

Localisation of neural progenitor cells and the influence of age in the African turquoise killifish

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Table of Contents

Acknowledgments	i
Table of Contents	ii
List of abbreviations	iv
Summary	v
1. Introduction.....	1
1.1 AGE-RELATED DISEASES	1
1.1.1 Ageing society	1
1.1.2 Neurodegenerative diseases.....	1
1.1.3 The mammalian hallmarks of ageing.....	2
1.1.4 Animal models for ageing research	3
1.2 THE AFRICAN TURQUOISE KILLIFISH: A MODEL FOR AGEING RESEARCH.....	4
1.2.1 The use of a teleost model	4
1.2.2 General characteristics of the African turquoise killifish	5
1.2.3 Life cycle and embryonic development	7
1.2.4 Ageing phenotype.....	8
1.3 ADULT NEUROGENESIS.....	9
1.3.1 Adult neurogenesis in mammals	9
1.3.2 Adult neurogenesis in teleost fish.....	10
1.3.3 Overview of the teleost brain and its neurogenic niches	12
1.3.4 Neural progenitor cells in the killifish telencephalon.....	13
1.4 TRANSGENESIS.....	16
1.4.1 The limited availability of transgenic killifish lines.....	16
1.4.2 Tol2 transposase system.....	16
1.4.3 Generation of transgenic killifish.....	17
2. Objectives.....	19
3. Materials and methods.....	20
3.1 FISH HUSBANDRY	20
3.2 CHARACTERISATION OF NEUROGENIC NICHES.....	20
3.2.1 Tissue preparation	20
3.2.2 Third generation hybridisation chain reaction (HCR v3.0)	20
3.2.3 Immunohistochemistry after HCR.....	21
3.2.4 Whole mount HCR.....	21
3.2.5 Whole-mount immunohistochemistry after HCR v3.0	22
3.2.6 Imaging, quantification and statistical analysis.....	22

3.3	TRANSGENIC LINES	23
3.3.1	<i>Generation of transgenic killifish lines</i>	23
3.3.2	<i>Genotyping ubb:CreERT2 line.....</i>	23
3.3.3	<i>Inbreeding of the Actb2:GTR line</i>	24
3.3.4	<i>Validation of the actb2:GTR transgenic reporter line</i>	24
3.3.5	<i>Tamoxifen experiment</i>	25
4.	Results.....	26
4.1	CHARACTERISATION OF NEUROGENIC NICHES.....	26
4.1.1	<i>Localisation of neural progenitor cells within the killifish telencephalon</i>	26
4.1.2	<i>The proliferation of neural progenitor cells declines with age</i>	31
4.1.3	<i>Effect of ageing on the spatial distribution of neural progenitor cells</i>	31
4.1.4	<i>Cell types identified in telencephalon are present in distinct niches in other brain regions</i> <i>32</i>	
4.2	TRANSGENIC REPORTER LINES	35
4.2.1	<i>Ubb:CreERT² can be integrated into the killifish genome</i>	35
4.2.2	<i>Generation of a homozygote actb2:GTR killifish reporter line.....</i>	35
4.2.3	<i>Transgenic Actb2:GTR fish mainly express GFP in neurons of the telencephalon</i>	36
4.2.4	<i>The GTR switch could not be established by tamoxifen treatment</i>	37
5.	Discussion.....	38
5.1	CHARACTERISATION OF NEUROGENIC NICHES.....	38
5.1.1	<i>Three neurogenic niches can be found in the adult killifish telencephalon</i>	38
5.1.2	<i>Neural progenitor subtypes are located in distinct niches</i>	39
5.1.3	<i>The effect of age on the proliferation of neural progenitor cells</i>	41
5.1.4	<i>Neural progenitor subtypes are also present in other regions of the killifish brain.....</i>	43
5.2	TRANSGENIC KILLIFISH REPORTER LINES	44
6.	Conclusion and future prospects	46
7.	References	47
8.	Addendum.....	1
8.1	RISK ASSESSMENT	1
8.2	SUPPLEMENTARY MATERIAL	3
8.2.1	<i>Buffer recipes for HCR v3.0.....</i>	12

List of abbreviations

BLBP	Brain lipid-binding protein
Ce	Cerebellum
CNS	Central nervous system
CSF	Cerebrospinal fluid
Dc	Central nucleus pallium
DG	Dentate gyrus
Di	Diencephalon
DI	Lateral nucleus pallium
Dm	Dorsomedial nucleus pallium
Dp	Posterior nucleus pallium
GFAP	Glial fibrillary acidic protein
GS	Glutamine synthetase
GTR	Green-to-red
H	Hypothalamus
IP	Intermediate progenitor
LV	Lateral ventricle
ML	Molecular layer
MO	Medulla oblongata
NGP	Non-glial progenitors
NSC	Neural stem cells
OB	Olfactory bulb
PCNA	Proliferating cell nuclear antigen
pHH3	Phospho-histone H3
PNS	Peripheral nervous system
RG	Radial glia
SGZ	Subgranular zone
SVZ	Subventricular zone
Tel	Telencephalon
TeO	Optic tectum
Vd	Dorsal nucleus subpallium
Vv	Ventral nucleus subpallium
WT	Wild type

Summary

All over the world, human populations are characterized by an increase in life expectancy. This leads to more people having to deal with age-related neurodegenerative diseases, such as Alzheimer's disease or Parkinson's disease. In the past decade, the African turquoise killifish has become an attractive model system to study the molecular aspects of ageing, due to its naturally compressed lifespan. In contrast to mammals, teleost fish are able to produce new neurons throughout their adult lives, a process known as adult neurogenesis. This results in an extremely high capacity for regeneration in the central nervous system after injury or neurodegeneration. Studying adult neurogenesis in a regeneration-competent vertebrate might unravel why adult neurogenesis in mammals, including humans, is so limited. Therefore, studies like this could bring us one step closer to finding potential treatments for age-related neurodegenerative diseases in the future.

In this project, we were interested in further characterizing the neurogenic niches within the killifish telencephalon. Single-cell sequencing data revealed that within the killifish telencephalon, four radial glia subtypes (RG1-RG4) and one type of non-glial progenitor (NGP) were present. Our aim was to localize these four RG subtypes and the NGPs within the killifish telencephalon. We were able to optimize specific HCR v3.0 markers to determine the location of each neural progenitor cell type. We found that RG1 was located on the pallial ventricular surface, while RG2 was located on the caudal side of the conjunction between the two hemispheres. RG4s were found to be present in the tela choroidea and were therefore suggested to be ependymal cells. We found that the NGPs were also present on the pallial ventricular surface, and that they were the most proliferating cell type within the entire killifish brain. Because we were not able to optimize HCR v3.0 to label RG3 specifically, we located this RG subtype via exclusion with other RG subtypes and NGPs. In addition, by optimizing whole mount analysis on killifish brains, we found that the number of proliferating cells within the entire telencephalon decreased with age.

To allow for future lineage tracing experiments, we tried to introduce the inducible Cre/Lox system in killifish. We were able to generate heterozygous *ubb:CreER^{T2}* driver killifish, together with homozygous *actb2:GTR* reporter fish. We then generated double mutant larvae (*ubb:CreER^{T2} actb2:GTR*) to validate the inducible Cre/Lox system via administration of the drug tamoxifen. This drug would then activate the CreER^{T2} recombinase that was present in the cytoplasm and would enable it to enter the nucleus in order to cause an inversion of the GTR switch gene. However, none of the double mutant larvae underwent a switch from green to red fluorescence upon addition of tamoxifen.

1. Introduction

1.1 Age-related diseases

1.1.1 Ageing society

Human populations across the world experience a remarkable increase in life expectancy (Division, 2019). This prolonged lifespan is mainly the result of improved living conditions combined with more efficient medical care (Division, 2019). As a consequence, the proportion of aged people within the world population rises drastically. According to the United Nations World Population Prospects (2019), the group of people above the age of 65 is growing the fastest. Projections show that the number of people within this group will double by 2050, while the group of people over 80 will even triple (Division, 2019). This means that by 2050, the number of aged people will exceed the number of young people for the first time in human history (Division, 2019).

However, an increase in lifespan certainly does not correlate with increased human health. Most living organisms, including humans, are affected by the process of ageing. Ageing is often defined as the time-dependent functional decline of an organism which ultimately leads to death (López-Otín et al., 2013; World Health Organization, 2015). This functional decline involves impaired vision, hearing, movement, and also an increased risk for chronic diseases (World Health Organization, 2015). Some relevant age-related diseases are cancer, stroke, type 2 diabetes, chronic lower respiratory disease, and neurodegenerative diseases (World Health Organization, 2015). Aged people also undergo psychological and social adaptations, as well as physical changes such as the loss of pigment in hair, wrinkling of the skin, and loss of muscles (World Health Organization, 2015; Kim et al., 2016). An interesting aspect of ageing is the variation between people. While some people can reach 70 years without severe health complications, others get age-related diseases earlier in life (World Health Organization, 2015).

1.1.2 Neurodegenerative diseases

One of the biggest concerns with an ageing society is the occurrence of age-related neurodegenerative diseases, the most common ones being Parkinson's disease and Alzheimer's disease. In general, neurodegenerative diseases are characterized by a progressive loss of neurons in the central nervous system (CNS) or peripheral nervous system (PNS). Parkinson's disease is caused by the progressive loss of dopaminergic neurons in the substantia nigra (National Health Service, 2019). As a result, less dopamine will be produced by the substantia nigra and signals towards the motor system will be slowed down or disturbed (National Health Service, 2019). Symptoms of Parkinson's disease are therefore often movement-related and patients are typically characterized by rigidity, tremor and slower

muscle movements (Armstrong & Okun, 2020). More than 6 million people were suffering from Parkinson's disease in 2020, and the available therapies mostly focus on reducing the symptoms, but cannot cure the disease itself (Armstrong & Okun, 2020). Treatments with dopamine are often used to improve motor movements, but are only effective in the early stages of Parkinson (Armstrong & Okun, 2020).

On the other hand, Alzheimer's disease (the most common cause of dementia) is characterized by the deposition of β -amyloid plaques and neurofibrillary tau proteins in and around neurons of the brain (Jack et al., 2018). Similar to other types of dementia, Alzheimer's disease shows a progressive course, affects cognitive abilities (e.g. memory and learning) and therefore seriously undermines a person's daily life (World Health Organization, 2017). It is estimated that 132 million people will suffer from dementia by 2050, of which 60% to 70% can be a result of Alzheimer's disease (World Health Organization, 2017). Until now, no treatment can reverse or stop the progression of this disease in a patient, although acetyl cholinesterase inhibitors are shown to slightly improve cognition initially (Johnson, 2015; Duggan et al., 2020). Once neurons of the mammalian CNS are damaged or lost, they can hardly be repaired or replaced, which makes these neurodegenerative diseases permanent. In adult humans, it remains unclear whether new neurons can be produced and incorporated in the brain at all (Duque et al., 2021). In human patients that suffer from neurodegenerative diseases, the amount of lost or non-functioning neurons will increase over time. This results in the disease getting worse and will ultimately lead to death of the patient.

1.1.3 The mammalian hallmarks of ageing

At the cellular level, nine hallmarks of ageing have been identified in mammals (Fig. 1) (reviewed in López-Otín et al., 2013). The primary hallmarks, which cause direct damage, include telomere shortening, DNA damage, epigenetic alterations and loss of proteostasis. When proteostasis is lost due to ageing, it may result in misfolded proteins (e.g. α -synuclein in Parkinson's disease), or the production of protein aggregates such as aggregates of hyperphosphorylated tau and amyloid- β in Alzheimer's disease (Hou, Yujun., 2019). The antagonistic hallmarks include mitochondrial dysfunction, deregulated nutrient sensing and cellular senescence. Integration of these primary and antagonistic hallmarks eventually leads to altered intercellular communication and stem cell exhaustion. Stem cell exhaustion is an important hallmark in relation to age-related neurodegenerative diseases. It is characterized by a reduction in the potential of NSCs to proliferate and differentiate, and contributes in several vertebrate species to impaired neurogenesis with ageing (reviewed in Nicaise et al., 2020; Tozzini et al., 2012). The potential to regenerate neurons can decrease with age due to deficient NSC proliferation, or as a result of depleted stem cell niches, caused by excessive proliferation (López-Otín et al., 2013).



Figure 1. Visualization of the nine mammalian hallmarks of ageing as described by López-Otín et al. (2013).

1.1.4 Animal models for ageing research

An ageing society thus comes with many challenges, and scientists are now attempting to expand the healthy years of life, instead of prolonging the unhealthy years even further. Effective treatments for neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease have not been discovered yet, but are urgently needed.

Fundamental ageing research is often performed on invertebrates, in particular yeast (*Saccharomyces cerevisiae*), worms (*Caenorhabditis elegans*) and fruit flies (*Drosophila melanogaster*), but vertebrate models, including zebrafish (*Danio rerio*) and mice (*Mus musculus*), are used as well (Kim et al., 2016). The extremely short lifespan of invertebrate models, ranging from several weeks to months, combined with a high amenability to genetic manipulations, makes these organisms ideal for high throughput genetic screenings (Kim et al., 2016). On the other hand, mice and zebrafish are unpractical because they are longer lived (respectively 3 – 4 and 5 years), yet allow extrapolation to humans because they share a typical vertebrate physiology (Kim et al., 2016). This includes an endoskeleton, blood, a spinal cord, an adaptive immune system, the process of tumorigenesis, but also many principal genes that are involved in the regulation of human ageing (e.g. APOE and IL8) (Harel et al., 2015; Kim et al., 2016).

Even though each of these models is essential for studying the mechanisms of ageing and/or neurogenesis, the translation from laboratory discoveries to effective clinical trials continues to fail (Johnson, 2015). According to Johnson (2015), this disappointing outcome is mainly the result of using relatively young vertebrate models, and thus non-aged nervous systems as study subjects for age-related diseases. New findings concerning the effect of age on the survival of damaged neurons could possibly link laboratory discoveries to successful clinical trials (Johnson, 2015). Development of a successful drug strategy against age-related diseases should thus include an aged vertebrate model organism.

1.2 The African turquoise killifish: a model for ageing research

1.2.1 The use of a teleost model

Since the 1960s, the use of teleost fish as experimental models has become extremely popular by the scientific community. Frequently used species are zebrafish, medaka (*Oryzias latipes*), guppy (*Poecilia reticulata*) and the African turquoise killifish (*Nothobranchius furzeri*) (Kim et al., 2016; Kuroyanagi et al., 2010; Mueller & Wullimann, 2009). Their success in the lab is mostly due to their amenability to genetic manipulations, lower maintenance costs in comparison with mice, and the production of many embryos (Kim et al., 2016). External fertilisation (all teleosts) in combination with transparent eggs (zebrafish) makes them very useful model organisms for developmental studies (Harel et al., 2016). Because teleosts are vertebrates, they share many organs and systems with humans, which make them exceptionally suitable for studying human diseases.

In order to develop therapies against age-related neurodegenerative diseases, two important features should be present in the model: mammalian-like ageing (more detail in section 1.2.4) and the potential to regenerate neurons (more detail in section 1.3.2). An ageing phenotype that resembles that of mammals is found in some teleost species (e.g. zebrafish and killifish). Some hallmarks of ageing have been proven to exist in the aged brain of zebrafish and African turquoise killifish, which makes these organisms favourable models for studying age-related diseases (reviewed in Van houecke et al., 2021; Van Houcke et al., 2015). All teleost species studied so far show the ability to regenerate lost neurons after injury, and to produce new neurons within the adult CNS (reviewed in Zupanc & Sîrbulescu, 2011). As neurons show a very limited regenerative ability in mammals, teleost fish might hold the key to successful regeneration of mammalian neurons.

1.2.2 General characteristics of the African turquoise killifish

To study age-related diseases, a suitable model organism for ageing is needed. The African turquoise killifish (*N. furzeri*) was first described by Jubb in 1971, and is suggested to be an ideal candidate (Cellerino et al., 2016). *N. furzeri* is a teleost fish (Actinopterygii), belonging to the order of the Cyprinodontiformes. Most species within this order belong to the genus *Nothobranchius* (family Nothobranchiidae), which includes 90 described species (Froese & Pauly, 2021). The distribution of *N. furzeri* is restricted to southeastern Africa (Fig. 2A), ranging from the Save river in the north to the Lebomo ridge in the south. The species inhabits temporary savannah pools that only contain water during a brief rainy season, called the monsoon (Fig. 2B) (Genade et al., 2005). These ephemeral pools are mostly located in floodplains and dry up during the summer period, leaving the killifish eggs behind in the muddy substrate (Fig. 2C) (Genade et al., 2005). The concerned regions are characterized by very unpredictable rainfall (200-600mm/year) and a high aridity (Reichard et al., 2009). The small isolated pools typically have turbid water with a lot of organic input, and are highly disturbed by large herbivores (Reichard et al., 2009).

In 2003, researchers became interested in using the African turquoise killifish for ageing research, mainly after the discovery of the extremely short lifespan that characterised the GRZ strain (Valdesalici & Cellerino, 2003). This inbred strain originates from the first *N. furzeri* collection by Jubb in the Gonarezhou National Park in Zimbabwe in 1968. Under strict laboratory conditions, the GRZ strain of *N. furzeri* has the shortest lifespan ever noticed in vertebrates, with a mean of 12 weeks (Fig. 2D) (Valdesalici & Cellerino, 2003). Several other *N. furzeri* strains are available as well, which are longer lived (6 – 8 months) and collected in Mozambique between 1998 and 2012 (Terzibasi Tozzini & Cellerino, 2020; Valenzano et al., 2015). The fact that these more recently collected strains have a longer lifespan, suggests that the extremely short lived phenotype of the GRZ strain is an exception, even within *N. furzeri*. Genetic mapping studies indicated that multiple genes are involved in lifespan determination, and that it is not a result of a single gene mutation (Kirschner et al., 2012). The African turquoise killifish, from here on referred to as killifish, is very small and has a typical body length of <7cm (Cellerino et al., 2016). There is a pronounced sexual dimorphism, with the males being quite colourful and the females being pale brown (Fig. 2E). The remarkable colouration of the males is probably due to a combination of intrasexual selection (dominant males are brightly coloured) and intersexual selection (females prefer colourful males) (Haas, 1976). The males come in a red and yellow morph, with the most prominent difference being the colour of the caudal fin. Female killifish are smaller than males, have iridescent scales and translucent fins. In the wild, killifish populations are female-biased, with a mean of 3 to 5 females for each male, although extremely biased populations also exist with cases up to 10 females for each male (Reichard et al., 2014).

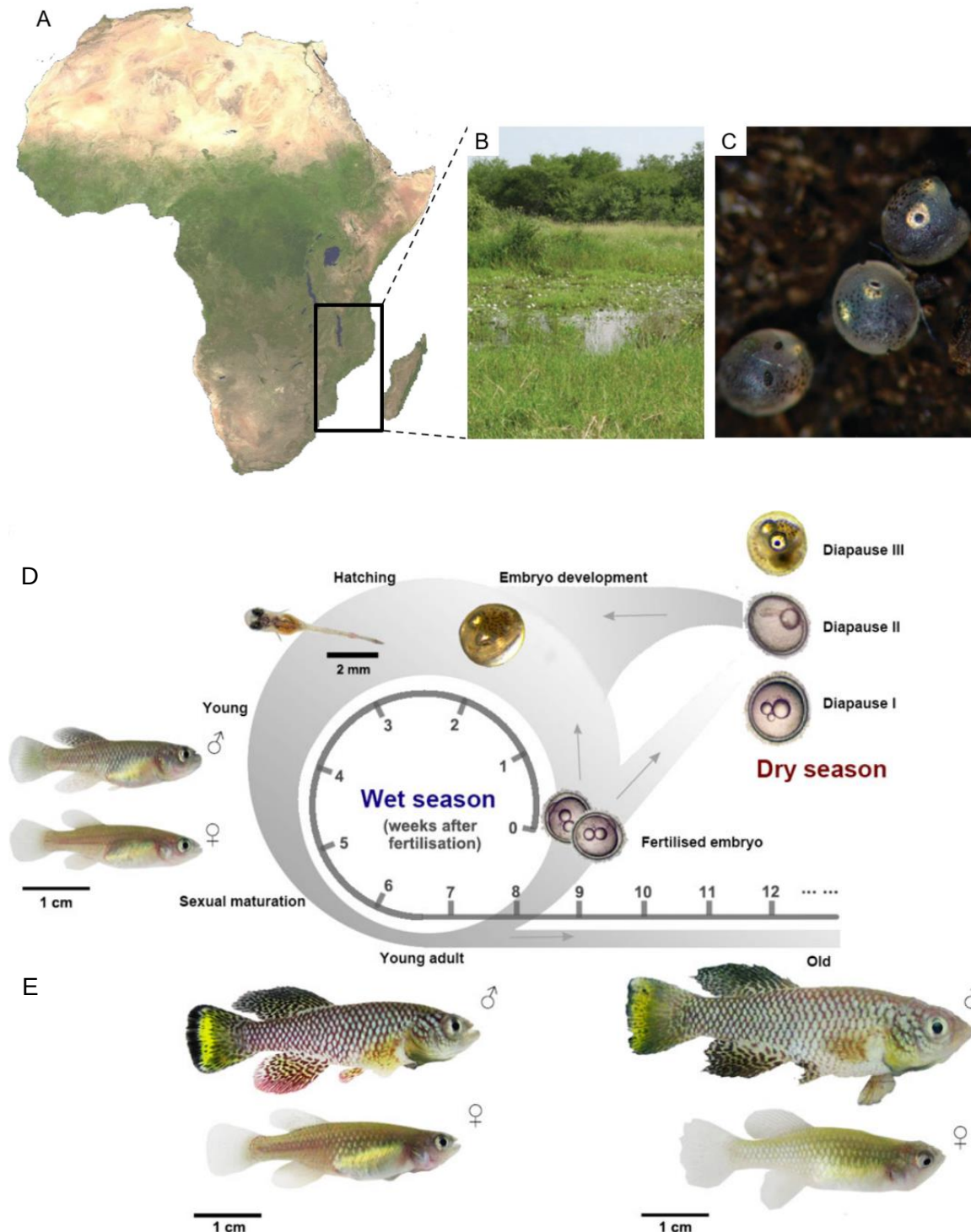


Figure 2. (A) The African turquoise killifish is found in south-eastern Africa. (B) The fish inhabits temporal savannah pools, that are only filled with water during the monsoon season. (C) Eggs of the African turquoise killifish are spawned into a muddy substrate, where they can survive in a drought-resistant state (diapause) for months (Furness, 2016). (D) A scheme of the life cycle of the African turquoise killifish. During the wet season, the embryos will hatch and start to grow very quickly. After 4 to 5 weeks, the fish will become sexually mature and start to reproduce. The female spawns the eggs into the muddy substrate, and the eggs can go into diapause at three different developmental stages to survive long periods of drought. (E) The killifish males are quite colourful while the females are pale brown. Some typical ageing characteristics can be recognized in the old killifish, such as maceration, larger eyes, spinal curvature and loss of pigment (Kim et al. 2016).

1.2.3 Life cycle and embryonic development

Due to their unique and unpredictable habitat, the life cycle of killifish is entirely adapted to surviving long periods of drought, and to having a very rapid sexual maturation (Fig. 2D). The short rainy season has an average duration of 75 days in semi-arid regions, which means that the killifish embryos have to spend almost 10 months in drought (Terzibasi Tozzini et al., 2013). The embryos therefore have the ability to enter diapause at three different embryonic stages, and are able to stay in this drought-resistant state for more than a year (Cellerino et al., 2016). Because the fertilized eggs of killifish are embedded in a muddy substrate, there is relatively little predation, which leads to limited selection for rapid embryonic development (Cellerino et al., 2016). The early embryonic phases in killifish therefore occur much slower compared to zebrafish. In killifish, cleavage occurs with an interval of 75 min, while in zebrafish this is on average 15 min (Cellerino et al., 2016; Kimmel et al., 1995). It was shown that this difference is partly caused by killifish only lacking the G1 phase during the first 5 cleavages, while zebrafish also lacks the G2 phase in early cleavage (Dolfi et al., 2014, 2019). Generally in teleosts, the cells divide synchronously within the blastodisc, a yolk-free region located on the animal pole. Only the cytoplasm of the blastodisc will eventually form the embryo, which is also referred to as discoidal cleavage.

