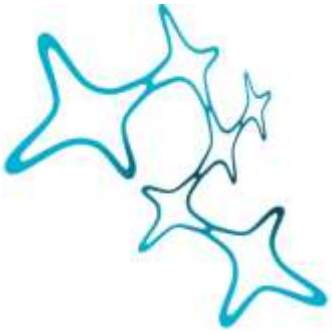
The Role of Neural Stem/Progenitor Cells in Mediating the Functional Recovery of Adult Zebrafish Brain After Traumatic Injury

Dissertation der Graduate School of Systemic Neurosciences der Ludwig-Maximilians-Universität München



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Abstract

The discovery of adult neural stem cells (NSCs) in humans raised hopes that they could be used in regenerative therapies to replace neurons which are lost upon neurodegenerative conditions, such as stroke or brain injuries. Despite a long-standing interest in adult NSCs and their putative application in regenerative therapies, little is known about their contribution in functionally replacing the neurons which are lost upon damage. In this study, the advantage of adult zebrafish widespread neurogenesis, coupled with the astounding regenerative capacity and knowledge of the circuitry mediating the optokinetic reflex (OKR) in larval zebrafish, were used to study the contribution of adult neural stem/progenitor cells in functionally replacing the lost neurons

In a first set of experiments, I demonstrated that OKR is a behaviour that can be reliably quantified in adult zebrafish and it is asymmetric. Afterwards, I mapped in adult zebrafish the pretectal neurons responsible for processing the horizontal OKR and established an injury model to kill them. Injury of the pretectal neurons in the right hemisphere resulted in the reduction of the OKR gain during CW stimulation and did not affect the OKR gain during CCW stimulation.

To investigate the capacity of adult zebrafish to recover an impaired behaviour, continuous measurements of the OKR gain were performed. This revealed that after this type of injury adult zebrafish can recover the OKR gain during CW stimulation at 13 days post injury (dpi) at the same levels as before injury, however not all animals recovered their OKR gain. To dissect the cellular basis for the observed behavioural recovery, I first tested the role of the uninjured hemisphere in mediating the recovery. Toward this aim, left eye enucleation experiments were performed to inhibit any retinal input in the injured hemisphere. This experiment suggested that the contralateral hemisphere does not mediate the OKR gain recovery during CW stimulation after injury.

Neural stem/progenitor cells located close to pretectal neurons reacted after injury by proliferating and generating neurons. Interference with their proliferation resulted in reduced neurogenesis and reduced the number of animals that recover an OKR gain, suggesting that the neurons generated by the neural stem/progenitor cells have a fundamental role in mediating the recovery after injury.

In conclusion, this work revealed for the first time that the adult zebrafish can functionally regenerate its brain after traumatic brain injury and shed light on the cellular components mediating this recovery during CW stimulation. Additionally, this work provides a vertebrate model where the cellular and molecular mechanisms which promote and inhibit brain functional regeneration can be studied.

1 Introduction

Adult neurogenesis is the process by which neural stem cells (NSCs) residing in restricted areas (stem cell niches) of the adult brain give rise to functional neurons which integrate into existing circuitries (reviewed in (Gage, 2019; Obernier and Alvarez-Buylla, 2019)). The discovery of adult neurogenesis also in humans (Boldrini et al., 2018; Eriksson et al., 1998; Ernst et al., 2014) raised hopes for using the adult NSCs as a therapy to replace neurons which are lost in response to injury, stroke or neurodegenerative conditions. To do so, the cellular and molecular mechanisms that direct the neuronal migration from neurogenic niches to the injury site, as well as the neuronal survival and integration into the existing circuitry, need to be better understood. In this regard, the main topic to be addressed in this thesis is the functional integration of newly generated neurons after stab wound injury in the brain of the adult zebrafish (Danio rerio).

In the following sections, the brain innate mechanisms to counteract injury-or disease-induced neuronal loss and the advances made so far to use the adult NSC in brain repair will be discussed.

1.1 Brain plasticity

Brain plasticity is the ability of the brain to reorganize neuronal connections and functions in response to intrinsic or extrinsic stimuli (reviewed in (Caleo, 2015; Grade and Götz, 2017; Murphy and Corbett, 2009)). Continuous remodeling of neuronal connections and cortical maps happens in response to adult neurogenesis, new experiences, acute neuronal loss such as traumatic brain injury (TBI) and stroke, or progressive neuronal loss such as Parkinson's disease (PD), Huntington disease (HD) and Alzheimer's disease (AD) (Quadrato et al., 2014). The mechanisms of brain plasticity in response to adult neurogenesis, during both acute and progressive neuronal loss will be discussed in the following sections.

1.1.1 Brain plasticity in response to neurodegenerative conditions

In neurodegenerative conditions associated with primary neuronal loss, a substantial amount of neuronal network restructuring takes place before the disease is

fully manifested, which reduces functional impairments or even masks the disease. PD is usually manifested only when 80% and 90% of the substantia nigra pars compacta (SNc) neurons projecting to the caudate nucleus and putamen, respectively, are lost (Hornykiewicz and Kish, 1987). The increase in the synthesis and release of dopamine (DA) from the spared SNc dopaminergic terminals mainly counts for this compensation (Zigmond et al., 1990). Similarly, the symptoms of HD are noticeable only when 95% of the GABAergic medium spiny projection neurons in the striatum are lost. In people at genetic risk for AD the magnitude and the extent of brain activation is increased during memory-retrieval tasks in regions affected by the disease, as compared to controls (Bookheimer et al., 2002).

1.1.2 Brain plasticity in response to acute neuronal loss

Small strokes and lesions are often followed by good recovery of function experimentally and clinically (Cramer, 2018). The recovery of functions after stroke is mostly confined to a 'critical period' extending for few months in humans and about a month in rodents (Cramer, 2008). The destruction of neural networks stimulates the reorganization of the remaining circuits, and this rewiring is thought to mediate functional recovery. These plastic phenomena involve the perilesional tissue in the injured hemisphere, but also the contralateral hemisphere, subcortical areas, and spinal cord (Alia et al., 2017; Nudo, 2013).

1.1.2.1 Brain plasticity from the contralesional hemisphere

Patient and rodent animal studies have shown that contralesional neuronal components and connections appear to be altered as a result of unilateral cortical damage (Dancause et al., 2015; Jones TA., 1999; Papadopoulos et al., 2006). Functional magnetic resonance imaging (fMRI) and positron-emission tomography (PET) studies in patients have reported an enhanced activity of the contralesional hemisphere 10 days post injury followed by an increased activity in the perilesional areas (3-6 months), in parallel with functional improvements (Marshall et al., 2000; Ward et al., 2003). Likewise, functional imaging studies in a middle cerebral artery occlusion (MCAO) rat model (which leads to functional sensorimotor impairments of the forelimb) have found that the activity of the healthy hemisphere was enhanced in the acute stage after stroke (when sensorimotor deficits are evident), followed by activation of the perilesional areas at later stages during the recovery phase. What was

striking from this report is that some of the areas activated in the healthy hemisphere are not normally involved in the sensorimotor function of the forelimb. One possible explanation of these results is that the contralateral healthy hemisphere may temporarily vicariate the function and then subsequent recovery depends on the relocalization of lost function in the infarct periphery.

Moreover, structural rearrangements at the level of individual neurons and of whole circuits have been observed after stroke in the somatosensory cortex of mice (Gonzalez et al., 2004; Takatsuru et al., 2009). In particular, a transient, localized increase in the dendritic fungiform (mushroom) spines, in a time period limited to 1 week post stroke was observed.

The above-mentioned studies demonstrate an involvement of the uninjured hemisphere after ischemic unilateral injuries. However, whether the uninjured hemisphere has a positive or a negative influence on functional recovery is highly debated (Murase et al., 2004; Di Pino et al., 2014). Approaches such as repetitive transcranial magnetic stimulation (rTMS) in stroke patients, to either enhance or abolish activity in the perilesional or contralesional areas have shown that in subjects with large unilateral lesions, lost functions are partly relocalized to the unaffected side (Grefkes and Ward, 2014; Di Pino et al., 2014). In those cases, lowering the excitation in the contralesional hemispheres dampens the functional restoration. In contrast, following minor strokes the healthy hemisphere appears to exhibit an excessive inhibitory role to the perilesional areas, thereby interfering with the plasticity and hampering the recovery of the forelimb control (Murase et al., 2004).

Similar results were also reproducible in rodent animal models of stroke. The effect of the contralesional hemisphere in mediating the recovery after unilateral brain damage strongly depends on the size of the infarct (Biernaskie et al., 2005). The contralesional hemisphere has a beneficial role in vicariating function and restitution of motor abilities following extensive damage to one hemisphere and a detrimental one in minor strokes (those that affect 5-15 % of the hemisphere) (Barry et al., 2014; Biernaskie et al., 2005; Mansoori et al., 2014).

1.1.2.2 Brain plasticity from the perilesional areas

Perilesional areas play a fundamental role in the recovery of function after minor strokes. FMRI and transcranial magnetic stimulation (TMS) studies have shown the reorganization of topographical maps in the cortex of patients after cortical stroke (Cicinelli et al., 1997; Liepert et al., 1998). Similar results have also been observed in animal models of stroke by using intracortical microstimulations (ICMS) (Alia et al., 2016; Nishibe et al., 2010; Nudo and Milliken, 1996) or optogenetic approaches (Harrison et al., 2013). Peri-infarct tissue that has a similar function vicariates lost or damaged functions (Harrison et al., 2013; Murphy and Corbett, 2009; Nudo and Milliken, 1996). For example, destruction of the mouse sensory cortex of the forelimb by targeted stroke can cause a new sensory representation to emerge in the territory normally occupied by forelimb motor cortex (Figure 1.1) (Harrison et al., 2013). Starkey and colleagues (Starkey et al., 2012) also showed another striking finding on how cortical areas reorganize at the subcortical level after ischemic injury to the rat forelimb motor cortex area. In this case the spontaneous recovery of forelimb function was correlated with hindlimb corticospinal neurons changing their target, anatomically and functionally, to the cervical, forelimb-related, spinal cord.

Despite these innate mechanisms of the brain to heal itself after small injuries, it largely fails to do so after extensive injuries where the brain accumulates dysfunction and patients acquire disabilities (reviewed in (Grade and Götz, 2017)). The discovery of human adult neurogenesis provides hope among scientists that it could be modulated to mediate the replacement of the lost neurons and the recovery of lost function after prominent brain injuries.

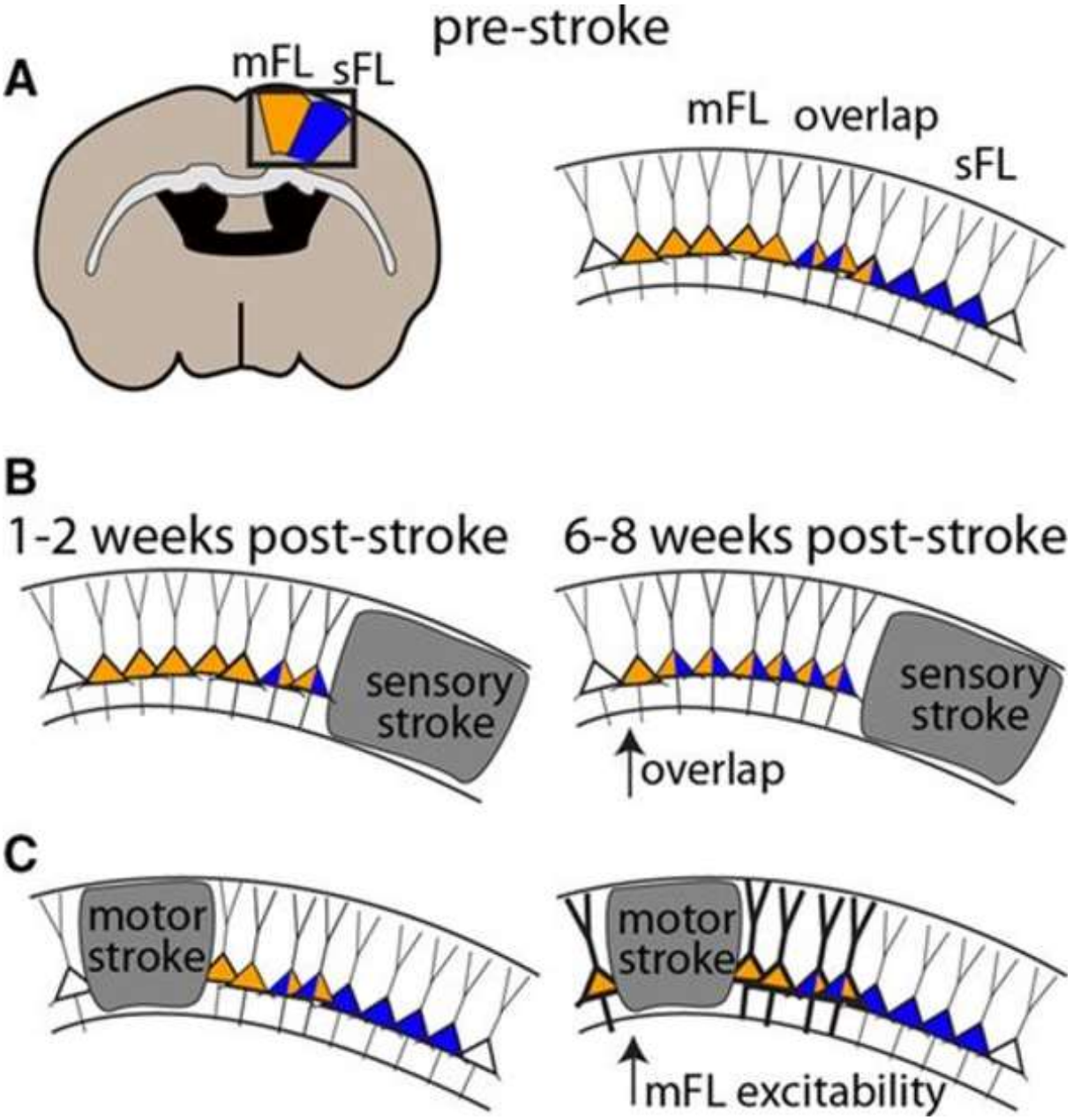

Figure 1.1 Reorganization of perilesional areas after targeted stroke in the sensory and motor maps in the mouse brain.

(A.) Sagittal section of the mouse brain, where the red box outlines the mouse motor forelimb (mFL) and sensory forelimb (sFL) maps in intact conditions. The sensorimotor map is comprised of overlapping populations of motor (orange) and sensory (blue) neurons. (B) After stroke in the sensory region, the sensory neurons die, but neurons in the overlapping region (represented with mixed orange/blue neurons) survive and expand to form the new representation of the sFL map. (C) Stroke in the motor cortex does not cause the motor map to expand into the sensory map. An increase in the excitability of peri-infarct neurons (indicated with bold lines) in the mFL compensates for the partial loss of mFL.

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1.2 Adult neurogenesis

The existence of adult neurogenesis has been described in most vertebrate species examined until now and is conserved in non-mammalian vertebrates (Adolf et al., 2006; Bernocchi et al., 1990; Cerri et al., 2009; Garcia-Verdugo et al., 1989; Grandel et al., 2006; Nottebohm, 1985; Parish et al., 2007; Tozzini et al., 2012; Zupanc et al., 2005) and almost all mammals analyzed so far, including non-human primates and humans (Boldrini et al., 2018; Bonfanti and Peretto, 2011; Braun and Jessberger, 2014; Eriksson et al., 1998; Ernst et al., 2014; Kornack and Rakic, 2001; Paredes et al., 2018; Sanai, 2004; Sanai et al., 2011; Spalding et al., 2013; Yuan et al., 2014)

Neurogenesis during post-embryonic stages depends on the presence of adult NSC (Doetsch et al., 1999). The distribution and the architecture of adult NSC niches differs within the brains of different vertebrate species between and even in different neurogenic regions within the brain of a single species (Figure 1.2) (reviewed in (Grandel and Brand, 2013; Kriegstein and Alvarez-Buylla, 2009)). These differences regarding the location and architecture of the NSC niches reflect the different physiological requirements in which these cells are employed in the various species and the different brain areas (Braun and Jessberger, 2014; Grandel and Brand, 2013).

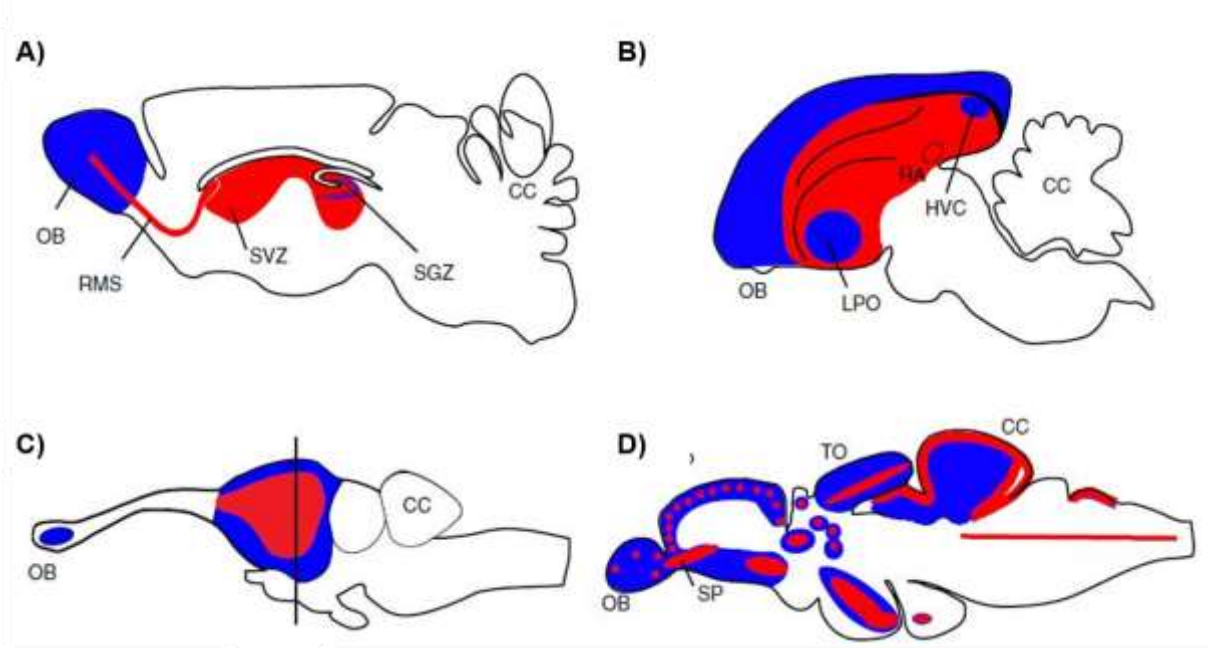

Figure 1.2 Distribution of proliferative (red) and neurogenic (blue) sites along the rostrocaudal axis of the brain of a:

(A) rodent (mouse),(B) bird (canary) (C) reptile (lizard) (D) fish (zebrafish). Abbreviations: OB-olfactory bulb, RMS-rostral migratory stream, SVZ- subventricular zone, SGZ-subgranular zone, CC-corpus cerebelli, LPO-lobus parolfactoris, HVC-high vocal center (nucleus engages in song learning and production, RA-robust nucleus of archistriatum, P-pallium (dorsal telencephalon, SP-subpallium (ventral telencephalon), TO-tectum opticum.

Adopted with permission from Springer Nature: Development Genes and Evolution (Grandel and Brand, 2013), 4663040870939, (2012). https://link.springer.com/article/10.1007%2Fs00427-012-0425-5.

Adult NSC are specified during embryonic development (Fuentealba et al., 2015), and as a consequence they are very similar to neuroepithelial (NE) and radial glia (RG) cells, the NSC during brain development (Kriegstein and Alvarez-Buylla, 2009). NE cells have several features in common with epithelial cells and are highly polarized along the apical-basal axis (reviewed in (Götz and Huttner, 2005)). They connect to the neighboring cells via adherens and tight junctions present at the most apical end of their plasma membrane (Aaku-Saraste et al., 1996; Götz and Huttner, 2005; Huttner and Brand, 1997; Weigmann et al., 1997) and contact the basal lamina via integrin α6 receptors concentrated at the basal membrane (Zhadanov et al., 1999). NE cells express the intermediate filament protein nestin, the transcription factor SRY (sex-determining region Y)-box 2 (Sox2), CD-133 (prominin-1), proteins that associate with adherence junctions such as zona occludens-1 (ZO-1), and brain lipid-binding protein (BLBP) (Götz et al., 2015). After the onset of neurogenesis, the NE cells lose some of their epithelial features and acquire astroglia properties such as the expression of glutamate-aspartate transporter (GLAST), glial fibrillary acidic protein (GFAP), and Vimentin, S-100β (Götz et al., 2015). This cell type, which exhibits astroglial and neuroepithelial features, is the RG cell.

The adult NSC of different species have features in common with embryonic NSC and express molecular markers such as GFAP, Prominin-1, Nestin, BLBP, S100β, Sox2, GLAST, and Vimentin, ZO-1 (Beckervordersandforth et al., 2010; Doetsch et al., 1997; Ganz et al., 2010, 2012; Götz et al., 2015; Kaslin et al., 2008; Kirkham et al., 2014; Lindsey et al., 2019; März et al., 2010b, 2010a). The adult NSC express the above mentioned molecular markers at a population level (Kirkham et al., 2014; März et al., 2010b, 2010a) while at a single cell level only some of the molecular markers are expressed (März et al., 2010a).

1.2.1 Adult neurogenesis in rodents

There are two major niches in the adult mammalian brain where NSC reside, the subependymal zone (SEZ) lining the lateral ventricles and the subgranular zone (SGZ) within the dentate gyrus (DG) of the hippocampus (Kriegstein and Alvarez-

Buylla, 2009). Adult NSC (also named Type B cells) in the SEZ are located beneath the ependymal layer, which separates the SEZ from the ventricle (Figure 1.3). Type B cells extend a single cilium through the ependymal layer and contact the cerebrospinal fluid (CSF) through a primary cilium that pokes through the ependymal layer (Mirzadeh et al., 2008). Type B cells give rise to transient amplifying progenitors (TAPs or Type C cells), which divide 3-4 times before becoming neuroblasts (Type A cells) (Calzolari et al., 2015). Neuroblasts form a chain and migrate 3-8 mm thorough the rostral migratory stream (RMS) to reach the olfactory bulb (OB), where they migrate radially and differentiate into a variety of periglomerular and granule interneurons and integrate into pre-existing circuits (Lledo and Valley, 2016). Newborn neurons in the olfactory bulb are involved in certain aspects of odor separation (Lepousez et al., 2013) and in maintaining tissue homeostasis (Imayoshi et al., 2008).

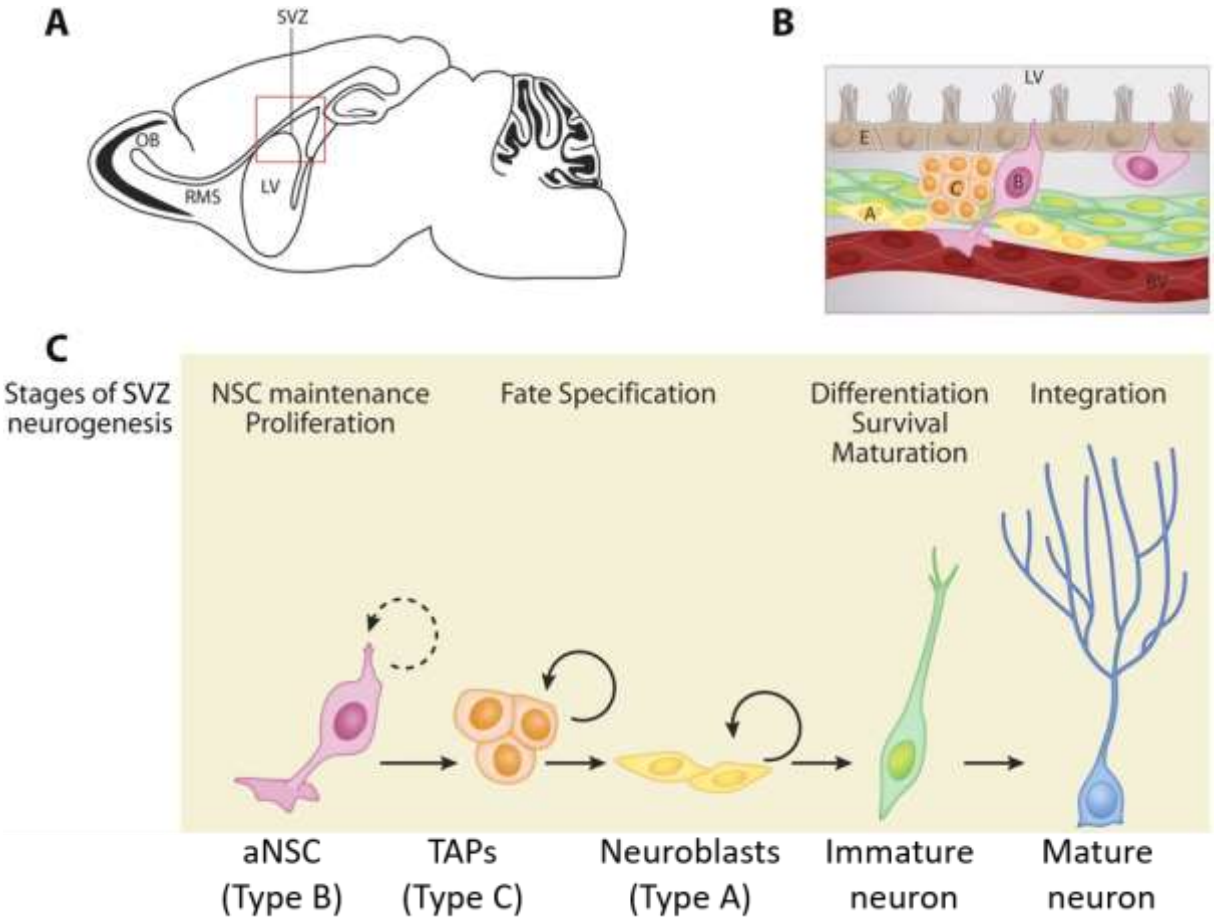

Figure 1.3 Adult neurogenesis in the SVZ/RMS/OB of rodents.

