waren an der geistigen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die Hilfe eines kommerziellen Promotionsberaters in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

4. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

5. Die Inhalte dieser Dissertation wurden in folgender Form veröffentlicht:

…………………………………………………………………………………………
…………………………………………………………………………………………
…………………………………………………………………………………………

6. Ich bestätige, dass ich die Promotionsordnung der Medizinischen Fakultät der Technischen Universität Dresden anerkenne.

Ort, Datum

Unterschrift des Doktoranden

Anlage 2

Hiermit bestätige ich die Einhaltung der folgenden aktuellen gesetzlichen Vorgaben im Rahmen meiner Dissertation

◊ das zustimmende Votum der Ethikkommission bei Klinischen Studien, epidemiologischen Untersuchungen mit Personenbezug oder Sachverhalten, die das Medizinproduktegesetz betreffen Aktenzeichen der zuständigen Ethikkommission

◊ die Einhaltung der Bestimmungen des Tierschutzgesetzes Aktenzeichen der Genehmigungsbehörde zum Vorhaben/zur Mitwirkung:

Tierversuchsantrag: TVV-52/2015, TVV-35/2016

◊ die Einhaltung des Gentechnikgesetzes Projektnummer

Az.:

◊ die Einhaltung von Datenschutzbestimmungen der Medizinischen Fakultät und des Universitätsklinikums Carl Gustav Carus.

Ort, Datum

Unterschrift des Doktoranden