After formation of the blastula, two types of cells will be produced in killifish: a layer of cells that forms the envelope, and blastomeres that will migrate under this envelope during epiboly. After 48h, this epibolic migration will be finished, and the completely dispersed blastomeres will go into cell cycle arrest followed by random migration at the surface of the yolk (Cellerino et al., 2016; Dolfi et al., 2014). This dispersed phase, also known as diapause I, lasts for about 5 days in killifish until the blastomeres will start reaggregation to form the embryonic axis (Cellerino et al., 2016). The killifish embryo can then enter a second cell cycle arrest, or diapause II, at a specific stage after the main neurulation and somitogenesis phases, but before initiation of the main organogenesis events (Dolfi et al., 2014). The embryo then remains in diapause II for at least 2 days, but some killifish eggs were hatched successfully even after 3 years (Cellerino et al., 2016). Temperature has been proven to influence the developmental regulation of killifish embryos. Incubation of the embryos at 28°C results in early hatching, at 12 days post-fertilisation, because diapause II is skipped (Cellerino et al., 2016). During diapause II, survival of the embryos is facilitated by a substantial (90%) decrease in both the rate of oxygen consumption, and heart rate of the embryo (Podrabsky & Hand, 1999).

After diapause II, killifish embryos will continue their development until they are ready to hatch. Embryos that completed their development (~12 – 18 days post fertilisation) are recognizable by the state of their eyes, which exhibit a typical golden iris (Fig. 2C) (Genade, 2007; Valenzano et al., 2011). In this ready-to-hatch state, the embryos can go into a final developmental arrest, or diapause III. When the rainy season starts and the pool is filled with water, extrinsic factors

can release the eggs from diapause III and trigger hatching. In the lab, hatching can be induced by transferring the golden-eyed embryos to a mixture of water and peat extract, and it can be further stimulated by motion (Genade, 2007).

After hatching, the juvenile killifish are adapted to grow rapidly in order to become sexually mature before the temporary pool dries up again. Under optimal laboratory conditions, the maturation time is about 3 – 4 weeks, which makes killifish the fastest maturing vertebrate known (Blažek et al., 2013). When killifish reach sexual maturity, the males will actively seek for females to start reproduction. Large males with bright colours are often dominant and therefore control the largest habitat or the superior substrates for spawning. Males are always ready to mate, and it is common that multiple males mate with multiple females (polygynandry). Females will lay their eggs with jerking movements, to make sure that they are covered within the muddy substrate. A female lays on average 20 to 50 eggs each day, but this number is highly dependent on the size and age of the female, as well as the available resources (Blažek et al., 2013). After spawning, the eggs remain present in the muddy substrate, also after dessication of the pool, and the developmental cycle can start.

1.2.4 Ageing phenotype

Because killifish are forced to mature very quickly in their habitat, they also evolved to having a very short life expectancy. The GRZ strain is characterized by a maximum lifespan of 4 – 6 months in captivity (Valenzano et al., 2015). As their short lifespan is mainly the result of accelerated ageing, killifish have the potential to become usefull model organisms for studying the mechanisms behind ageing and age-related diseases (Di Cicco et al., 2011). The ageing phenotype in killifish has been found to resemble that of mammals, including humans. Some morphological characteristics of aged killifish are curvature of the spine, loss of pigment (especially in males), maceration and larger eyes (Fig. 2E) (Lucas-Sánchez et al., 2011). Spinal curvature and loss of pigment in hair and skin are also typical features of human ageing. In terms of behaviour, aged killifish generally have reduced locomotion and impaired learning, which are often seen in humans as well (Genade et al., 2005; Terzibasi et al., 2008).

Within the brain, some interesting cellular phenotypes can be observed in the aged killifish. First of all, the proliferative activity of NSCs seems to decrease drastically within the aged killifish brain, a phenomenon that is also observed in ageing mammals, which leads to a reduced neurogenic capacity (Tozzini et al., 2012; Van houcke et al., 2021). In addition to this, most of the newborn neurons in the killifish telencephalon can no longer reach the site of injury (Van houcke et al., 2021). Degeneration of neurons was demonstrated in several experiments regarding the killifish species *N. furzeri* and *N. guentheri*, and was found to start already at the age of 9 weeks in *N. furzeri* (GRZ strain) (Liu et al., 2012; Terzibasi et al., 2009; Valenzano et al., 2006). Research by Matsui et al. (2019) revealed that killifish also exhibit an accumulation

of α -synuclein in the brain, together with an age-related decline in dopaminergic and noradrenergic neurons. Because both of these features are distinctive for human patients with Parkinson's disease, this suggests that killifish can be used to unravel the mechanisms behind the development of this disease (Matsui et al., 2019). Gliosis, which is the fibrous proliferation of glial cells, and a high inflammatory reaction are also observed within the aged killifish brain, which are known to be typical characteristics of glial injury in mammals (Tozzini et al., 2012; Van houecke et al., 2021). Furthermore, the nine hallmarks of ageing, which characterize the mammalian brain, can also be found in the aged killifish brain (reviewed in Van houecke et al., 2021).

There is thus promising evidence that the killifish can be an excellent model organism for ageing research. Studying ageing phenotypes in the lab can be much more practical by using killifish, due to their extremely short lifespan and mammalian-like ageing phenotype. The killifish could fill the gap between the commonly used short-lived invertebrate models (e.g. *c. elegans*) and relatively long-lived mammals (e.g. mice). As the limited regenerative capacity of the adult mammalian brain can also be found in the aged killifish brain, the use of killifish as ageing models can be a huge step forward in the development of treatments against age-related neurodegenerative diseases (Van houecke et al., 2021).

1.3 Adult neurogenesis

1.3.1 Adult neurogenesis in mammals

Until the late 1900s, the central dogma stated that neurons in the mammalian brain could not be generated during adulthood, and that neurogenesis in mammals only occurred during embryonic development (Oppenheim, 2019). The first evidence of adult neurogenesis in mammals was reported by Altman & Das (1965), in a study that re-examined this dogma by injecting thymidine- H^3 in the brains of adult rats. However, general acceptance of adult neurogenesis in mammals, including primates, only happened in the 1990s to 2000s (Gross, 2000).

Neurogenesis during embryonic development occurs via NSCs, so called neuroepithelial cells. These neuroepithelial cells will renew themselves via symmetrical division (production of two daughter cells with identical cellular fates) on the ventricular surface. After closure of the neural tube, the neuroepithelial cells will differentiate into radial glia cells (RGs), which are characterised by long radial fibres. RGs can generate new neurons by asymmetrical division (production of two daughter cells with different cellular fates, e.g. one stem cell and one differentiated cell) and will guide the migration of these new neurons along their radial fibres, towards their target site. Once embryonic neurogenesis is finished, RGs will differentiate into multiple glial cell types including ependymal cells, astrocytes and oligodendrocytes.

Although adult neurogenesis has been proven to exist in some mammals, the process is restricted to the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG), and the subventricular zone (SVZ), a region that lines the lateral ventricle (LV) (Fig. 3) (Kornack & Rakic, 1999, 2001). In the SGZ and SVZ, activated NSCs will proliferate and produce neural progenitor cells, which have glial characteristics (Fig. 4). These neural progenitors will then form neuroblasts which start to migrate and differentiate into specific neural cell types (Fig. 4). Migration of these newly formed neurons occurs until they reach their target site, where they can mature and incorporate into the existing neural circuits (Fig. 4). In rodents, neural progenitors in the SGZ give rise to granule cells that will integrate into the granular cell layer (Cameron et al., 1993). On the other hand, the SVZ contains different types of progenitors (neuroblasts and astrocytes) that give rise to a variety of cell types that will eventually migrate towards the olfactory bulb (OB) in order to become inhibitory interneurons (Menn et al., 2006). In other regions of the brain, NSCs are mostly quiescent. Nowadays, scientists are still debating the functionality of these newly formed neurons in the adult mammalian brain (Oppenheim, 2019).

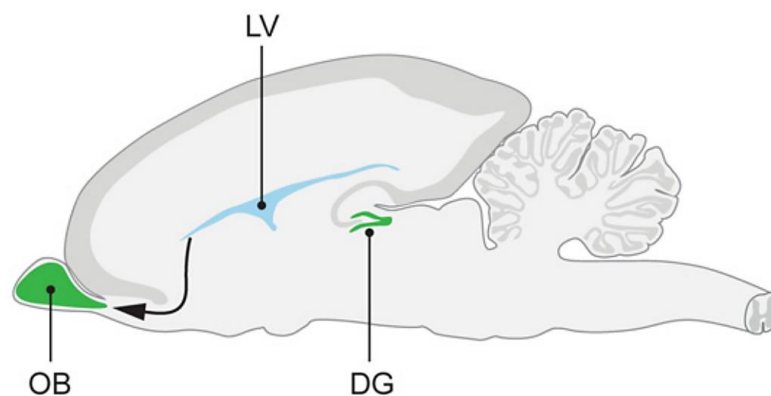


Figure 3. Left lateral view of the adult mouse brain. In rodents, NSCs within the SVZ and SGZ will proliferate and produce neural progenitor cells. The SGZ is located in the dentate gyrus (DG), while the SVZ lines the lateral ventricle (LV). Neural progenitors will migrate (black arrow) towards the olfactory bulb (OB). Here, the cells will differentiate and incorporate into the existing neural circuits (green = new neurons) (Ernst & Frisén, 2015).

1.3.2 Adult neurogenesis in teleost fish

In contrast to mammals, teleost fish do have the amazing ability to both produce new and regenerate damaged neurons within the CNS throughout their lives. The extent to which adult neurogenesis and neuroregeneration occur in teleosts is, until now, unseen in any other vertebrate model (reviewed in Ganz & Brand, 2016). While other regenerative models, such as frogs, are only able to regenerate tissues before metamorphosing into an adult, teleosts retain this ability during adulthood (Lust & Tanaka, 2019). A difference with mammalian neurogenesis is that newly formed neurons in teleosts are long-lived and gliosis is rarely seen, which can contribute to the high neurogenic capacity in their CNS (Baumgart et al., 2012).

It is suggested that the main driver behind their widespread neurogenic capacity, is the fact that teleosts continuously grow and have no limit in body size (Ganz & Brand, 2016). As unlimited body growth occurs, the input of primary sensory information will increase and the fish will have to expand their sensory systems (Kaslin et al., 2008). This persistent brain growth allows teleosts to continuously generate new neurons in every region of the brain, and consequently, to regenerate lost neurons when necessary (Ganz & Brand, 2016; Kaslin et al., 2008).

Adult neurogenesis in teleosts is, like in mammals, accomplished by proliferation of NSCs (Fig. 4). The rate of proliferating NSCs has been studied in the brown ghost knifefish (*Apteronotus leptorhynchus*) and zebrafish by labelling the cells in S-phase with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU). It was found that, in the brown ghost knifefish, on average 100.000 cells are in S-phase within 2 hours, while in zebrafish, 6000 cells enter the S-phase in 30 minutes (Zupanc & Horschke, 1995; Hinsch & Zupanc, 2007). This corresponds to respectively 0.2% and 0.06% of the total amount of brain cells present in these species (Zupanc & Horschke, 1995; Hinsch & Zupanc, 2007). On the contrary, the rate of neural proliferation in the SVZ of adult mice was much slower, with approximately 30.000 new cells per day (Lois & Alvarez-Buylla, 1994).

After NSC division, the young neurons can stay near their location of birth, or they can migrate towards the site where they will differentiate (Zupanc & Zupanc, 2006). Migration of newly formed neurons happens along the fibres of RGs, until they eventually are incorporated into the existing neural circuits of the CNS (Diotel et al., 2020). In the zebrafish cerebellum, it takes about 10 days for progenitor cells to migrate from the proliferation zone towards their target layers (Zupanc et al., 2005).

To determine the number of newly formed cells that eventually differentiate into neurons, Zupanc et al. (2005) performed double-labelling experiments on the dorsal telencephalon of zebrafish. The detection of DNA replication was done via BrdU labelling and was 9 months later combined with immunohistochemical staining for Hu-C and Hu-D, which are neuron-specific marker proteins (Zupanc et al., 2005). This revealed that approximately 50% of the newly born cells eventually differentiate into neurons within the dorsal telencephalon (Zupanc et al., 2005). Adult neurogenesis in teleosts shows highly plastic characteristics, and is known to be influenced by growth factors, epigenetics and microRNAs, as well as environmental factors (Tozzini et al., 2012).

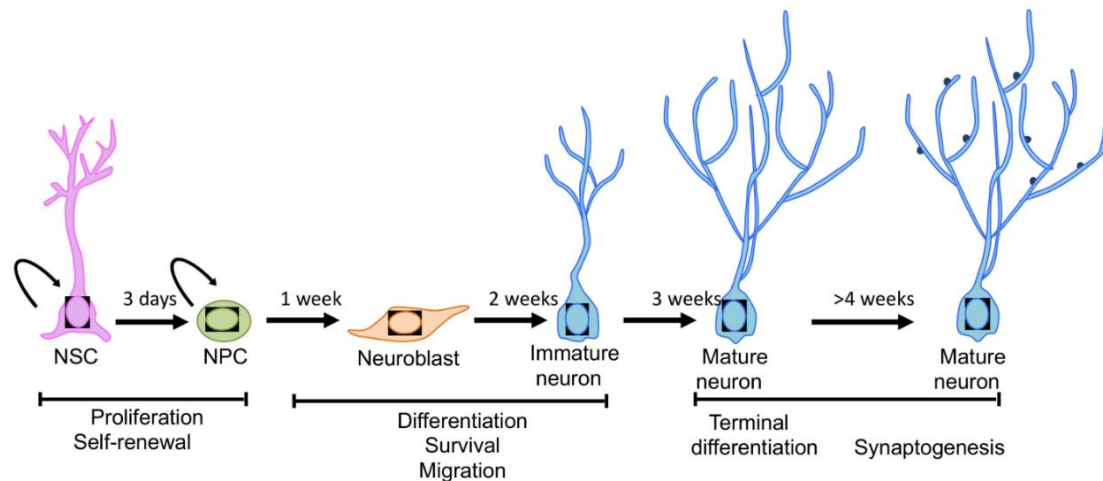


Figure 4. Schematic representation of adult neurogenesis in mammals. Neural stem cells (NSCs) will proliferate and produce neural progenitor cells (NPCs) which can proliferate to form neuroblasts. These neuroblasts will migrate and differentiate, until they are mature neurons that can incorporate into the existing neural circuits of the brain (Niklison-Chirou et al., 2020).

1.3.3 Overview of the teleost brain and its neurogenic niches

Similar to other vertebrates, three regions can be distinguished within the teleost brain, namely the forebrain, midbrain and hindbrain. The general structure and organisation of these regions is also comparable to other vertebrates. The forebrain in teleost fish contains the telencephalon (Tel) and diencephalon (Di), whereas the hindbrain is subdivided into the metencephalon (pontine area and cerebellum) and myelencephalon (medulla oblongata, MO) (Fig. 5A) (Yamamoto, 2009). The midbrain is known to contain the optic tectum (TeO), a multi-tissued structure that serves as the main visual centre in non-mammalian vertebrates. The majority of cognitive information in vertebrates is processed in the most rostral part of the brain, the telencephalon, which consists of two hemispheres that join each other at the septum (Yamamoto, 2009). In contrast to mammals, the telencephalic vesicles in teleosts develop by eversion (outward folding), and not from invagination (inward folding) (Mueller & Wullimann, 2009). This results in a brain topology where the brain parenchyma and proliferative ventricular zones are surrounded by the ventricle (Mueller & Wullimann, 2009). Hereby, teleosts retain a T-shaped ventricle in the centre of the telencephalon, which is surrounded by a membrane called the tela choroidea (Nieuwenhuys, 2009).

Within each hemisphere of the telencephalon, two major regions can be distinguished, namely the dorsal area (pallium) and ventral area (subpallium) (reviewed in Ganz & Brand, 2016). Both of these areas can be further subdivided into smaller regions, depending on their rostro-caudal position (Fig. 5B). The subpallium consists of the ventral nucleus (Vv) and the dorsal nucleus (Vd) (Wullimann, 2009). The pallium is further subdivided into the lateral nucleus (DI), the dorsomedial nucleus (Dm), the central nucleus (Dc) and the posterior nucleus (Dp) (Wullimann, 2009). The DI, Dp and Dm are often described as homologues to the hippocampus

in mammals, more specifically, the SGZ of the dentate gyrus (Ganz & Brand, 2016; Wullimann, 2009). On the other hand, the Vv is suggested to be equivalent to the SVZ of the lateral ventricle in the mammalian brain (Mueller & Wullimann, 2009). As the SGZ and SVZ are the only known regions within the mammalian brain where adult neurogenesis occurs, their homologs within the teleost brain are interesting targets for studying the process of adult neurogenesis. In addition to this, it has been found that NSCs are extensively present in almost every brain region of teleosts (Ekström et al., 2001). In general, 5 main neurogenic regions have been described in the teleost brain, namely the dorsal telencephalon, ventral telencephalon, cerebellum (Ce), hypothalamus (H) and optic tectum (TeO) (reviewed in Ganz & Brand, 2016). Studies on adult medaka and zebrafish brains resulted in the identification of 16 proliferative niches that were in common (Grandel et al., 2006; Kuroyanagi et al., 2010). Whole mount analysis suggested that these niches are present in the killifish brain as well (Tozzini et al., 2012). This suggests that the location of NSCs may be conserved between teleost species, and that adult neurogenesis is a conserved vertebrate trait (reviewed in Ganz & Brand, 2016). Generally, the NSCs in teleosts can be of both neuroepithelial and glial nature, of which RGs are the most common glial cell type. Activated NSCs are mostly associated with the ventricular system, where neurogenesis will take place in specialized neurogenic niches. Research on the killifish telencephalon in particular, revealed three distinct neurogenic niches (Fig. 5B) (Tozzini et al., 2012). Niche I is part of the subpallium, contains a dense mass of fast proliferating cells and can be found at the rostro-ventral conjunction between the two hemispheres (Tozzini et al., 2012). On the other hand, neurogenic niches II and III have a pallial origin and both show less proliferating cells than niche I (Tozzini et al., 2012).

1.3.4 Neural progenitor cells in the killifish telencephalon

In the adult zebrafish pallium, RGs cover the entire pallial ventricular surface and act as primary neural progenitor cells that sustain adult neurogenesis (Dirian et al., 2014). They are characterised by cell bodies that form a monolayer covering the ventricular surface, which are in contact with the cerebrospinal fluid (CSF) (Than-Trong & Bally-Cuif, 2015). The RGs have long processes that extend towards the parenchyma of the pallium, where they reach large blood vessels via the pial surface of the brain (Than-Trong & Bally-Cuif, 2015).

Coolen et al. (2020) reported that within the killifish pallium, adult neurogenesis is mainly driven by another type of progenitor cells, namely the non-glial progenitors (NGPs), and thus not by RGs as previously described in zebrafish. NGPs have a similar morphology as RGs, with their cell bodies located at the apical surface and a process that extends towards the brain parenchyma, but they are less branched (Coolen et al., 2020). Several primary progenitor markers are expressed by NGPs, such as SOX2, MSI1 and NESTIN (Coolen et al., 2020). On the other hand, the expression of RG markers (e.g. vimentin, glutamine synthetase (GS), glial

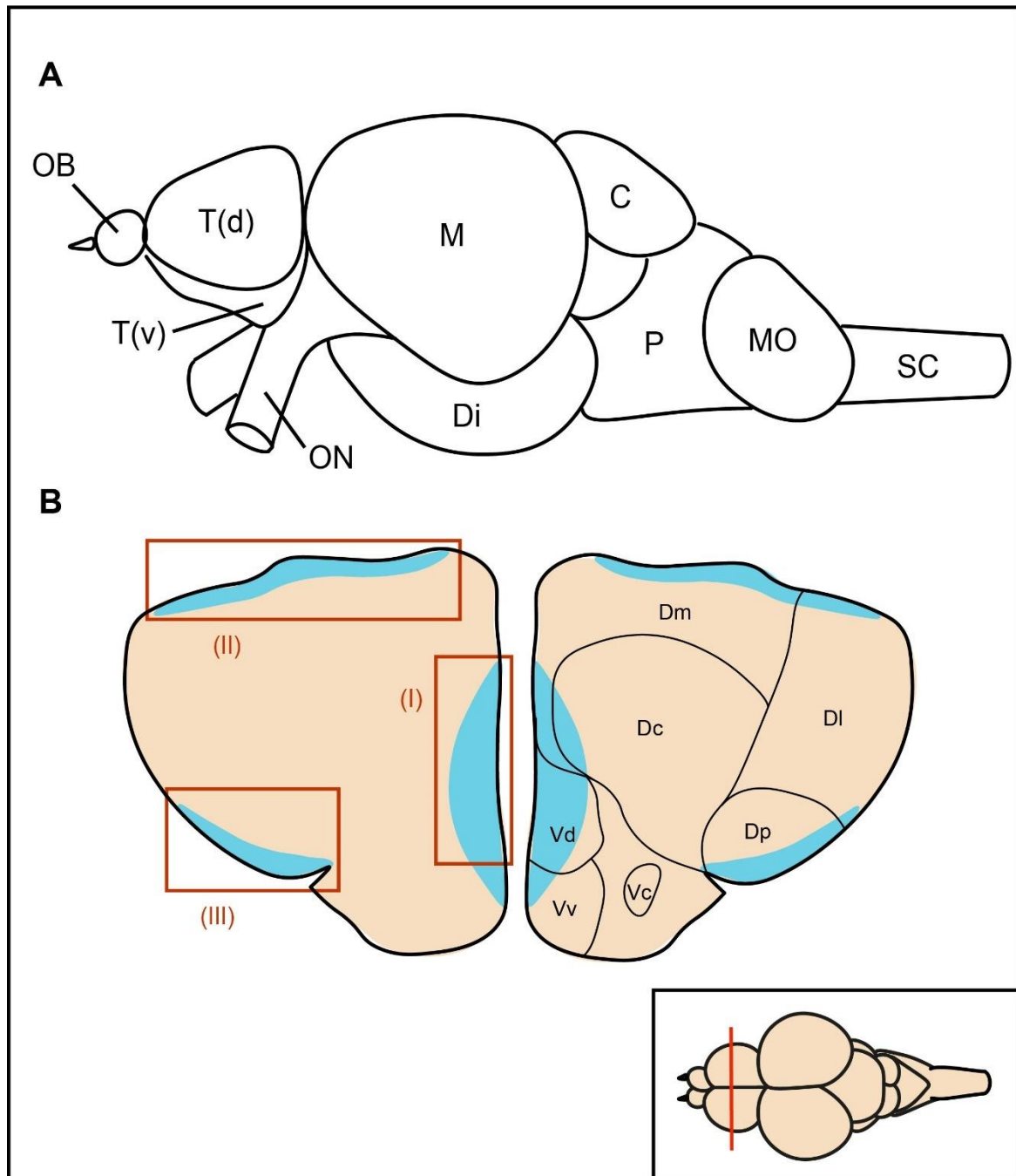


Figure 5. (A) Illustration of the teleost brain from a left lateral view. OB = olfactory bulb, T(d) = dorsal telencephalon, T(v) = ventral telencephalon, ON = optic nerve, Di = diencephalon, M = midbrain, P = pons, MO = medulla oblongata, C = cerebellum, SC = spinal cord. (B) Illustration of a coronal section of the killifish telencephalon. The three neurogenic niches (I-III) within the killifish telencephalon are visualized in blue. Region I (subpallium) is found at the rostro-ventral conjunction between the two hemispheres. Region II and III have a pallial origin. The subdivisions of the pallium (D) and subpallium (V) are visualized on the right (Dm = dorsomedial nucleus, Dc = central nucleus, Dl = lateral nucleus, Dp = posterior nucleus, Vv = ventral nucleus, Vd = dorsal nucleus).

fibrillary acidic protein (GFAP) and brain lipid-binding protein (BLBP)) is lacking in NGPs, which gives them neuroepithelial characteristics (Coolen et al., 2020; Kaslin et al., 2009).