Sagittal view of the rodent brain with the red boxed area marking the SVZ next to the lateral ventricle (LV). (B) Schematic depicting of the SVZ niche with ependymal cells (E), blood vessels (BV) and distinct stem/progenitor cell types (type B,C, and A). (C) The SVZ niche is composed of astrocyte-like neural stem cells (type B, pink),which reside along the ependymal layer and extend a radial process to contact the blood vessels. They also extend a single cilium through the ependymal cells to contact the cerebrospinal fluid in the ventricular space. Type B cells generate TAPs (type C, orange), which turn into neuroblasts (type A, yellow). Neuroblasts migrate to the RMS to

reach the OB where they differentiate into immature neurons (green) and later integrate into the OB pre-existing neuronal circuits (mature neurons (blue).

The SGZ niche is located between the granule cell layer and the hippocampus hilus (Gage, 2000) (Figure 1.4). Unlike the adult NSC in the SVZ, the NSC in the SGZ (Type-1 cells) do not have contact with the CSF or ependymal layer (Seri et al., 2001). Type 1 cells produce intermediate progenitors (IPs, Type-2a cells), which divide only once and generate Type-2b cells which undergo multiple rounds of division, acting as the main transit amplifying progenitors of the DG (Bonaguidi et al., 2011; Encinas et al., 2011; Lugert et al., 2012; Pilz et al., 2018). Type -2b cells give rise to type 3 cells (neuroblasts), which develop into immature neurons and migrate radially into the granule cell layer to differentiate into dentate granule neurons (Bond et al., 2015; Lugert et al., 2012). The newly generated neurons aid hippocampal function in distinguishing highly similar events or environments and in storing the experiences as separate memories (reviewed in (Toda et al., 2018)).

In the mouse brain, neurogenesis is also reported to occur in the hypothalamus, however it seems to be restricted only to postnatal stages (Robins et al., 2013).

What is remarkable about adult mammalian neurogenesis is that species-specific differences also exist (reviewed in (Amrein, 2015)). Dolphin and whales have relatively small hippocampi and apparently generate no new neurons in this region (Patzke et al., 2013). Also, adult hippocampal neurogenesis is absent in some species of bats (Amrein et al., 2007).

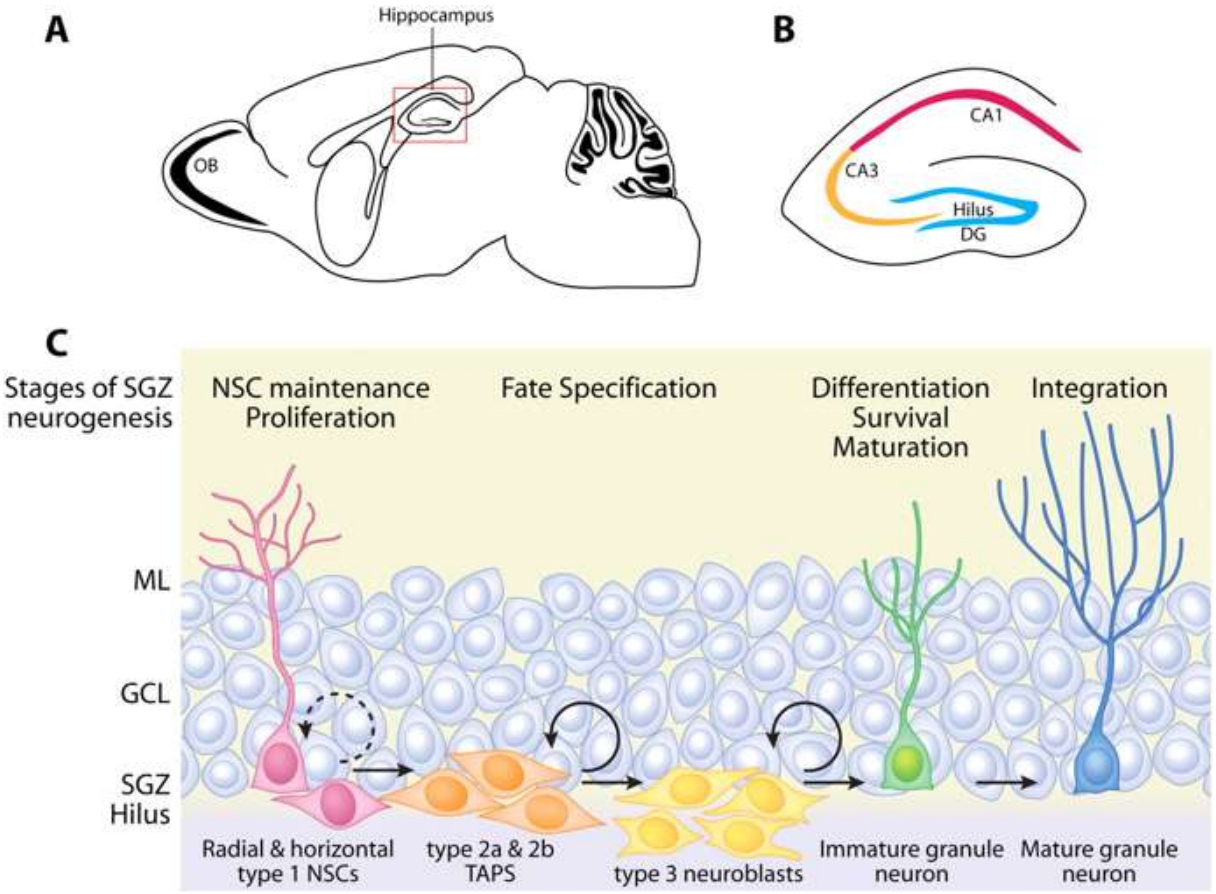

Figure 1.4 Adult neurogenesis in the subgranular zone (SGZ) of the dentate gyrus (DG) in rodents.

(A**)** Sagittal view of the adult rodent brain with the red boxed area outlining the hippocampus. (B) Schematic of the hippocampus with the CA1, CA2 and hilus region. (C) Radial glia-like neural stem cells (Type 1 cells) extend a process through the granular cell layer (GCL) of the DG and the molecular layer (ML).

1.2.2 Adult neurogenesis in humans

The main reason behind a continued interest in understanding mammalian adult neurogenesis is the fact that a similar process might be happening in humans. Whether neurogenesis in humans exists has been a matter of intense debate and research (reviewed in (Gage, 2019; Kempermann et al., 2018; Rakic, 1985, 2002)). Similar to most other mammalian species, in adult humans the NSC niches are located in the SVZ of the lateral ventricle and in the SGZ of DG in the hippocampus (Figure 1.5). However, profound differences exist regarding the structure of the SVZ niche, the migration route of the neuroblast, and the subtypes of neurons generated. In contrast to rodents, the human adult NSC of the SVZ niche are not adjacent to the ependymal

cells lining the ventricle, but are separated from them by a hypocellular gap layer and it is currently not known if they are in contact with the CSF (Sanai, 2004). Moreover, while new neurons born in the SVZ of many adult mammalian species (Bonfanti and Peretto, 2011; Lois et al., 1996), including non-human primates (Kornack and Rakic, 2001), migrate in chains through the RMS to reach the OB, this process does not seem to happen in adult humans (Sanai, 2004; Sanai et al., 2011; Wang et al., 2011). Indeed, a retrospective birth dating study of OB cells using carbon 14 (^{14}C), showed that no neurons are added to the OB of adult humans (Bergmann et al., 2012). A later study suggested that the neuroblasts which are generated in the lateral ventricle wall neurogenic niche in humans might migrate to the striatum where they generate striatal interneurons (Ernst et al., 2014).

Regarding human adult hippocampal neurogenesis, for a long time it was thought that it would not be possible because the adult human brain has to favor stability over plasticity in order to accomplish its computational tasks (Rakic, 1985). This dogma persisted until Eriksson and colleagues (Eriksson et al., 1998), provided strong evidence for the birth of neurons in brains of cancer patients. This finding was later supported by another study which took advantage of birth-dating ^{14}C techniques and showed that approximately 700 neurons are added every day to each hippocampus, resulting in a median turnover rate of 1.75% per year during adulthood within the renewing population (Spalding et al., 2013). Recently, two opposite publications brought back the debate concerning adult human hippocampal neurogenesis (Boldrini et al., 2018; Sorrells et al., 2018). Sorrells and colleagues (Sorrells et al., 2018) using post-mortem and fresh tissue, concluded that the DG of adult human hippocampus is devoid of neurogenesis and that the hippocampus of humans must function differently from that of other species where neurogenesis persists. In contrast, Boldrini and colleagues (Boldrini et al., 2018) demonstrated the exact opposite and reported lifelong adult hippocampal neurogenesis. Further studies and more precise techniques regarding the processing of human brain samples will be needed to confirm the presence of adult hippocampal neurogenesis in humans (also discussed in (Paredes et al., 2018; Snyder, 2019; Tartt et al., 2018)).

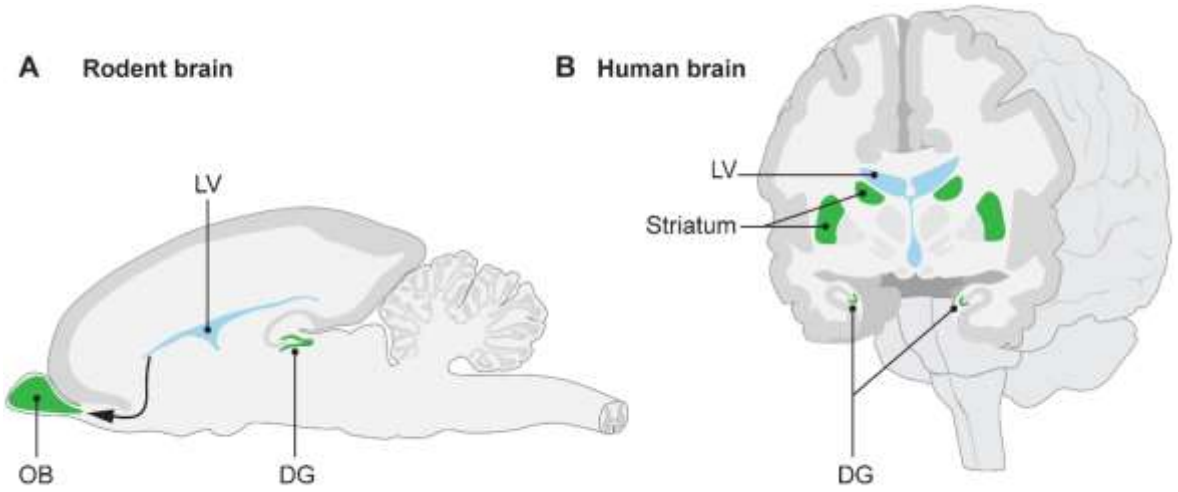

Figure 1.5 Schematic depicting the adult neurogenesis differences between human and rodent brain.

(A) In rodents, neuroblasts (indicated in green in both A and B) generated in the SVZ of the lateral ventricle (LV) migrate through the rostral migratory stream (curved arrow in A) and integrate in the olfactory bulb (OB),a structure essential for olfaction. (B) In humans, neuroblasts are also generated in the SVZ, however this is different from rodents in that they do not migrate in OB. Data from Ernst and colleagues (Ernst et al., 2014), suggest that these cells might migrate and integrate in the adjacent striatum, which plays a role in movement coordination, emotional and motivational control. Hippocampal neurogenesis in the dentate gyrus (DG) is thought to be a common feature of both rodents and humans.

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1.2.3 Role of neurogenesis after brain insults in mammals

The persistence of NSC in the adult brain together with advances in stem cell biology raised the possibility of using adult NSC as treatment for replacing lost neurons after stroke (reviewed in (Lindvall and Kokaia, 2015)).

Evidence for brain insult induced neurogenesis has been obtained in different rodent brain areas where new neurons normally do not form, such as striatum (Parent et al., 2002; Thored et al., 2006, 2007), cerebral cortex (Brill et al., 2009; Jiang et al., 2001; Zhang et al., 2006), and hippocampal pyramidal cell layer (CA1 region of hippocampus) (Nakatomi et al., 2002) (Figure 1.6) . Different studies have established that the neuroblasts migrating toward damaged brain areas originate in the SVZ of the lateral ventricles (Kreuzberg et al., 2010; Osman et al., 2011; Yamashita et al., 2006). Moreover, recent evidence also indicated that stroke in the striatum in addition to SVZ neurogenesis also unlocks the neurogenic potential of striatal astrocytes (Magnusson et al., 2014) (also reviewed in (Magnusson and Frisén, 2016)). Although a large

number of neuroblasts reach the damaged areas, few of them appear to differentiate into mature neurons. Most adult born neurons appear to die (Parent et al., 2002), probably from a failure to integrate or due to the highly inflammatory environment (Hoehn et al., 2005). The surviving cells differentiate into neurons, but the nature of the neurons that persist long term in the brain is controversial. Some studies showed that SVZ derived neuroblasts can give rise to medium spiny neurons that are not normally generated in the SVZ (Parent et al., 2002) and other studies showed that only interneurons are generated (Liu et al., 2009; Teramoto et al., 2003).

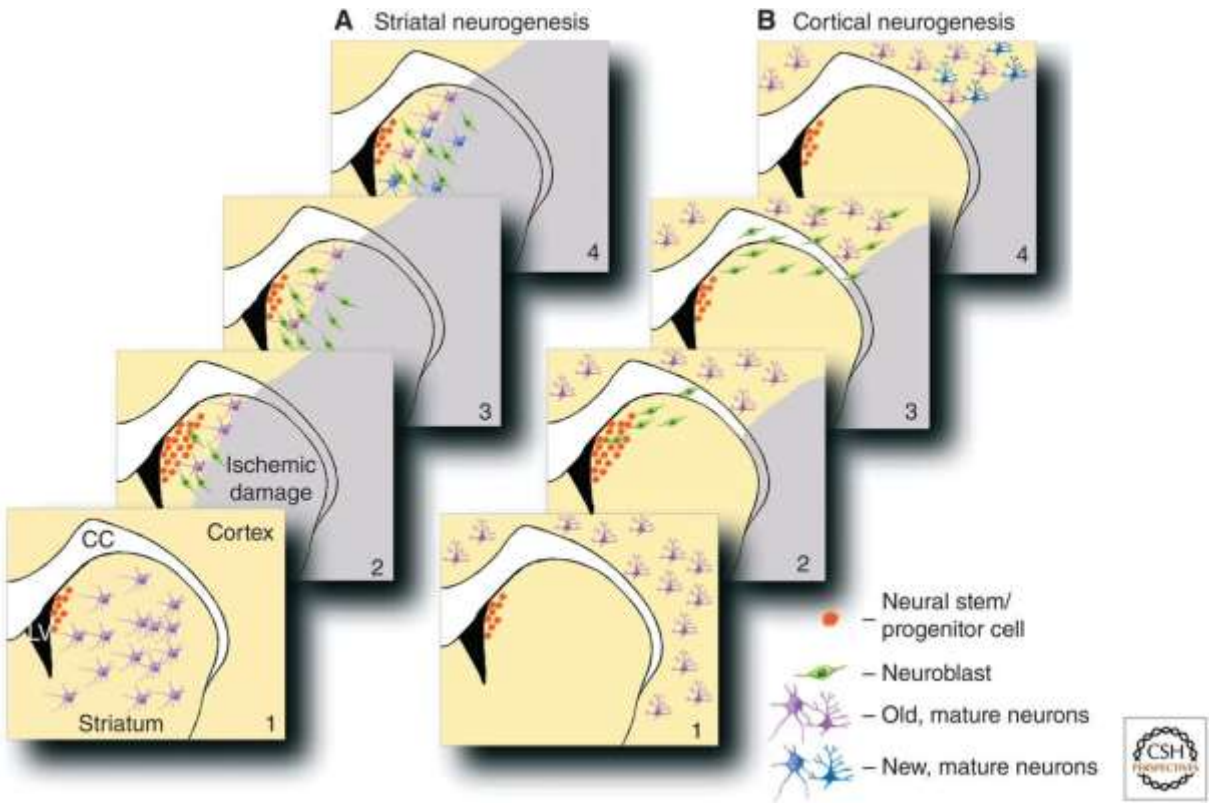

Figure 1.6 Schematic representation of stroke-induced neurogenesis in rodents in the striatum and cerebral cortex.

(A1 and B1) Neural stem/progenitor cells reside in the subventricular zone (SVZ) of the lateral ventricle (LV). (A2 and B2) Ischemic stroke in the striatum and cerebral cortex leads to pronounced neuronal loss and increased proliferation of the progenitors in the SVZ of LV. (A3) Neuroblasts migrate to the damaged part of the striatum, where some of them manage to mature and differentiate into striatal neurons (A4). (B3) Following ischemic stroke in the cerebral cortex, neuroblasts from the SVZ of the LV migrate towards the damaged cortical region and they express markers of mature neurons in the peri-infarct area.

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https://cshperspectives.cshlp.org/content/7/11/a019034.long

At present it is not clear whether the limited numbers of surviving adult born neurons replace lost cells by integrating appropriately and whether this improves recovery after brain damage. To have an answer for this critical biological question, organisms with distinguished brain regenerative abilities such as zebrafish need to be studied. In the following sections the current state of the art regarding brain regeneration findings in zebrafish will be discussed.

1.2.4 Adult neurogenesis in zebrafish

Compared to other vertebrates, teleost fish, a taxonomic class to which zebrafish belongs to, display the most widespread adult neurogenesis. 12-16 neurogenic zones have been identified along the rostrocaudal axis in adult teleosts (Adolf et al., 2006; Fernández et al., 2011; Grandel et al., 2006; Kuroyanagi et al., 2010; Maruska et al., 2012; Olivera-Pasilio et al., 2014; Strobl-Mazzulla et al., 2010; Teles et al., 2012; Zupanc and Horschke, 1995; Zupanc et al., 2005). It is still unclear why teleost fish have such a widespread neurogenesis. However, the proposed model is that constitutive neurogenesis in teleost fish allows brain growth which is required to match the concomitant body growth and the accompanying increase in primary sensory input.

Zebrafish adult neurogenesis occurs in all brain subdivisions along the rostrocaudal axis (Figure 1.7) (Adolf et al., 2006; Grandel et al., 2006; Zupanc et al., 2005). Niches are populated by heterogenous neural stem/progenitor cells and are dominated largely by NE-like and RG-like cells which differ in respect to the fate of their progeny.

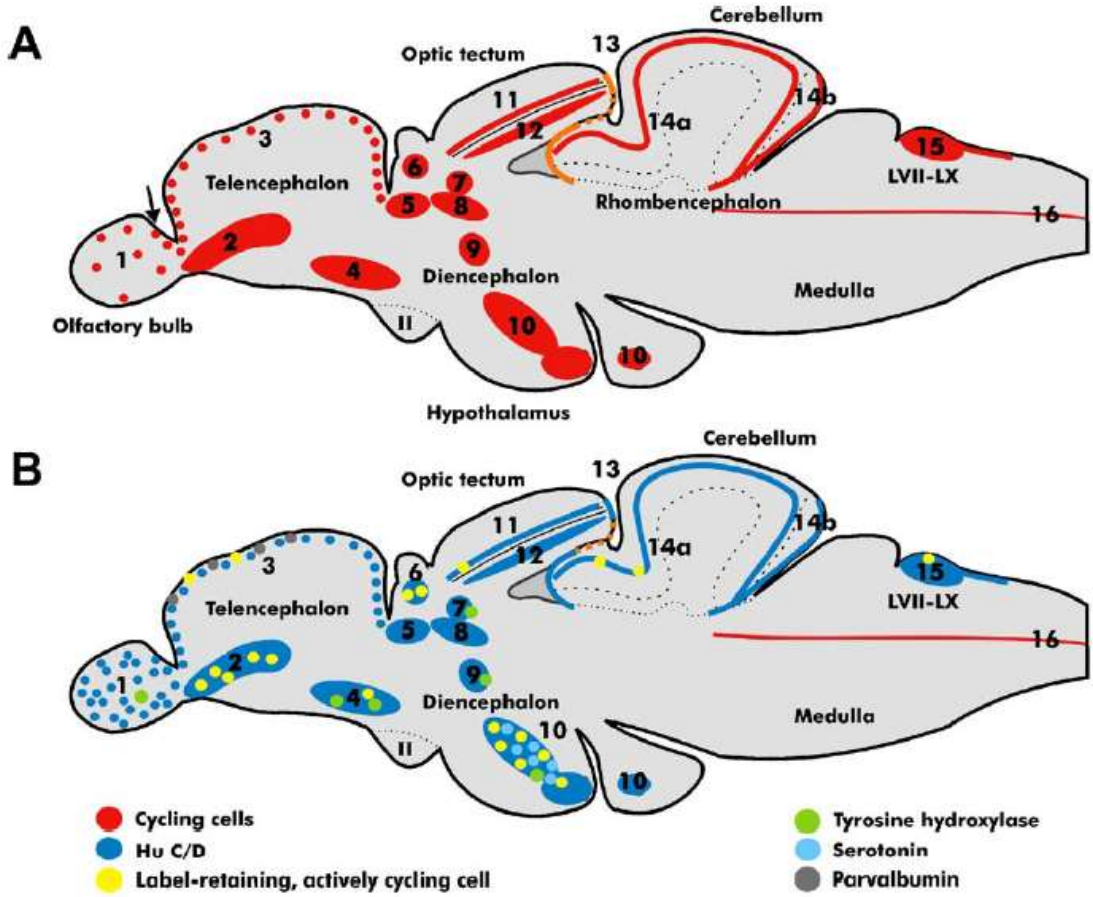

Figure 1.7. Proliferation and neurogenic sites in the adult zebrafish brain.

(A) Along the rostrocaudal axis 16 proliferation sites (red areas) are present. Olfactory bulb: (1) scattered proliferation in the olfactory bulb. Arrow points at the accumulation of cells at the junction between telencephalon and the olfactory bulb. Telencephalic proliferation sites : (2) ventral (subpallial) and (3) dorsal (pallial) telencephalic proliferation sites. Diencephalic proliferation sites: (4) preoptic, (5) ventral thalamic, (6) habenular, (7) pretectal, (8) dorsal thalamic, (9) posterior tubercular and (10) hypothalamic proliferation sites. Mesencephalic proliferation sites: (11) tectal and (12) torus longitudinalis proliferation sites. (13) Posterior mesencephalic lamina connects the tectum to the cerebellum. It starts dorsally at the proliferative tectal margin, continues as non-proliferative lamina and becomes proliferative again as it touches the cerebellar surface. Cerebellar proliferation zones: (14a) molecular layer proliferation site extending through the valvular and corpus cerebelli. (14b) Proliferation zone of the cerebellar caudal lobe extending from the ventricular lumen through the granular layer to its surface. Proliferation zones in the medulla oblongata: (15) proliferation sites in the facial (LVII) and vagal l (LX) lobes extending caudally into the nucleus of Cajal. (16) Romboncephalic ventricular proliferation site extends into the spinal cord. (B) Neuronal precursors generated in the proliferation sites migrate and integrate in the respective HuC/D positive neuronal nuclei (blue areas). Depending on the niche newborn neurons can differentiate into parvalbumin, tyrosine hydroxylase (TH)-, serotonin-positive neurons.

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The neurogenic niches of the adult zebrafish telencephalon are so far the best characterized during homeostasis and injury (Adolf et al., 2006; Barbosa et al., 2015, 2016; Baumgart et al., 2012; Ganz et al., 2010, 2012; Di Giaimo et al., 2018; Grandel et al., 2006; Kishimoto et al., 2011, 2012; Kroehne et al., 2011; März et al., 2010b, 2010a, 2011; Skaggs et al., 2014) due to the homology of the zebrafish telencephalon with mammalian forebrain structures (Mueller, 2012). As the zebrafish telencephalon

forms by eversion, the VZ is located not only internally but also at the outer surface of telencephalon, below the brain pia (Folgueira et al., 2012). Differently from the mouse, in the adult zebrafish, there is no ependymal cell layer that separates the NSC from the ventricle lumen, so the NSC are directly facing the ventricle. In the telencephalon two proliferative zones can be distinguished; a dorsal zone (called pallium) and a ventral zone (called subpallium) (Figure 1.8).

The ventral proliferative zone is more similar to the SEZ of mammals and studies conducted so far have suggested that the neural stem/progenitors of this niche generate GABA-ergic and tyrosine hydroxylase interneurons, which integrate in the OB (Adolf et al., 2006; Grandel et al., 2006; Kishimoto et al., 2011). Similar to rodents, the PSANCAM-positive neuroblasts generated in this niche migrate to the OB, through RMS-like structures guided by aligned blood vessels, but differently from rodents the neuroblasts are not enwrapped by a sheet of astrocytes. In contrast to rodents, the subpallium niche also generates neurons that migrate in the adjacent parenchyma, an area thought to be homologous to the mammalian striatum (Mueller, 2012). The generated neurons express TH, Paired box protein 6a (pax6a), and glutamic acid decarboxylase isoform with molecular weight kDa (gad67) (Adolf et al., 2006). Given that some mammalian species such as humans and rabbits generate neurons in the striatum during adulthood it is impressive that the zebrafish has common features of adult neurogenesis with all mammalian species.

The subpallial niche of adult zebrafish is one of the most heterogenous regarding the progenitor cell types it contains. It contains cells that proliferate and express the typical markers of RG in the dorsal part of the niche, while most of the cells within the ventral part have NE-like features (Ganz et al., 2010; Lindsey et al., 2012; März et al., 2010a). Moreover, at mid-telencephalic levels in the ventral part of the subpallial niche, cells are present which are positive for Olig2, an oligodendrocyte/oligodendrocyte precursor (März et al., 2010b). The presence of Olig2 and PSANCAM positive cells in proximity to the subpallial niche suggests that the Olig2-positive cells in this niche might give rise to oligodendrocyte precursors that migrate in the parenchyme (März et al., 2010b).

The pallial niche of the adult zebrafish telencephalon consists of radial glia-like cells and progenitor cells. The radial glia-like cells express markers which are expressed from RG cells during development (Adolf et al., 2006; Ganz et al., 2010;

März et al., 2010a). In addition to the radial glia-like cells, more committed non-glial progenitors positive for Sox2 and PSANCAM (also called Type III) are observed in the pallial niche. The neurons that are generated from this niche migrate 1-2 cell diameters below the ventricular surface to the adjacent and subjacent telencephalic nuclei. The extent of differentiation of neural progenitors in the dorsal pallium during homeostatic conditions still remains unclear, however one study reported that some of them differentiate into parvalbumin interneurons (Ganz and Brand, 2016; Grandel et al., 2006)

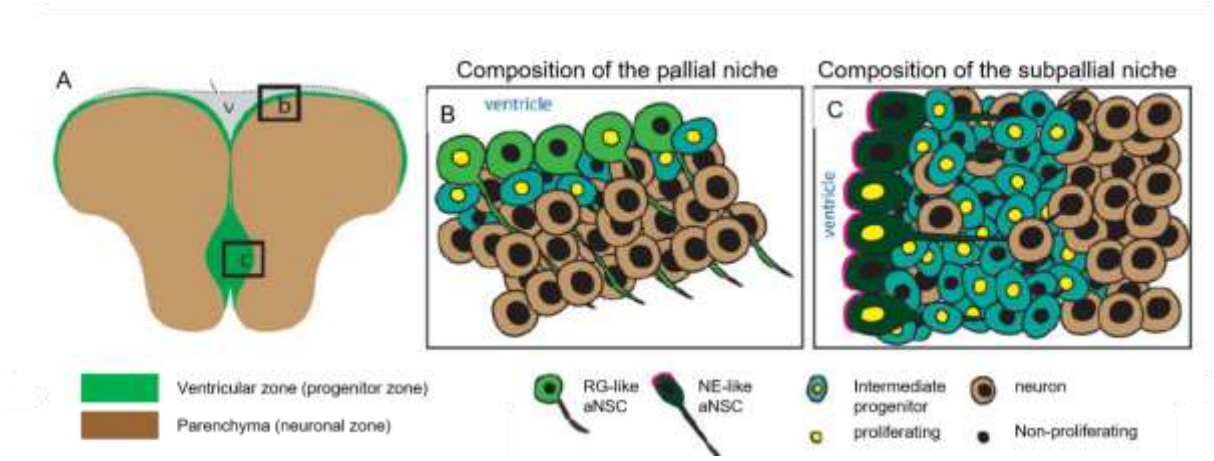

Figure 1.8 Neural stem /progenitor niches in the adult zebrafish telencephalon.

(A) Schematic representation of a coronal section through the zebrafish telencephalon. The ventricular zone where the neuronal progenitors reside is depicted in green, while the parenchyma that mostly contains neurons in brown. (B) Scheme depicting the neuronal progenitors in the dorsal niche (pallial niche). This niche is composed of radial glia-like (RG-like) cells, intermediate progenitors which are intermingled with neurons. The neurons generated in this niche migrate 1-2 cells diameters and integrate in the adjacent and subjacent telencephalic nuclei (C) Schematic depicting the neural progenitors in the ventral niche (subpallial niche), which is mostly composed neuroepithelial-like (NE-like) cells and intermediate progenitors. Neurons generated in the subpallial niche migrate through a reminiscent structure of mammalian rostral migratory stream in the olfactory bulb and also in the adjacent telencephalic nuclei.

https://www.tandfonline.com/doi/full/10.1080/23262133.2016.1148101

In the cerebellum, two distinct populations of neural stem/ progenitor cells are established during development, but only one remains active during adulthood (Kaslin et al., 2009, 2013, 2017). The actively cycling population displays neuroepithelial-like characteristics and primarily produces granule neurons for the granule cell layer of the cerebellum (Kaslin et al., 2009, 2013, 2017). The other population is located at the VZ, has radial glia-like characteristics, and produces inhibitory neuronal cell lineages and glia in the juvenile cerebellum. This population becomes quiescent in the adult fish,

concomitantly with the ceased production of all the inhibitory cell lineages in the adult zebrafish (Kaslin et al., 2013).

The optic tectum is the most prominent structure of the dorsal mesencephalon and corresponds to the superior colliculus of the mammalian midbrain. It has a multi-layered structure and the majority of neurons are packed in layer 1, the periventricular grey zone. (PGZ) (R. NieuwenhuysH. J. ten DonkelaarC. Nicholson, 1998). The proliferating cells are located in the caudal part of the PGZ and they have neuroepithelial features (Ito et al., 2010). BrdU labelling studies have shown that the neuroepithelial-like cells in this region give rise to neurons which integrate in the superficial layer of the PGZ and glial cells which integrate in the deepest layer of PGZ (Figure 1.9), (Ito et al., 2010). The deepest layer of the PGZ contains cells which have radial glia morphology and express radial glia markers, but these cells are largely quiescent and in normal conditions have only structural and supportive function for the optic tectum neurons (Ito et al., 2010; Lindsey et al., 2019; Shimizu et al., 2018).

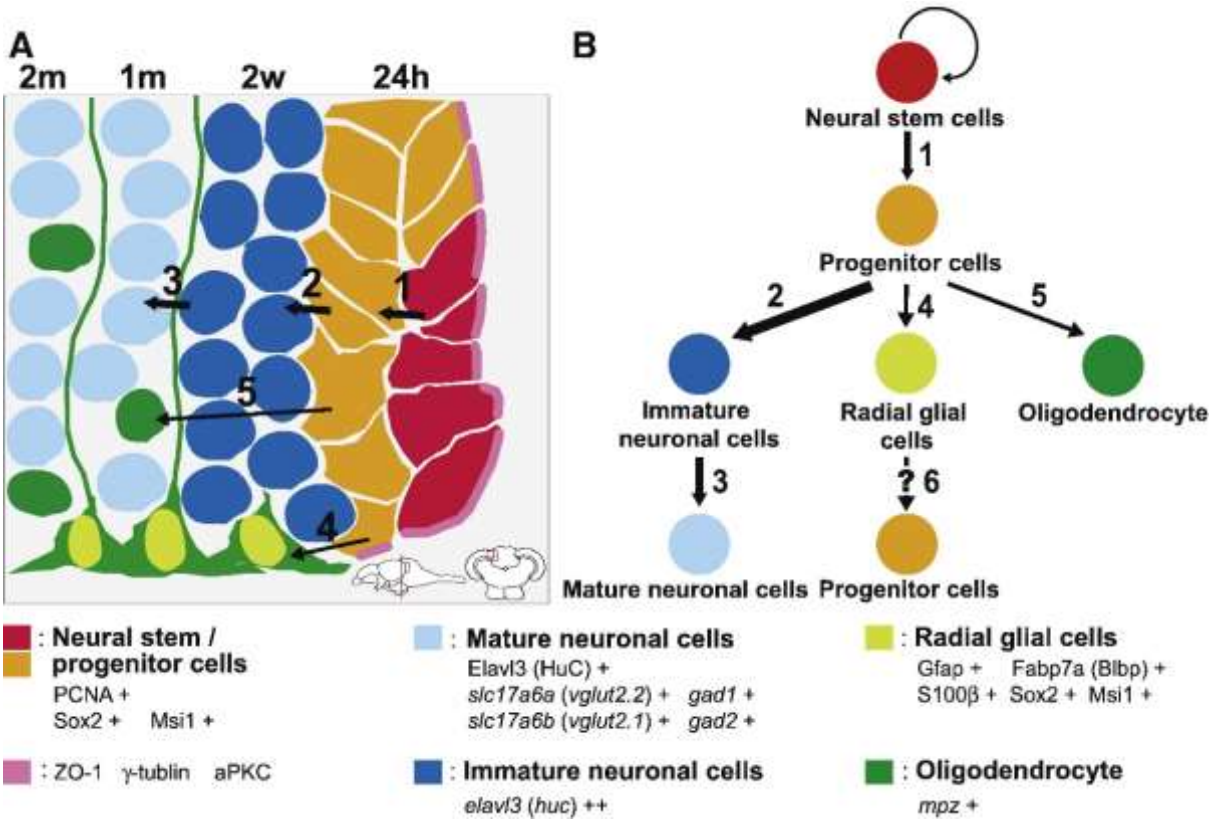

Figure 1.9 Neural stem /progenitor niches in the periventricular grey zone (PGZ) of adult zebrafish optic tectum.

(A) Schematic depicting the distribution of neural stem cells (red) with neuroepithelial-like (NE-like) features (purple apical surface) and progenitors in the caudal part of PGZ. Next to the neural stem/progenitor niche are immature neuronal cells (dark blue), mature neuronal cells (light blue), oligodendrocytes (green) and quiescent radial glia-like (RG-like) cells. The neural stem cells in the caudal PGZ have the potential to give rise to immature neuronal cells

(arrow 2), RG-like cells (arrow 4), oligodendrocytes (arrow 5). The immature neuronal cells migrate in the PGZ layer and further differentiate into GABA-ergic and glutamatergic neurons.

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1.2.5 Regenerative potential in the adult zebrafish brain

Since stem cell niches of the adult zebrafish telencephalon are the best characterized, the first studies in adult zebrafish brain sought to determine the regenerative capacities of this structure after traumatic injuries (Ayari et al., 2010; Baumgart et al., 2012; Kishimoto et al., 2012; Kroehne et al., 2011; März et al., 2011), excitotoxic injuries (Skaggs et al., 2014) and in a Amyloid-β-42 induced experimental Alzheimer's disease model (Bhattarai et al., 2016, 2017a, 2017b; Cosacak et al., 2019; Kizil and Bhattarai, 2018). There are two current models of traumatic brain injuries in the adult zebrafish telencephalon: 1) Injury by pushing sharp tools (syringe needle, glass capillary, or cannula) through the fish nostrils, which damage mostly the pallium, but spare the VZ where the neural stem/progenitors cells reside (Ayari et al., 2010; Baumgart et al., 2012; Kroehne et al., 2011); and 2) injury by pushing a syringe needle through the telencephalon's skull, which results in damage of VZ and pallium (März et al., 2011) The immediate response, characteristic of traumatic and excitotoxic injuries, is a wave of neuronal death which recedes at 3dpl (Kroehne et al., 2011; Skaggs et al., 2014). Common to traumatic and excitotoxic injuries is an increased proliferation of microglia at the injured hemisphere and accumulation of microglia and leukocytes at the injury site (Ayari et al., 2010; Baumgart et al., 2012; Kroehne et al., 2011; Kyritsis et al., 2012; März et al., 2011; Skaggs et al., 2014). Another type of response that these injuries have in common is proliferation of RG-like cells and upregulation of GFAP expression, however these cells do not migrate toward the injury (Kroehne et al., 2011; März et al., 2011; Skaggs et al., 2014). A typical response after traumatic injury, where the VZ is damaged, is a prolonged accumulation of oligodendrocytes/oligodendrocytes progenitors at the injury site, however this is only a temporary effect and is resolved by 35 dpl (März et al., 2011). In all the models of traumatic injury in the adult zebrafish, telencephalon acute inflammatory and gliosis take place, but no chronic inflammation or scarring (Kroehne et al., 2011; März et al., 2011; Skaggs et al., 2014). The zebrafish brain is devoid of stellate shape astrocytes

present in the mammalian brain (reviewed in (Lyons and Talbot, 2015)). In mammals, in response to invasive injury conditions, such as traumatic brain injuries and stroke, astrocytes react by becoming hypertrophic and upregulating most prominently the intermediate filaments GFAP and vimentin (Robel et al., 2011). Moreover, a distinct subset of astrocytes, whose somata are directly apposed to blood vessels (juxtavascular astrocytes) assume proliferation (Bardehle et al., 2013). It seems like the only GFAP expressing cell type (radial glia-like cells) characterized so far in the adult zebrafish telencephalon share some common features with the mammalian astrocytes, such as proliferation and GFAP upregulation, in response to traumatic injury. However, in contrast to mammals this response is acute in the adult zebrafish. Noticeably, depending on the injury size, the telencephalon of adult zebrafish appears histologically recovered already 7 days after small size injuries (Baumgart et al., 2012) and 30 days after bigger injuries (Kishimoto et al., 2012; Kroehne et al., 2011). In conjunction with the rapid decrease of cell death, gliosis, inflammation and regenerative neurogenesis takes place. Newborn neurons are seen in the brain telencephalon parenchyma already from the second week and survive for a long time in the brain parenchyma (with 90 days post injury being the latest studied time point) (Baumgart et al., 2012; Kroehne et al., 2011). Cre-lox lineage tracing technology and *in-vivo* imaging studies have determined that the radial glia-like cells in the pallial niche give rise to neurons that enable neuronal repair (Barbosa et al., 2015; Di Giaimo et al., 2018; Kroehne et al., 2011; Skaggs et al., 2014). Moreover, the radial glia-like cells switch their division mode towards neurogenic symmetric divisions and deplete themselves (Barbosa et al., 2015). The inflammatory response after injury is required and is sufficient to enhance their proliferation (Kyritsis et al., 2012).

A diversity of neuronal subtypes is generated after injury which are marker positive for TH, calretinin, neural differentiation 1 (NeuroD1), parvalbumin, GABA, and Prospero homeobox protein 1 (Prox 1). These neurons mature, form synaptic contacts, and send projections into the uninjured hemisphere (Kroehne et al., 2011; Skaggs et al., 2014). From all these studies three main questions emerged:

1. Are all the lost neuronal subtypes recovered after injury?
2. Do the cell types generated by radial glia-like/progenitor cells differ between homeostatic and injury conditions?

3. Do the newly generated neurons functionally replace the ones that were lost?

The first two questions were recently answered by Kaslin and colleagues (Kaslin et al., 2017). After studying in detail which neurons are generated from embryo to adulthood in the zebrafish cerebellum and from which stem cell/progenitor population (Kaslin et al., 2013), they performed stab wound injury to test if all the lost neuronal subtypes could be regenerated and if the stem/cell progenitor cells progeny differs between homeostatic and injury conditions. A striking finding from this study was that not all the lost neurons could be regenerated and only the cell types produced during homeostatic conditions are also produced during the regenerative response. Moreover, despite the fact that this injury induces the proliferation of VZ radial glia-like cells, this does not have as a consequence the production of neuronal cell lineages produced by this population during adulthood and juvenile stages (Kaslin et al., 2017). This study challenged for the first time the assumption that the highly regenerative zebrafish can regenerate all the cell types. Regardless of the lack of regeneration of all neuronal subtypes in the cerebellum, the swimming behaviour of the animals recovered within 4 weeks (Kaslin et al., 2017). The role of the newly generated neurons in the recovery of behaviour was not addressed in this study and it remains to be elucidated (Kaslin et al., 2017). Furthermore, it remains to be elucidated if this also applies to other brain regions. The circuitry and cell types of the zebrafish telencephalon are not known in detail, so the possibility that some cell types regenerate poorly in these structures cannot be excluded. In the future, accurate analysis of the zebrafish brain subpopulations and connections will be fundamental to address this question.

Another study from the Becker lab (Caldwell et al., 2019) tackled the question of how different populations of dopaminergic neurons regenerate through the rostrocaudal axis of the adult zebrafish brain. They showed an unexpected remarkable heterogeneity in the regenerative capacity of dopaminergic neurons. TH-positive neurons with long spinal projections failed to regenerate and consequently the shoaling and mating behaviour for which the non-regenerated populations are responsible for also never recovered (Caldwell et al., 2019).

This study showed the detrimental consequences of the lack of brain regeneration but again did not address whether the newly generated neurons can functionally replace the lost neurons by integrating into the pre-existing circuitries and recoveran impaired behaviour.

1.2.6 Role of latent progenitors in CNS repair

In some non-mammalian species, a regenerative response involves latent progenitors (reviewed in (Alunni and Bally-Cuif, 2016)). Latent progenitors are cells which have a neurogenic potential but their potential is hidden during homeostatic conditions and may be activated in response to stimuli such as injury and stroke (Berg et al., 2011a; Magnusson et al., 2014)

The best characterized example of regeneration mediated by latent progenitors is the zebrafish retina (reviewed in (Goldman, 2014)). The zebrafish retina grows during the entire life by addition of new neurons and the neuroepithelial-like cells located at the ciliary marginal zone (CMZ) of the retina mediate this growth (Raymond et al., 2006). In addition, the differentiated zebrafish retina also contains cells with radial glia-like morphology, the Müller glia cells. Müller glia are the only type of cells to span all the retinal layers and have processes that contact neighbouring neurons. Because of this, Müller glia are well positioned to monitor retinal homeostasis. During homeostatic conditions they have structural and supportive functions and divide slowly and sporadically to produce fate-restricted rod progenitors (Raymond et al., 2006). Following retinal injuries, Müller glia divide asymmetrically (Nagashima et al., 2013) and produce all the retinal neuronal subtypes (Figure 1.10) (Ramachandran et al., 2010).

Another example of successful regeneration from latent progenitors is the spinal cord of adult zebrafish. These latent progenitors are called ependymoglia since they line the central canal of the spinal cord and have radial glia. They are also positive for BLBP and the transcription factor Olig2. Under normal conditions they show very little proliferation and neurogenesis, however following a lesion they re-enter the cell cycle, migrate and differentiate into motor neurons (Reimer et al., 2008, 2013).

Rigorous studies regarding the role of latent progenitors in repairing the CNS have been conducted in the midbrain of newt (Berg et al., 2011b; Parish et al., 2007). Following lesion of dopaminergic neurons in this area, homeostatically quiescent cells lining the ventricle of the midbrain which have radial glia-like morphology (ependymoglia) re-enter the cell cycle and generate TH-positive neurons (Berg et al., 2011b). Two separate studies also tested the role of latent progenitors in the adult zebrafish midbrain after optic tectum injury (Lindsey et al., 2019; Shimizu et al., 2018). Injury in this area activates the quiescent radial-glia like cells present in the deep layer of the PGZ, however it is not clear whether this activation has as a final output of generation of neurons contributing to neuronal repair. Based on BrdU tracing studies, one group concluded that activation of these cells is not followed up by neuronal production and the neuroepithelial-cells present at the caudal PGZ niche are responsible for the repair (Lindsey et al., 2019), while the other group showed the exact opposite. Lineage tracing using the Cre-lox technology would be essential in the future to precisely determine the contribution and the neuronal subtypes produced by this population.

Latent but reactivatable progenitor cells exist in all the subdivisions of mammalian brain, however, their reactivation does not result in successful CNS repair (Barnabé-Heider et al., 2010; Carlén et al., 2009; Götz et al., 2015; Magnusson et al., 2014; Sirko et al., 2013). A possible reason for this might be the highly gliotic and inflammatory environment present in the mammalian central nervous system (Burda and Sofroniew, 2014), which interferes with successful neuronal integration and axonal growth.

Cell-intrinsic factors could also be another cause for the lack of regeneration from latent progenitors in mammals. Comparative transcriptomics of latent progenitors from regenerative species (e.g. newts and zebrafish) and poorly regenerative species (mammals) during different injury time points would be of vital importance to rule out this question.

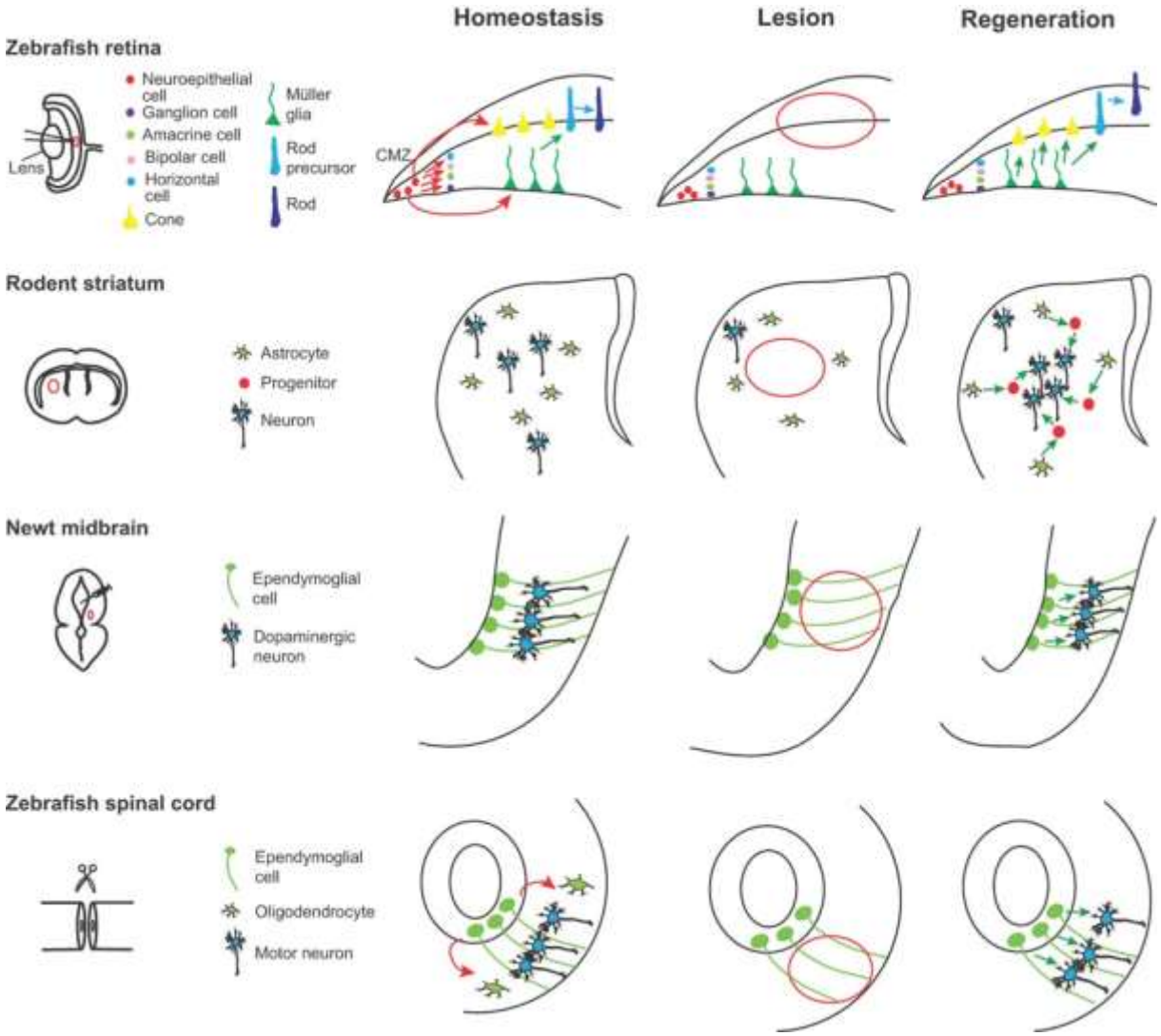

Figure 1.10. Contribution of latent progenitors in repairing the CNS in non-mammalian and mammalian species.

Müller glia in the adult zebrafish retina during homeostatic conditions produce only rod precursors (left panel, green arrow). Following lesion contexts (red outline, central panel), Müller glia re-enter the cell cycle, divide asymmetrically and give rise to all the missing retinal neuronal subtypes (right panel green arrows). Astrocytes in the adult rodent striatum are not neurogenic. After a striatal stroke (red outline, center panel), some astrocytes in this area activate a latent neurogenic program and give rise to a limited number of neurons (green arrows, right panel). Ependymoglial cells lining the ventricle of the newt hindbrain are highly quiescent during homeostatic conditions (right panel). Loss of dopaminergic neurons caused by intraventricular injection of 6-OHDA (red outline, central panel), induces the proliferation of ependymoglial cells, which produce new dopaminergic neurons (green arrows, right panel). Ependymoglial cells lining the central canal of adult zebrafish spinal cord, in homeostatic conditions divide sporadically to generate oligodendrocytes (red arrows, left panel), however after an injury (red outline, central panel), these cells divide and generate motor neurons (green arrows, right panel).