Single-cell RNA sequencing of the adult killifish telencephalon revealed seventeen different cell types, including neuronal cells, glial progenitors and NGPs (Ayana et al., 2021). Sub-clustering analysis of the progenitor cell population has led to the identification of four RG subtypes (RG1-RG4), one type of NGP, and two cell clusters that featured the transition between progenitor cells and neuronal cells (Fig. 6A) (Ayana et al., 2021). Both STMN1A (stathmin 1A) and HMGB2A (high mobility group box 2A) are found to be highly expressed in the NGP cell population (Fig. 6B) (Ayana et al., 2021). SLC1A2 (solute carrier family 1 member 2) is found to be specifically expressed in RG1 (Fig. 6B) (Ayana et al., 2021). KRT18 (keratin 18) is highly expressed in RG2 and RG3, although the proportion of KRT18 expressing cells is higher in the RG3 population (Fig. 6B) (Ayana et al., 2021). In addition to KRT18, a neuroepithelial marker, the RG3 population also expresses EMX2 and WNT8B, which are typical markers for radial glia self-renewal, and HEPACAM (Ayana et al., 2021). CX43 (gap junction protein alpha 1) is highly expressed in both RG1 and RG2, with a higher proportion of expression in the RG2 population (Fig. 6B) (Ayana et al., 2021). High expression of EPD (ependymin) was found in the RG4 cell population (Fig. 6B) (Ayana et al., 2021). Although four RG subtypes are described based on differential gene expression, the single-cell sequencing data confirms that the NGP population is the main proliferating cell type within the adult killifish telencephalon (Ayana et al., 2021). However, the RG3 cell population is also found to be proliferative, although less than NGPs, and suggested to be an apical progenitor as well (Ayana et al., 2021). Lineage inference analysis suggests that RG3 might be at the start of the NGP lineage, which could be the origin of the three other RG subtypes (Ayana et al., 2021).

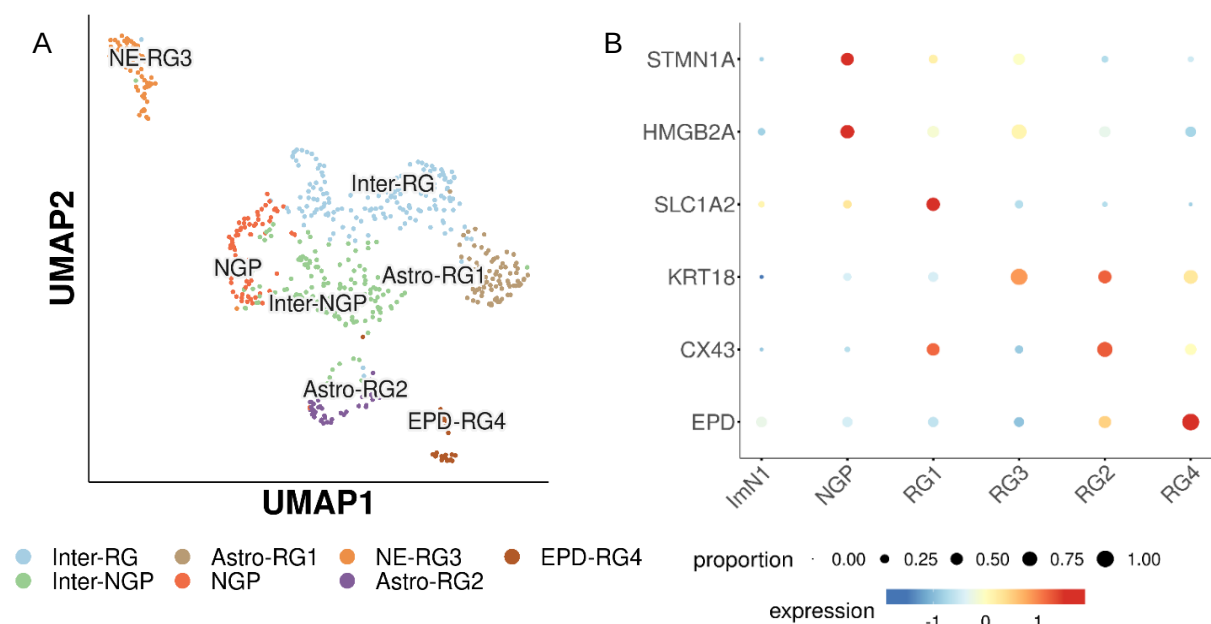


Figure 6. (A) UMAP plot displaying the progenitor cell types as a cluster of points, each with a unique colour. (B) Bubbleplot showing the expression profiles of some highly expressed genes (dark red) within each progenitor cell type. The size of the bubble reflects the proportion of cells within each cell type that expresses the gene.

1.4 Transgenesis

1.4.1 The limited availability of transgenic killifish lines

Although killifish could be an ideal model organism to study the aspects of ageing, the use of killifish in the lab is often hampered by the absence of transgenic lines and the limited tools for genetic modification. Nevertheless, the use of killifish does have some advantages for the rapid generation of stable transgenic lines. Their very fast sexual maturation (~3 to 4 weeks from hatching) allows an extremely short generation time of only 5 to 7 weeks, starting from fertilized egg (Harel et al., 2016). Furthermore, the genome of the killifish has been sequenced recently and a platform for efficient gene targeting was created (Harel et al., 2015; Valenzano et al., 2015). This platform offers a great resource for the analysis of genes that underlie the unique features of the killifish, including the fast ageing process and embryonic diapause. The dataset and tool provided by Ayana et al. (2021) includes cell-specific molecular markers for seventeen celltypes found within the killifish telencephalon, and can be used for detailed investigations regarding the adult killifish brain.

1.4.2 *Tol2* transposase system

The *Tol2* transposase is one of the most widely used transposon systems for transgenesis (Fig. 7A). It has been used previously to generate transgenic lines of zebrafish and *Xenopus*, alongside some individual, genetically modified mice (Gou et al., 2020; Lane et al., 2013; Pawar et al., 2016; Yang et al., 2016). The *Tol2* system was also used successfully *in vitro* on primordial germ cells of chicken and primary T-cells of humans (Huang et al., 2010; Macdonald et al., 2012). The *Tol2* transposon element was discovered in medaka fish, where it got its name “Transposable element of *O. latipes*, number 2”, and is an autonomous DNA element that encodes a fully functioning transposase protein (Koga et al., 1996). Together with the *Sleeping Beauty* transposon and I-SceI meganuclease, *Tol2* transposase is one of the most effective tools for genetic modification in fish (Grabher & Wittbrodt, 2007; Kawakami, 2007). To generate a *Tol2* transgenic fish, the mRNA of the *Tol2* transposase is injected together with a plasmid that contains the DNA of interest, flanked by terminal inverted repeats. The transposase will bind recognition sites in the terminal inverted repeats, and consequently facilitate the random integration of the sequence of interest from the donor plasmid into the target DNA via a “cut and paste” system.

In 2011, the first transgenic killifish line was established by Valenzano et al. (2011), based on the *Tol2* transposase system. The transgenic killifish expressed green fluorescent protein (GFP) under the control of the zebrafish heat-shock protein 70 promoter or the ubiquitous *Xenopus* cytoskeletal actin promoter, and it was shown that the transgene could be transmitted to the next generations (Valenzano et al., 2011). More recently, Dolfi et al. (2019) generated a

stable transgenic killifish line that can be used to study developmental cell cycle dynamics, with diapause in particular. The transgenic line expresses both a red and green fluorescent reporter, to respectively label the G1 and S/G2/M phase (Dolfi et al., 2019). To generate this double reporter line, two different transgenic Fucci (Fluorescent Ubiquitination-based Cell Cycle Indicator) lines were crossed (Dolfi et al., 2019). These included the “Fucci green” reporter line and the “Fucci red” reporter line, which were both created by using the *Tol2* transposase system (Dolfi et al., 2019).

Another effective method for transgenesis is the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) associated Cas9 nuclease. This technology for targeted mutagenesis is based on the bacterial defense mechanism type II from *Streptococcus pyogenes* against foreign nucleic acids (e.g. plasmids or viruses). The CRISPR/Cas9 method has already been proven to work in many organisms, including yeast, worms, flies, zebrafish, killifish, mice, rabbits and human cells (see Sander & Joung, 2014 for a detailed list).

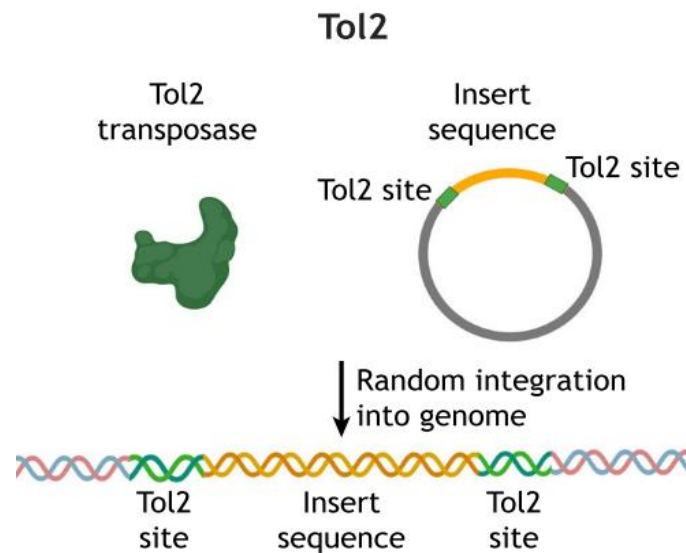


Figure 7. Scheme of the mechanism underlying the *Tol2* transposase techniques. The *Tol2* system is based on random integration of the sequence of interest into the host genome. *Tol2* transposase will bind the *Tol2* recognition sites and mediate the transposition of the insert sequence from the donor plasmid into the host DNA (figure from Poeschla & Valenzano, 2020).

1.4.3 Generation of transgenic killifish

Introducing the components for genetic modification to a zygote allows direct integration of the sequence of interest into the genome of the embryo, and hence, the presence of the transgene in every cell of the organism, including germ cells (Harel et al., 2016). To evaluate germline transmission, the F0 chimeras (resulting directly from the genetic modification) can be crossed with wild-type (WT) animals to create the F1 generation (Harel et al., 2016). Further backcrossing with the desired animals can result in stable transgenic lines, and minimize off-target mutations (Harel et al., 2016). In the case of killifish, each generation takes about 5 to 7

weeks to develop, which creates the opportunity to generate stable transgenic lines incredibly fast (2 – 3 months) (Harel et al., 2016).

Although the availability of transgenic killifish lines is still extremely limited, other fish species, in particular zebrafish, have been used widely to create transgenic lines for *in vivo* studies. Interesting methods to conditionally activate transgenes in zebrafish include the yeast GAL4/UAS system, the Tet-On and the Cre/Lox mediated system (Hans et al., 2009; Kesavan et al., 2018; Knopf et al., 2010). These 'split systems' allow a spatiotemporal regulation of the transgene *in vivo*, and can also be used for *in vivo* cell lineage tracing. By using the ligand-inducible Cre variant (CreER^{T2}), one can achieve very precise temporal regulation of the transgene expression. In normal conditions, CreER^{T2} recombinase is only present in the cytoplasm and should be activated by administration of tamoxifen (TAM) or its active metabolite (4-hydroxytamoxifen, 4-OHT) (Hans et al., 2009). Tamoxifen will bind the estrogen receptor (ER) that is fused to the Cre-recombinase, which will result in a conformational change of the enzyme and allows it to enter the nucleus. Once inside the nucleus, CreER^{T2} recombinase will specifically recognize the lox sites gene of interest which results in site-specific recombination.

In zebrafish for instance, a transgenic line was made that expresses CreER^{T2}, coupled to an mCherry reporter, under the control of the *myelin basic protein a (mbpa)* promoter (Pinzon-Olejua et al., 2017). Combining this zebrafish line with a Cre-dependent enhanced GFP (EGFP) reporter line, allows spatiotemporal control over the expression of *mbpa* (Pinzon-Olejua et al., 2017). Addition of TAM results in EGFP expression in oligodendrocytes in the brain, optic nerve and spinal cord, in both larvae and adult zebrafish (Pinzon-Olejua et al., 2017).

The development of a genomic platform for the African turquoise killifish and the optimization of transgenesis tools (e.g. *To/2* and CRISPR/Cas9), in combination with the naturally occurring short lifespan of this species, provides the scientific community with a very promising model system for studying vertebrate ageing.

2. Objectives

Reactivation of adult neurogenesis in humans could be an effective therapy to combat age-related neurodegenerative diseases in the future. Unravelling the distribution of neural progenitors within the killifish telencephalon, and how their proliferation changes with age could help us to understand the limited abilities for adult neurogenesis in mammals. In this thesis, two main objectives will be covered. One side of the project will try to further characterize the three neurogenic niches that were previously described in the killifish telencephalon. Based on specific marker genes, we will try to identify the location of these progenitor cell types within the telencephalon, and investigate how their distribution or proliferation are influenced by ageing. Because neural progenitor cells in teleosts are known to be located at the ventricular surface, we assume that both the non-glial progenitors (NGPs) and the four radial glia subtypes (RG1-RG4) will be distributed here. We expect that the NGPs will be located within the three neurogenic niches, as this cell type is known to be the main proliferating cell type in killifish. Based on previous studies, we also expect that the amount of proliferating cells will decrease with age. Determining the spatial distribution of each progenitor subtype can make it more efficient to study one particular subtype in the future. In addition, spatial differences between subtypes also suggest that there are possibly functional differences.

As a second objective, we will try to generate transgenic killifish lines based on the inducible Cre/Lox system that is used in zebrafish and mice. A Cre-driver line will be made via the *To12* transposase system, which expresses the CreER^{T2} gene under the control of a ubiquitous promoter. A Cre-dependent green-to-red (GTR) fluorescent reporter line was generated previously via the *To12* transposase system. Combination of these two transgenic lines will result in offspring that expresses a red fluorescent protein (mCherry) in all cells when tamoxifen is added. Introduction of the inducible Cre/Lox system, for the first time ever in killifish, creates a foundation for developing more specific fluorescent reporter lines in the future. Transgenic killifish lines, which are fluorescently labelled for specific neural progenitor cell types, could then be generated, and would enable detailed lineage tracing in future experiments. It would allow *in vivo* studies of the origin, migration and differentiation of neural progenitor cells in a regeneration competent vertebrate. Lineage tracing of neural progenitor cells could inform us about which cell types respond to injury, which ones change because of age or what happens during certain diseases. New insights about the role of each progenitor cell type could bring us one step closer to finding treatments against age-related neurodegenerative diseases.

3. Materials and methods

3.1 Fish husbandry

All experiments were performed on African turquoise killifish (*N. furzeri*) of the inbred GRZ-AD strain, originating from the Leibniz Institute on Aging, Fritz Lipmann Institute, Jena, Germany. The killifish were housed under standardized conditions; water temperature 28°C, pH 7, conductivity 600µs and a 12h/12h light/dark cycle. Adult fish were fed twice a day with red bloodworms (*Chironomidae* larvae, Ocean Nutrition) while fry was fed with brine shrimp (*Artemia salina*, Ocean Nutrition) until the age of three weeks. Each male was housed with three females in a multilinking ZebTEC aquarium system (Tecniplast). Experimental fish were kept in 3,5L tanks, while breeding pairs were housed in 8L tanks provided with sandboxes for spawning. All experiments were approved by the KU Leuven ethical committee in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU) and the Belgian legislation (KB of 29 May 2013).

3.2 Characterisation of neurogenic niches

3.2.1 Tissue preparation

Fish at 6 and 18 weeks of age were euthanized in 0.1% buffered tricaine (MS-222, Sigma-Aldrich) and perfused via the heart with PBS and 4% paraformaldehyde (PFA, Sigma-Aldrich) in PBS as described by Mariën et al. (2022). The brains were extracted, postfixed overnight in 4% PFA in PBS at 4°C, followed by three times washing in PBS. For whole-mount brains, dehydration was performed in a series of graded methanol (MeOH)/PBST (0.1% Tween 20 in PBS) washes on ice as described in Choi et al. (2016). Whole-mount brains were then stored at -20°C for at least 16h, until further use. For cryosections, brains were immersed in 30% sucrose in PBS and embedded in 1.25% agarose in PBS, containing 30% sucrose. Both the coronal and sagittal sections (10µm thickness) were made on a CM3050 S Cryostat (Leica) using Tissue-Tek® OCT (compound 4583) to fix the embedded brain. Sections were collected on SuperFrost Plus Adhesion slides (10149870, Thermo Fisher Scientific), kept at room temperature (RT) for one hour and then stored at -20°C until further use.

3.2.2 Third generation hybridisation chain reaction (HCR v3.0)

Probe pairs for fluorescent in situ mRNA visualisation were generated by using `insitu_probe_generator` (Null & Özpolat, 2020), and specificity of the probe pairs was validated by using `Blastn` (www.NCBI.com). DNA oPools were ordered from Integrated DNA Technologies and dissolved in ultrapure water (Invitrogen) (Table S1). The HCR v3.0 was carried out according to the protocol described by Deryckere et al. (2021), which was based on the work of Choi et al. (2016, 2018), and adjusted for cryosections as described in Van houecke et al. (2021). In brief, cryosections were dried for 30 min at 37°C to improve adhesion to the glass slides and were washed three times in PBS-DEPC followed by 10 min in TBS (0.3% Triton-X-100 in PBS). To permeabilize the tissue, slides were incubated in proteinase K

solution (Roche, 1:3000 in PBS-DEPC) for 10 min at 37°C and this reaction was stopped by fixating the slides in 4% PFA in PBS for 5 min. Both the proteinase K and 4% PFA steps were skipped in case the tissue was to be used further for immunostaining. Slides were washed twice in PBS-DEPC and 10 min in 5x SSCT (0.1% Tween 20, biorad, in 20x SSC, Invitrogen). A pre-hybridisation step was performed by incubating the slides in probe hybridisation buffer at 37°C for 30 min. Hereafter, probe solution (1.2 pmol of each probe in probe hybridization buffer) was incubated overnight at 37°C. The next day, excessive probes were removed by incubating the slides at 37°C in a graded 5x SSCT/probe wash buffer solution for 15min each. A pre-amplification step was performed by adding amplification buffer to the slides for 30 min. Separate hairpin solutions (4.5 pmol for H1 and 4.5 pmol for H2) were made by snap-cooling the hairpins and adding them to 75µL amplification buffer. Snap-cooling included heating the hairpins at 95°C for 90 s, followed by 5 min cooling on ice and leaving them 30 min at RT. The hairpin solutions were incubated overnight and excess hairpins were removed the next day by washing the slides 3x10 min in 5x SSCT. Slides were incubated with DAPI (1:1000 in 5x SSCT), followed by 5 min in 5x SSCT and mounted in Mowiol. The latter step was skipped for slides that were used for immunohistochemistry after HCR. The slides were stored at 4°C, protected from light until microscopy.

3.2.3 Immunohistochemistry after HCR

Cryosections were washed three times in PBST and blocked for one hour by using pre-immune goat serum (PIG) in Tris-NaCl blocking buffer (TNB). Primary antibodies were diluted in TNB and incubated for 48h at RT. The primary antibodies used in this experiment were Rabbit anti-PCNA (1:500, GeneTex) and Rabbit anti-SOX2 (1:1000, Sigma Aldrich). Sections were washed three times in PBST followed by 2h incubation with secondary antibodies diluted in TNB. Secondary antibodies used were Goat anti-Rabbit Alexa fluor 488 (1:300). After three times rinsing in PBS, the slides were incubated with DAPI (1:1000 in PBS, Thermo Fisher Scientific), to stain the cell nuclei, and mounted in Mowiol and a cover glass (Menzel Gläser, Thermo Fisher Scientific).

3.2.4 Whole mount HCR

Whole-mount HCR on killifish brains was performed as described in Elagoz et al. (2022), which was based on the HCR v3.0 protocol for whole-mount mouse embryos (*Mus musculus*) provided by Choi et al. (2016, 2018). Probe sets were designed and ordered as explained above. In brief, whole-mount killifish brains were thawed on ice for 15 min and then moved to RT for another 15 min. The brains were rehydrated in a series of graded MeOH/PBST washes for 10 min each at RT, and permeabilized for 15 min with proteinase K (Roche, 75µg/mL) at RT. Brains were washed twice in PBST and postfixed for 20 min in 4% PFA in PBS, followed by three washes in PBST. Both the proteinase K and post-fixation steps were skipped in case the brain was to be used further for immunostaining against PCNA. Brains were pre-hybridized

for 30 min with 100 μ L probe hybridization buffer at 37°C and incubated overnight at 37°C with probe solution (0.4pmol probe in 100 μ L probe hybridization buffer). Excessive probes were removed by washing the brains in probe wash buffer for 4x15 min and twice in 5x SSCT. Brains were pre-amplified in 100 μ L of amplification buffer for at least 30 min at RT. Hairpin solution was prepared by adding 2 μ L of snap-cooled hairpins (3pmol of H1 and 3pmol of H2 separately) to 100 μ L amplification buffer at RT. After overnight incubation with the hairpin solution, excessive hairpins were removed by washing twice in 5x SSCT for 5 min, two washes in 5x SSCT for 30min followed by a final wash in 5x SSCT for 5 min. Brains were then stained with DAPI (1:1000) for 2h and washed in SSCT for 5 min. Brain samples were cleared for at least 3 days in Fructose-Glycerol Clearing solution (29,72 g fructose in 33 mL glycerol and 7 mL distilled water) as described in Dekkers et al. (2019) and Elagoz et al. (2022). The samples were stored at 4°C, protected from light until microscopy. The nuclear staining with DAPI and the clearing step were skipped for whole-mount brains that were used for immunohistochemistry after HCR.

3.2.5 Whole-mount immunohistochemistry after HCR v3.0

Immunohistochemistry on whole-mount killifish brains was performed according to the protocol provided by Elagoz et al. (2022). The entire IHC protocol was carried out at 4°C. Whole-mount brains were incubated with the primary antibody and diluted in 5x SSCT for two consecutive days after HCR. The primary antibody used in this experiment was Rabbit anti-phospho-histone H3 (Ser10) (1:100). Brains were washed three times for 2h with 5x SSCT and were then incubated overnight with the secondary antibody diluted in 5x SSCT. The secondary antibodies used were Goat anti-Rabbit Alexa-488 (Invitrogen). The excessive secondary antibodies were removed by washing the tissue twice in 5x SSCT for 2h, and the brains were incubated with DAPI (1:1000) for 2h. After nuclear staining, the samples were washed in 5x SSCT twice for 5min, and cleared in Fructose-Glycerol clearing as described above.

3.2.6 Imaging, quantification and statistical analysis

Tile scans (20X) were captured using a Leica DM6 microscope and stitched using LAS X software (Leica Microsystems). Snaps were taken at 20X for greater detail. Figure configurations were made in Adobe Illustrator (Adobe Systems). Whole mount imaging was done by using a Zeiss Z1 light sheet microscope (Carl Zeiss AG, Germany). Reconstructions in 3D were made by using Arivis Software (Vision4D, Zeiss Edition 3.1.4). Quantification was done by manually counting the pHH3 positive cells by using the cell counter tool in Image J (Fiji). To include the effect of size differences between the telencephali of different individuals, the depth of each telencephalon was calculated by multiplying the number of planes with the interval between the planes (Table S8). The fraction of pHH3 positive cells per area was then calculated for each brain. Statistical analysis was done via GraphPad Prism (version 9.0). Data was first tested for normal distribution (Shapiro-Wilk) and later analysed (unpaired t-test).

3.3 Transgenic lines

3.3.1 Generation of transgenic killifish lines

To establish transgenic killifish, microinjection of killifish embryos was performed as described previously by Hartmann & Englert (2012). Killifish eggs were collected from the sandbox after a fertilisation period of 2h, to ensure development until the one-cell stage. Embryos that were characterized by a clearly visible blastodisc were then selected and fixed in a 1.5% agarose (Invitrogen) injection plate. The embryos were reorientated in order to face the injection needle with their blastodisc. Borosilicate glass needles (GC100F-10, OD 1.0mm, ID 0.58mm) were used to penetrate the chorion of the embryos and microinjection was carried out by using a FemtoJet (Eppendorf) pressure injector ($P_i = 200\text{hPa}$, $P_c = 130\text{hPa}$, time = 1s), a micromanipulator (Saur) and a stereomicroscope (Leica S9i). The injection mixture contained the plasmid of interest (30ng/ μL) (Fig. S2), Tol2 transposase mRNA (25ng/ μL) together with phenol red dye, and was injected directly into the blastodisc of one-cell stage killifish embryos. After injection, the eggs were stored at 28°C.

The *actb2:GTR* injected embryos were checked with an inverted fluorescence microscope (Leica, DM IL LED) for endogenous EGFP expression 4 to 6 days after injection. Fish that were expressing EGFP had integrated the GTR gene and were considered as F0 embryos of the *actb2:GTR* line. They were then transferred to coconut fibre and stored on 28°C until hatching. To create the *ubb:CreERT²* transgenic fish, the injection mixture contained an additional component, EGFP mRNA (30ng/ μL), to allow selection for successfully injected embryos 24h after injection. Embryos with EGFP expression were transferred to coconut fibre and stored at 28°C until hatching.

Embryos that were ready to hatch were selected based on the presence of the golden-eye stage. Hatching was done in a small volume of cold humic acid solution (53680, Sigma-Aldrich, 1g/L in system water) and further stimulated by motion. The larvae were kept in tanks with continuous oxygenation and the water volume was doubled every day, for four days in a row, by adding fresh system water.

3.3.2 Genotyping *ubb:CreERT2* line

Once the F0 offspring was large enough (after 4-5 weeks), a fin clip was performed on the caudal fin while the fish was anesthetised in 0.03% tricaine. To recover from anaesthesia, the fish were placed in fresh system water and provided with oxygen. Genomic DNA was extracted by digesting the fin in lysis buffer (0.1 M Tris-HCl pH 8.5, 0.05 M EDTA, 0.2% SDS, 0.2 M NaCl) and proteinase K (Roche, 10 $\mu\text{g}/\text{mL}$) at 55°C overnight. The samples were precipitated in isopropanol and incubated in 10mM Tris buffer (pH 8.5) at 55°C for at least 1h. Amplification of genomic DNA was done by polymerase chain reaction (PCR), using GoTaq DNA polymerase together with specific forward and reverse primers (Table S3). The amplified DNA samples were finally loaded on a 1.5% agarose gel (1x TAE). Fish that were positive for the

CreER^{T2} gene could then be used for backcrossing with wild-type (WT) fish. After 4-5 weeks, the F1 offspring was genotyped to check for germline transmission.

3.3.3 Inbreeding of the *actb2:GTR* line

As the F2 generation of *actb2:GTR* transgenic reporter fish was already generated in the lab, we decided to generate a homozygous F3 generation. Since the F2 generation could exist of both hetero- and homozygous individuals, we wanted to select the homozygous individuals for inbreeding. Backcrossing was done via housing each male or female of the *actb2:GTR* F2 generation with a WT individual of the opposite sex. Couples were made based on their age and size, to avoid large physical differences between the partners and increase the chances for successful mating. Each couple was placed in a separate tank and provided with a sandbox for spawning. One week later, the eggs of each tank were collected in a sterile way, put in 96-well plates (one egg per well) and checked for fluorescence by using an inverted fluorescence microscope (Leica, DM IL LED). Embryos maintained in 96-well plates were prevented from bacterial growth by adding Penicillin Streptomycin to the medium. According to Mendelian inheritance, eggs that resulted from a homozygous *actb2:GTR* parent should all be heterozygous and expressing EGFP. On the other hand, eggs that were the result from a heterozygous *actb2:GTR* parent should only express EGFP in 50% of the cases. In this way, we were able to select the homozygous F2 individuals and use them for the generation of a homozygous F3 generation. Tanks were then set-up with both a homozygous F2 male and female. Their eggs were then collected weekly, put on coconut fibre and stored at 17°C.

3.3.4 Validation of the *actb2:GTR* transgenic reporter line

Fish of the *actb2:GTR* transgenic reporter line were euthanized in 0.1% buffered tricaine (MS-222, Sigma-Aldrich) and perfused via the heart with PBS and 4% paraformaldehyde (PFA, Sigma-Aldrich) in PBS as described by Mariën et al. (2022). The brains were extracted and in all steps protected from direct light to prevent loss of endogenous fluorescence. They were postfixed overnight in 4% PFA in PBS at 4°C, followed by three times washing in PBS. Brains were immersed in 30% sucrose in PBS and embedded in 1.25% agarose in PBS, containing 30% sucrose. Coronal sections (10µm thickness) were made on a CM3050 S Cryostat (Leica) using Tissue-Tek® OCT (compound 4583) to fix the embedded brain. Sections were collected on SuperFrost Plus Adhesion slides (10149870, Thermo Fisher Scientific), kept at room temperature (RT) for one hour and then stored at -20°C until immunohistochemistry. Cryosections were dried for 30 min at 37°C, followed by a 5 min wash in aqua destillata and 5 min wash in PBS. Sections were blocked for one hour by using pre-immune goat serum (PIG) in Tris-NaCl blocking buffer (TNB). The primary antibody Mouse anti-HuC/D (1:100, Invitrogen) was incubated at 4°C for 48h and diluted in Pierce Immunostain Enhancer (46644, Thermo Fisher Scientific). Sections were washed three times in PBST followed by 2h incubation with the secondary antibody Goat anti-Mouse Alexa-594 (1:300) diluted in TNB. After three times

rinsing in PBS, the slides were incubated with DAPI (1:1000 in PBS, Thermo Fisher Scientific), to stain the cell nuclei, and mounted in Mowiol and a cover glass (Menzel Gläser, Thermo Fisher Scientific).

3.3.5 Tamoxifen experiment

The set-up for the tamoxifen treatment was based on previous work by Than-Trong et al. (2020). *CreER^{T2};actb2:GTR* double transgenic larvae of 8 days post-hatching were placed overnight in beakers containing 0.5µM tamoxifen (Sigma Aldrich) dissolved in 100 mL system water. Continuous oxygenation of the beakers was provided via a pump-generated air flow. The next morning, larvae were transferred to fresh system water and fed twice a day with *Artemia salina* (Ocean Nutrition). Before taking the larvae into the fish facility, they were rinsed with fresh system water for at least three times over 48 hours. Larvae that died during the experiment were postfixed in 4%PFA for 24 hours. Larvae were checked for GFP and RFP expression with an inverted fluorescence microscope (Leica, DM IL LED), 2 days and 8 days after the treatment. At 4 weeks of age, fish were euthanized in 0.1% buffered tricaine (MS-222, Sigma-Aldrich) and postfixed overnight in 4% PFA in PBS at 4°C. Larvae were washed three times in PBS and immersed in 30% sucrose in PBS. Coronal sections (14 µm thickness) of the larvae head were made on the cryostat as described before (section 3.3.4). Immunohistochemistry was performed as described previously (section 3.3.4), with primary antibodies Rabbit anti-Cre recombinase (1:500 in TNB) and Mouse anti-RFP (1:1000 in TNB). Secondary antibodies used in the experiment were Goat anti-Rabbit Alexa-594 (1:300) and Goat anti-Mouse Alexa-594 (1:300).

4. Results

4.1 Characterisation of neurogenic niches

4.1.1 Localisation of neural progenitor cells within the killifish telencephalon

To investigate the distribution of the neural progenitor cell types within the killifish telencephalon, we used single-cell RNA sequencing data provided by Ayana et al. (2021) to select highly expressed genes for each cell type (Fig. 8). We visualized the mRNA for each cell type via third generation hybridisation chain reaction (HCR v3.0) on sections of 6-week old killifish telencephali. To localize RG1 and RG2, we selected CX43 and SLC1A2 as candidate marker genes (Fig. 8b). Because CX43 is expressed both in RG1 and RG2, and SLC1A2 is highly expressed only in RG1 (Fig. 8b), we were able to distinguish these two cell types from each other via HCR 3.0 multiplexing.

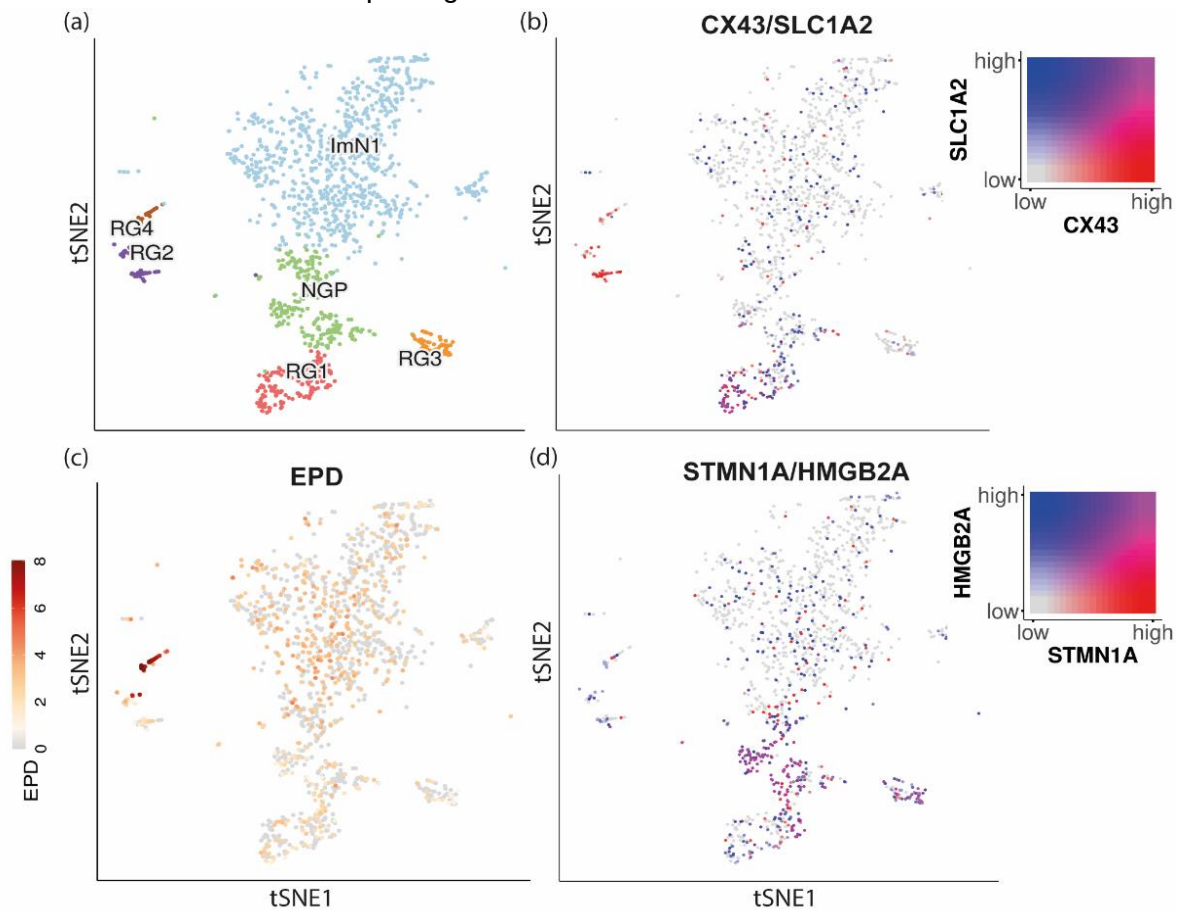


Figure 8. (a) tSNE plot visualizing clusters of neural progenitor cell types, each with a unique colour. (b-d) tSNE plots showing the expression profiles of CX43 and SLC1A2 in RG1 and RG2 (b), EPD in RG4 (c) and STMN1A and HMGB2A in NGPs (d) (figures made with the tool provided by Ayana et al. (2021)).

RG1 (CX43⁺ SLC1A2⁺) was found to be present at the pallial ventricular surface, while RG2 (CX43⁺ SLC1A2⁻) was found to be located caudally between the two hemispheres (Fig. 9A-C). RG1 and RG2 were found to have a typical radial glia morphology, with their cell bodies located at the ventricular surface and long fibres projecting towards the parenchyma (Fig. 9D-F). However, it is possible that the fibres are longer than visualized by the HCR v3.0 signal, as the mRNA is not necessarily translocated in the entire fibre.

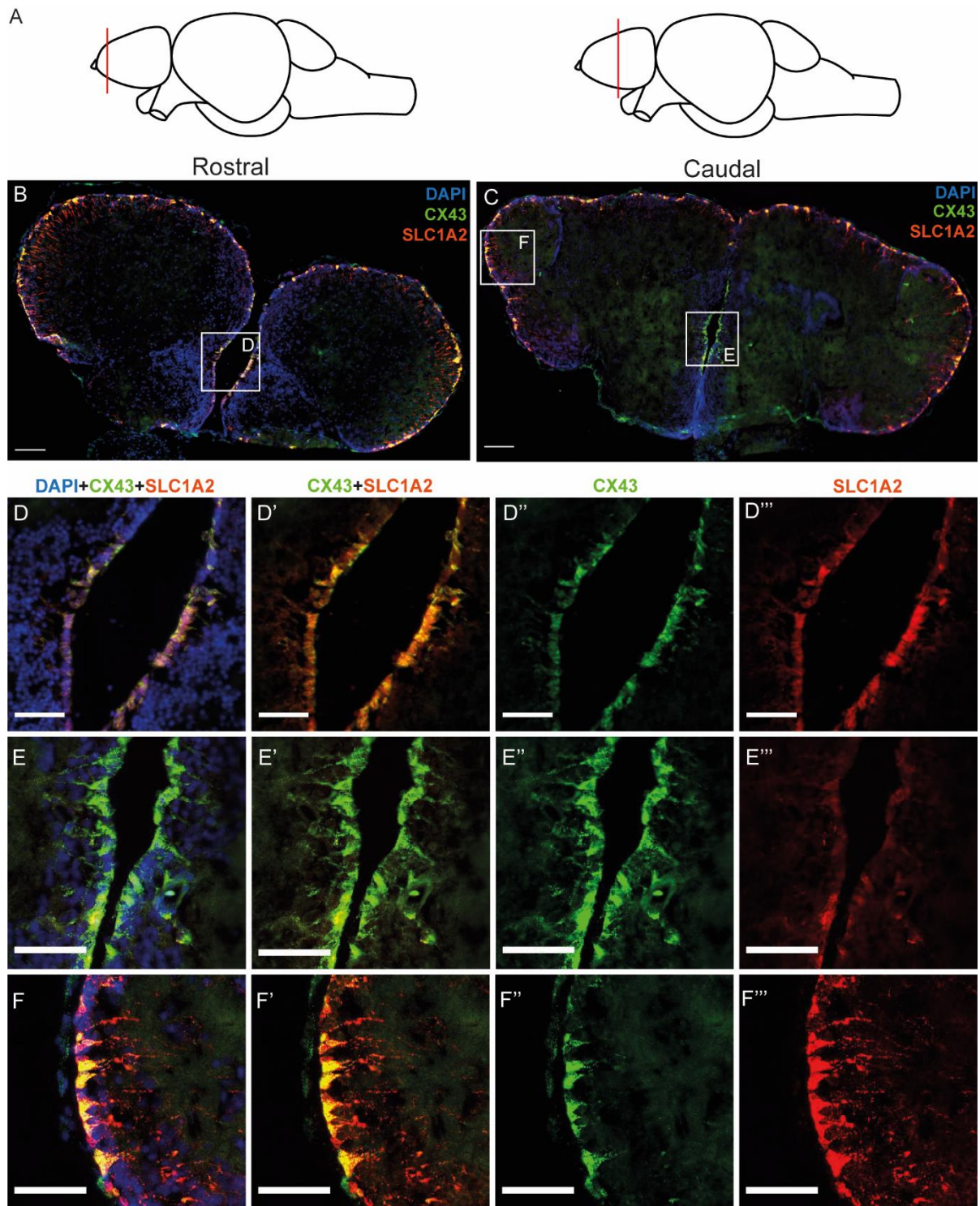


Figure 9. RG1 and RG2 are located in distinct niches on the ventricular surface of the killifish telencephalon. (A) Schematic overview of *N. furzeri* brain from the left lateral side. The red lines indicate the location within the telencephalon where the sections were taken. Sections were selected more rostrally (left) or caudally (right). (B-C) Coronal sections of 6-week-old *N. furzeri* telencephalon visualizing the expression of CX43 (green) and SLC1A2 (red) with DAPI (blue). (D) At the rostral side, CX43 is co-expressed with SLC1A2, suggesting that RG1 is mainly located at the rostral side of the conjunction between the two hemispheres. (E) At the caudal side, expression of SLC1A2 decreases which suggests that RG2 is located more caudally at the conjunction. (F) Co-expression of CX43 and SLC1A2 occurs everywhere at the ventricular surface, both on rostral and caudal sides, which demonstrates the presence of RG1 at the ventricular surface. Scale bars: in (B-C): 100µm, in (D-F): 50µm.

To visualize the location of RG3, we selected genes that were highly expressed within this cell type (e.g. HEPACAM, WNT8B, EMX2 or KRT18) for HCR v3.0. However, none of these marker genes could be optimized for HCR v3.0, as the fluorescent signal was not present, undetectable or not specific for RG3. Therefore, we decided to determine the location of RG3 based on exclusion with other neural progenitors. We performed triple labelling for CX43, NGP markers and SOX2, a common marker for neural progenitor cells (Fig. 10A). In niche I, a cluster of RG3 was found at the ventral side (Fig. 10B). RG3s (SOX2⁺ CX43⁻ NGP⁻) were found to be present scattered at the entire ventricular surface (Fig. 10C-D).

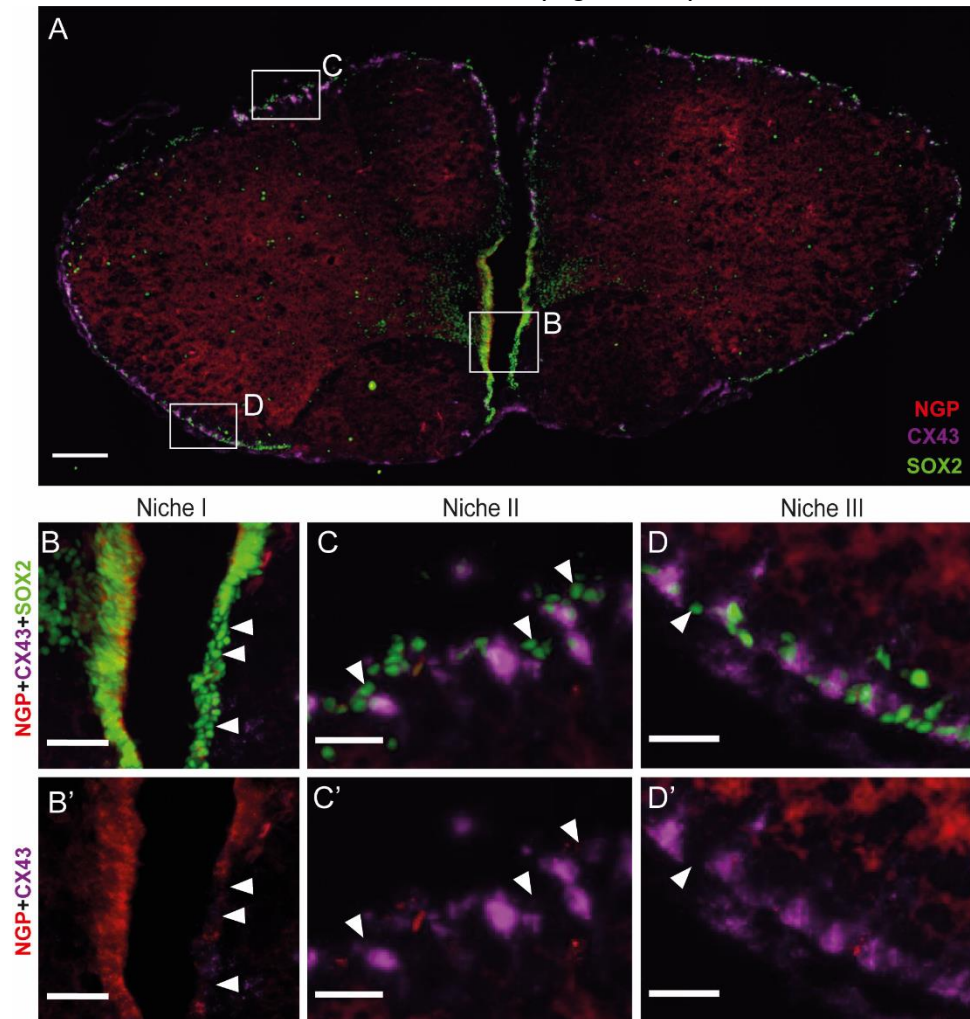


Figure 10. Localization of RG3 within the killifish telencephalon. (A) Coronal section of a 6-week-old killifish telencephalon visualized for NGP (red), CX43 (magenta) and SOX2 (green). (B) A cluster of RG3 is found at the ventral side of neurogenic niche I. (C-D) RG3s were found to be scattered along the entire ventricular surface. Scale bars: (A): 100µm, (B-D): 25µm.

To determine the location of RG4, we chose EPD as a specific cell type marker (Fig. 8c). We show that EPD was expressed specifically in the tela choroidea, a thin cell layer surrounding the ventricle, also known as the ependyma (Fig. 11). This suggests that RG4 could be ependymal cells, also referred to as ependymoglia because of their glial nature. RG4 were also found to express CX43 (Fig. 11).