1.3 OKR as a behavior to study the brain function recovery

The OKR is a reflexive innate behavior that can be reliably elicited and quantified in zebrafish starting from the 4th day after fertilization (Easter and Nicola, 1997) and persists throughout adulthood (Mueller and Neuhauss, 2010; Zou et al., 2010). OKR is elicited in zebrafish by translational motion and serves to stabilize images on the retina during the movements of the animals or of its visual environment (Masseck and Hoffmann, 2009). It can be evoked by rotating a drum fitted with stripes around the animal and consists of two components: A slow following movement in the direction of the perceived motion (the slow phase of the OKR) and, after the eyes have reached the maximum deflection angle, a fast resetting movement in the opposite direction (saccade) .What is peculiar about OKR is that it can be elicited in zebrafish even when the animals are immobilized (Brockerhoff, 2006; Mueller and Neuhauss, 2010; Zou et al., 2010). Due to this peculiarity, and to its reliability, OKR was widely used in genetic screens to identify mutations affecting several aspects of visual development and function (Brockerhoff et al., 1995, 1997; Muto et al., 2005; Rick et al., 2000). During these OKR behavioral screenings several mutants with impaired OKR performance were identified and at the same time the interest for uncovering the neural circuitry underlying it in zebrafish started to grow (Allwardt et al., 2001; Rick et al., 2000). In particular, one zebrafish mutant line, which displays no OKR, the *no optokinetic response c* (*nrc*) mutants (Allwardt et al., 2001), shed light on the type of retinal ganglion cells (RGCs), which mediate the OKR. An electrophysiological study of this line suggested that the ON RGCs mediate the OKR perception, since *nrc* mutants lack the ON RGCs and pharmacological inhibition of the ON RGC in wildtype zebrafish results in no OKR phenotype (Emran et al., 2007). This is a shared trait also with other species such as rabbits (Ariel et al., 1988) and frogs (Yucel et al., 1989). Another mutant line, *belladonna (bel)* mutants (Huang et al., 2006; Rick et al., 2000), showed that the contralateral projection of the RGCs is essential for a correct OKR. About 40% of the homozygous mutants in *bel* line are achiasmatic and these achiasmatic mutants display reversed OKR upon visual stimulation. The RGCs innervate the contralateral zebrafish brain through 10 arborization fields (AFs) (Burrill and Easter, 1994; Robles et al., 2014). For a long time it was unknown which of the AFs mediates the OKR in zebrafish. Only recently, by combining the UAS-Gal4 system (Asakawa and Kawakami, 2008) and genetically encoded calcium indictors (GECIs) technology (Akerboom et al., 2012; Chen et al., 2013) in the RGCs, Kramer and colleagues

showed that in larval zebrafish the AF5 mediates the OKR (Kramer et al., 2019). In lower vertebrates the horizontal OKR is asymmetrical (Jardon et al., 1992; Qian et al., 2005). The eye that perceives the drum's motion in the temporal- nasal (T-N) direction (e.g during a clockwise rotation of the drum (CW)) is the left eye and the right eye during counter-clockwise rotation (CCW) is more efficient in evoking the OKR, than the eye that perceives the stimulation in the nasal-temporal direction (Jardon et al., 1992; Qian et al., 2005). Scientists were also intrigued by how the OKR is further processed in the zebrafish brain. In most vertebrates the contralateral RGCs projections convey information to the contralateral accessory optic system (AOS), a nuclear structure in the midbrain (Cochran et al., 1984; Liu et al., 2016; Masseck and Hoffmann, 2009). Two main nuclei in the AOS, the dorsal terminal nucleus (DTN) and the nucleus of the optic tract (NOT) provide the neuronal information for processing the OKR in the horizontal plane. The neuronal information is further conveyed to extraocular motor neurons, either through a direct pathway or indirectly via circuits that implement a velocity storage mechanism (vsm) and velocity to position neural integrator (vpni). The abducens nucleus (ABN) drives ipsiversive eye movements via the lateral rectus muscle (LR) and sends projections, via internuclear neurons, to the contralateral oculomotor nucleus (OMN) to drive contraversive movements of the stimulated eye via the medial rectus muscle (MR) (Cochran et al., 1984; Lopez-Barneo et al., 1982; Masseck and Hoffmann, 2009). These studies served as solid fundaments for the scientists, who were keen on discovering the neural circuits mediating OKR in zebrafish and recent studies in zebrafish suggest that the circuitry might similarly function in zebrafish (Kubo et al., 2014; Portugues et al., 2014; Roeser and Baier, 2003; Schoonheim et al., 2010). Unilateral optogenetic stimulation of the pretectal neurons resulted in conjugate eye movements which resemble the OKR (Kubo et al., 2014). In contrast, optogenetic inhibition of the contralateral pretectal neurons during monocular stimulation caused lack of slow-phase eye movements of the OKR (Kubo et al., 2014). 3D reconstruction of pretectal neurons in larval zebrafish suggested that these neurons also project caudally toward hindbrain motor centers (Kramer et al., 2019).

Three main aspects make the OKR a suitable behavior to study the capacity of newly-generated neurons in mediating the functional recovery of the brain after stab-wound injury:

1) It can be reliably measured in larval and adult zebrafish (Brockerhoff, 2006; Mueller and Neuhauss, 2010; Zou et al., 2010).

2) OKR asymmetry has been proven in larval zebrafish (Qian et al., 2005). An asymmetric behavior is very important, not only because it reduces the number of control experiments needed for an experiment, but also serves as an internal control. Interference with the neuronal components, which mediate the OKR during the CW stimulation, does not influence the OKR performance during CCW stimulation.

3) Knowledge of the neuronal components mediating it in larval zebrafish (Kramer et al., 2019; Kubo et al., 2014; Portugues et al., 2014; Roeser and Baier, 2003; Schoonheim et al., 2010). Precise location of the neuronal components mediating the OKR in adult zebrafish brain is very important, since this allows specific ablation of the neurons mediating it. While the neuronal circuitry mediating the OKR in larval zebrafish has begun to be fully elucidated, it is not known where the neuronal components mediating it in adult zebrafish reside. To be able to use OKR as a behavioural readout for studying functional regeneration of the adult zebrafish brain from the newly generated neurons, mapping of the circuitry in the adult zebrafish brain is a necessity. The findings about the neuronal circuitry in adult zebrafish will serve as groundwork toward this aim.

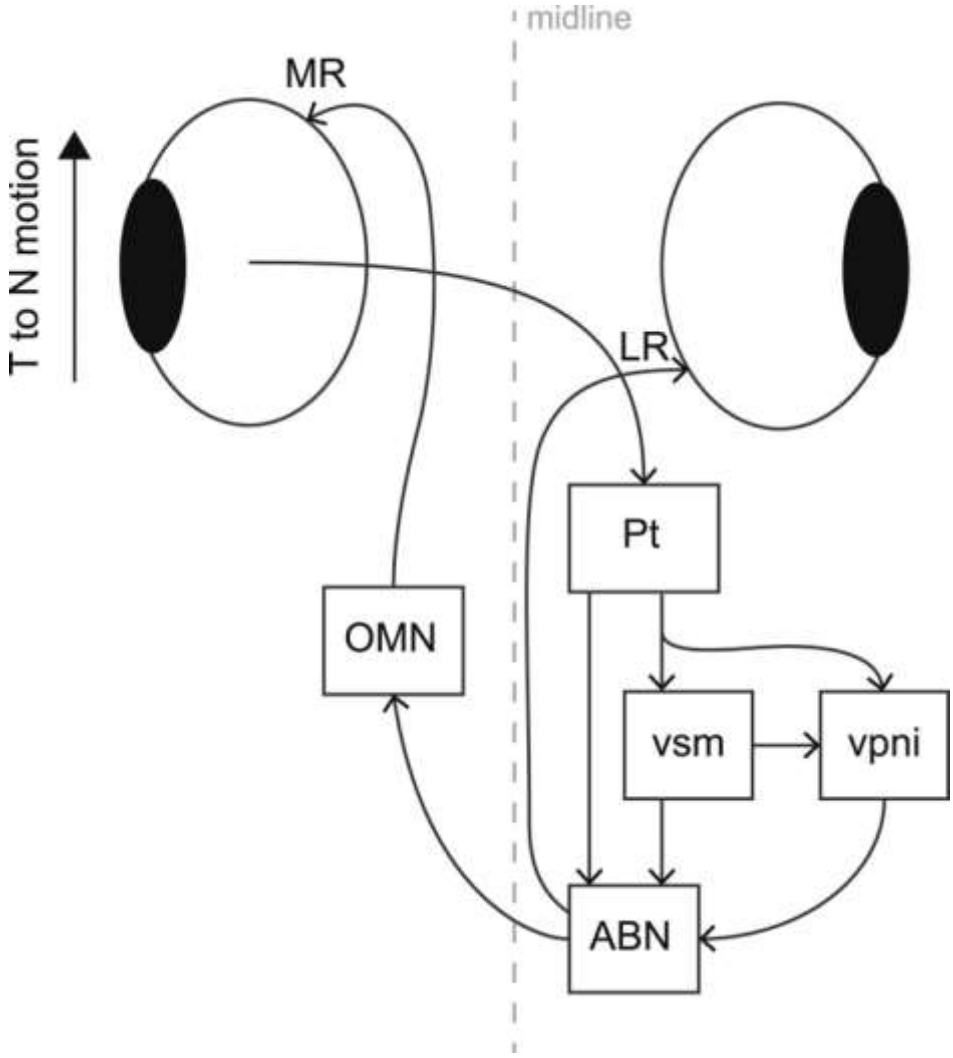

Figure 1.11 Schematic model the OKR circuitry in lower vertebrates.

Temporal-to-nasal (T to N) motion to one eye is conveyed via direction-selective retinal ganglion cells to the contralateral pretectal area (Pt) and is relayed to extraocular motor neurons (abducens nucleus (ABN), either through a direct pathway or indirectly via circuits that implement a velocity storage mechanism (vsm) and velocity to position neural integrator (vpni). The abducens nucleus (ABN) drives ipsiversive eye movements via the lateral rectus muscle (LR) and sends projections, via internuclear neurons, to the contralateral oculomotor nucleus (OMN) to drive contraversive movements of the stimulated eye via the medial rectus muscle (MR**).**

Adapted with permission from Elsevier, Neuron, (Portugues et al., 2014) 4713071085179, (2015).

https://www.cell.com/neuron/fulltext/S0896-6273(14)00050-6?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS0896627314000506%3Fshowall%3Dtrue

2 Aims of my thesis

The main aim of this thesis was to study the capacity of neurons generated after stab wound injury in the zebrafish brain to functionally replace the ones that were lost. Toward this aim, I first mapped the location of the pretectal neurons which mediate the OKR in adult zebrafish brain and then performed unilateral injuries, in order to affect the OKR performance during stimulation in a specific direction. The OKR behaviour was chosen not only for the characteristics mentioned in section 1.3, but also for the fact that pretectal neurons are close to constitutive neurogenesis niches (Ito et al., 2010) and to a niche that contains latent progenitors (Shimizu et al., 2018). Therefore, the chosen model should be able to unravel several aspects of functional regeneration: 1) Is the adult zebrafish brain able to restore a behaviour after injury? 2) If yes, how long does it takes to functionally regenerate the brain after stab wound injury? 3) More importantly, what is the cellular basis for the observed functional brain regeneration? Is this due to rearrangement of the existent neuronal connections or to the newly generated neurons? 4) If zebrafish cannot functionally regenerate the brain after stab wound injury, what are the reasons for the lack of brain regeneration? 5) How do the latent progenitors react to this type of injury?

3 Experimental Procedures

3.1 Commonly used solutions

10X Phosphate-Buffered Saline (PBS)

400g NaCl (Sigma)

10g KCl (Roth)

10g KH_2PO_4 (Sigma)

58, 75g Na_2HPO_4 (Merck)

Dissolve in 5l Millipore (Milli-Q) water, adjust to pH=7.4 and autoclave. For all the experiments PBS 1X was used which was obtained by diluting 10X PBS 1:10 in autoclaved Milli-Q water.

4% Paraformaldehyde (PFA)

40g PFA (Roth)

3 NaOH pellets (Merck)

100 ml of 10X PBS

900 ml Milli-Q water

Dissolve 40g PFA in 800ml Milli-Q water by gentle heating while stirring with the addition of 3 NaOH pellets to enable PFA powder to dissolve. Cool down the solution to room temperature and adjust the pH with the addition of HCL extremely slowly drop by drop. When the pH drops to 1-3, quickly add 100ml of 10X PBS. Adjust the pH to 7.2-7.3 and add Milli-Q water to final volume 1l. Filter the solution through paper filter and store at 4°C.

10X PO4 buffer

13,8g NaH_2PO_4 x H_20 (Sigma-Aldrich)

3,0 g NaOH (Merck)

40ml double-distilled water (ddH_2O)

Adjust the pH=7.2-7.4.

Storing solution

30% (v/v) glycerol (Sigma-Aldrich)

30% (v/v/ Ethylene glycol (Sigma-Aldrich)

10% (v/v) 10X PO_4 buffer

30% (v/v) ddH_2O

Adjust pH=7.2-7.4.

0.1M Sodium Borate Buffer

40.2g $Na_2B_40_7$ (Merck)

1000 ml Milli-Q water

Adjust the pH =8.5 with HCl 37% (Roth).

0.2 % Tricaine Methanesulfonate (MS-222)

1g MS-222 (Biomol)

500 ml of ddH_2O

Adjust the pH=7. 0.02% MS-222 was prepared by diluting 0.2% MS-222 in ddH_2O.

10 % Triton

10% (v/v) Triton X-100 (Sigma)

90% (v/v) PBS 1X

2N HCL

1/3 (v/v) HCl 37% (Roth)

2/3 (v/v) ddH_2O

3% Hydrogen peroxide (H_2O_2)

10% (v/v) H_2O_2 30% (Roth)

90% (v/v) ddH_2O

3.2 Fish maintenance and strain

Zebrafish were maintained under standard conditions (Westerfield Monte, 2000) in the Helmholtz Zentrum and Biocenter fish husbandry facility. In all experiments, animals used were between 3 and 5 months old. The strains/transgenic animals used were wild – type AB/EK hybrid, *brassy* (Kelsh et al., 1996), Tg(gfap:GFP)^{mi2001} (Bernardos and Raymond, 2006) crossed with Brassy. To identify transgenic animals,

embryos at 24 hours post fertilization (hpf) or 48hpf were screened for positive signal under a stereomicroscope (Stereo Lumar.V12, Zeiss and F-View SZX12, Olympus).

All animal experimental procedures were performed in accordance with the German and European Union guidelines and were approved by the institutional Animal Care and Use Committee (IACUC) and the Regierung von Oberbayern/Government of Upper Bavaria under license number: AZ 55.2-1-54-2532-09-16.
All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.3 Mapping of the neuronal components of the optokinetic reflex

3.3.1 Optic nerve labelling

Fish were anaesthetized in 0.02% MS-222 and put in a MS-222 soaked sponge. A small hole was created in the cornea using forceps (Cat. Nr. 11255-20, Fine Science Tools) to allow the dissection of the lens. The lens of the left eye was dissected out using forceps. After this procedure, fish were awakened in aerated tanks and 1h after were anaesthetized again and the retina was removed. With the help of a pulled and cut glass capillary (A. Hartenstein), biotinylated dextran amines (BDA) crystals coupled with different fluorophores (488, 546,647) (Cat. Nr. D22910, D22911, D22914, Life technologies) were placed in the exposed optic nerve. The removal of retina layers is a crucial step since it enables the uptake of BDAs, which have a molecular weight of 10kDa and cannot enter the membranes by passive diffusion (Nicole J. Yang and Marlon J. Hinner, 2015; Stephens and Pepperkok, 2001). Fish were awakened again in aerated tanks and kept in husbandry conditions until sacrifice that was after 24 hours.

3.3.2 DiI labelling of the pretectal neurons

To process the brains for the 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI, Cat. Nr. D3911, Molecular Probes) labeling of the pretectal neurons, animals were first sacrificed with 0.2% of MS-222. Then, the brain was dissected in PBS 1X. Immediately after the dissection, DiI crystals were placed in the abducens nucleus. After this step brains were first fixed with 4% PFA for 3 h at 4°C and then with 1% PFA for 72h at 37°C to accelerate the diffusion of DiI.

3.3.3 Stab wound injury of the pretectal neurons

Before injury, fish were anaesthetized in 0.02 % MS-222 for 30 to 45 s. Fish were placed in a Tricaine-soaked sponge to keep them firmly in place for the injury yet also allowing accessibility for the head. With the visual aid of a dissecting microscope (MZ6, Leica), a microdriller (Cat. Nr. 78001, RWD) was inserted into the zebrafish brain between the right optic tectum and the right telencephalic hemisphere border, resulting in the injury of the right pretectal neurons. After injury, fish were placed in tanks with aerated water to insure complete recovery from the anaesthetic.

3.4 Optokinetic reflex

3.4.1 Setup

For optokinetic reflex experiments animals of both sexes only in the AB/EK background were used. To elicit the optokinetic reflex (OKR) in the adult zebrafish, the animals were first restrained from any head movement. To restrain the animals from head movements, freely swimming animals were captured with a net and placed in a homemade fish restraining device (FRD) with the help of blunt ended forceps (Cat. Nr. 11252-20, Fine Science Tool). The FRD, which consisted of a conic shape plastic tube (made from 3,5 ml transfer pipettes, Cat. Nr. 86.1171, Sarstedt), which was stably fixed in a 6 cm petri dish (Cat. Nr. 150288, Nunclon® Δ Multidishes), was filled with fresh husbandry water during the OKR stimulations. Plastic tubes having different sizes were made, since the animals have different body sizes. The FRD was placed on top of a circle and was surrounded by a black and white stripped drum.

For behavioral recordings, a CCD camera (Grasshopper 0.3 MP Color FireWire 1394b, Cat. Nr. GRAS-03K2C-C, FLIR Systems), mounted 20cm above the FRD, allowed online tracking of horizontal eye movement by custom-written software (Beck et al., 2004).

3.4.2 Stimulus paradigm

Horizontal eye movements were elicited by large-field visual stimuli. The OKR inducing stimuli consisted of a black and white stripped pattern drum, with a spatial frequency of 0.03 cycles/degree rotating in clockwise (CW) or counter clockwise

(CCW) direction with a velocity of 20 deg s^{-1}. For measuring the OKR in one specific direction the animals were kept in the FRD for 35 seconds. After each OKR measurement the animals were released to swim again. 5 minutes was the minimum amount of time between the measurements of a specific time point for each animal. The OKR measurements were always done between 12:00h-18:00h. After the OKR measurements of a specific time point fish were kept in their normal husbandry conditions until the next time point of OKR measurements.

The OKR of the experimental animals was measured before injury, 2 days post injury (dpi), 6dpi and after 6dpi was measured every other week, according to the purpose of each experiment. For every time point the OKR in CW and CCW direction was measured.

3.4.3 OKR gain quantification

To extract horizontal eye movements from the top-down video recordings, images were binarized using a manually chosen threshold level to separate the eye from the white background. Since the cornea is transparent, the iris of the eye then represents the boundary between the background and the eye. Two points on the iris, one on either side of the eye lens, were chosen manually to track the orientation of the iris within the image plane. Horizontal eye movements caused a change in the iris' orientation, and thus, a change in the relative location of the two chosen points. The orientation of the eye was computed in each frame by computing the angle of the line connecting the two tracked points of the iris.

To compute OKR gain, the resulting eye position traces in each trial were low-pass-filtered using a gaussian low-pass filter with a cut-off frequency of 10Hz. Then, optokinetic fast-phases were detected in the eye-position trace as all periods within the recording in which the eye velocity was greater than 20°/s. This threshold ensured that all slow-phase following eye movements were below threshold and, thus, not erroneously identified as fast phases.

Subsequently, the average horizontal eye velocity of each slow-phase segment between two successive fast phases was computed by evaluating the slope of a linear fit to the eye position trace in each segment. From this average velocity, the OKR gain was then computed as the ratio of horizontal eye velocity and stimulus velocity

3.5 Eye nucleation experiments

Before eye nucleation, fish were anaesthetized in 0.02% MS-222 for 30-45 s. For the eye enucleation experiments, first the eye muscles were cut using forceps (Cat. Nr. 11255-20, Fine Science Tools) and then the optic nerve. Left or right eyes were enucleated according to the experimental purpose. After eye enucleation, animals were placed in aerated water to ensure complete recovery from the anaesthetics and were kept in normal husbandry conditions for 24 h. After 24 h the OKR measurement took place and immediately after, the animals were sacrificed.

3.6 Cytosine arabinoside treatment

To interfere with the DNA (deoxyribonucleic acid) replication (S-phase of the cell cycle) animals were let to swim for 72h in a tank containing 1% cytidine analogue cytosine arabinoside (Ara-C, Cat. Nr.16069, Cayman Chemical Company). Ara-C was dissolved in fish husbandry water by continuous stirring for 30 min at room temperature (RT). This solution will be named hereafter as Ara-C water.

To estimate the effect of Ara-C treatment on OKR behaviour recovery and on the generation of new neurons, animals were let to swim in Ara-C water from the third to the sixth day after injury. Ara-C water was changed every 24h to make sure that the activity of Ara-C was not light-deactivated. During the remaining survival time animals were swimming in normal water.

3.7 5-bromo-2’-deoxyuridine (BrdU) labelling

To label proliferating cells in the S-phase of the cell cycle, for the analysis of newly generated neurons after pretectal neurons injury (Figure 4.17), fish were placed in a tank containing 0.3% thymidine analogue 5-bromo-2’-deoxyuridine (BrdU, Cat. Nr. B5002-5G, Sigma-Aldrich). BrdU was dissolved in fish husbandry water by continuous stirring for 30 min at RT. This solution will be named hereafter as BrdU water. The animals were allowed to swim in the BrdU water overnight (O/N) (20:00h-08:00h) from the third to the sixth day after injury. BrdU water was changed every 48h. During the remaining time animals were swimming in normal water.

To label proliferating cells in the S-phase of the cell cycle during Ara-C treatment in telencephalon (Figure 4.19), after 60h of swimming in 1% Ara-C, fish were allowed

to swim in water containing 1% Ara-C and 0.3% BrdU dissolved in fish husbandry water (Ara-C-BrdU water). Animals were immediately sacrificed and further processed for immunohistochemistry.

To label proliferating cells in the S-phase of the cell cycle for the analysis of newly generated neurons after pretectal nucleus injury during the Ara-C treatment (Figure 4.20), fish were let to swim in Ara-C-BrdU water from the third to the sixth day after injury. Ara-C-BrdU water was changed every 24h. During the remaining time animals were swimming in normal water.

3.8 Immunohistochemistry

3.8.1 Tissue preparation

To process brains for sectioning, animals were sacrificed by deep anaesthesia with 0.2 % MS-222. Brains were dissected in 1X PBS and immediately after were fixed with 4% PFA at 4°C for 3h. For vibratome sectioning, after fixation brain tissue was washed in 1X PBS for some hours and then embedded in 3% (w/v) agarose dissolved in 1X PBS. After agarose polymerization, 100µm horizontal or coronal sections were cut in a vibrating blade microtome (Leica VT1000 S, Leica Biosystems and Microm 650 V Thermo Fisher Scientific) and collected in 1X PBS. For long term preservation, vibratome sections were kept in storing solution at -20°C.

3.8.2 Immunostaining

Primary antibodies (see Table 3.1 and Table 3.2 for the concentrations of the antibodies used and the companies from which they were purchased) were diluted in staining solution made of 1X PBS containing 0.5% Triton-X-100 and 10% normal goat serum (NGS, Cat. Nr. S1000, Vector Laboratories) and sections were incubated with this solution O/N at 4°C with constant gentle shaking, to respectively allow specific antibody binding and penetration. The following day sections were washed in 1X PBS 3 times each 10 min at RT. Afterwards sections were incubated for 2h at RT with subclass specific secondary antibodies diluted in staining solution. To visualize the cells nuclei 4',6-diamidino-2-phenylindole (DAPI, Cat. Nr. D9542, Sigma 20mg/ml) was added to the secondary antibody solution. After complete staining, sections were washed 3 times with 1X PBS and placed by the help of brushes on glass slides (Cat.

Nr. J1800AMNZ), dried shortly and mounted in Aqua Poly/Mount (Cat. Nr.18606-20, Polysciences Inc.).

Although this general protocol worked for many of the antigens, some antigens required specific retrieval since they were masked during tissue preparation. Different retrieval procedures used in this thesis are summarized in the next paragraph.

Table 1. Inventory of all the primary antibodies used in this thesis with the respective information.

Antigen	Species, Isotype	Working dilution	Pre-treatment	Company (Cat. Nr.)
Anti-BrdU	*Rat*	*1:200*	*2N HCl 30min*	*Abcam (ab6326)*
Anti-GFP	*Chicken*	*1/1000*	*-*	*Avės Labs (GFP-1020)*
Anti-HuC/D	*Rabbit*	*1:500*	*-*	*Abcam (ab210554)*
Anti-PCNA	*Mouse, IgG_{2a}*	*1:200*	*3% H_2O_2 30 min*	*Santa Cruz Biotechnology (sc-56)*
Anti-Sox2	*Rabbit*	*1:500*	*.-*	*EMD Millipore (AB5603)*

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Table 2. Inventory of all the secondary antibodies used in this thesis with the respective information.