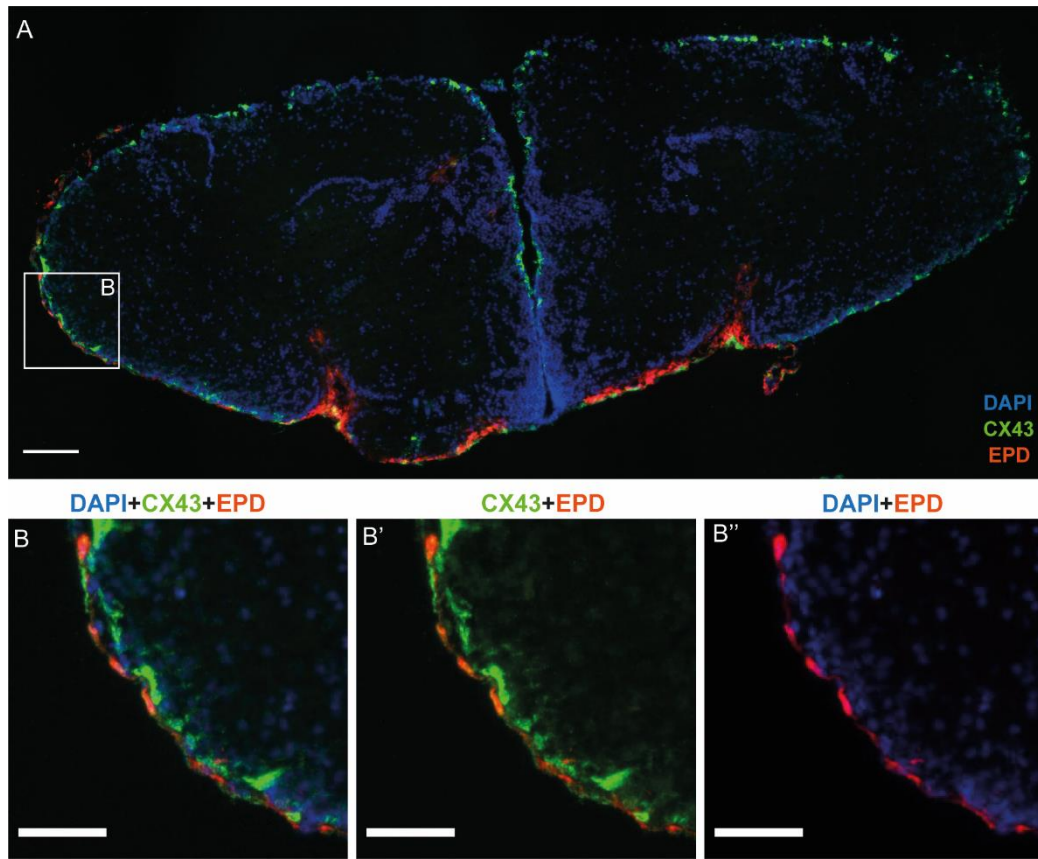


Figure 11. RG4 is located within the tela choroidea. (A) Coronal section of 6-week-old *N. furzeri* telencephalon visualizing the expression of CX43 (green) and EPD (red) with DAPI (blue). EPD is expressed specifically in the tela choroidea, a thin layer that surrounds the ventricle, and suggests the presence of RG4 within this layer. (B-B'') Higher magnification of the tela choroidea, showing the specific expression of EPD within the tela choroidea. (B-B') CX43 expression in radial glia visualizes the ventricular surface. Scale bars: in (A): 100μm, in B-B'': 50μm.

In addition to the four RG subtypes, we also wanted to localize the NGPs via HCR v3.0. We selected two genes with high expression within the NGP cell population, namely *STMN1A* and *HMGB2A* (Fig. 8d). However, the number of probe pairs that we could design for each gene was too limited for the HCR v3.0 to efficiently label the mRNA. Therefore, we decided to mix these two probe pools in order to intensify the fluorescent signal within each cell. This NGP probe mix revealed that the NGP cell population was present on the pallial ventricular surface, mainly within the three neurogenic niches (I-III), although some NGPs were scattered along the ventricular surface (Fig. 12A). We observed that the dorsal part of niche I mainly contained CX43⁺ cells (Fig. 12B'), while the density of NGPs was higher at the ventral part of niche I (Fig. 12B''). In niche II and III, the number of NGPs was lower and they were located in between CX43⁺ cells at the ventricular surface (Fig. 12C-D).

To confirm that the NGPs are the main proliferating cell type, we combined the HCR v3.0 multiplex for the NGP probe mix and CX43 with an immunostaining for proliferating cell nuclear antigen (PCNA), a common marker of DNA synthesis. This revealed a dense cluster of proliferating cells (PCNA⁺) at the conjunction between the two hemispheres (Vv), which corresponds to the highly proliferative neurogenic niche I (Fig. 12B'''). Many PCNA⁺ cells were

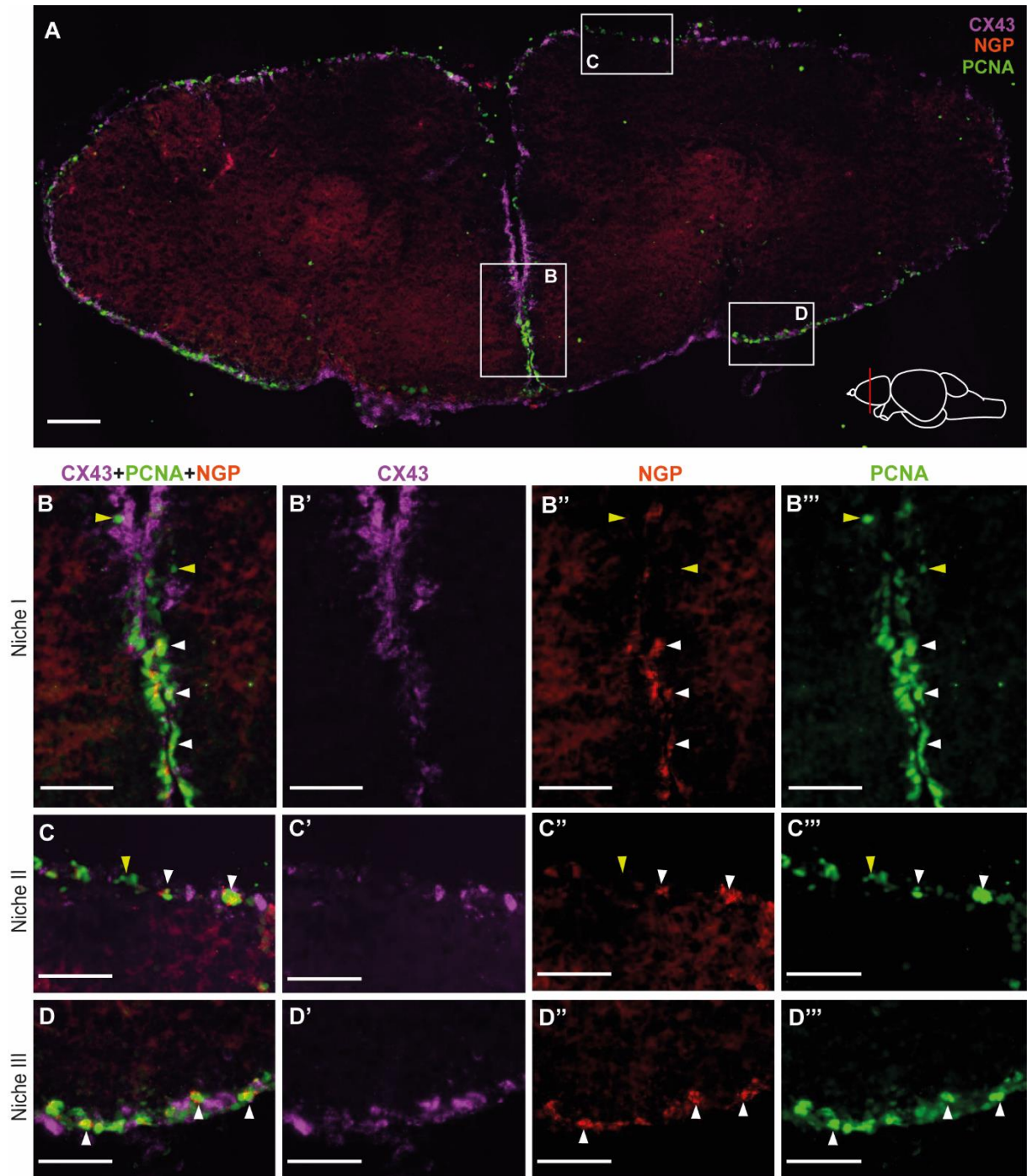


Figure 12. Proliferation mainly occurs by the NGPs in three distinct neurogenic niches. (A) Coronal section of the 6-week-old *N. furzeri* telencephalon, visualizing CX43 expression (magenta), expression of NGP markers STMN1A and HMGB2A (both red) combined with a staining for PCNA (green). Proliferating cells (PCNA⁺) are mainly found within the three neurogenic niches (I-III). (B-D) Cells expressing NGP markers are also PCNA⁺ (white arrows), while cells expressing CX43 are PCNA⁻, which verifies that the NGPs are the main proliferating cell type within the *N. furzeri* telencephalon. Cells that are PCNA⁺, but show no NGP marker expression are suggested to be RG3, as it is known that this cell type can also proliferate (yellow arrows). (B) Neurogenic niche I contains a dense cluster of proliferating cells (PCNA⁺), while niche II (C) and III (D) show less proliferative cells compared to niche I. Scale bars: in (A): 100µm, in (B-D): 50µm.

also found at the ventricular surface on the dorsal and latero-ventral side, corresponding respectively to neurogenic niche II and III (Fig. 12C-D). However, niche II showed less PCNA⁺ cells compared to niche III. Triple labelling showed that the PCNA⁺ cells were mainly NGPs, while none of the CX43 expressing cells were PCNA⁺ (Fig. 12B-D). Cells that were PCNA⁺ and NGP⁻ were considered as RG3, as they are also known to proliferate (Fig. 12B-D).

4.1.2 The proliferation of neural progenitor cells declines with age

Since we expected that the proliferation rate would decline with age, we decided to investigate this via quantifying the number of proliferating cells within young and old killifish telencephali. Whole mount immunostaining for phospho-histone H3 (pHH3), a marker of mitosis, was performed on both young (6w-8w) and old (18w-20w) killifish brains. This showed a decrease in the absolute number of proliferating cells within the aged killifish telencephalon, although it was not significant ($p=0.56$) (Fig. 13B). To correct for the size of the telencephalon, we computed the depth of each telencephalon and calculated the number of pHH3⁺ cells per depth. However, this also showed no significant difference in pHH3⁺ cells between young and old killifish ($p=0.30$) (Fig. 13C). In both young and old telencephali, a cluster of pHH3⁺ cells was seen on the rostral side of the conjunction between the hemispheres, which corresponds to neurogenic niche I (Fig. 13D-E, video V1, video V2). At both ages, pHH3⁺ cells were also scattered on the dorsal side of the ventricular surface, known as neurogenic niche II, although the abundance seemed less in the old telencephalon (Fig. 13D-E, video V1, video V2). In the young killifish telencephalon, a cluster of pHH3⁺ cells was seen on the ventral side of the right hemisphere, corresponding to neurogenic niche III (Fig. 13D). This cluster of pHH3⁺ cells was not observed in the aged killifish telencephalon (Fig. 13E).

4.1.3 Effect of ageing on the spatial distribution of neural progenitor cells

To investigate the potential effect of age on the spatial distribution of the neural progenitor cells, we performed HCR v3.0 on sections of both 6- and 18-week-old killifish telencephali. We found that RG1 (CX43⁺ SLC1A2⁺), RG2 (CX43⁺ SLC1A2⁻) and RG4 (EPD⁺) cell populations were distributed the same in both young (6w) and aged (18w) killifish (Fig. S4-S5). However, while the NGPs were found in the three neurogenic niches in young killifish, less NGP⁺ cells were observed in old killifish. The latter contained only a small cluster of NGPs in niche I and only a few NGPs in niche III (Fig S6). In addition, whole mount analysis of the killifish telencephalon allowed us to investigate the spatial distribution of RG1 and RG2 and the effects of age in a three dimensional space. In both young (6w) and old (18w) killifish, whole mount analysis revealed the presence of RG1 (CX43⁺ SLC1A2⁺) on the ventricular surface, while a cluster of RG2 (CX43⁺ SLC1A2⁻) was present on the ventral side of each hemisphere and on the caudal side of the hemispheric conjunction (video V1, video V2).

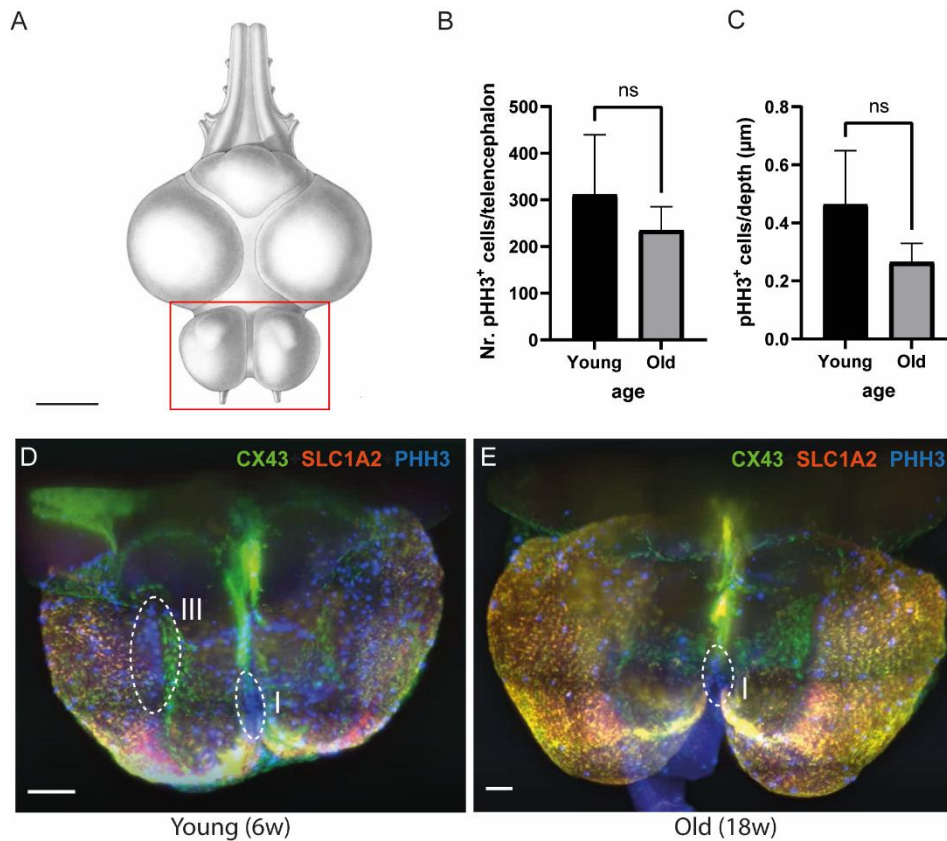


Figure 13. Proliferation of neural progenitor cells decreases with age. (A) Illustration of the dorsal view of the killifish brain. The red square indicates the telencephalon (adapted from D'Angelo, 2013). (B) The absolute number and (C) the fraction of pHH3+ cells within the telencephalon decreased in old (18w-20w, $n = 4$) killifish compared to young (6w-8w, $n = 3$) killifish, although not significant (unpaired t-test, $p = 0.56$, $p = 0.30$). Values are mean $\pm$ SEM. (D-E) Whole mount telencephalon of a 6-week-old (D) and 18-week-old (E) killifish, visualized for CX43 (green), SLC1A2 (red) and pHH3 (blue). The observed number of pHH3+ cells is higher in the young telencephalon (D) compared to the old telencephalon (E). In both the young and old telencephalon, a cluster of pHH3+ cells is found at the most rostral side of the conjunction between the hemispheres (niche I, dotted ellipse). (D) Within the young telencephalon, a cluster of pHH3+ cells is visible on the right hemisphere (niche III, dotted ellipse). (E) This cluster of pHH3+ cells is not observed in the old telencephalon. Scale bars: in (A): 200μm, (D-E): 100μm.

4.1.4 Cell types identified in telencephalon are present in distinct niches in other brain regions

To investigate to what extent the neural progenitor cell types populate different brain areas, we performed HCR v3.0 on sagittal sections of the entire killifish brain (Fig. 14). We were able to localize RG1 (CX43+ SLC1A2+), RG2 (CX43+ SLC1A2-) and RG4 (EPD+) in other regions of the brain, apart from the telencephalon. RG1 was found to be present in the midbrain (M), cerebellum (Ce), diencephalon (Di), medulla oblongata (MO), hypothalamus (H) and an undefined region located on the caudal side of the cerebellum (Fig. 14B). In the midbrain, RG1s were covering the entire surface of the periventricular grey zone (PGZ), which is located at the ventral side of the optic tectum (TeO) (Fig. 14B). Even though CX43+ SLC1A2+ cells were found in almost every region of the brain, we observed differences in gene expression levels. Expression of both genes was the highest in the MO and PGZ, and was similar to the expression level observed in the telencephalon (Fig. 14B). Other regions, such as the Ce and Di, showed lower expression levels of CX43 and SLC1A2 (Fig. 14B). The only cells that were

SLC1A2⁻, and thus considered to be RG2, were located at the most rostral part of the MO (Fig. 14B).

As expected, cells that were EPD⁺ were found within the tela choroidea (Fig. 14C). Because the tela choroidea was very hard to preserve on sections, not every region remained intact during the HCR v3.0 experiments. However, the parts of the tela choroidea that remained intact all showed EPD expression. This suggests that RG4 might be found in the entire tela choroidea that surrounds the brain, and not only the part that covers the telencephalon. Cells within the tela choroidea that were EPD⁺, were often also expressing CX43. This was expected based on the single-cell sequencing data, as this already revealed that RG4 also expressed CX43 in addition to the high expression of EPD.

We were also able to localize NGPs by performing HCR v3.0, using the same probe pool mix as before to label the mRNA of both STMN1A and HMGB2A. We combined this with a staining for PCNA to investigate whether NGPs are also the proliferating cell type within other regions of the brain. Double positive cells (NGP⁺ PCNA⁺) were observed in the Ce, in the caudal part of the PGZ and in the hypothalamus. More specifically within the cerebellum, dividing NGPs were found in a saw-like pattern within the molecular layer (ML), reflecting the internal structure of the ML. No CX43⁺ cells were observed to express PCNA, and all NGPs were PCNA⁺, which confirms that NGPs are the main proliferating cell type within the entire brain.

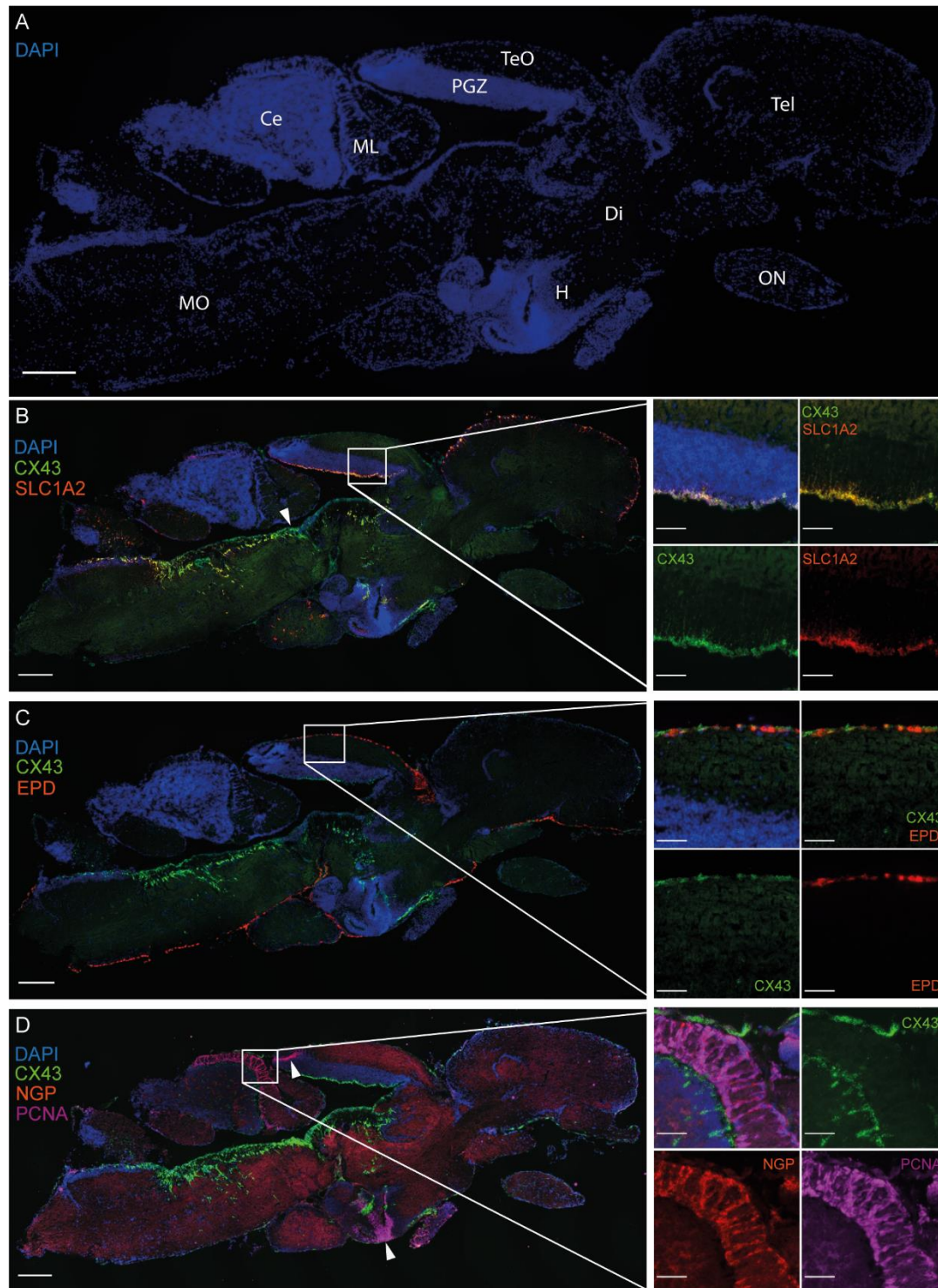


Figure 13. Cell types identified in telencephalon are also present in other brain regions. (A) Sagittal section of 6-week-old killifish brain stained with DAPI (blue). Tel = telencephalon, ON = optic nerve, Di = diencephalon, TeO = optic tectum, PGZ = periventricular grey zone, Ce = cerebellum, ML = molecular layer, MO = medulla oblongata, H = hypothalamus. (B) Sagittal section of the 6-week-old killifish brain visualizing CX43 (green) and SLC1A2 (red) expression with DAPI (blue). A more detailed view of the PGZ is provided on the right. Expression of CX43 is colocalized with SLC1A2 in the PGZ and the dorsal part of the MO, suggesting that RG1 is present within these regions. Expression of CX43 in the more rostral part of the MO suggests the presence of RG2 in this region (white arrow). (C) Sagittal section of the killifish brain visualizing CX43 (green) and EPD (red) expression with DAPI (blue). A more detailed view of the TeO is provided on the right. Expression of EPD is restricted to the tela choroidea. (D) Sagittal section of the killifish brain visualizing CX43 (green) and NGP markers STMN1A and HMGB2A (red) combined with a staining for PCNA (magenta). Cells that express NGP markers are also PCNA⁺, and are found in a saw-like pattern within the molecular layer of the cerebellum. White arrows indicate the two other proliferative regions, namely the caudal part of the PGZ and the hypothalamus. Scale bars: in (A-D): 200µm, right panel: 50µm.

4.2 Transgenic reporter lines

4.2.1 *Ubb:CreER^{T2}* can be integrated into the killifish genome

To establish the *ubb:CreER^{T2}* transgenic killifish, one-cell staged embryos were injected with the *CreER^{T2}* DNA construct and Tol2 transposase mRNA. The embryos were checked for successful microinjection after 24h, and we observed that the survival percentage was about 77% (Table 1). We found that 51% was expressing EGFP (Table 1). Non-surviving embryos were mostly the result of damage by the injection needle, abortive development or mold. The remaining embryos were then incubated for three weeks at 28°C until hatching. Despite the relatively high survival percentage after microinjection, many embryos died during these three weeks of incubation, which led to a mean survival percentage of only 6% until the golden-eye stage. However, once the embryos reached the golden-eye stage, their chances for survival drastically increased, as successful hatching took place in almost all of the cases. Genotyping of the *ubb:CreER^{T2}* F0 generation revealed that the *CreER^{T2}* gene was integrated in the killifish genome in 7 out of 19 individuals.