Antigen	Working dilution	Company (Cat. Nr.)
Alexa Fluor 488 Goat Anti-Chicken IgG	1:1000	Life Technologies (A11039)
Alexa Fluor 488 Goat Anti-Mouse IgG	1:1000	Life technologies (A11029)
Alexa Fluor 488 Goat Anti-Mouse IgG_{2a}	1:1000	Life technologies (A21131)
Alexa Fluor 633 Goat Anti-Rabbit IgG	1/1000	Life technologies (A21070)

Alexa Fluor 546 goat Anti-Rat IgG	1:1000	Life technologies (A11081)

3.8.2.1 Antigen retrieval procedures

Since BrdU is incorporated into the DNA, its detection required a special treatment where DNA is denatured. For immunostaining against BrdU, sections were pre-incubated for 30min at RT in 2N HCl, followed by neutralization of the pH with 2 washes, each 15 min with 0.1M borate buffer (pH=8.5). Afterwards sections were washed in 1X PBS 2 times each 10min (this protocol will be referred to hereafter after as HCL treatment). Subsequently the incubation with the primary antibody solution was conducted.

To access the epitope to which the anti-proliferating cell nuclear antigen (PCNA) antibody binds, sections were first pre-incubated in 3% H_2O_2 for 30 min at RT, washed in 1X PBS 3 times each 10 min (this protocol will be referred hereafter as H_2O_2 treatment). At the end sections were incubated with the primary antibody solution.

3.8.2.2 Sequential immunostaining

When sections were co-stained with anti-PCNA and anti-BrdU primary antibodies, which were respectively produced in mouse and rat, a sequential immunostaining procedure was performed to avoid the cross-reactivity of the mouse IgG secondary antibody. To reveal the antigens to which the primary antibodies bind, the protocol involved the sequential application of the HCl and H_2O_2 treatment. At the completion of the pre-treatments, sections were incubated with mouse anti-PCNA primary antibody diluted in staining solution O/N at 4°C. The next day sections were washed in 1X PBS 3 times each 10 min at RT and subsequently incubated with Goat Anti-Mouse IgG and Alexa Fluor 488 Goat Anti-Mouse IgG_{2a} diluted in staining solution for 2h at RT. After staining, sections were washed in 1X PBS 3 times each 10 min at RT and later incubated with rat anti - BrdU primary antibody diluted in staining solution O/N at 4°C. The following day sections were washed in 1X PBS 3 times each 10 min at RT and then incubated with Alexa Fluor 546 goat Anti-Rat IgG diluted in staining solution for 2h at RT. DAPI was also diluted 1:500 in the staining solution to visualize

the cell nuclei. At the end, sections were washed 3 times with 1X PBS and placed by the help of brushes on glass slides, dried shortly and mounted in Aqua Poly/Mount.

3.8.3 Image acquisition and quantification

Fluorescent labelled sections were photographed with Olympus FV1000 confocal laser-scanning microscope (Olympus), using the FW10-ASW 4.0 software (Olympus).

All the quantifications were done in a minimum of 3 animals per experimental condition. For the evaluation of newly generated neurons after pretectal nucleus injury (Figure 4.17) at least 2 sections / animal were used. In this case the area of the parvocellular superficial pretectal nucleus (PSp) in the intact hemisphere was used as a reference to quantify the newborn neurons in the injured hemisphere. To quantify the number of PCNA+ positive cells after Ara-C treatment in the telencephalon (Figure 4.18) ≥3 sections along the rostro-caudal axis were used and only the cells that were distant 25µm from the telencephalic ventricular zone were considered. Assessment of co-staining for two or-more markers for which the quantifications were done using the ImageJ-win32.exe software, by analysing all the individual z-planes of an optical z-stack.

3.9 Statistical analysis

GraphPad Prism (6.0) software was used to test the normal distribution of the data, to generate the dot plots and to test for statistical significance. The number of biological replicates can be seen on the dot plots and on the figure legends. The normally distributed data are shown as mean ± standard deviation (SD), while the non-normally distributed data are shown as median ± interquartile range (IQR). Statistical significance was tested only in the experiments with ≥4 biological replicates and the critical level for all the statistical comparisons were considered *$p<0.05$. Statistical tests were conducted according to the distribution of the data.

4 Results

4.1 Eliciting and quantifying OKR in adult zebrafish

OKR can be reliably elicited in zebrafish by combining the following approaches:

1. Restraining the animal from body and head movements
2. Rotating a stripped drum in front of the animal's eyes

OKR has been used extensively as a high-throughput screening technique for vision mutants in the zebrafish larvae due to the ease of restraining the larval fish. To measure the OKR in larval fish, larvae are first anaesthetized and then embedded in viscous methylcellulose or low-melting agarose solution. Since at this life stage the scales are lacking, larvae are sufficiently oxygenated through their skin (Brockerhoff et al., 1995; Easter and Nicola, 1997) Doing the same procedure in the adult zebrafish is not possible, because the agarose would cover the gills which are the main breathing organ during this life stage and the animals would die of asphyxia. The studies published so far in this regard overcome this technical hurdle by first anesthetizing the adult zebrafish and then clamping them between two pieces of sponge or by pinning them in a sponge (Cameron et al., 2013; Mueller and Neuhauss, 2010; Zou et al., 2010). Throughout this thesis, to restrain adult zebrafish animals, a fish restraining device (FRD) (Figure 4.1A) (explained in detail in Materials and Methods section) which does not require prior anesthesia of the animals, was developed. By restraining the animals in the FRD and simultaneously rotating in front of their eyes a stripped drum with a constant velocity of 20°/s in CW or CCW direction for 37s the OKR was elicited in the adult zebrafish animals (Figure 4.1A and Figure 4.2).

Two different parameters of the OKR were analyzed to select for the most reliable parameter to quantify the OKR performance: the frequency of saccades (number (No.) of saccades/min) and the performance of stimuli tracking (OKR gain = Slow Phase Velocity/ Stimuli Velocity). Toward this aim the OKR of 15 animals was elicited in CW or CCW every week through 5 weeks (Figure 4.1B). Eye movements of each animal were recorded and analyzed for every session. Analysis of the No. of saccades/min and of the OKR gain showed that the OKR gain is a more reliable parameter, since no statistically significant differences in the OKR gain were observed between the different recordings in the CW or CCW direction (Figure 4.1 C-F). Given

these results, the OKR gain was chosen as the OKR parameter to quantify the OKR performance for all the experiments done in this thesis.

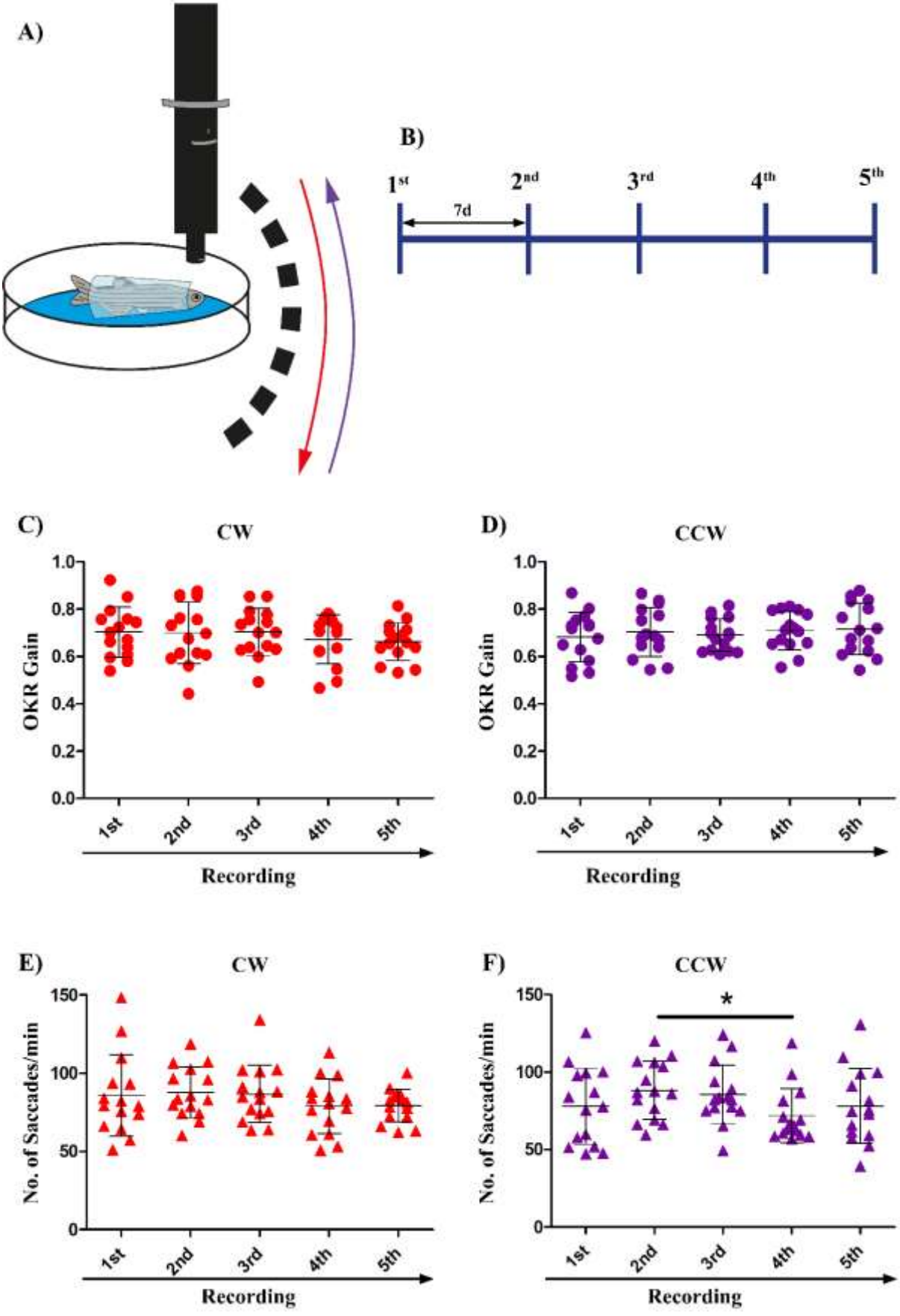

Figure 4.1 Gain is a reliable parameter to quantify the OKR in adult zebrafish.

(A)Schematic drawing of the apparatus used to elicit the OKR and record the eye movements. (B) Experimental paradigm to test the reliability of OKR parameters. (C-D) OKR gain in the CW and CCW direction of the stimuli, respectively. (E-F) Number of saccades/min in the CW and CCW direction of the stimuli. (C-F) Data shown as Mean ±SD; n (number of animals) =15; *p<0.05); Paired ANOVA test. Single dots represent individual animals indicating biological replicates.

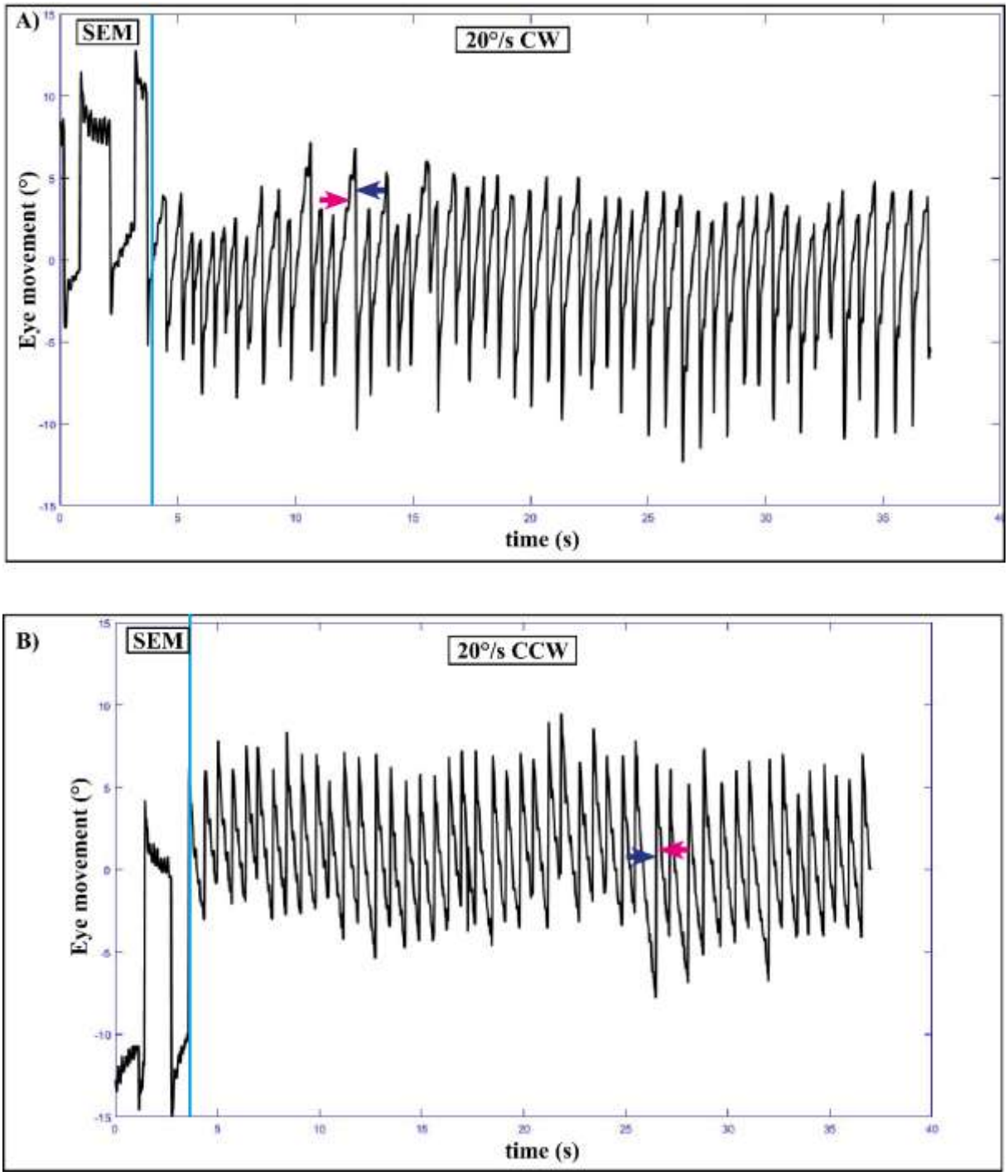

Figure 4.2. Examples of graphical representations of right eye movements of adult zebrafish before and during OKR stimulation.

(A) Right eye movements of adult zebrafish during OKR stimulation in the CW direction. The blue line indicates the separation between the spontaneous eye movements (SEM) and the evoked eye movements by the OKR stimuli. The pink and the blue arrow point at the slow and at the fast resetting phase of the OKR, respectively. (B) Right eye movements of adult zebrafish during OKR stimulation in the CCW direction. The blue line indicates the

separation between the spontaneous eye movements (SEM) and the evoked eye movements by the OKR stimuli. The pink and the blue arrow point at the slow and at the fast resetting phase of the OKR, respectively.

4.2 Lack of directional asymmetries in the OKR gain of adult zebrafish during binocular stimulation

The asymmetry of the OKR was one important reason why OKR was chosen as the behavior to study the aims of this thesis. While the asymmetry of OKR has been studied in larval zebrafish ((Qian et al., 2005), little is known about it in adult zebrafish (Mueller and Neuhauss, 2010). In response to gratings with low spatial frequencies both eyes of larval zebrafish exhibit an asymmetric response to the rotating drum, in other words, they are more responsive to temporal-to-nasal rotation than to nasal-to-temporal motion (Qian et al., 2005). This pattern is shifted when gratings with higher spatial frequencies are used (eyes become more responsive to nasal-temporal direction).

The directional asymmetry of the OKR in adult zebrafish was tested under binocular viewing conditions by using a drum which rotates with an angular velocity of 20°/s and spatial frequency of 0.03 cycles/degree in CW or CCW direction. No directional asymmetries were found for the left eye, since no statistically significant differences were found in the OKR gain between the temporal-to-nasal (CW) and the nasal-to-temporal (CCW) stimulation (Figure 4.3). The same results were also observed for the right eye, with no statistically significant differences found between the nasal-to-temporal stimulation (CW) and the temporal-to-nasal stimulation (Figure 4.3). Moreover, both eyes were equally responsive to the CW stimulation and CCW stimulation (Figure 4.3). These results suggest that differently from larval stage, during adulthood the zebrafish develops a symmetric OKR response during binocular stimulation at the velocity and spatial frequency tested.

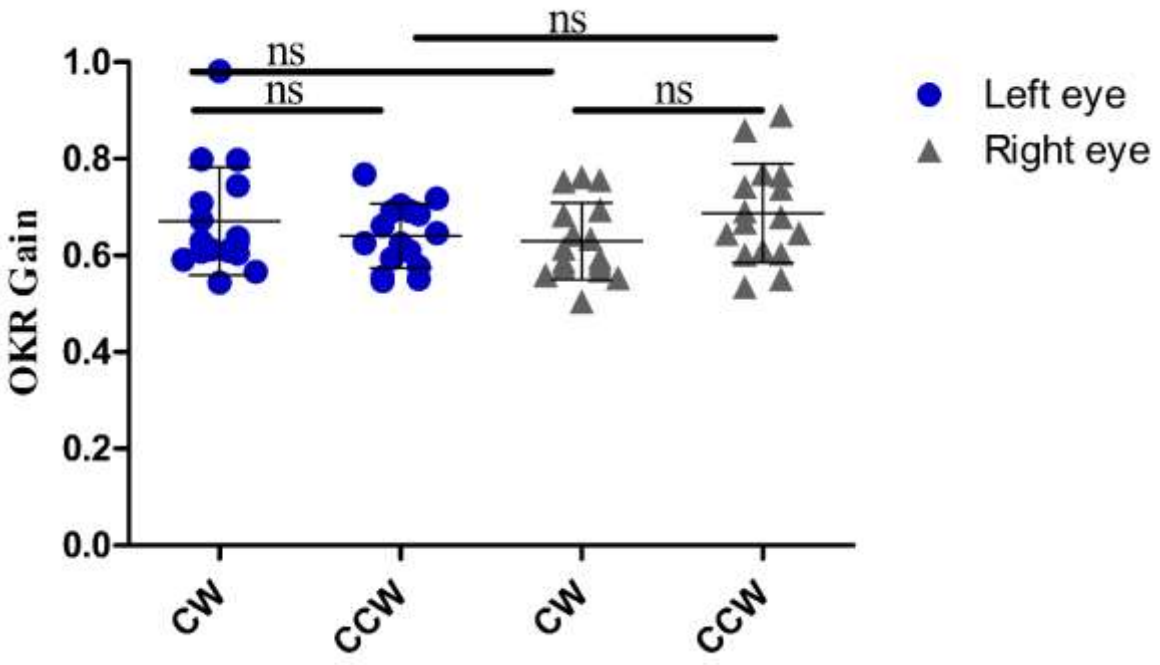

Figure 4.3. Lack of directional asymmetries in the OKR of adult zebrafish during binocular stimulation.

Graph depicting the OKR gain of left eye (blue circles) and right eye (grey triangles) in the CW and CCW stimulations. Data shown as mean ± SD; n=16; ns (non-significant), *p<0.05; Paired t-test. Single dots represent individual animals indicating biological replicates.

4.3 Asymmetry of OKR gain in adult zebrafish during monocular stimulation

The above-mentioned results (section 4.2) show that during binocular stimulation, both the eyes of adult zebrafish perform equally at the tested velocity and speed, however the most rigorous way to test OKR asymmetry is during monocular stimulation. To test the OKR asymmetry of adult zebrafish animals during monocular stimulation, enucleations of the left eye or right eye were performed and the OKR gain during CW or CCW stimulation was quantified before and after eye enucleation (Figure 4.4A). The eye enucleation was preferred compared to the monocular stimulation, in order to exclude any bias resulting from inadequacy in covering the eye.

After right eye enucleation, the animals were able to perform OKR in both direction of the stimulation (Figure 4.4C,D). Although no statistically significant change was observed in the OKR gain during CW stimulation before and after right eye nucleation (Figure 4.4D), the OKR gain after right eye enucleation in CCW direction

was significantly reduced compared to the OKR gain in CCW direction before the right eye enucleation (Figure 4.4D).

After left eye nucleation, the animals were also able to perform the OKR in both directions of the stimulation. However, after left eye nucleation the OKR gain in the CW stimulation was significantly affected compared to the OKR gain before left eye stimulation, while the OKR gain in the CCW stimulation after left eye enucleation was not statistically significant compared to the OKR gain in CCW stimulation before left eye-nucleation.

These results show that in adult zebrafish, as in larval zebrafish, the input through each eye is sufficient to evoke the OKR response during the CW or CCW stimulation. Nevertheless, only during the temporal-to-nasal stimulation are they capable of performing an OKR with similar performance as the binocular stimulation, suggesting that in adult zebrafish the OKR is asymmetric.

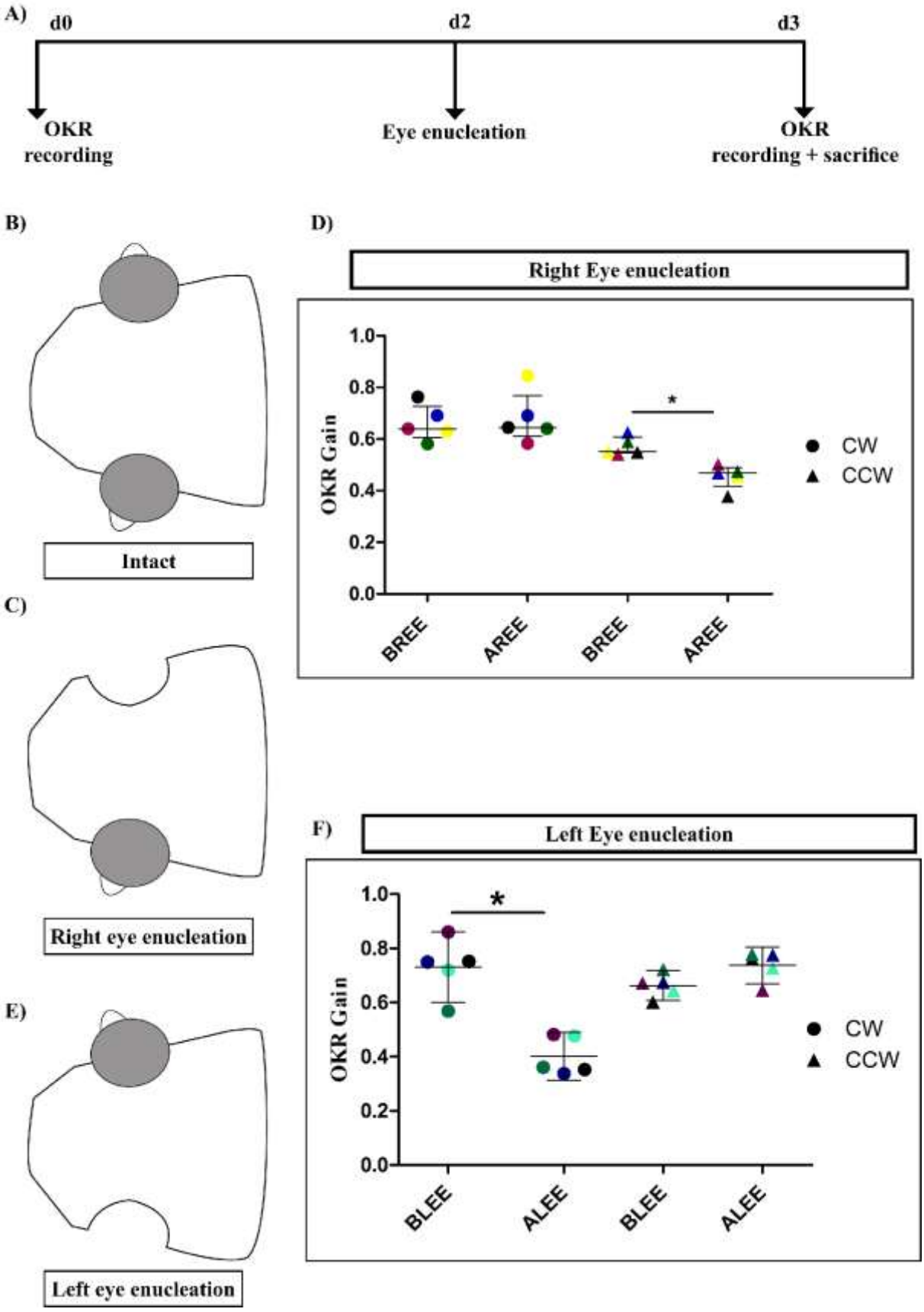

Figure 4.4. Directional asymmetries in the OKR of adult zebrafish

(A) Experimental paradigm to test the directional asymmetries in the OKR of adult zebrafish. (B-C) Schematics depicting zebrafish head before (intact) and after right eye enucleation, respectively (D) Graph depicting the OKR

gain of the left eye during CW or CCW stimulations before and after right eye enucleation. (E) Schematic depicting zebrafish head after left eye enucleation. (F) Graph depicting the OKR gain of the right eye during CW or CCW stimulations before and after left eye enucleation. Data shown as mean ± SD; n=5 for both experiment; *p <0.05; Paired t-test. Color indicates the same animal at different conditions. Abbreviations: BREE-Before Right Eye Nucleation, AREE- After Right Eye Nucleation, BLEE-Before Left Eye Nucleation, ALEE-After Left Eye Nucleation.