Table 1. The number of embryos that were able to survive after microinjection with the *CreER^{T2}* construct.

Batch	Total eggs injected	Survived 24h	GFP expression	Golden-eye	Hatched
1	102	79	65	2	2
2	114	81	63	19	17
3	103	84	35	0	0
Survival %	100%	77%	51%	6%	6%

4.2.2 Generation of a homozygous *actb2:GTR* killifish reporter line

In order to introduce the inducible Cre/Lox system in killifish, a stable homozygous killifish reporter line should be generated. A transgenic *actb2:GTR* F2 generation was already obtained by previous work in the lab. In this project, the focus was on generating a stable homozygous *actb2:GTR* line. To select which individuals of the F2 generation were homozygous, we backcrossed F2 fish with WT. In total, 10 males and 7 females of the F2 generation were backcrossed with WT. The eggs of each couple were then checked for fluorescence. Homozygous parents, recognized when 100% of the offspring was fluorescent, were selected and put in tanks for further inbreeding. We determined 5 individual F2 fish as homozygous (3 females and 2 males). Each homozygous F2 male was then housed with either one or two homozygous F2 females for further inbreeding. In this way, we were able to generate a homozygous *actb2:GTR* transgenic killifish line, which could be used to collect and store eggs for future experiments.

4.2.3 Transgenic *actb2:GTR* killifish mainly express GFP in neurons of the telencephalon

Next, we were interested in whether the *actb2:GTR* reporter fish expressed EGFP in all cells of the telencephalon. The inserted GTR switch gene is controlled by a regulatory sequence consisting of the *Xenopus* EF1alpha enhancer and the zebrafish *actb2* promoter. As the *actb2* promoter is known to be active in actin filaments within the cytoskeleton, we expected that EGFP would be expressed in all cells of the *actb2:GTR* reporter fish. However, we immunostained for EGFP and noticed that the expression was not present in all cells of the telencephalon. Instead, we observed that expression of EGFP was restricted to a small amount of neurons within the telencephalon. These EGFP expressing cells were often located at the ventricular surface. To investigate whether the EGFP was indeed present in neurons, we stained for HuCD, a commonly used pan-neuronal marker (Fig. 14A). Although the number of cells with endogenous EGFP expression was relatively low, we found that they overlapped with HuCD staining (Fig. 14B). This confirms that the *actb2:GTR* reporter line expressed EGFP in a subpopulation of neurons within the telencephalon.

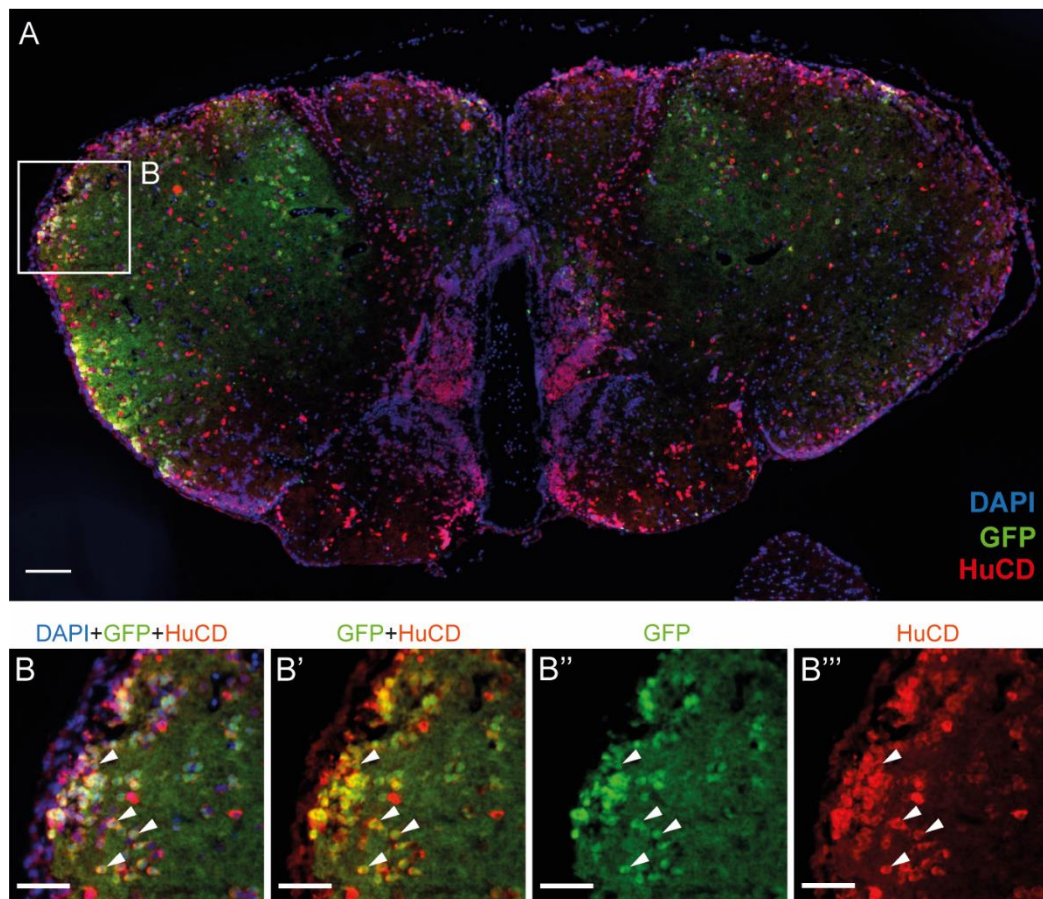


Figure 14. Transgenic *actb2:GTR* killifish mainly express EGFP in neurons of the telencephalon. (A) Coronal section of an adult killifish telencephalon which visualizes endogenous EGFP expression (green) combined with a staining for HuCD (red) and DAPI (blue). (B-B''') Cells expressing EGFP are often HuCD⁺, which suggests that the *actb2:GTR* mutant mainly expresses EGFP in neurons of the telencephalon (white arrows). Scale bars: in (A): 100µm, in (B-B'''): 50µm.

4.2.4 The GTR switch could not be established by tamoxifen treatment

As the GTR switch was already proven to work *in vitro* (Fig. S7), we now intended to validate the inducible Cre/Lox system *in vivo*. Therefore, we crossed the *CreER^{T2}*-driver and *actb2:GTR* reporter line to generate double mutants. Addition of tamoxifen would activate the *CreER^{T2}* recombinase which would result in the expression of red fluorescence in all cells (Fig. 15A). To induce this switch to red fluorescence, 12 *CreER^{T2}*; *actb2:GTR* double mutant larvae were placed overnight in tamoxifen solution. Only 5 larvae were able to survive after the tamoxifen treatment. Both the living and the dead larvae were checked for EGFP or RFP (mCherry) expression at 2 days and 8 days after the treatment. At both timepoints, EGFP was clearly expressed in the entire larvae, both living and dead, while RFP expression was not observed in any of the individuals. To investigate the presence of *CreER^{T2}* recombinase or RFP in more detail, we sacrificed the 5 remaining double mutants at 4 weeks of age and stained for Cre recombinase or RFP (Fig. 15B-E). We observed that each of the 5 double mutants showed endogenous EGFP expression, mainly in neurons of the telencephalon and muscle fibres (Fig. 15D). However, no signal was observed for Cre recombinase or RFP staining (Fig. 15E). None of the double mutants could thus be used to validate the inducible Cre/Lox system of the transgenic killifish reporter lines.

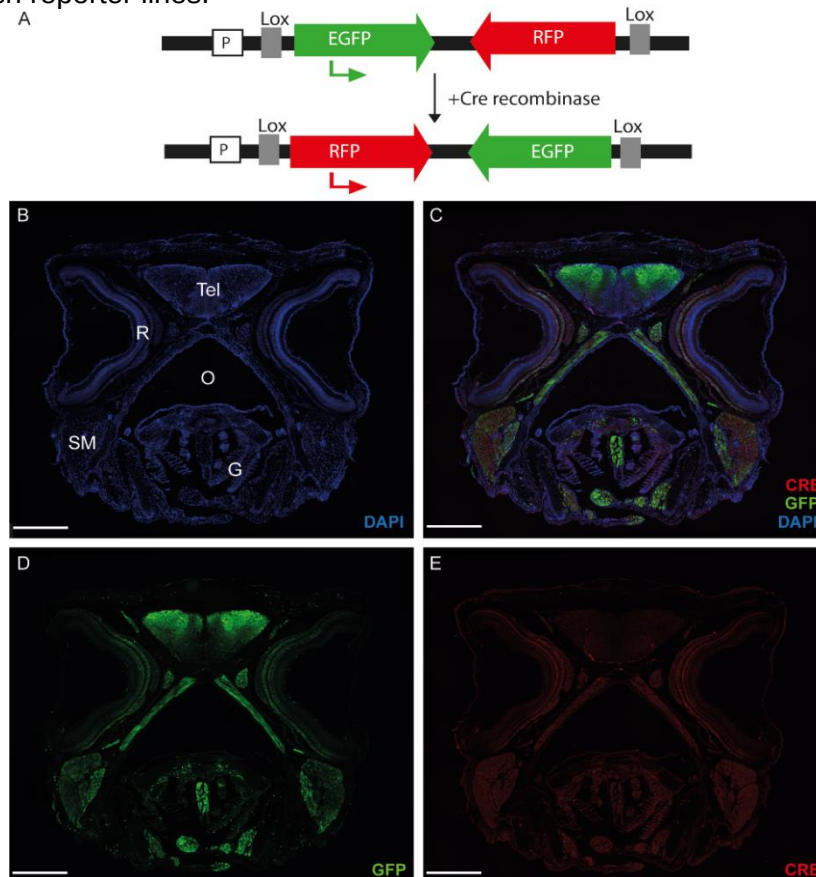


Figure 15. Expression of Cre recombinase was not observed in any of the tamoxifen treated larvae. (A) Simplified scheme of the Cre/Lox system. (B) Coronal section of an entire killifish head stained with DAPI (blue). Tel = telencephalon, R = retina, O = oral cavity, SM = skeletal muscle, G = gills. (C-E) Coronal sections of entire killifish head, visualized for endogenous EGFP expression (green) (D) and stained for *CreER^{T2}*-recombinase (red) (E) and DAPI (blue).

5. Discussion

5.1 Characterisation of neurogenic niches

5.1.1 Three neurogenic niches can be found in the adult killifish telencephalon

In this study, we aimed to gain a better understanding of how neural progenitor cells are distributed within the brain of adult African turquoise killifish. Based on previous work by Tozzini et al. (2012), we expected that there would be three distinct neurogenic niches (I-III) within the killifish telencephalon. Niche I should be located at the conjunction between the two telencephalic hemispheres, while niche II and III would be present on the dorsal and ventrocaudal side of the pallial ventricular surface, respectively (Tozzini et al., 2012). Tozzini et al. (2012) also described that niche I was the most proliferative region, while niche II was the least proliferative. Niche III was found to have a proliferation rate in between niche I and II (Tozzini et al., 2012). To investigate the distribution of proliferating cells, we performed an immunostaining for PCNA on sections of adult killifish telencephali, and for pHH3 on whole mount killifish brains. Our results indeed confirmed the presence of three distinct neurogenic regions within the adult killifish telencephalon. We found a high proliferative region between the two telencephalic hemispheres (niche I), a less proliferative region on the ventral side of the ventricular surface (niche III) and a region with the least proliferation on the dorsal side of the ventricular surface (niche II).

Generally in teleost fish, neurogenic niches within the brain are known to be exceptionally conserved (reviewed in Ganz & Brand, 2016). In zebrafish for instance, a cluster of tightly packed proliferating cells is present along the ventricular surface of the conjunction between the two hemispheres (Vv) (Ganz et al., 2010). This cluster of proliferating cells can be compared to neurogenic niche I in killifish, and is thought to correspond with the subventricular zone (SVZ) in mammals. The dorsal telencephalon in zebrafish contains small clusters of proliferating cells scattered along the entire ventricular surface, although distinct niches similar to killifish (niche II-III) have not been described yet (Ganz et al., 2010). These proliferating cells along the ventricular surface in teleosts are thought to correspond with the subgranular zone (SGZ) in mammals.

In humans and sheep, the SVZ is characterized by a high number of proliferating cells while the SGZ has a rather low proliferation rate (Curtis et al., 2005; Low et al., 2013). This would suggest that the SVZ in mammals, which also corresponds to the highly proliferative Vv in teleosts, is usually characterized by the highest proliferation rate of the brain. However, rodents are characterized by a high proliferation rate in both the SVZ and SGZ. This indicates that the proliferation rate of each region in the brain can differ between species, and also indicates the importance of studying these neurogenic regions in multiple species.

5.1.2 Neural progenitor subtypes are located in distinct niches

To investigate whether the differences in proliferation between niches might depend on the distribution of neural progenitor cells within that niche, we determined the location of each neural progenitor cell type. By optimization of HCR v3.0 for specific marker genes, based on transcriptome data provided by Ayana et al. (2021), we were able to identify the location of the NGPs and the RG subtypes (RG1-RG4). We optimized HCR v3.0 specifically for NGPs (STMN1A/HMGB2A⁺), RG1 (CX43⁺ SLC1A2⁺) and RG4 (EPD⁺), while RG2 (CX43⁺ SLC1A2⁻) and RG3 (SOX2⁺ CX43⁻ NGP⁻) could be localized via exclusion.

As NGPs are known to be the main proliferating cell type in the adult killifish brain, we expected these cells to be highly abundant within the three neurogenic niches (I-III) of the telencephalon (Coolen et al., 2020; Van houcke et al., 2021). We find that most NGPs are indeed located within the three neurogenic niches (I-III), although some NGPs were scattered around on the ventricular surface between niche II and III. Double labelling for NGP markers and PCNA confirmed that NGPs are the main proliferating cell type within the killifish telencephalon. This is different from zebrafish, where adult neurogenesis is mainly driven by dividing radial glia or neuroepithelial-like progenitors (Ganz et al., 2010).

We observed that neurogenic niche I contained the highest number of NGPs, while niche III contained fewer, and niche II even less NGPs. This correlates with the overall number of proliferating cells in each niche, as described above. We therefore suggest that the capacity for proliferation might depend on the abundance of NGPs within a particular niche, although more research is certainly needed to confirm this hypothesis. This also raises the question whether the second type of neural progenitor cells, namely the RGs, would also have different abundances in each niche.

In niche I, which contained the most proliferating NGPs, we observed that RG1 was present at the rostral side of the hemispheric conjunction, while a cluster of RG2 was located at the caudal side. We also noticed a dorsoventral pattern, with CX43⁺ cells located at the dorsal part of niche I, while NGPs were located in the centre, and RG3 were located at the most ventral area. The less proliferative regions, namely niche II and III, were both located on the pallial ventricular surface. We found that RG1 and RG3 were covering the entire pallial ventricular surface, and that the abundances of RG1 and RG3 were very similar between niche II and III, even though both niches contained a different number of NGPs.

A difference in spatial organisation between RG subtypes might suggest that they have distinct functions. By exploring the transcriptome of adult killifish neural progenitor cells, Ayana et al. (2021) suggested that RG1 (CX43⁺, SLC1A2⁺) and RG2 (CX43⁺, SLC1A2⁻) could possibly have astrocytic functions in the killifish, since both cell types expressed common markers for mature astrocytes in mammals (e.g. CX43, SLC1A2, SCARA3 and S100A10).

SLC1A2 (GLT1) encodes a protein which is predicted to be involved in the activation of the transmembrane L-glutamate transporter. This might explain why the fluorescent signal is visible in the RG1 fibres, as the mRNA of SLC1A2 is translocated to the fibres for maintaining synaptic signalling in neurons. In mammals, GLT1 is known to be a high-affinity glutamate transporter, found in astrocytes of the adult brain. CX43 (GJA1) is a member of the connexin gene family and encodes a protein that is a component in gap junctions. In mammals, CX43 is the primary component of astrocytic gap junctions in the adult brain.

Astrocytes in mammals have been well studied, and are known to rely on their typical star-shaped morphology with many fine branches to interact with other glial cells, blood vessels or neurons in the CNS. However, it has remained controversial whether true astrocytes can be found within the teleost brain or not. Chen et al. (2020) were the first to visualize cells with dense cellular processes, similar to astrocyte morphology in mammals, in both the zebrafish brain and spinal cord. Although they observed a different morphology between astrocytes in the spinal cord and radial astrocytes in the hindbrain, the expression of molecular markers was similar in both cell types, which suggests functional similarity (Chen et al., 2020). Radial astrocytes in the hindbrain were found to have a long process between the cell body on the surface and the fine branches in the parenchyma (Chen et al., 2020). Although we did not investigate the RG1 and RG2 morphology in such detail, we observed a typical radial glia morphology, with cell bodies located at the ventricular surface and a main process extending into the parenchyma. Previous research by Coolen et al. (2020) investigated the morphology of both RGs and NGPs in the killifish telencephalon. They found that the morphology of both cell types was relatively similar, but that RGs were much more branched. This is consistent with the idea that RG1 and RG2 may have astrocytic functions, as this would require many fine branches to interact with other cell types in the CNS. How astrocytic RGs might function in killifish, and how similar this is to mammals remains a topic for further investigation.

In addition, Ayana et al. (2021) proposed that RG3s could be neuroepithelial-like progenitors, based on their expression of typical neuroepithelial markers (e.g. KRT18, SDC4 and HES1). In mammals, neuroepithelial cells are the main neural stem cells during embryonic neurogenesis (Götz and Huttner, 2005). This is different in adult mammals, where neuroepithelial cells only reside in the SVZ and SGZ, which results in a very limited number of regions that can contribute to adult neurogenesis. In zebrafish, neuroepithelial-like progenitors are, together with RGs, the main proliferating cell type in the brain (Ganz et al., 2010). Recent single-cell transcriptomics revealed 9 subtypes of neural progenitor cells (PC 0 - PC 8) in the zebrafish telencephalon, of which PC 6 and PC 8 expressed neuroepithelial-like markers (e.g. KRT8) (Cosacak et al., 2019). These neuroepithelial-like progenitors were restricted to the ventral nucleus of the subpallium (Vv) (Cosacak et al., 2019). This is different from killifish, where we also found RG3s at the pallial ventricular surface. Ayana et al. (2021) suggested that the neuroepithelial-like RG3 cell population in killifish might give rise to NGPs, which in

their turn proliferate and form the other RG subtypes. Therefore, we expected to find RG3 in the same regions as NGPs. As we find that both NGPs and RG3s are present in all three neurogenic niches (I-III), the hypothesis that RG3 could be the origin of the NGP cell line is reinforced.

The only progenitor cell subtype that was not present in one of the neurogenic niches, was RG4. This cell type is known to have high expression of apolipoproteins (e.g. APOA1, APOA2, CLU/APOJ and APOEB) and EPD, a gene encoding for ependymin (Ayana et al., 2021). Because ependymin is a glycoprotein found in the cerebrospinal fluid (CSF) of vertebrates, the high expression levels found in RG4 suggest that this progenitor cell subtype is possibly ependymal cell-like. Generally in vertebrates, ependymal cells are glial cells that form a neuroepithelium lining the ventricular surface in the brain and spinal canal. Indeed, our results confirmed that the expression of EPD in the killifish telencephalon was restricted to the tela choroidea, a thin cell layer that surrounds the ventricle.

In other vertebrates, ependymal cells are known to contain cilia to maintain circulation of the CSF within the ventricles. In zebrafish in particular, single-cell transcriptomics suggested that PC 7 are ciliated ependymal cells in the zebrafish telencephalon (Cosacak et al., 2019). To the contrary, Ayana et al. (2021) proposed that the RG4 population within the killifish telencephalon are non-ciliated ependymal cells, as they lacked expression of ciliogenesis-associated genes. However, more research should be done to conclude whether RG4 are ciliated or non-ciliated ependymal cells.

5.1.3 The effect of age on the proliferation of neural progenitor cells

Because previous studies described an age-related decrease in proliferating cells in the optic tectum and dorsal telencephalon of killifish, we were curious to find whether this could be due to changes in the distribution of neural progenitor cells (Tozzini et al., 2012; Van houcke et al., 2021). To investigate the effect of ageing on the proliferation and distribution of neural progenitor cells, we optimized HCR v3.0 and immunohistochemistry on whole mount killifish telencephali. Consistent with these previous studies, we noticed a decrease in the absolute number of proliferating (pHH3⁺) cells as a result of ageing, although it was not significant ($p=0.56$). Similar to our observations on sections stained for PCNA, we could distinguish the three neurogenic niches (I-III) within the killifish telencephalon, based on the abundance of pHH3⁺ cells. Keeping in mind that the number of pHH3⁺ cells would be about 8 times lower compared to PCNA⁺, as pHH3 stains the relatively short M-phase (~1h), while PCNA stains the long lasting S-phase (~8h). We observed a decrease in the number of proliferating cells in all three neurogenic niches (I-III). We also noticed that niche I remained the most proliferative region in the old killifish telencephalon, while niche II and III drastically decreased in proliferation. We then investigated whether this decrease could be due to a reduction of the number of NGPs within a niche. When investigating the effect of age on the abundance of

NGPs, we observed less NGPs in the old killifish. However, this might be due to a decreased expression of the selected marker genes (STMN1A and HMGB2A). STMN1A is predicted to regulate tubulin polymerisation and depolymerisation, which are known to play a central role in mitosis. As the proliferation of NGPs is shown to decrease with age (Van houce et al., 2021), this might be correlated with decreased expression of STMN1A. HMGB2, the human ortholog of HMGB2A, was identified as one of the key genes involved in cellular senescence in human fibroblasts (Aird et al., 2016). It was found that the expression of HMGB2 drastically decreased due to ageing (Aird et al., 2016), which might explain why we see a decreased expression of HMGB2A in NGPs of aged fish. Therefore, we cannot draw any conclusions about the effects of ageing on the distribution of NGPs within the killifish telencephalon.

Overall, we found more proliferating cells within the young, and thus relatively small, killifish brain, compared to the old and thus larger one. Because of this size difference between young and old brains, we expected that our results would become significant if we would include this difference. Therefore, we normalized the absolute number of proliferating cells for the depth of each telencephalon. This however did also not result in a significant difference between young and old ($p=0.30$). We assume that our results were influenced by the small sample size and the manual quantifications. A larger sample size combined with quantification software might have resulted in a significant difference of the number of proliferating cells between young and old killifish telencephali. In future experiments, it may also be more accurate to adjust the number of proliferating cells for the entire area and volume of the telencephalon, rather than just for depth.

In addition, the age categories (young and old) in our research contained fish that were not exactly the same age. Young fish were ranging from 6 weeks to 8 weeks, while old fish were ranging from 18 weeks to 20 weeks, which might have led to variation within each age category. We noticed a decrease in the number of pHH3⁺ cells in the 8 week-old compared to 6-week-old killifish. However, this decreasing trend was not observed between 18-week and 20-week-old individuals. One batch of both young and aged telencephali also had less pHH3⁺ cells compared to the other batches. This can be due to a poor immunostaining, although the protocol and used concentrations were identical. In future experiments, using fish of exactly the same age will give more accurate results, as well as performing the experiment in one batch instead of multiple. We also noticed that the probes or antibodies were not penetrating very deeply into the tissue. This was not an issue when studying these progenitor cells, because they are located on the ventricular surface. However, if future research wants to focus on other cell types, optimization and standardisation of HCR v3.0 on whole mount killifish brains will be necessary.