4.4 Neuronal circuitry mediating the OKR

Pretectal neurons play an important role in eliciting the OKR. The broad activation of the pretectum is sufficient to evoke the OKR in larval zebrafish, while experimental inactivation suppresses this behavior (Kubo et al., 2014). Pretectal neurons receive retinal input from the direction selective retinal ganglion cells (DS-RGCs) (Kramer et al., 2019) and send their projections to abducens motoneurons which in turn generate conjugate eye movements. Given their central role in the OKR circuitry, they represent a crucial target to study the functional regeneration of the zebrafish brain after stab wound injury. While advances have been made in elucidating OKR neuronal circuitry in larval zebrafish (Kramer et al., 2019; Kubo et al., 2014; Portugues et al., 2014), the precise location of the neuronal components mediating the OKR in adult zebrafish remains unknown. In adult zebrafish there are several pretectal nuclei and until today it is unknown which of them mediates the OKR (Wulliman M, Rupp B, 1996). In order to precisely lesion the pretectal neurons, knowledge of their exact location in the adult zebrafish brain is essential for the aims of this thesis. The mapping of pretectal neurons was carried out, under specific reasoning that they should receive axonal projections from the RGCs and should send efferent projections to the abducens motoneurons at the same time.

4.4.1 Anterograde labelling of the optic nerve

Visual information is transmitted to the vertebrate brain through the DS-RGCs. In larval zebrafish, the axons of RGCs project mainly to the optic tectum, hypothalamus, thalamus and the pretectum through 10 AFs named AF1-AF10 (Burrill and Easter, 1994; Robles et al., 2014).

BDAs conjugated with 647 or 488 fluorophores were applied inside the eye lobe. Animals were sacrificed after 24h and their brain was further processed to look at the optic nerve projection pattern.

In agreement with what has been previously published in larval zebrafish (Burrill and Easter, 1994; Robles et al., 2014), optic nerve projections were also present in the optic tectum (Figure 4.5 A-F''), pretectal nucleus (pink dashed circle Figure 4.5 B, B', C, C', D, D'), thalamus (yellow dashes ellipse Figure 4.5 B, B', C, C'), and hypothalamus (green dashed circle Figure 4.5 D, D'). Moreover, ipsilateral optic nerve projections were also observed. In contrast to previous studies done in zebrafish larvae, which observed ipsilateral axonal projections of RGCs projecting to AF1 only in the hypothalamus (Burrill and Easter, 1994), with this technique optic nerve projections only in the optic tectum were observed (Figure 4.5 D, D'). However, compared to the contralateral projections (Figure 4.6B, B', B'') these were fewer. Some unspecific labelling of BDA 647 was also observed in the surface of the optic tectum (Figure 4.6 A, A'', A'''). These cells are probably zebrafish Fluorescent Granular Perithelial cells (FGPs), which are well known for their capacity to 'scavenge' extracellular molecules and heavy molecular weight particles including India ink, horseradish peroxidase and ferritin, take them up, and incorporate them into their intracellular vacuoles (Galanternik et al., 2017). Altogether, these results show that BDAs reliably label the axonal projections of RGCs in the adult zebrafish brain and can be used in combination with DiI to trace the pretectal neurons processing the horizontal OKR.

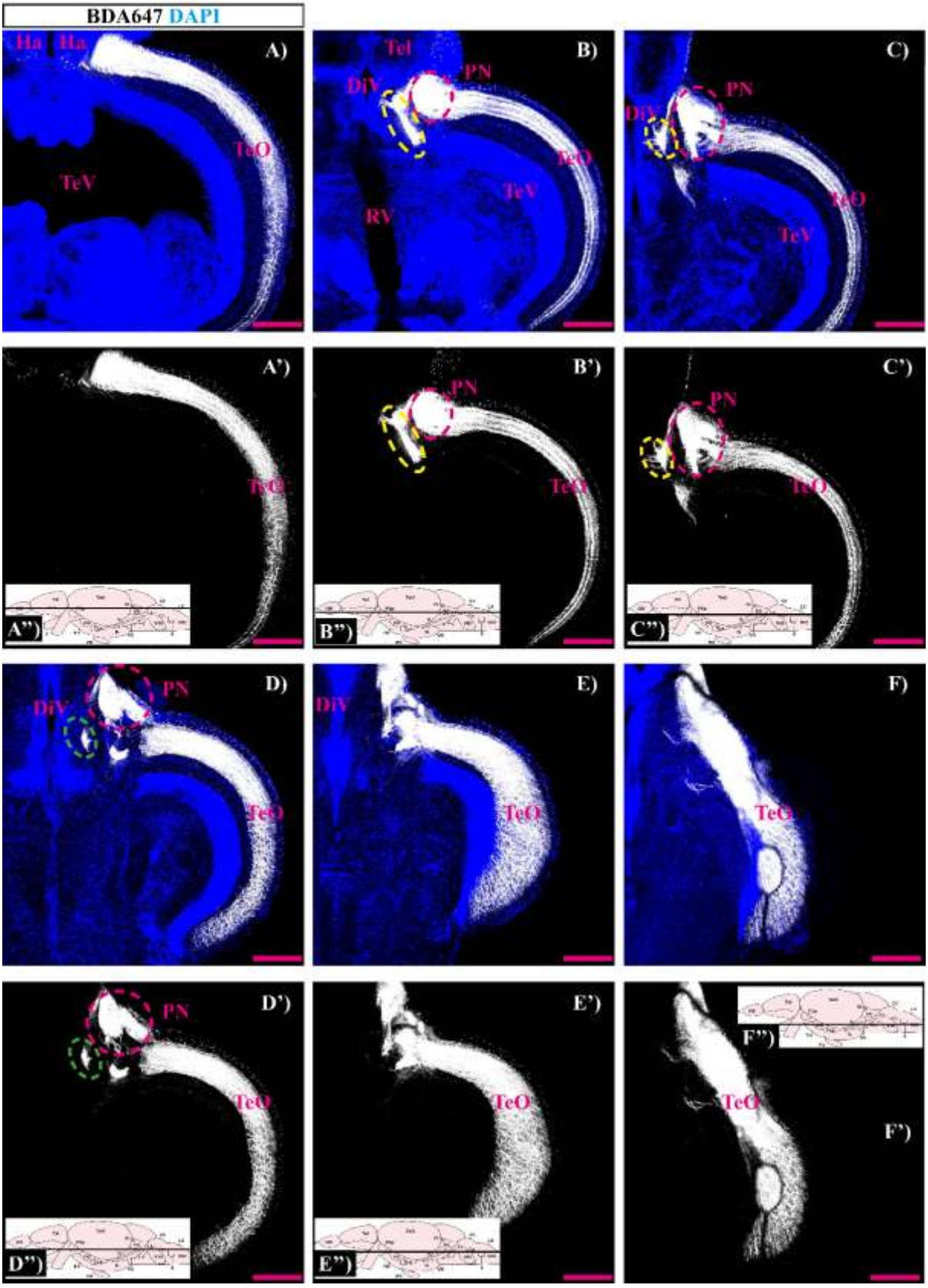

Figure 4.5. RGC axonal projections in the adult zebrafish brain.

Sections in this figure are consecutive brain sections of the adult zebrafish in dorsal-ventral direction. (A and A') RGC axonal projections in the optic tectum. (B, B', C, C'), pretectal nucleus (dashed pink ellipse) and thalamus (yellow dashed ellipse). (D and D') Optic nerve projections in the optic tectum, pretectal nucleus (dashed pink circle),

hypothalamus (green dashed ellipse). (E, E', F, F') Optic nerve projections in the most ventral sections. (A'', B'', C'', D'', E'', F'') Schematic drawings where the black line indicates the brain level the sections belong to. Abbreviations: Ha-Habenula, TeV-Tectal Ventricle, TeO-Tectum Opticum, Tel-Telencephalon, DiV-Diencephalic Ventricle, RV-Romboncephalic Ventricle, PN- Pretectal Nucleus. Scale bars-200µm. 10 animals were analyzed.

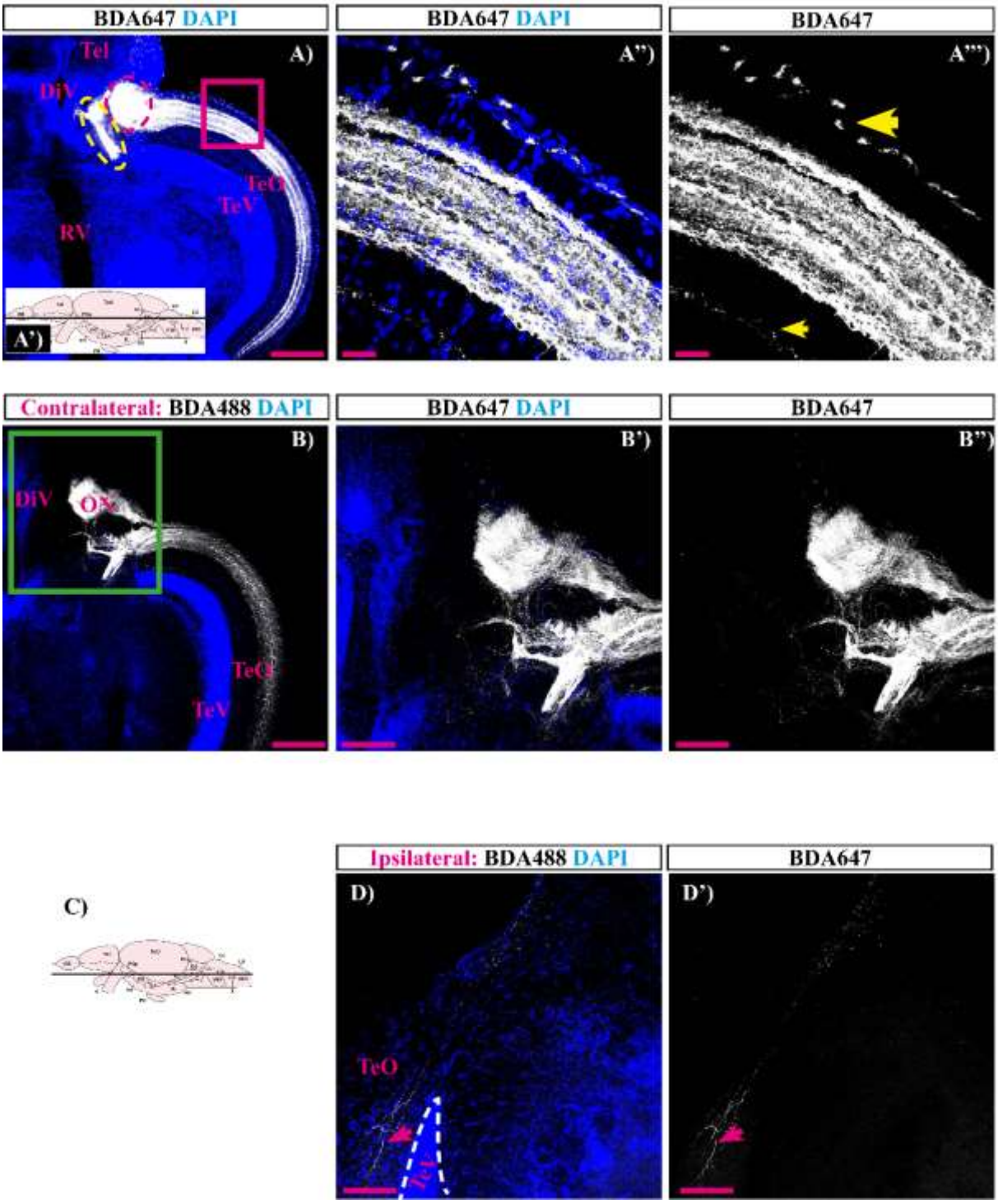

Figure 4.6. Contralateral projections of the adult zebrafish optic nerve.

(A) Sections with RGC axonal projections in the optic tectum, pretectal nucleus (pinked dashed ellipse), thalamus (pinked yellow ellipse). (A') Schematic drawing of the brain level where section A belongs to. (A'') Magnification of the boxed area in A. (A''') BDA 647 labelling in the optic tectum. The big yellow arrow points at the BDA 647 labelling in the cells at the brain surface, while the small yellow arrow points at the optic nerve projections inside the optic tectum. (B) Contralateral optic nerve projections in one ventral section of the optic tectum. (B'') Magnification of the

boxed area in B. (B''') Contralateral optic nerve entrance in the optic tectum. (C) Schematic drawing indicating the brain level where section B and D belongs to. (D and D') Ipsilateral optic nerve projections. The pink arrow points at the RGC axonal projections in the ispilateral optic tectum. Scale bars: A, B-200µm, B', B'', D, D'-100µm, A'', A'''-20µm. Abbreviations: TeV-Tectal Ventricle, TeO-Tectum Opticum, Tel-Telencephalon, DiV-Diencephalic Ventricle, RV-Romboncephalic Ventricle, PN- Pretectal Nucleus.

4.4.2 Tracing of pretectal neurons mediating the OKR

After the axonal projections of RGCs were reliably labelled in the adult zebrafish brain with BDAs, the next obvious step was to combine this technique with the DiI retrograde labelling from the abducens motoneurons, in order to map the pretectal neurons that mediate the OKR in the adult zebrafish brain (Figure 4.7 B). Towards this aim, we first labelled *in-vivo* the axonal projections of left-eye RGCs by using BDAs conjugated with 488 fluorophore as described in section 4.4.1. Afterwards, the animals were sacrificed and DiI crystals were placed in the exit of the right abducens nerve in the hindbrain. At the end of the third day the brain was horizontally sectioned with a vibratome and stained with DAPI to determine the tissue architecture (Figure 4.7 A).

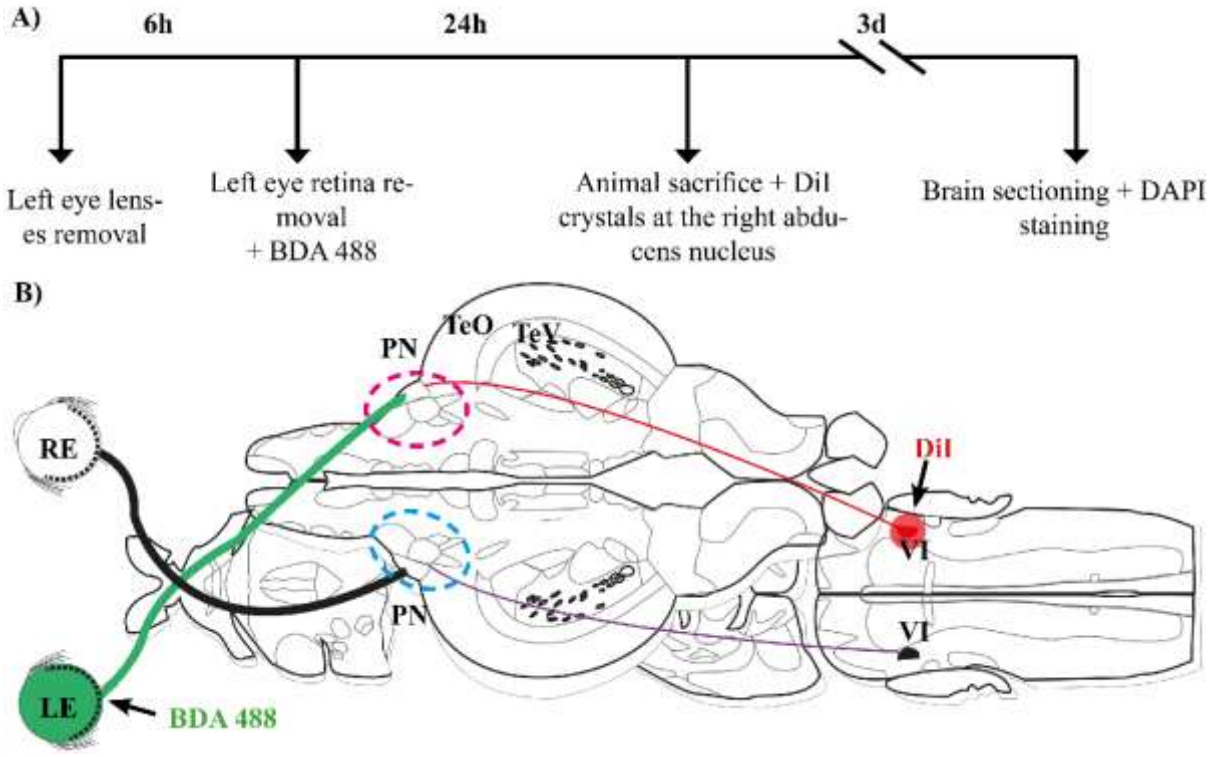

Figure 4.7. Strategy to map the pretectal neurons mediating the OKR in the adult zebrafish brain.

(A) Experimental paradigm to map the pretectal neurons mediating the OKR. (B) Scheme depicting the strategy to map the pretectal neurons. Abbreviations: LE-Left Eye, RE-Right Eye, PN-Pretectal Neurons, TeO-Tectum Opticum, TeV-Tectal Ventricle, VI-VI cranial nerve (abducens motoneurons)

Scheme modified with permission from Prof. Dr. Jovica Ninkovic.

Cells with neuronal morphology fulfilling the above mentioned criteria, meaning they receive axonal projections from RGCs and send efferent axonal projections to the abducens motoneurons, were found in only 2 sections and belonged to the parvocellular superficial nucleus (PSp) (Figure 4.8 A, A', B, B'). Immediately after, in sections containing the traced neurons, the cellular processes of these neurons were visible (Figure 4.8 C, C'). In the sections containing the cellular processes, two projections directed towards different brain areas were observed. As expected, one of the projections was directed caudally, most probably towards the abducens motoneurons (yellow arrowhead in Figure 4.8 D, E). However, it was difficult to trace these projections up to the hindbrain where the abducens motoneurons reside since the DiI labelling had spread in the entire hindbrain. The second projection crossed the midline and projected contralaterally (white arrowhead Figure 4.8 D, E anterior part), potentially to the contralateral pretectal nucleus area. These results make the PSp nucleus a very good candidate for being involved in the OKR neuronal circuitry. The next two obvious steps were to determine if the traced neurons project in the hindbrain and also to determine their contribution in the performance of horizontal OKR.

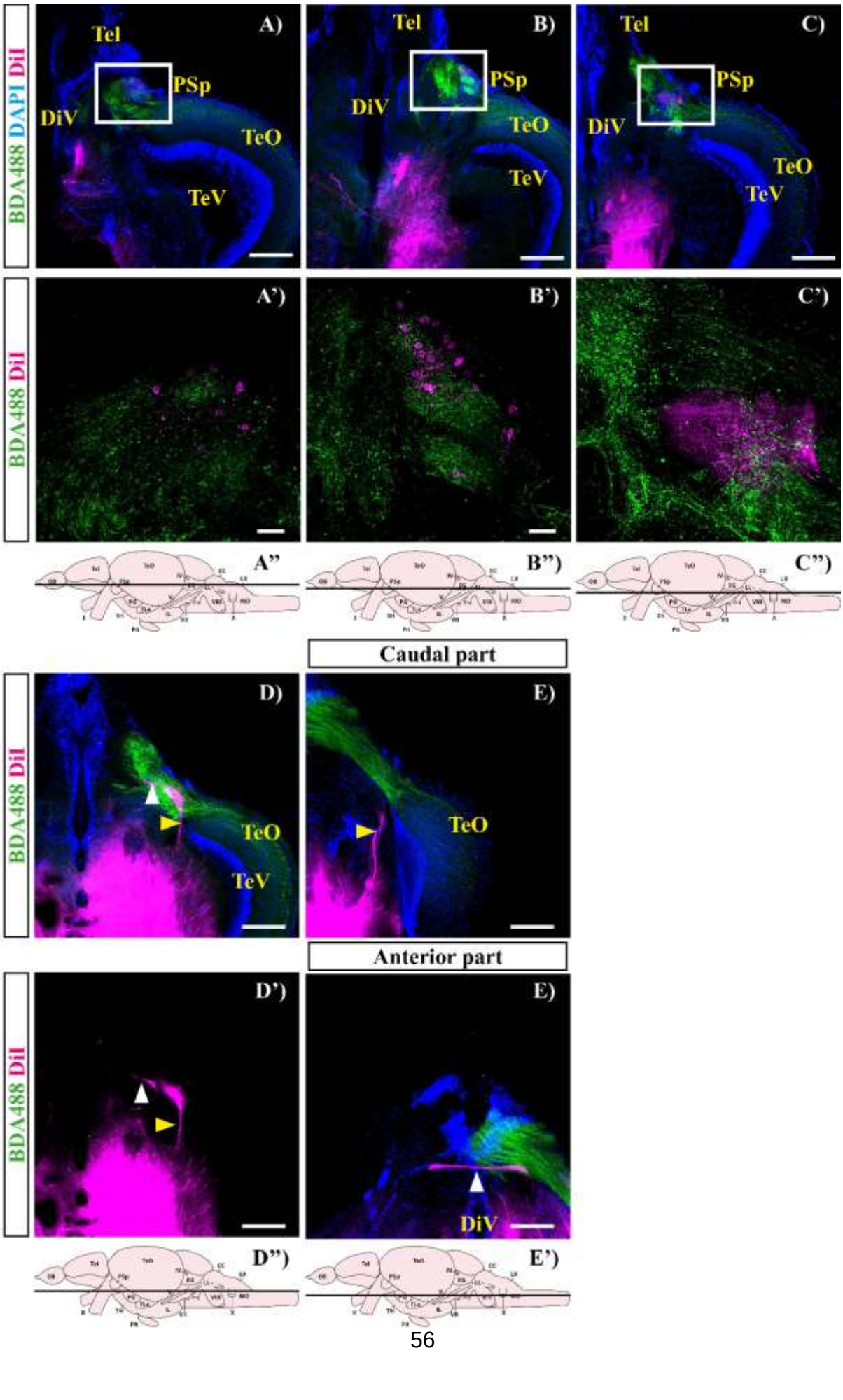
BDA488 DAPI DiI
A)
Tel
PSp
DiV
TeO
TeV
B)
Tel
PSp
DiV
TeO
TeV
C)
Tel
PSp
DiV
TeO
TeV
BDA488 DiI
A')
B')
C')
A"
B")
C")
Caudal part
BDA488 DiI
D)
TeO
TeV
E)
TeO
Anterior part
BDA488 DiI
D')
E)
DiV
D")
E')

Figure 4.8. PSp is the pretectal nucleus which receives retinal input and sends efferent axonal projections to the abducens motoneurons.

Sections in this figure are consecutive brain sections of the adult zebrafish in dorsal-ventral direction. (A, B) Cells traced with DiI which also receive retinal input were found in the area of PSp nucleus (white rectangular area in A and B. (A', B') Magnifications of the white rectangular area in A and B, showing neurons labelled with DiI and receiving retinal projections. (C) Cellular processes of the traced neurons in A and B. (C') Magnification of the white rectangular area showing the cellular processes of the traced neurons. (D, D') 2 main projections emanate from the PSp nucleus. White arrowhead points at the projection that crosses the midline and projects contralaterally (anterior part E), while the yellow arrowhead points at the projection which projects caudally (Caudal part E). (A'', B'', C'', D'', E') Schematic drawings where the black line indicates the brain level from which the sections were obtained. Scale bars: A, B, C, D, E - 200µm, A', B', C'-20µm. n (number of animals with traced pretectal neurons) =3. Abbreviations: TeV-Tectal Ventricle, TeO-Tectum Opticum, Tel-Telencephalon, DiV-Diencephalic Ventricle, RV-Romboncephalic Ventricle, PSp-Parvocellular Superficial pretectal nucleus.

4.4.3 Neurons from the PSp nucleus project to the brainstem

To determine if the PSp neurons project to the hindbrain where the abducens motoneurons reside, DiI crystals were placed at the right PSp nucleus of the dissected brain (Figure 4.9 A, B). The PSp nucleus can be accessed from the surface of the brain, so tracer was placed at the location where PSp neurons reside.