5.1.4 Neural progenitor subtypes are also present in other regions of the killifish brain

In this research, we also investigated the presence of neural progenitor cells and adult neurogenic niches in other regions of the killifish brain. We identified neurogenic niches within the killifish brain based on the proliferative status via PCNA labelling. Similar to other teleosts, three main neurogenic regions were found in addition to the dorsal and ventral telencephalon that we discussed earlier. We found a cluster of proliferating cells in the periventricular grey zone (PGZ), the hypothalamus and the molecular layer of the cerebellum.

The PGZ is the first and most ventral layer of the optic tectum. It is known to contain the majority of tectal neurons and to be the main player in integrating spatial aspects received by visual or other sensory inputs. We found a cluster of highly proliferative NGPs at the most caudal part of the PGZ. This region has been referred to before as neurogenic niche V and VI by Tozzini et al. (2012). We found a layer of RG1 covering the entire surface of the tectal ventricle, also known as the deep layer of the PGZ. The presence of RG1s in a region that contains many neurons is reasonable, since RG1 is suggested to have astrocytic functions and can therefore be expected in close proximity with neurons. Studies in zebrafish described very similar distributions of proliferative and non-proliferative cells. In the adult zebrafish brain, proliferating neuroepithelial-like progenitors are present in a crescent-shaped zone at the caudal part of the PGZ, corresponding to niche V and VI in killifish (Ito et al., 2010). In zebrafish, radial glia were also found in the deep layer of the PGZ, and these radial glia were found to be non-proliferative, which is also the case in killifish (Ito et al., 2010). It is unclear whether this layer of radial glia in the zebrafish PGZ could have astrocytic characteristics, as found in killifish (Ayana et al., 2021; Ito et al., 2010).

The second cluster of proliferating cells was found in the killifish hypothalamus, which is consistent with findings by Tozzini et al. (2012). Within these proliferating cells, we found mainly proliferating NGPs, but proliferating neuroepithelial-like RG3 were also present. The fact that these two cell types coexist in the same niche again reinforces the idea that RG3 may be the origin of the NGP cell lineage. We also found some RG1s that were lining the killifish hypothalamus. However, more detailed investigations of the killifish hypothalamus are needed to understand the different subdivisions and cell types present. In zebrafish, the hypothalamus contains proliferating RGs instead of the neuroepithelial-like progenitors that are found in the PGZ (reviewed in Ganz & Brand, 2016). This is similar to mammals, where radial glia-like cells are found to slowly proliferate in the adult hypothalamus (reviewed in Yoo & Blackshaw, 2019). The third region of the killifish brain where proliferating NGPs were present, was the molecular layer of the cerebellum. Tozzini et al. (2012) already described this neurogenic niche in the adult killifish brain and referred to it as niche VII. The neurogenic niche of the cerebellum has been studied extensively in zebrafish, and was found to contain neuroepithelial-like progenitors instead of RGs (Kaslin et al., 2009). In contrast, mammals are lacking proliferating cells in the

adult cerebellum (Ming & Song, 2011). More research on this cerebellar neurogenic niche of teleosts is necessary to get insight in why this niche has lost its proliferative ability in adult mammals.

In addition to the presence of neural progenitors in these three neurogenic regions, we also noticed a cluster of RG1s lining the medulla oblongata, and a small cluster of RG2s at the most rostral part of the medulla oblongata. As the medulla oblongata serves as a connection centre between neurons of the brain and spinal cord, RG1s and RG2s are again located in close proximity with neurons, which is expected if they would have astrocytic functions.

Finally, RG4 was also detected in the tela choroidea surrounding other brain regions. This confirms that the entire tela choroidea contains these ependymoglia-like RG4, and not only the part that surrounds the telencephalon.

5.2 Transgenic killifish reporter lines

To create a foundation for detailed cell lineage tracing experiments in the future, we wanted to introduce and optimize the inducible Cre/Lox system in killifish. Therefore, we generated transgenic Cre-driver killifish, together with green-to-red (GTR) reporter killifish. The Cre-driver line is regulated by the general *ubb* (ubiquitin B) zebrafish promoter. This was expected to result in the expression of the gene of interest, namely *CreER^{T2}*, in every cell of the killifish. The GTR reporter line is regulated by a combination of the *Xenopus EF1-alpha* (elongation factor 1 alpha) promoter and the zebrafish *actb2* (actin beta 2) promoter, which was expected to drive general expression of the GTR switch gene in every cell of the killifish.

In order to validate the Cre/Lox system *in vivo*, we crossed both transgenic lines to generate *ubb:CreER^{T2};actb2:GTR* double mutants. We expected to induce a switch from green to red fluorescence after administration of tamoxifen, as *CreER^{T2}* recombinase would then become activated, enter the cell nucleus and cause an inversion of the GTR switch gene. Since the GTR-switch gene was already validated in an *in vitro* experiment, we assumed that the GTR gene construct would also switch *in vivo*. However, we were not able to detect red fluorescence in any of the *ubb:CreER^{T2};actb2:GTR* double mutants after administration of tamoxifen. This might be due to a variety of reasons.

First of all, when exploring the expression pattern of the *actb2:GTR* fish via immunostaining, we observed that EGFP expression was not ubiquitous, but restricted to neurons in the telencephalon and certain muscles. We ran into the same problem with the *ubb:creER^{T2}* transgenic fish, as an immunostaining for *CreER^{T2}* recombinase on the double mutants revealed that the enzyme was not present in any of the tissues. However, this can be due to the fact that the protocol for the *CreER^{T2}* recombinase immunostaining was not yet optimized for killifish brains. In addition, the double mutants were the result of a homozygous *actb2:GTR* male and a heterozygous *ubb:CreER^{T2}* female. This resulted in only 50% of the offspring

having received the *CreER^{T2}* gene. This, in combination with a small sample size, drastically reduced the chance for finding a double mutant larvae that would successfully switch from green to red fluorescence. Even though each of the *ubb:CreER^{T2}* females were genotyped before breeding, it could be that the primers that we used for the PCR showed non-specific binding and therefore false positive females were used for breeding.

Both the Cre-driver line and the GTR reporter line were generated via the Tol2 transposase system. Although Tol2 transposase is known to be a very efficient transgenesis tool in fish, the random integration site might result in unwanted silencing by the upstream region (Harel et al., 2016; Kawakami, 2007; Mukherjee & Liao, 2018; Valenzano et al., 2011). One can avoid this in future experiments by using targeted gene strategies, such as CRISPR-cas9, to make sure that the upstream region allows a ubiquitous expression pattern of the inserted gene.

Another reason for the non-ubiquitous expression of both transgenes, can be the fact that we used zebrafish and *Xenopus* promoters. Several studies showed that promoters still showed the same expression pattern in phylogenetically distant species (Ruvinsky & Ruvkun, 2003; Wang et al., 2007; Yoshinari et al., 2012). Therefore, we expected that the zebrafish and *Xenopus* promoters would also drive ubiquitous expression of the *CreER^{T2}* and GTR switch gene. Using a general promoter that is endogenous to killifish may result in expression of the transgenes in every cell of the killifish.

The overnight tamoxifen treatment should also be optimized for future experiments. In this experiment, we treated larvae of 8 days post-hatching overnight with 0.5µM tamoxifen. Only 5 out of 12 embryos survived the overnight treatment, which indicates that either the concentration or duration of the experiment should be lowered. Depending on whether you want to study killifish embryos, adults or aged individuals, the tamoxifen concentration and the duration of the experiment should be optimized. Low doses of tamoxifen (0.05 µM) allow mosaic labelling of single cells, while high doses (5 µM) allow for high RFP expression (Hans et al., 2009). In addition, several experiments with Cre-controlled mutagenesis in zebrafish did not use tamoxifen, but its active metabolite 4-hydroxytamoxifen (4-OHT) to induce conditional gene activation (Hans et al., 2021; Mukherjee & Liao, 2018; Than-Trong et al., 2020). Tamoxifen first has to be metabolized in the liver, which takes about 6 to 12 hours in zebrafish (Hans et al., 2009). As it is unknown how killifish metabolize tamoxifen, it could be more efficient to directly treat the fish with 4-OHT in future experiments.

6. Conclusion and future prospects

In this research, we identified the location of the four radial glia subtypes (RG1-RG4) and non-glial progenitors (NGPs) within the killifish brain. We found that, within the killifish telencephalon, RG1-RG3 were located in distinct niches on the ventricular surface, while RG4 was present in the tela choroidea. We were able to optimize HCR v3.0 to visualize RG1, RG4 and the NGPs specifically, while we could localize RG2 and RG3 via exclusion. Specific HCR v3.0 markers for RG2 and RG3 are not optimized yet, but will certainly be beneficial for future research. We found that the NGPs were located on the ventricular surface as well, and that they mainly proliferate within three neurogenic niches in the telencephalon. Other proliferative regions in the killifish brain were the cerebellum, hypothalamus and periventricular grey zone of the optic tectum. We also noticed that the number of proliferating cells decreases in old killifish telencephali, which confirms previous studies. It would be interesting to quantify the number of proliferating cells within each neurogenic niche, and investigate how this is affected by ageing.

Recently, NGPs were described as the main proliferative cell type in killifish, however, it still remains uncertain what the functions of the RG subtypes are. As we found that the four RG subtypes are present in distinct niches, we expect that they might have distinct functions as well. It might be that heterogeneity within the neural progenitor cell population reflects differences in regenerative abilities and brain plasticity between cell types. However, this remains a topic for future research. To unravel the function of each RG subtype, one could generate killifish mutants with knock outs for specific RG subtypes and investigate their phenotype. Lineage tracing analysis with RG subtype specific lines will also be interesting to reveal the origin, migration route or progeny of each progenitor subtype. In order to perform such detailed lineage tracing in the future, we tried to introduce the inducible Cre/Lox system in killifish. We managed to generate a heterozygous *ubb:creER^{T2}* driver line and a homozygous *actb2:GTR* reporter line. However, none of the *ubb:creER^{T2}; actb2:GTR* double mutant larvae showed a switch from green to red fluorescence upon administration of tamoxifen. Experiments in the near future should focus on optimizing the concentration of tamoxifen and duration of the treatment. It will also be interesting to investigate the potential difference between treatments with tamoxifen versus 4-hydroxytamoxifen. In addition, optimization of CRISPR-cas9 based transgenesis in killifish will certainly be useful to target specific genes for the generation of specific killifish reporter lines. In this way, it would be possible to follow specific RG subtypes or NGPs and investigate the effects of ageing or disease on the distribution or function of these neural progenitor subtypes. This would be a great step for understanding the mechanisms of adult neurogenesis in the killifish brain, which has the potential to elucidate new therapies against age-related neurodegenerative diseases in humans.

7. References

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8. Addendum

8.1 Risk assessment

General precautions for working in the lab are wearing a lab coat and protective gloves at any time. When entering the killifish facility, clean gloves and a separate lab coat should be worn, in order to protect yourself against pathogenic bacteria that can be present in the system water. Shoe covers are also obliged, to make sure hazardous material does not enter the facility via contaminated footwear. Separate lab coats and clean gloves should also be worn in L2 labs, where experimental cell lines are used, to prevent biological contamination.

To protect yourself from physical risks, all hazardous material should be handled with caution and machines should always be used as instructed by the manufacturer. When dissecting fish or microinjecting embryos, special caution should be taken with sharp objects (e.g. surgical blades or injection needles). If tissue or other material is stored in a freezer of -80°C, always wear gloves and sleeves that cover your entire arm to prevent frostbites. When using the cryostat, one should be very careful when handling the microtome cutting blade. The cryostat should always be locked when it is not used, to prevent other people from having accidents. Furthermore, when using ultraviolet radiation (e.g. for GelRed® visualisation), make sure the lid of the transilluminator is always closed when turning the UV-light on. This prevents damage to the cornea of the eye, and also makes sure that your skin is not exposed to UV-light for too long. When using fluorescence microscopes, never look into the laser as this can also damage your cornea. Also make sure that the laser had time to cool down before turning it on again. Lastly, centrifuges should always be balanced before using them.

In addition to physical risks, one should also avoid chemical dangers. A 4% PFA solution was used to fixate fish or tissues. PFA (paraformaldehyde) is dangerous when inhaled or taken in via the mouth. It is also suspected to be carcinogenic. Some buffers used in HCR v3.0 contained formamide, which is known to be hazardous when inhaled or taken in via the mouth. It is suggested to cause reproductive damage and may also harm the unborn child. Therefore, working under the hood and wearing a FFP2 mask against organic solvents is strongly recommended when working with both PFA and formamide. GelRed® was used to visualize DNA after gel electrophoresis. Although GelRed® is considered to be safer than ethidium bromide, it is still recommended to be extremely careful when dealing with this chemical. Material that was in contact with the gel electrophoresis bench, should not be taken back into the lab due to possible contamination with GelRed®. To test the inducible Cre/Lox system, fish were treated with tamoxifen, a hazardous drug which is known to be carcinogenic in humans. Wearing a lab coat, gloves and working under the hood is therefore strongly recommended. Fish that were treated with tamoxifen were washed separately with fresh system water for 4 times, to prevent tamoxifen to contaminate the ZebTEC aquarium system. System water that was contaminated with tamoxifen was discarded in a separate waste category. For whole

mount analysis, methanol was used to dehydrate or rehydrate the brains before HCR v3.0. Methanol is known to be flammable, and can be dangerous when inhaled, taken in via the mouth or when it comes in contact with the skin. Therefore, working under the hood is necessary when handling methanol.

In general, chemical or biological waste should always be discarded in the correct waste category. Specialized containers are present for glass capillaries or other sharps (e.g. contaminated injection needles, glass cover slips). Leftover fish that were treated with 4% PFA should be discarded separately from other dead fish.

8.2 Supplementary material

Table S1. Nucleotide sequences of HCR probes

B1_STMN1A	GAGGAGGGCAGCAAACGGaaAATATTTGTTTTCTGGCGTTGATA
B1_STMN1A	TTGCCTGCGACAACATAAAATAAATtaGAAGAGTCTTCCTTTACG
B1_STMN1A	GAGGAGGGCAGCAAACGGaaGAACTTCTCGTTGAGAGCCGCCATC
B1_STMN1A	ACATCATTAGGAGCTCACCTTCTCtaGAAGAGTCTTCCTTTACG
B1_STMN1A	GAGGAGGGCAGCAAACGGaaATCTTCTGATTGAGCTTCTCTTGTG
B1_STMN1A	GCGCTGCGGTTCTCTTTGTTGCCTtaGAAGAGTCTTCCTTTACG
B1_STMN1A	GAGGAGGGCAGCAAACGGaaTGGCCTTCTGGATGACCTCCTTCTC
B1_STMN1A	GTTTGCTAAAGTTGCTGTTTTCTCtaGAAGAGTCTTCCTTTACG
B1_STMN1A	GAGGAGGGCAGCAAACGGaaAAACCTGTAAGCTGCATTTGTTTCC
B1_STMN1A	AAGGAATTACATTAGAGCGAAAGTtaGAAGAGTCTTCCTTTACG
B1_STMN1A	GAGGAGGGCAGCAAACGGaaCCTGTTGCGGGACAGCAGGAAGTCC
B1_STMN1A	AATCTCATCCAGTGATGTTTCCTTtaGAAGAGTCTTCCTTTACG
B1_STMN1A	GAGGAGGGCAGCAAACGGaaATGACCTCGAACGCCTGGCCTGATG
B1_STMN1A	TTAGCATCGGGGGTGGAGGGGCTCtaGAAGAGTCTTCCTTTACG
B1_STMN1A	GAGGAGGGCAGCAAACGGaaCTGGAAACAGGAAACTTCTTTAGTC
B1_STMN1A	GCTTGTTCACTCTTCACCTGAATtaGAAGAGTCTTCCTTTACG
B1_STMN1A	GAGGAGGGCAGCAAACGGaaTCATGGACCATCTCAGCTATGTTCA
B1_STMN1A	AACCAAGTATTAGAGAGGACTAAAGtaGAAGAGTCTTCCTTTACG
B1_STMN1A	GAGGAGGGCAGCAAACGGaaCAAATGATCGGTGTAAGTCTAGAAA
B1_STMN1A	ATGTTTGTGTATGAACAGCTGATCtaGAAGAGTCTTCCTTTACG
B1_STMN1A	GAGGAGGGCAGCAAACGGaaTGATGTAAACGGATACTTTACACGT
B1_STMN1A	ATCAAAAACACCAATTTGCAGTAAAtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaGACAAGCGTGCACTCCGCAGCAGAG
B1_slc1a2	TTATTCGTGTTTCAACGGTTCCTCtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaTCTACTAAGATAGAATTGTTAATTG
B1_slc1a2	TTCGTGGCTGAGGTTACCTTGCAATTtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaAGCTCATCCAGCTCAGCTTTGAAA
B1_slc1a2	TCATCAGATTTAGCCATGTGCGCGTtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaCGCCCACCACATTACCGAGGTCCT
B1_slc1a2	GGTACACGATTCCAGCTCCGTAGGAtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaGTCACCAGCCCGCGCTGGGAATAC
B1_slc1a2	CCGACAGCAGTCAGAATCAACAGCAtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaGCGCCGACAGGGAGCACAAAACGAG
B1_slc1a2	AAAGCAGTTCCGTCCATGTTTATGGtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaATAGCAAAGTAGATGCTGGGCAGGA
B1_slc1a2	AAGAAAGTGAAGGGATTTTTCTGGtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaGGAGATGATCTTACCACAAATCAGA
B1_slc1a2	CCTTGCTACAACCTCCAAGTCTGGtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaACCATGATGACAATTTTCATCACA
B1_slc1a2	GCAATGCCAAAGGGAGAGTACCACAtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaACCAATCAGACCCAAAACATTCATG
B1_slc1a2	CATGCAGATGCCAATGCAATAAAGtaGAAGAGTCTTCCTTTACG

B1_slc1a2	GAGGAGGGCAGCAAACGGaaATGATCTGAGGCTCCTTGGTCATGT
B1_slc1a2	CTCTTGAAGTGAAGGGACTTTTTGAtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaGAAACAAGCTTGACACCAGATTTTCT
B1_slc1a2	TTTCTTTGTGACTGTTTGGATCTGTaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaGCATCCAAGCTAGACACATCCTCAT
B1_slc1a2	AACAGATTTCTGATCAGATCGAAGAtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaGTTTGGGGTTGCCCGGGTGAATGAC
B1_slc1a2	CCTGGCCTTCGCCCAGATTCTCCTTtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaAATAACAGTAGTTGTCATGTAGTAG
B1_slc1a2	CACCAGAATGACTCCCAGAACAGCAtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaGGATCAACATTTTCAGCATCCTCAT
B1_slc1a2	TGATTAACTGGAGATGATGAGTGGtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaGACAATATCTGGATGGATTGGAGAA
B1_slc1a2	GATGTCTCCAGGAAAGGCGATCACctaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaCCCAAGATCACACCGAGGACGGTGA
B1_slc1a2	ACACGAAGCAACATCCCTGCCACAGtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaCTCGTGCATCCTCACCTCCACTTGT
B1_slc1a2	CCTGGCTTCGATGGGCTCCAGGTGGtaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaAACCTCCAGACTTTGGTAAGTGAAA
B1_slc1a2	TCCTGGAGGAGATCAGATTGGTGActaGAAGAGTCTTCCTTTACG
B1_slc1a2	GAGGAGGGCAGCAAACGGaaTCACAAAAGCTCCATCCAGAACTCT
B1_slc1a2	AATTCGAGAACAAAGTGTCAGCTGtaGAAGAGTCTTCCTTTACG
B3_CX43	GTCCCTGCCTCTATATCTttGTGCTGGCCAGCCGGCTCTGCTCA
B3_CX43	GGAGTTAGAAATAGTGCTTCCTGCAttCCAACCACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTttGTAACTGGGGGGGACATAGGTGA
B3_CX43	CGGTCCCCCTCTCCCCGTGGCGAGttCCAACCACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTttCCGCTGGCGTAGAGCGCGGGCTGCT
B3_CX43	TCTTTGGGAGTGGGACTCAACGTTctCCAACCACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTttTAAAGAACACGTAGAAGAGCTCGAT
B3_CX43	TCCCTTCACGCGGTCTTAATCCTttCCAACCACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTttAGCCGTACATGTACCACTGGATCAC
B3_CX43	CACACGTGTAGACGGCCGACAGGCTttCCAACCACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTttGAAGATGCTGACTACGTAGGTTCTG
B3_CX43	GAAGCCAACCTCAAACAGGGATTTGttCCAACCACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTttCTTCTGCTCCTTCTGTGCAGGTAG
B3_CX43	CTTAAGCTCCTCCTCCTTGCGGTTGttCCAACCACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTttATTTGTCGTAGCAGACGTTCTCGCA
B3_CX43	AGAAGCGAACGTGGGAGATAGGGAAttCCAACCACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTttAAGATGAAGAGGACCGAAAGCCAGA
B3_CX43	ACGGCCGTCCCCAGCACCAGGATCctCCAACCACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTttGATGTTCTTCAGAGCTCTGAACCTG
B3_CX43	GGCGAGTTTTCTAAAATCAAGCGTttCCAACCACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTttCCTAGACCCATAAAGTCGGTCCTGT
B3_CX43	TTCAGCACATTTAAACATCGTTTCTttCCAACCACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTttGCCATGCTTTAATCTGGGATCCATC
B3_CX43	ACAGCTCGGCTGACAGAACGCCGTGttCCAACCACTTTAACCCG

B3_CX43	GTCCCTGCCTCTATATCTtAAAAACAGCAGGAAAGTGGAGCTCGT
B3_CX43	ACGCATCAGATCAGGACACCTCAACtCCACTCAACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTtTGAATTTAAGATTTAAGACACAGAT
B3_CX43	GGCCAGCTGGGTAAAAGTTTCGGTtCCACTCAACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTtACGCAGCTGAGACCTAAAGATCCTG
B3_CX43	TTTGAAAAGTATCATTAACAGTAAACtCCACTCAACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTtGAAACCAGAGCCAGGTCACCTTCAG
B3_CX43	AGAGTCGTCATCAGAGCAGGACCTtCCACTCAACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTtTCACCGGCCGTGTTGTCCACGGATC
B3_CX43	CGTAGGGCTGGTCTCCACACCGGAtCCACTCAACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTtATTCATCTGAGTTCTTCTGTAGTTT
B3_CX43	ACAGCCCGTAAACCCGGCGTTGGCTtCCACTCAACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTtTCCTGATCTGTACCTGCGAGCGTGT
B3_CX43	CTGGTTTCTGCTGCGGAGCTGAATtCCACTCAACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTtGCGACCCGTTCCGTTACTCTGTCAAG
B3_CX43	CTGTACAGAGGCACAGCAGCCGGTtCCACTCAACTTTAACCCG
B3_CX43	GTCCCTGCCTCTATATCTtGAACAGGGAGCCGGTTCTGTAAAG
B3_CX43	CTGTTATCCGCGTGTGGGACCGGAGtCCACTCAACTTTAACCCG
B4_HMGB2A	CCTCAACCTACCTCCAACaaTCGCCAAGCTTCTTGGAACATCTC
B4_HMGB2A	TCTTTGGAGCCTTGTGTGGACCACAaTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaTGCGGGGACGGTGGTCGGAACAGAA
B4_HMGB2A	TGGAGATTCCAGGGTCTCTTCCTTatTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaGTTTTATCTCCCGATCATATCGAA
B4_HMGB2A	TTCTTGGCACCTTTAGGAGGGACGTatTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaTCACCTTCTCCTTGGCTGACATGGT
B4_HMGB2A	TGTCAGTCTTGGCCATCTCATCAAAaTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaCTCTGCAAAGTTCACGCTGGTTCCT
B4_HMGB2A	CCATCTTTCGGAACATTTCTTGGAGatTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaTAACAAAATGTAAAAAAGAAGA
B4_HMGB2A	GGCTGCGAATTATCTAACAACCGGAaTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaCAACTAAATTCTGTGTTAATTGGGG
B4_HMGB2A	AAAGAGCTGCGCGAGACTAGTAAAAaTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaGACTCCATTTGTATCTTCCCGCCT
B4_HMGB2A	TTCTGCCTCTCCGGGACAAAAAGACatTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaACTGAACCTAAACACAAATTACAACA
B4_HMGB2A	CGCCATCTGCGCCGAAACTTCCAATatTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaATAGTATGAACGCGACGGAAACGAA
B4_HMGB2A	TTCAAATATCCGCGATGAAACTCTTatTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaCATCAGAACGTCGGTATTTGAATG
B4_HMGB2A	CACAACCGATCGAACTGCTTCAAAAaTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaATTAAATCGATTATATGCAACGTTG
B4_HMGB2A	AACATTAGCAAACCTACTTATTACCCatTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaCAACTTAACTGTGTCTATATAATTA
B4_HMGB2A	TTTATCACGTAGGCAAAAAAAGTCAaTCTCACCATATTCGCTTC
B4_HMGB2A	CCTCAACCTACCTCCAACaaAAACAGTATTTCTTACCTGTTCTC
B4_HMGB2A	GCTTAAAAACGCGGAGTTATTGATatTCTCACCATATTCGCTTC