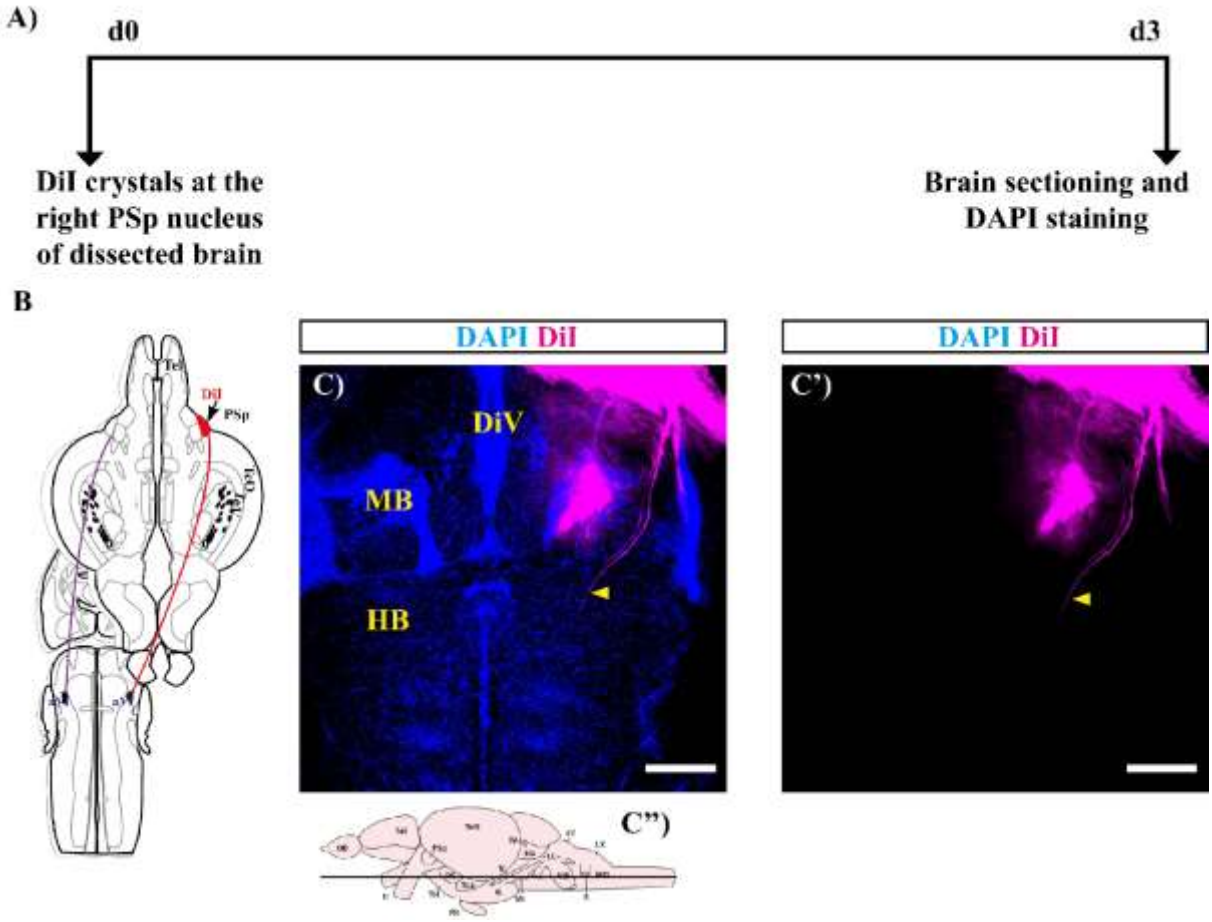

Figure 4.9: PSp nucleus sends efferent projections to the hindbrain.

(A) Experimental design to investigate the caudal projection of PSp nucleus. (B) Scheme depicting the strategy to map the caudal projection of PSp nucleus. (C, C') Section showing the projection of the PSp nucleus in the brainstem, indicate by the yellow arrowhead. C'' Schematic drawing where the black line indicates the brain level from which the section was obtained. Scale bars: C, C'-200µm. Abbreviations: DiV-Diencephalic Ventricle, MB-midbrain, HB-hindbrain.

Analysis of the confocal sections from this experiment suggested that the PSp neurons project their axons caudally towards the brainstem.

4.4.4 Injury of the PSp nucleus reduces the OKR gain during the CW stimulation

The next step to determine if the neurons of the PSp nucleus contribute to the OKR behavior was to injure them and quantify performance of the OKR. The PSp neurons were injured by inserting a microdriller in the right pretectal nucleus area (Figure 4.10A). To ensure that this type of injury kills the PSp neurons, immediately after the injury the animals were sacrificed and the tissue architecture was assessed

by DAPI staining (Figure 4.10B, C). This type of injury clearly damages the area where the PSp neurons reside, because altered tissue architecture was observed after the injury in the ipsilateral pretectal area (Figure 4.10 C), in contrast to the contralateral pretectal neurons (Figure 4.10 B).

To determine if the injury of the PSp neurons had an effect on the OKR performance, the OKR gain of the injured animals was quantified at 2 days after injury (Figure 4.10 D). Injury of the PSp neurons remarkably reduced the OKR gain during the CW stimulation at 2dpi since a statistically significant reduction of the OKR gain for both eyes was observed (Figure 4.10 E). On the other side, the OKR performance during the CCW stimulation was not affected by injury of the PSp neurons since a statistically significant change in the OKR gain was not observed (Figure 4.10F).

These results further reinforce the finding that the PSp nucleus is the pretectal nucleus mediating the OKR and set the foundations for answering the main questions of this thesis. Does the zebrafish brain recover the OKR performance after traumatic brain injury? If yes, how is this recovery mediated? What are the main players enabling it? If not, what are the reasons behind that?

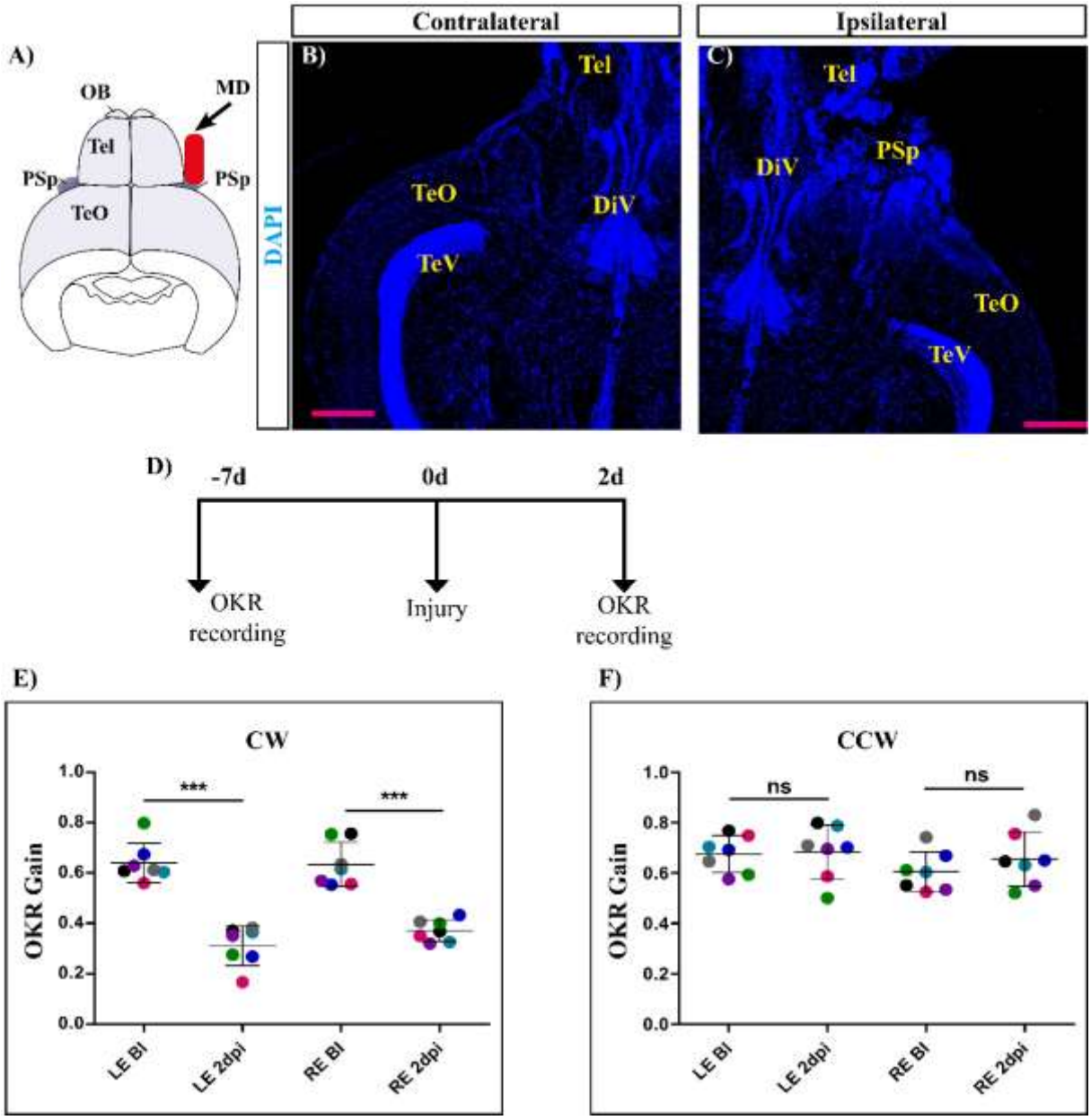

Figure 4.10 Injury of the right PSp nucleus causes a reduction of the OKR gain during CW stimulation.

(A) Scheme depicting the injury at the right PSp nucleus. The red cylinder depicts the injury tool. (B) Contralateral (left) side of the brain containing the intact pretectal nucleus. (C) Brain architecture after injury of the right PSp nucleus. (D) Experimental design to test the OKR performance after right PSp injury. (E) Graph depicting the OKR gain for both eyes during the CW stimulation before and after injury of the right PSp nucleus. (F) Graph depicting the OKR gain for both eyes during the CCW stimulation before and after injury of the right PSp nucleus. Data shown as mean ± SD; n=7; *** <0.001; Paired t-test. Abbreviations: LE-Left Eye, RE-Right Eye, BI-Before Injury, OB-Olfactory Bulb, PSp-Parvocellular Superficial Nucleus, TeO-Tectum Opticum, TeV-Tectal Ventricle, Tel-Telencephalon, DiV- Diencephalic Ventricle. MD-microdriller.

4.5 OKR performance recovers after stab wound injury of the pretectal neurons

In order to monitor the OKR performance after stab wound injury, the OKR gain from the right eye of the animals during the CW and CCW stimulation was quantified before injury, at 2dpi, 6dpi and after this timepoint every other week up to 28dpi (Figure 4.11 A). 5 month old zebrafish were used in this experiment.

According to what is reported in section 4, injury of the pretectal neurons significantly reduced the OKR gain during the CW stimulation at 2dpi (Figure 4.11 A, Figure 4.12 B). At 6dpi the OKR gain during the CW stimulation remained low for all the animals. Conversely, at 13 dpi some animals (3/10) recovered their OKR gain during the CW stimulation to the same levels as before injury (dashed black circles Figure 4.11 B, Figure 4.12 C).

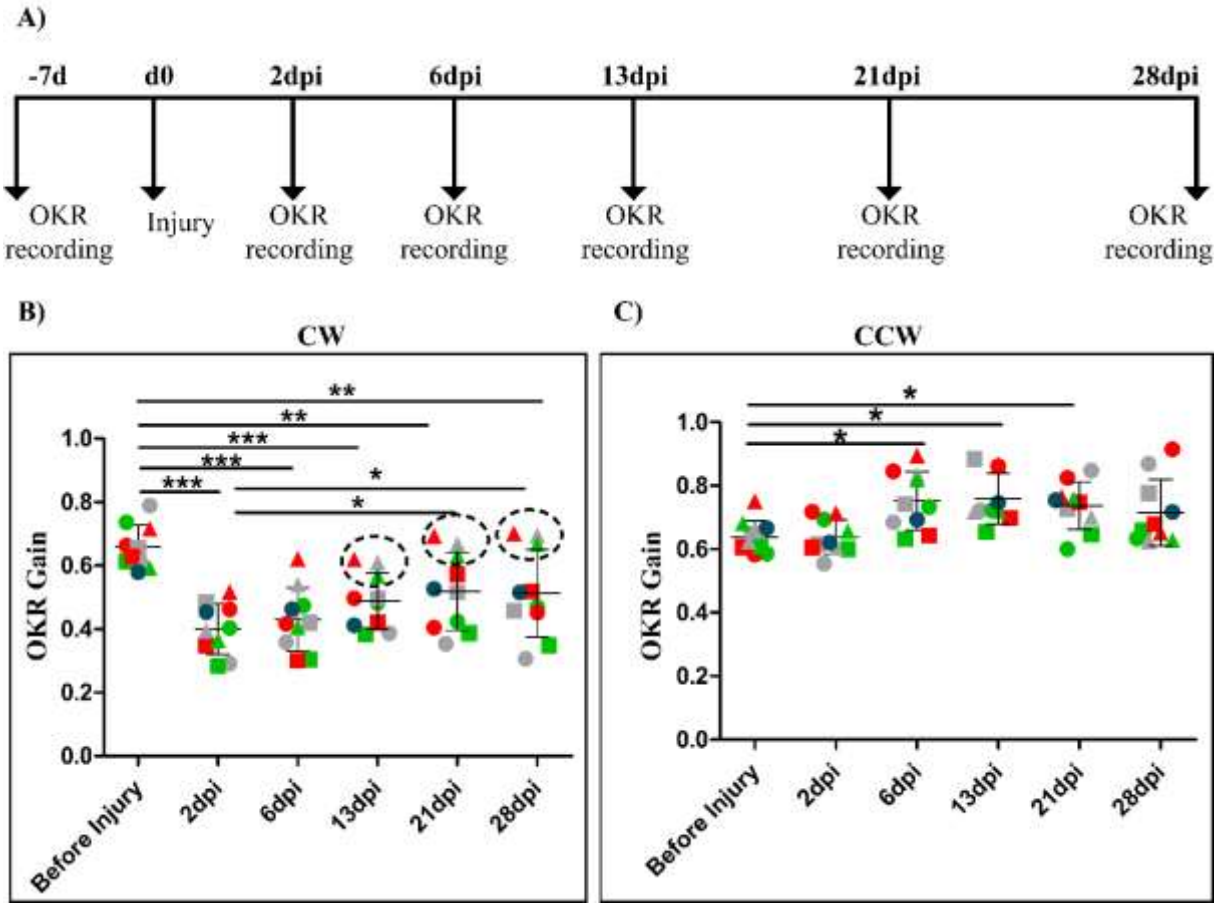

Figure 4.11. Adult zebrafish recover the OKR performance during CW stimulation at the same level as before injury.

(A) Experimental design to monitor the OKR performance recovery in adult zebrafish. (B) Graph depicting the OKR gain during the CW stimulation up to 28 days after pretectal nucleus injury. (C) Graph depicting the OKR gain during

the CCW stimulation up to 28 days after pretectal nucleus injury. Shapes with the same color represent the OKR gain of the same animal. Data shown as mean ± SD; n=10;Paired ANOVA test: *p≤0.05.

Once the OKR gain of the animals was recovered it remained stable during the recordings done at 21dpi and at 28dpi (dashed black circles Figure 4.11A). However, the animals that did not recover the OKR performance (7/10) to the same levels as before injury at 13dpi and also did not recover the OKR gain at the other timepoints. However, a significant increase in the OKR gain at 21 days after injury compared to 2dpi was observed suggesting that a partial recovery takes place (Figure 4.11 B).

A big surprise was OKR performance during CCW stimulation after stab wound injury of the pretectal neurons. As we expected at 2dpi a statistically significant change in the OKR gain during the CCW stimulation compared to before injury was not observed (Figure 4.11 C and Figure 4.13 B). However, already at 6dpi a statistically significant increase in the OKR gain was observed compared to before injury (Figure 4.11 C). This increase in the OKR gain during the CCW stimulation persisted up to 21 days post injury (Figure 4.11 C).

The above-mentioned results show that the adult zebrafish brain can functionally recover the behavior after stab wound injury and at the same time raise the following questions:

How is this recovery mediated? Is it mediated by compensation from the other circuitries or is it mediated by newly generated neurons? Why is the recovery heterogenous between the animals? Why is the OKR gain during the CCW stimulation increased at 6dpi? How is this increase in OKR gain during the CCW stimulation mediated?

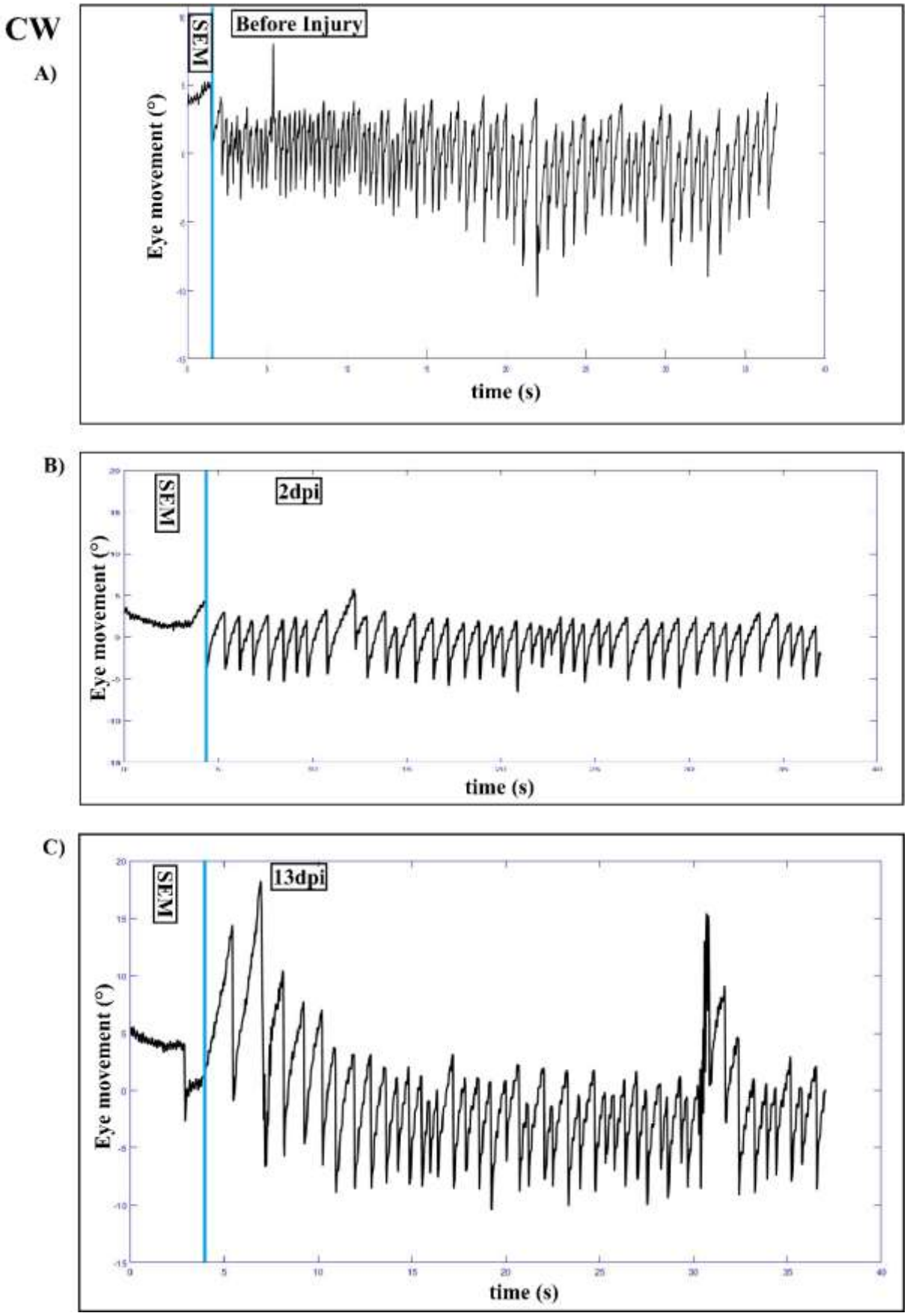

Figure 4.12. Examples of graphical representations of right eye movements of adult zebrafish before and during OKR stimulation in the CW direction before and after injury.

(A) Right eye movements during the OKR stimulation in the CW direction before injury. (B) Right eye movements during the OKR stimulation in the CW direction at 2dpi. (C) Right eye movements during the OKR stimulation in the CW direction at 13dpi.

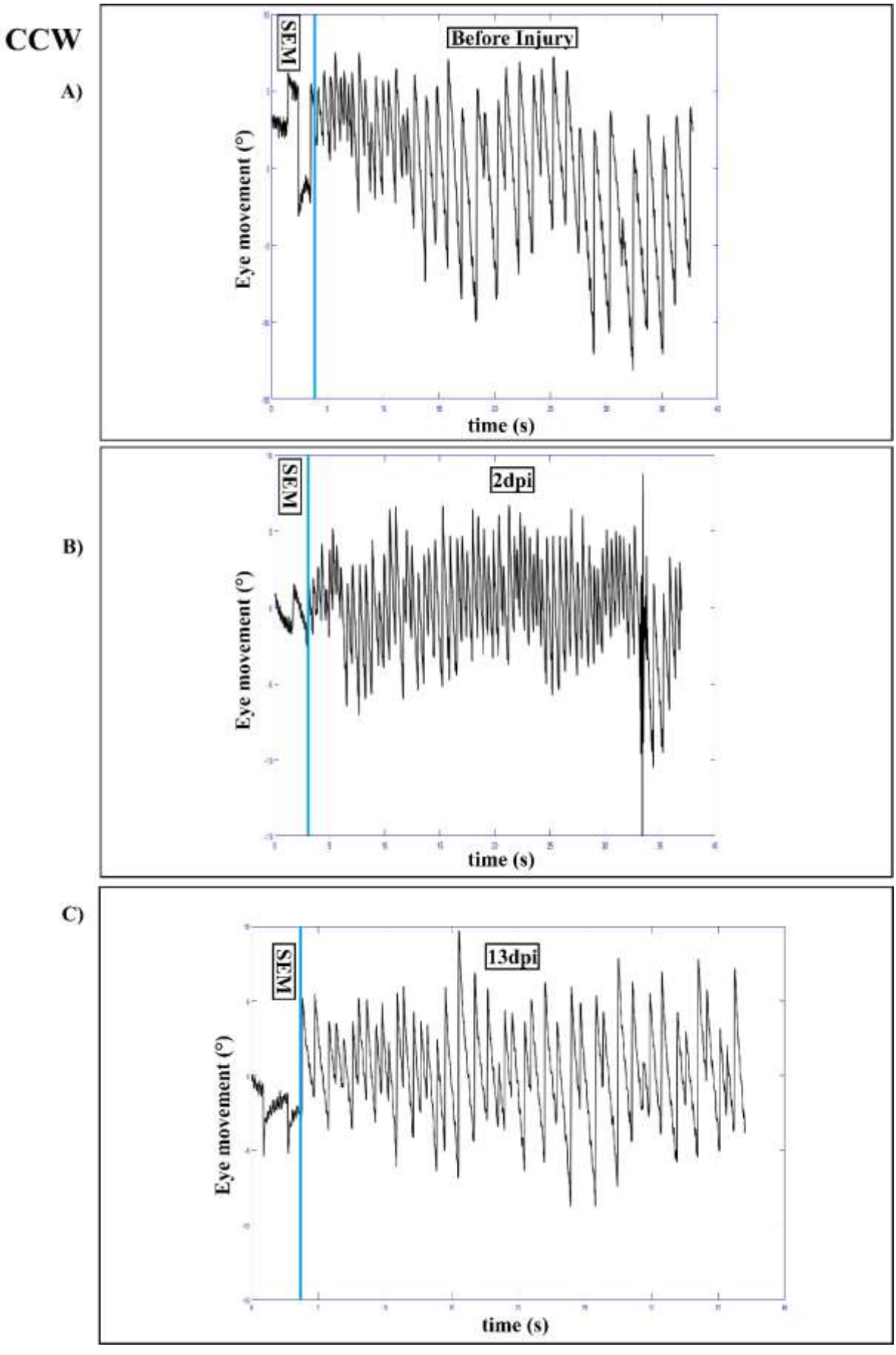
CCW
A)
SEM
Before Injury
Eye movement (°)
time (s)
B)
SEM
2dpi
Eye movement (°)
time (s)
C)
SEM
13dpi
Eye movement (°)
time (s)

Figure 4.13. Examples of graphical representations of right eye movements of adult zebrafish before and during OKR stimulation in the CCW direction before and after injury.

(A) Right eye movements during the OKR stimulation in the CCW direction before injury. (B) Right eye movements during the OKR stimulation in the CCW direction at 2dpi. (C) Right eye movements during the OKR stimulation in the CCW direction at 13dpi. Abbreviations: SEM-Spontaneous Eye Movements.

4.6 The recovery of OKR performance is not due to compensation by the uninjured side of the brain

To assess the effect of compensation from the uninjured side of the brain in the recovery of the OKR gain, first the injury of the right pretectal nucleus was performed and the OKR gain of the injured animals was quantified at 2dpi to confirm that the injury led to the reduction of the OKR gain. Afterwards, at 13 dpi the OKR gain of the animals was quantified to screen for those that recovered the OKR gain to the same levels as before injury. The next day the left eye of all the animals was enucleated, in order to allow visual input in the zebrafish brain only to the intact side. If the recovery of the OKR gain was due to the uninjured side of the brain, then the left eye enucleation (LEN) is not expected to influence the recovered OKR gain. To assess the effect of LEN on OKR performance, the OKR gain was quantified 1 day after LEN experiment. (Figure 4.14 A). The animals used in this experiment were 3.5 months old.

Injury of the right pretectal nucleus led to a reduction in the OKR gain during CW stimulation at 2dpi and 57% (4/7) of the animals recovered their OKR gain during the CW stimulation at the same levels as before injury at 13dpi (Figure 4.14 B), while the OKR gain during CCW stimulation was not affected at any timepoint (Figure 4.14 C). After LEN, 86% (6/7) of the animals reduced their OKR gain during the CW stimulation compared to 13dpi. These results support the idea that the recovery of the OKR gain is not due to compensation from the intact side of the brain, since it alone cannot support the OKR gain recovery.