B4_EPD	CCTCAACCTACCTCCAACaaTACTGTCCCCTCAGGAGTCTCCTCC
B4_EPD	CTACATGAACTCATTGAGGAACTGatTCTCACCATATTCGCTTC
B4_EPD	CCTCAACCTACCTCCAACaaACAAGGAGATCTGGATCCTTGACCT
B4_EPD	GGCTTTCCATGACAAAATGAGGGAAatTCTCACCATATTCGCTTC
B4_EPD	CCTCAACCTACCTCCAACaaATGAGGTGGTTTCAGTGAAATAGAA
B4_EPD	GTTCAACATCCGTGTTGCTGAAATAatTCTCACCATATTCGCTTC
B4_EPD	CCTCAACCTACCTCCAACaaAGACATGGAGTAGGAGCCTTTCATG
B4_EPD	GCTGACAGGCAAACATCCCATAGTTatTCTCACCATATTCGCTTC
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B4_EPD	CCTCAACCTACCTCCAACaaACAGCTCTGGTTTTTGCTGTCAATC
B4_EPD	CAAGTCCATTTGCAGTTTCTTTTCatTCTCACCATATTCGCTTC
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B4_EPD	TAAAAAATCCCCTCTTCAAAGAGCAatTCTCACCATATTCGCTTC
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B4_EPD	CGGGGTGGCTCTCGTTGGATCTGAAatTCTCACCATATTCGCTTC
B4_EPD	CCTCAACCTACCTCCAACaaCTTTAGGGTCATCACTCGCATTAAT
B4_EPD	GAATGCACCGACTGCTCTCATCTCAatTCTCACCATATTCGCTTC
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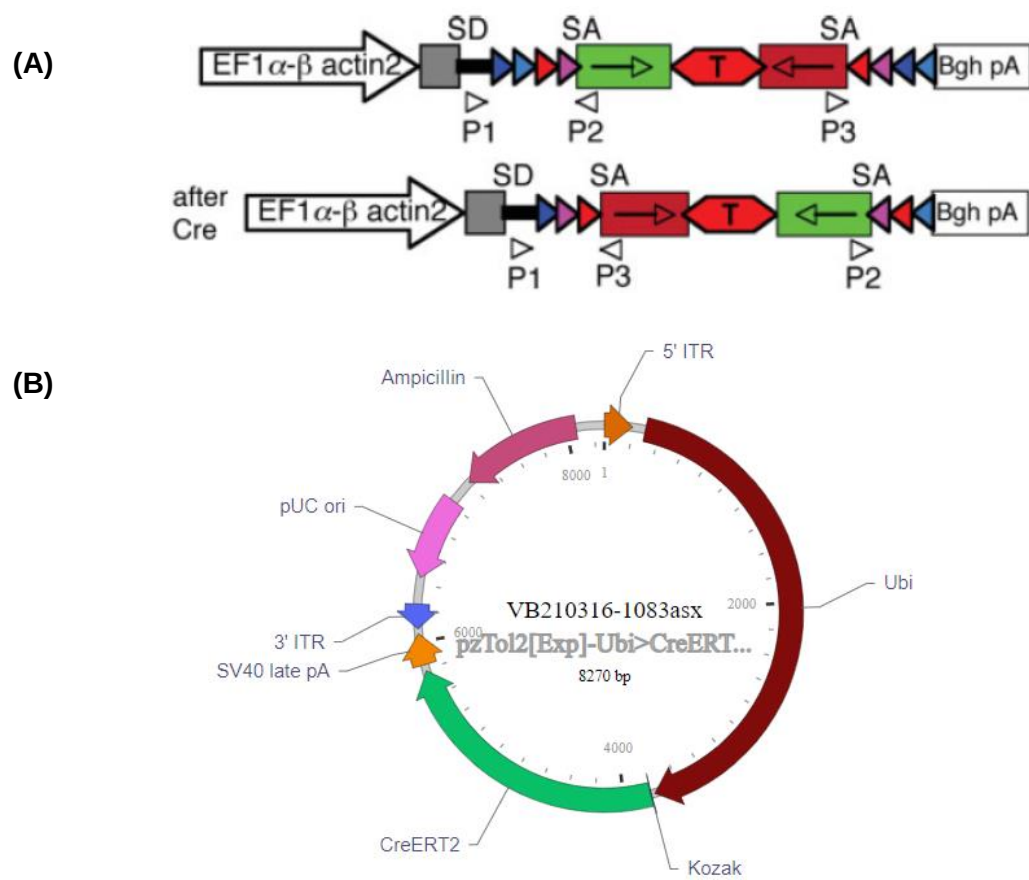


Figure S2. Overview of the injected DNA constructs to create transgenic killifish. (A) GTR switch gene under the control of the *Xenopus* EF1 α and zebrafish *actb2* promoters. (B) CreERT2 recombinase under the control of the ubiquitous zebrafish promoter *ubb*.

Table S3. Nucleotide sequence of primers used to amplify fragments of the CreERT2 gene

	Forward (5' -> 3')	Reverse (5' -> 3')
<i>CreERT²</i>	GTCCACTGTCAGCCATTGTT	AATCCCCAGAAATGCCAGAT

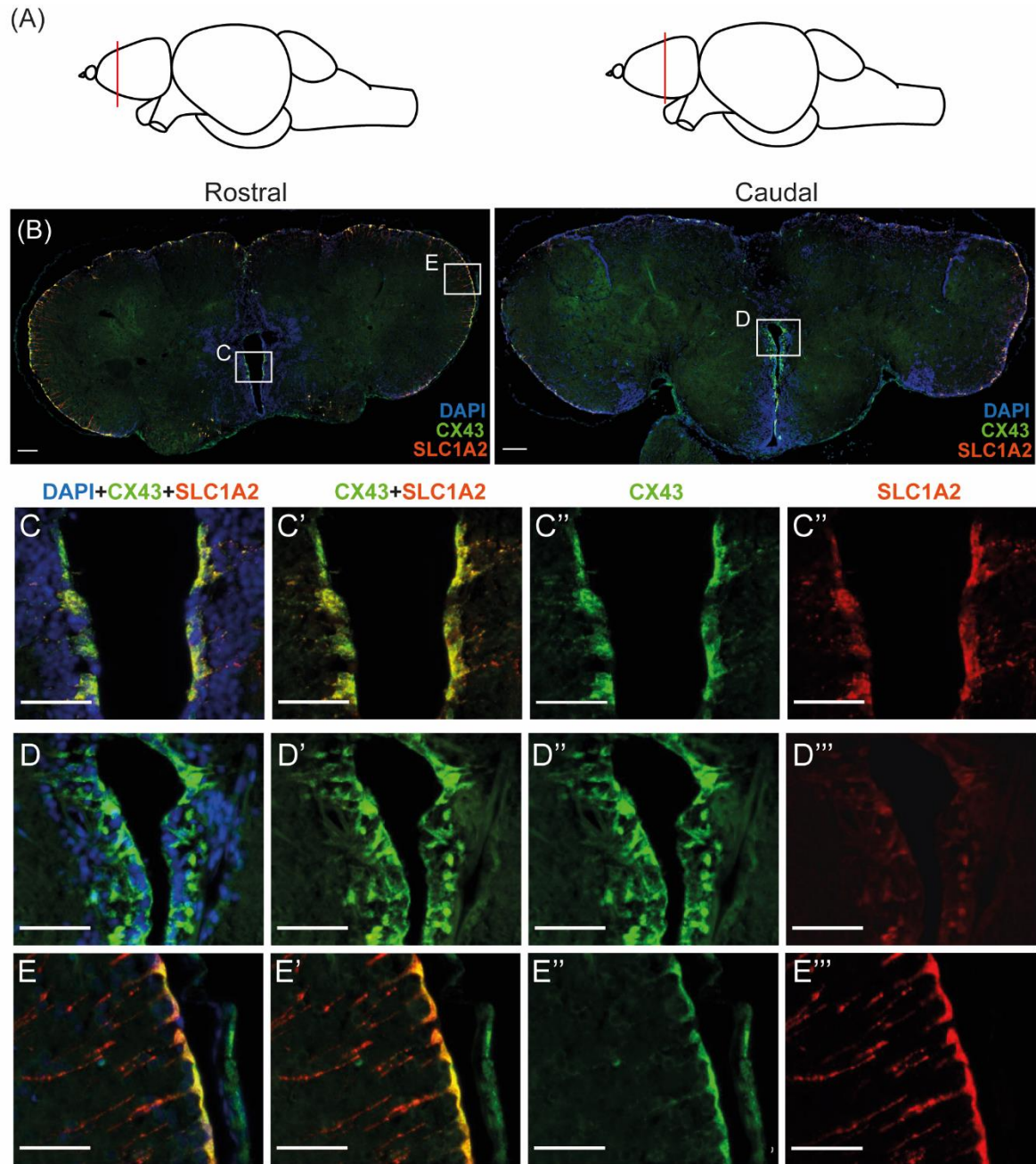


Figure S4. The location of RG1 and RG2 does not change with age. (A) Schematic overview of the killifish brain from the left lateral side. The red lines indicate the location within the telencephalon where the sections were taken. Sections were selected more rostrally (left) or caudally (right). (B) Coronal sections of 18-week-old killifish telencephalon visualizing the expression of CX43 (green) and SLC1A2 (red) with DAPI (blue). (C-E) At both the rostral and caudal sides of the conjunction between the hemispheres, and the ventricular surface, expression of CX43 and SLC1A2 is similar to 6-week-old killifish. This suggests that the location of RG1 and RG2 remains the same in old killifish. Scale bars: in (B-C): 100 μ m, in (D-F): 50 μ m.

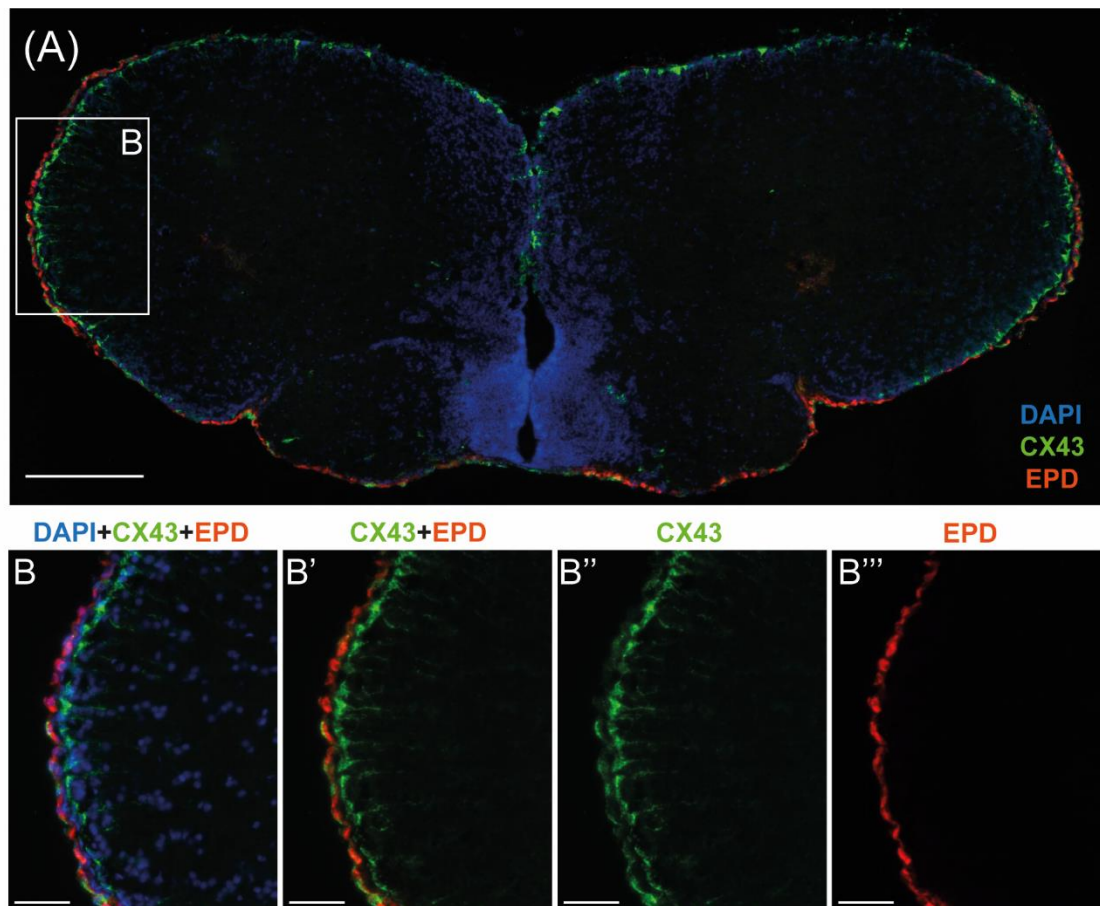


Figure S5. RG4 is located within the tela choroidea, also in old killifish (A) Coronal section of a 18-week-old killifish telencephalon visualizing the expression of CX43 (green) and EPD (red) with DAPI (blue). EPD is expressed specifically in the tela choroidea, which is similar to our observations in young killifish (B-B''') Higher magnification of the tela choroidea, showing the specific expression of EPD within the tela choroidea. Scale bars: in (A): 100 μ m, in B-B''': 50 μ m,

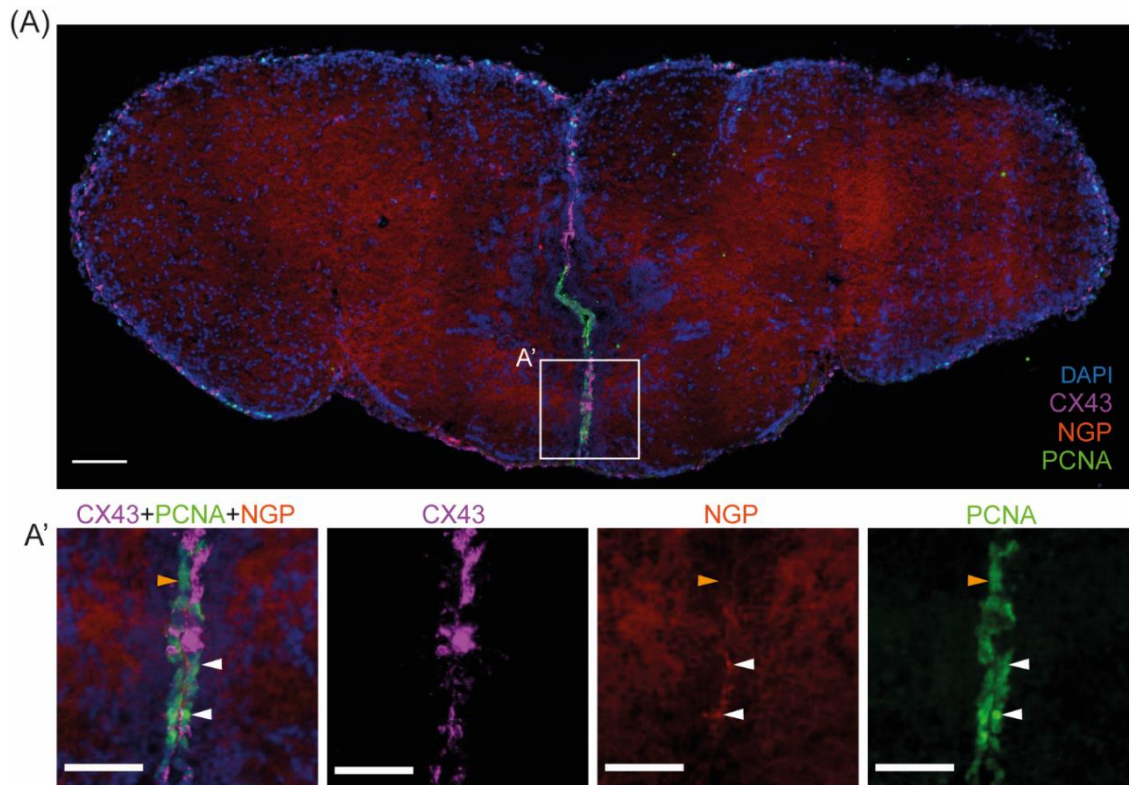


Figure S6. The signal of the NGP probe mix is not very clear in old killifish. (A) Coronal section of an 18-week-old killifish telencephalon, visualizing NGP probe mix (red), CX43 (magenta), PCNA (green) with DAPI (blue). (A') NGP positive cells were only visible at the conjunction between the hemispheres. PCNA signal was found in all three neurogenic niches.

Supplementary Video V1 and V2 can be found at

https://kuleuven-my.sharepoint.com/:f/g/personal/ine_jacobs1_student_kuleuven_be/Er2g6c3gCzJEU132FxyBKdAB-YYQ7mTQ6ZzoVDd4zGnTRg?e=bp7qqX

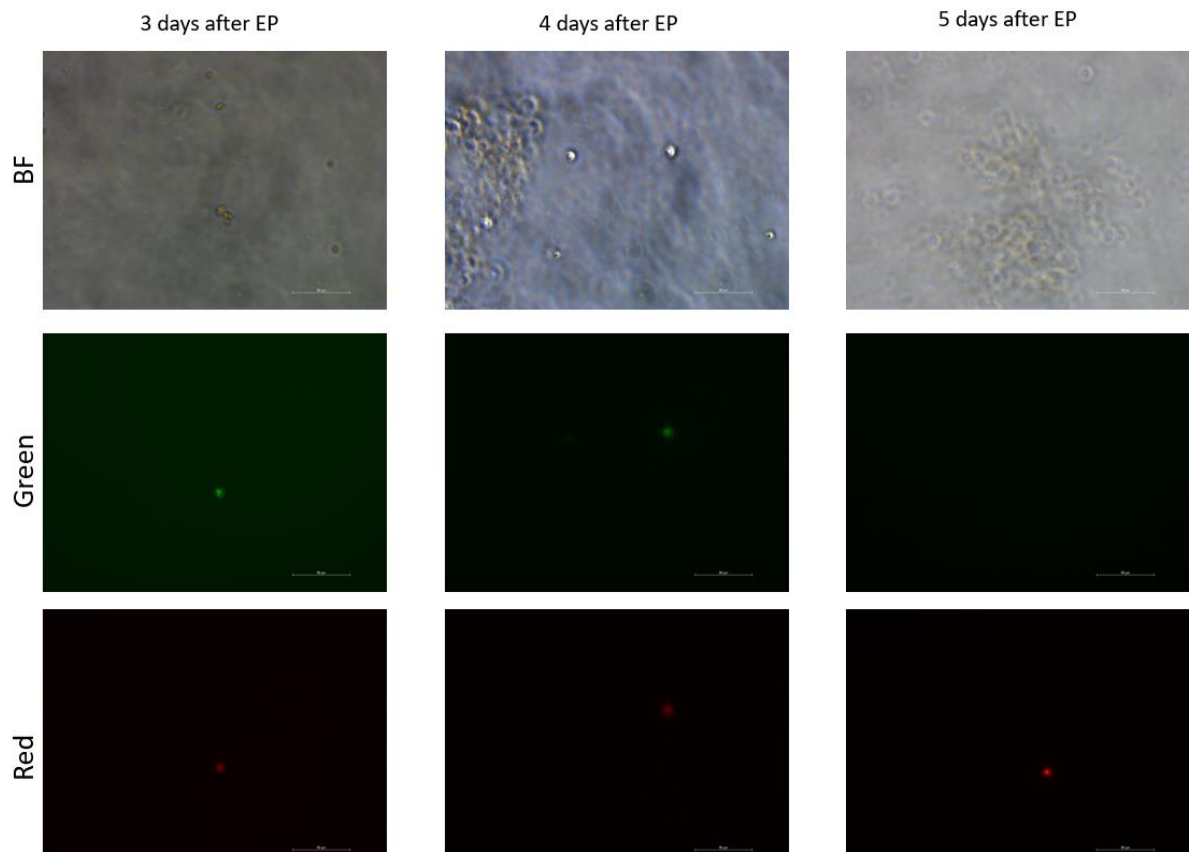


Figure S7. Results of a previous experiment with *in vitro* electroporation (EP) of pCAGGS-Cre vector in a killifish brain cell suspension. This experiment validated the GTR switch *in vitro*, as one cell completely switched to red fluorescence after 5 days, without any leftovers of green fluorescent signal.

Table S8. Absolute number of pHH3+ cells and depth for each telencephalon that was used for the experiment.

Sample	Age	pHH3+	Depth (μm)	pHH3+/depth
1	6w	523	650	0.80
2	6w	80	515	0.16
3	8w	331	770	0.43
4	18w	182	921	0.20
5	18w	353	803	0.44
6	18w	126	916	0.14
7	20w	280	991	0.28

8.2.1 Buffer recipes for HCR v3.0

Probe hybridization buffer (-20°C)

30% formamide
5x SSC
9 mM citric acid pH 6
0,1% Tween 20
50 ug/ml heparin
1 x Denhardts solution
10% dextran solution

12 ml formamide
10 ml of 20 x SSC
360 ul 1 M citric acid pH 6
400 ul of 10% Tween 20
200 ul of 10 mg/ml heparin
800 ul of 50x Denhardt's solution
8 ml of 50% dextran sulfate
Fill up to 40 ml with ultrapure H2O

Probe wash buffer (-20°C)

30% formamide
5x SSC
9 mM citric acid pH 6
0.1% Tween 20
50 ug/ml heparin

120 ml formamide
100 ml of 20 x SSC
3,6 ml of 1 M citric acid pH 6
4 ml of 10% Tween 20
2 ml of 10 mg/ml heparin (4°C)
Fill up to 400 ml with ultrapure H2O (170,4 mls)

Amplification buffer (4°C)

5 x SSC
0,1% Tween 20
10% Dextran sulfate

10 ml of 20 x SSC
400 ul of 10% Tween 20
8 ml of 50% Dextran sulfate
Fill up to 40 ml with ultrapure H2O

5x SSCT (RT)

5x SSC
0,1% Tween 20

250 ml of 20 x SSC
10 ml of 10% Tween 20
Fill up to 1L with ultrapure H2O (740 ml)

50% Dextran sulfate (-20°C)

20 g of dextran sulfate powder
Fill up to 40 ml with ultrapure H2O.

10 mg/ml Heparin (4°C)

500 mg heparin
Fill up to 50 ml with ultrapure H2O.
Filter 0.2 uM and store at 4°C.

1M citric acid pH 6

10.5 g in 30 mls

Fill up to 50 ml

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