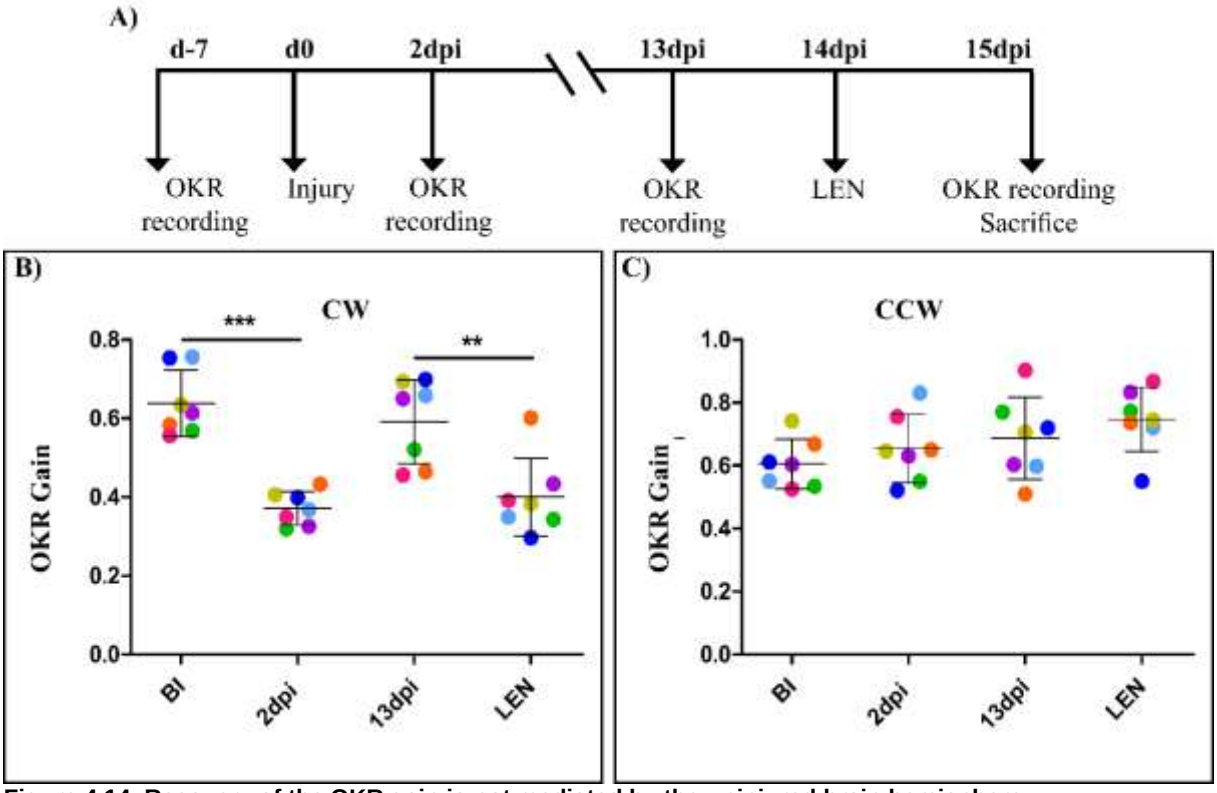

Figure 4.14. Recovery of the OKR gain is not mediated by the uninjured brain hemisphere.

(A) Scheme representing the experimental design to test the recovery of the OKR gain from the intact side of the brain. (B, C) Dot plot showing the OKR gain of 7 animals during the CW stimulation (B) and during the CCW stimulation (C) before injury (BI), at 2dpi, at 13dpi and after left eye nucleation (LEN**)..** Data shown as mean ± SD; *p≤ 0.05 (Paired ANOVA test). Single dots represent individual animals (differently color coded) indicating biological replicates in the dot plots.

4.7 Newly generated neurons mediate the OKR gain recovery after injury of the pretectal neurons

4.7.1 PSp neurons reside close to the radial glia-like cells

Based on previous studies, neurogenesis after injury and circuit remodeling of the brain were the first two possibilities considered to mediate the recovery of the OKR gain after the injury of the pretectal nucleus in the right hemisphere of the brain (Bareyre et al., 2004; Baumgart et al., 2012; Berg et al., 2011a, 2013; Joven and Simon, 2018; Kroehne et al., 2011; Lazarini et al., 2014; Parish et al., 2007; Reimer et al., 2008; Takatsuru et al., 2013, 2009).

The first requirement for considering reactive neurogenesis as a factor mediating the recovery of OKR gain is the presence of niches containing neural stem/neural progenitors cells close to the PSp nucleus. To assess the presence of niches containing neural stem cells, the PSp neurons were retrogradely traced with DiI

in the reporter line for glial fibrillary acidic protein (GFAP, Tg(*gfap*:GFP)^{mi2001}), which labels the RG-like cells in the adult zebrafish brain (Barbosa et al., 2015, 2016; Baumgart et al., 2012).

Analysis of confocal images of the sections containing the PSp neurons retrogradely traced with DiI revealed the presence of RG-like cells (GFP+ cells) in the diencephalic ventricle (DiV) and in the tectal ventricle (TeV) (Figure 4.15 B, B', B'', C, C'). These cells have their cell soma in close contact with the tectal ventricle and have radial processes, typical of RG-like cells in the zebrafish telencephalon (Figure 4.15 C, C'). In particular, the RG-like cells adjacent to the TeV project their processes towards the most superficial layers of the optic tectum (Lindsey et al., 2019). These RG-like cells are quiescent in homeostatic conditions and only after injury in their proximity (such as optic tectum) they become highly proliferative and differentiate toward the neuronal lineage (Grandel et al., 2006; Lindsey et al., 2019; Shimizu et al., 2018).

Moreover, the presence of the cells GFP+ processes probably coming from the radial glial-like cells were observed at the PSp nucleus (Figure 4.15 D). It has been shown in rodents that these processes continuously receive environmental cues and respond to them by modifying their proliferative and differentiative behavior (reviewed in (Obernier and Alvarez-Buylla, 2019)), which strengthens the hypothesis that they might do the same in zebrafish. These findings show that the RG-like cells reside in proximity to the PSp neurons and raise the next questions: Do the RG-like cells increase their proliferation after injury of the right PSp nucleus and do they increase their neuronal output?

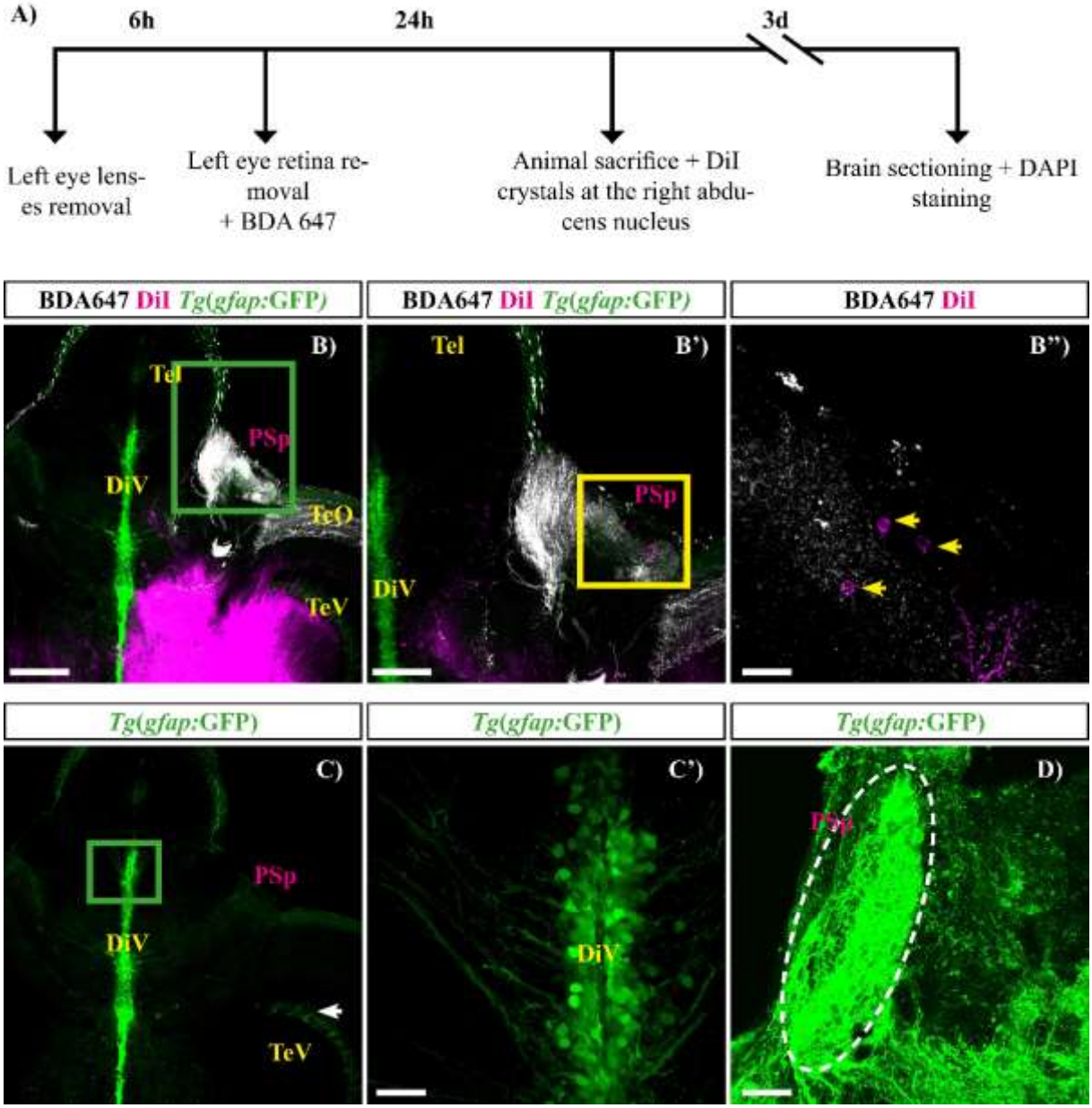

Figure 4.15. Radial glia-like cells reside close to the PSp nucleus.

(A) Schematic representation of the experimental procedure to trace the PSp neurons in the transgenic line Tg(*gfap*:GFP)^{mi2001}. (B) Horizontal section of the zebrafish brain containing the PSp neurons traced with DiI. (B') High magnification of the green boxed area in B, showing the labelled PSp neurons (yellow rectangle) and the radial glia cells in the diencephalic ventricle (DiV). (B'') High magnification of the yellow boxed area in B' showing the PSp neurons retrogradely traced with DiI (yellow arrows). (C) Single channel image showing the niches with radial glia cells close to the PSp nucleus. The radial glia cells in the tectal ventricle (TV) are pointed by the white arrow. (C') High magnification picture of the green boxed area in C showing the stem cells in the diencephalic ventricle. D) Confocal image of the PSp nucleus (white dashed circle) in the Tg(*gfap*:GFP) animals showing GFP+ processes. Abbreviations: TeV-Tectal Ventricle, TeO-Tectum Opticum, Tel-Telencephalon, DiV-Diencephalic Ventricle, PSp-Parvocellular Superficial Pretectal nucleus.

4.7.2 Radial glia-like cells at the tectal ventricle proliferate after injury of the PSp nucleus

In response to stab wound injury in the adult zebrafish telencephalon, the RG-like cells lining the ventricular zone (VZ) of the telencephalon increase their proliferation and change their division modes compared to homeostatic conditions to produce more neurons which are needed for brain repair after injury (Barbosa et al., 2015; Baumgart et al., 2012; Di Giaimo et al., 2018; Kroehne et al., 2011; März et al., 2011; Skaggs et al., 2014).

To analyze the injury induced proliferation of the RG-like/progenitor cells in proximity to the PSp nucleus, first the injury of the right pretectal nucleus was performed in Tg(gfap:GFP) animals and then the proliferation of RG-like/progenitor cells at the TeV was examined at 3dpi, which is a timepoint where the proliferation of quiescent radial glia peaks based on previous reports (Lindsey et al., 2019; Shimizu et al., 2018).

At 3dpi the number of proliferating cells (PCNA+ cells) was increased 15-fold in the TeV ipsilateral to the injured PSp nucleus compared to the TeV ventricle of intact animals (Figure 4.16 A, A',B, B', D) . Surprisingly also the number of proliferating cells in the TeV contralateral to the injured PSp nucleus was increased to almost the same levels as in the TeV ipsilateral to the injured PSp nucleus (Figure 4.16 C, C', D). In the control animals, only 30% of total PCNA+ cells were also Sox2+, while in the TeV ipsilateral and contralateral to the injured hemisphere Sox2+ cells made up 89% and 100% respectively of total PCNA+ cells (Figure 4.16 E). Consistent with previous publications from (Lindsey et al., 2019; Shimizu et al., 2018) no one of the cells positive for PCNA+ and Sox2+ were GFP+, further confirming that the radial glia-like cells in this region are highly quiescent (Figure 4.16 F). Conversely, in the TeV ipsilateral and contralateral to the injured hemisphere, 66% and 81% respectively of PCNA+ Sox2+ cells were also GFP+.

All together these results show that injury in the right PSp nucleus induces the proliferation of otherwise quiescent RG-like cells which may act in turn as a source of new neurons.

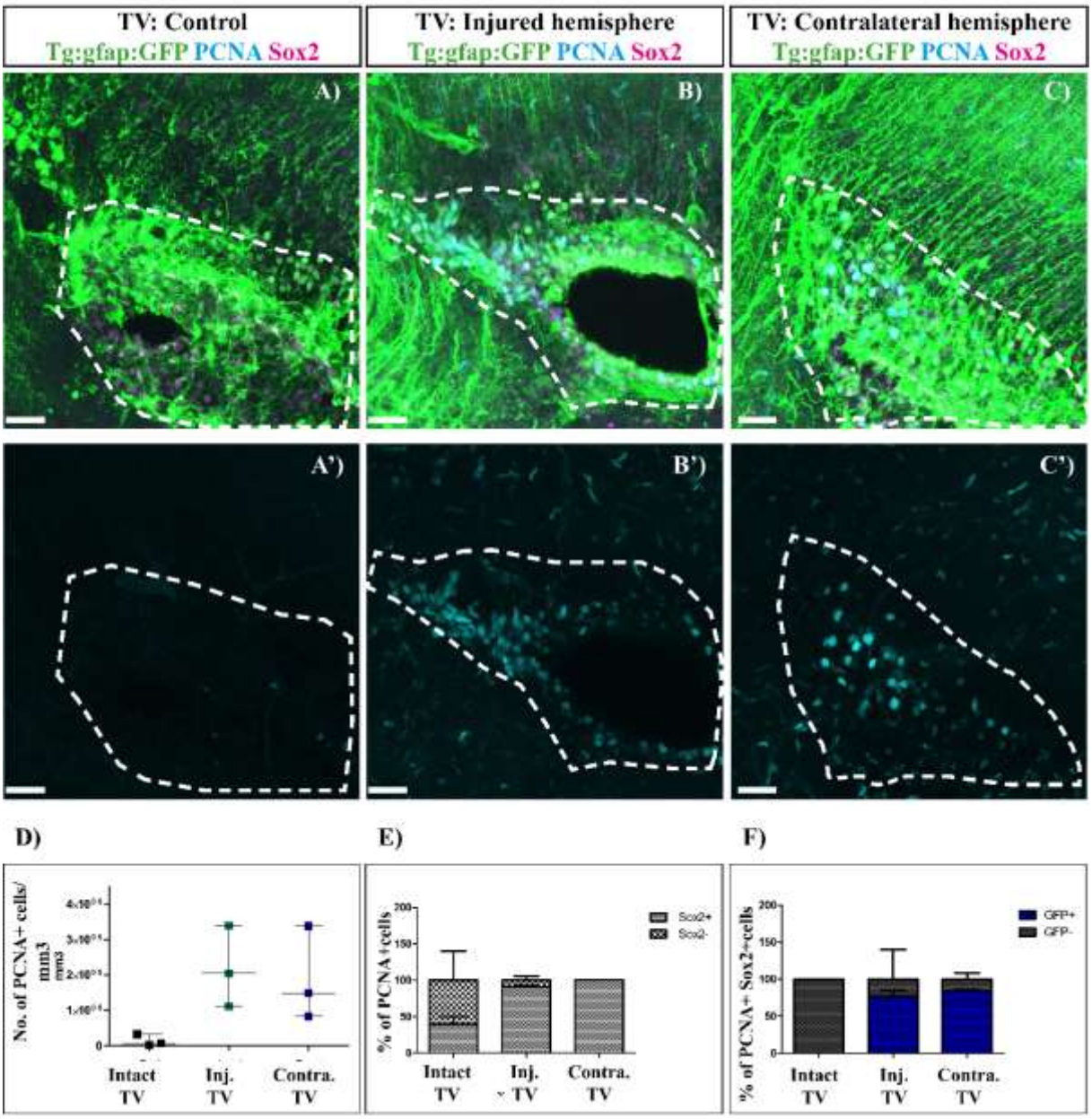

Figure 4.16.Proliferation of neural stem/progenitor cells in the tectal ventricle after injury at the PSp nucleus.

(A) Representative picture of the tectal ventricle in the intact gfap:GFP adult zebrafish brain stained with PCNA (cyan) and Sox2 (pink). (A') Single channel image of A showing the proliferation of stem cells and of their progenitors in the TV. Dashed area indicates the area was used to perform the quantification I D and E. (B,C) Representative picture of the tectal ventricle in the injured (B) and uninjured brain hemisphere (C) of adult gfap:GFP zebrafish at 3dpi stained with PCNA (cyan) and Sox2 (pink). (B', C') Single channel image of B and C showing the proliferation of stem cells and of their progenitors in the TV the injured hemisphere (B) and uninjured hemisphere. Dashed areas indicate the areas that were used to perform the quantifications in D, E and F. (D) Dot plot depicting the density of PCNA-positive cells in the TV of intact brain, of the TV ipsilateral to the injured PSp nucleus and contralateral to the injured PSp nucleus. (E) Graph depicting the percentage (%) of PCNA-positive cells positive for Sox2 or not. Data shown as median ± IQR. Single dots represent individual animals. (F) Graph depicting the % of PCNA-positive and Sox-2 positive cells positive for GFP or not. n=3 for each condition; Data shown as Median ±IQR.

4.7.3 New neurons are generated after injury of the PSp neurons

To investigate the effect of neurogenesis on the recovery of the OKR gain, the animals that were tested for compensation from the intact side of the brain were let to swim in water containing the thymidine analogue BrdU for 3days overnight (O/N) (from 3dpi-6dpi), in order to label as many proliferating cells as possible. The animals were sacrificed at 15 dpi (Figure 4.17 A). This is a timepoint when the animals had recovered their OKR gain and also the compensation from the intact side of the brain was tested, so a presence of newly generated neurons (BrdU+/HuC/D+ cells) could be linked to the recovery of the OKR gain.

Analysis of BrdU+/HuC/D+ cells at the injured PSp nucleus and at the contralateral PSp nucleus (Figure 4.17 B, B', B'', B''', C, C', C'', C''') revealed an increase of these cells around the injured PSp nucleus, indicating that new neurons are generated after injury of the right PSp nucleus (Figure 4.18 D). The presence of these cells at 15dpi makes neurogenesis a good candidate responsible for the recovery of the OKR gain.

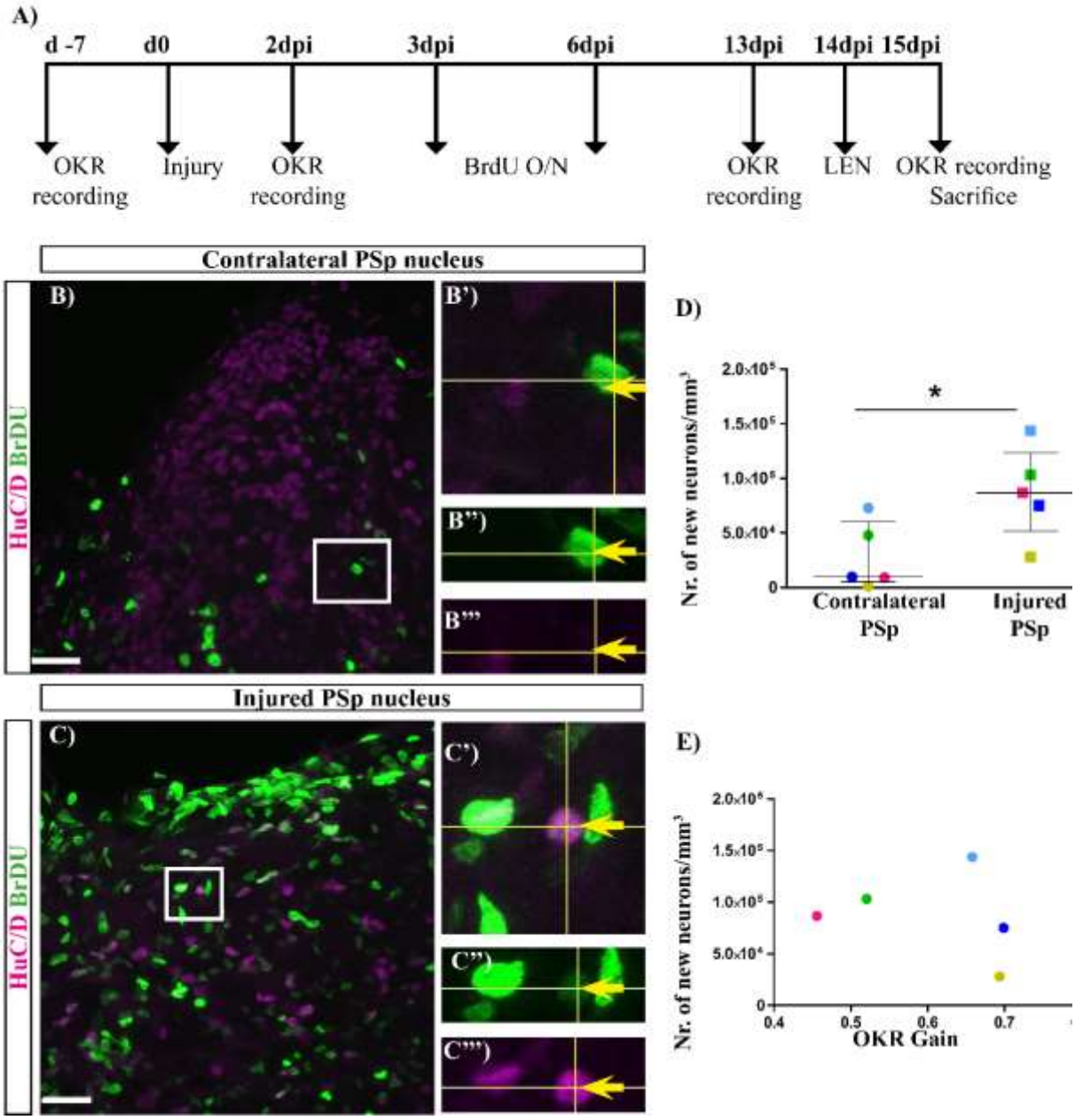

Figure 4.17. Neu neurons are generated around the PSp nucleus after injury

(A) Scheme showing the experimental outline to assess the components mediating the OKR recovery. (B,C) Confocal images showing BrdU positive cells on the contralateral side of the brain (B) and on the injured side of the brain (C) at 15dpi. High magnification images with orthogonal projections depict representative BrdU+/ HuC/D- (white boxed area B, B', B'', B''') and BrdU+/HuC/D+ (white boxed area C, C', C'', C''') cells. Scale bars B, C=20µm. (D) Dot plot showing the density of BrdU+/HuC/D+ cells in the contralateral PSp nucleus and in the injured PSp nucleus. Data shown as median ±IQR; *p<0.05 (paired Mann-Whitney test). (E) Dot plot showing the lack of correlation between the OKR gain and the density of newly generated neurons. n=5. Single dots represent individual animals (which are differently color coded) indicating biological replicates in the dot plots.

4.7.4 Cytosine arabinoside inhibits the proliferation of the stem cells

An important question prompted by the findings above is whether the newly generated neurons after the injury are responsible for the recovery of the OKR gain. One way to investigate the role of the newly generated neurons in the recovery of the OKR gain is to suppress their generation. Zebrafish generate new neurons after injury mainly through division of the neural stem cells (Barbosa et al., 2015). Therefore, one strategy to inhibit the generation of new neurons would be to interfere with the division of stem cells and their fast dividing progeny. In order to inhibit the generation of new neurons an advantage of the cytidine analogue Ara-C was taken, which inhibits DNA synthesis and consequently cell division (Doetsch et al., 1999; Kufe et al., 1984; Li et al., 2017; Paulovich et al., 1997).

To use the Ara-C in adult zebrafish to inhibit the division of neural stem/progenitor cells in the PSp injury, first a protocol was established, since no existing protocols are available up-to-date. Toward this aim, the animals were allowed to swim in water containing 1% Ara-C for 72h and in the last 12h the effect of Ara-C was tested by letting the animals swim in water containing Ara-C 1% and BrdU 0.3% (Figure 4.18 A). Afterwards, the animals were sacrificed and the number of proliferating PCNA+ and BrdU+ cells was analyzed in the VZ of telencephalon. The reason why the VZ of the telencephalon was selected to perform the analysis is because this area mainly contains proliferating neural stem cells in homeostatic conditions which contribute to constitutive neurogenesis and the effect of Ara-C can immediately be observed (Adolf et al., 2006; Barbosa et al., 2015; Grandel et al., 2006; Rothenaigner et al., 2011).

Analysis of BrdU+ cells revealed that the telencephalic VZ of animals treated with Ara-C was devoid of BrdU+ cells (Figure 4.18 B'', C''), indicating a defective DNA synthesis of the neural stem/progenitor cells compared to control brains. On the other side, the number of PCNA+ cells was increased in Ara-C treated animals compared to controls (Figure 4.18 D), suggesting an accumulation of cells arrested in the S-phase of the cell cycle.

These data show that within the established protocol Ara-C is effective in inhibiting the DNA synthesis in neural stem/progenitor cells in the VZ of telencephalon

and suggest that it can be used to inhibit the proliferation of neural stem/progenitor cells after injury at the PSp nucleus.

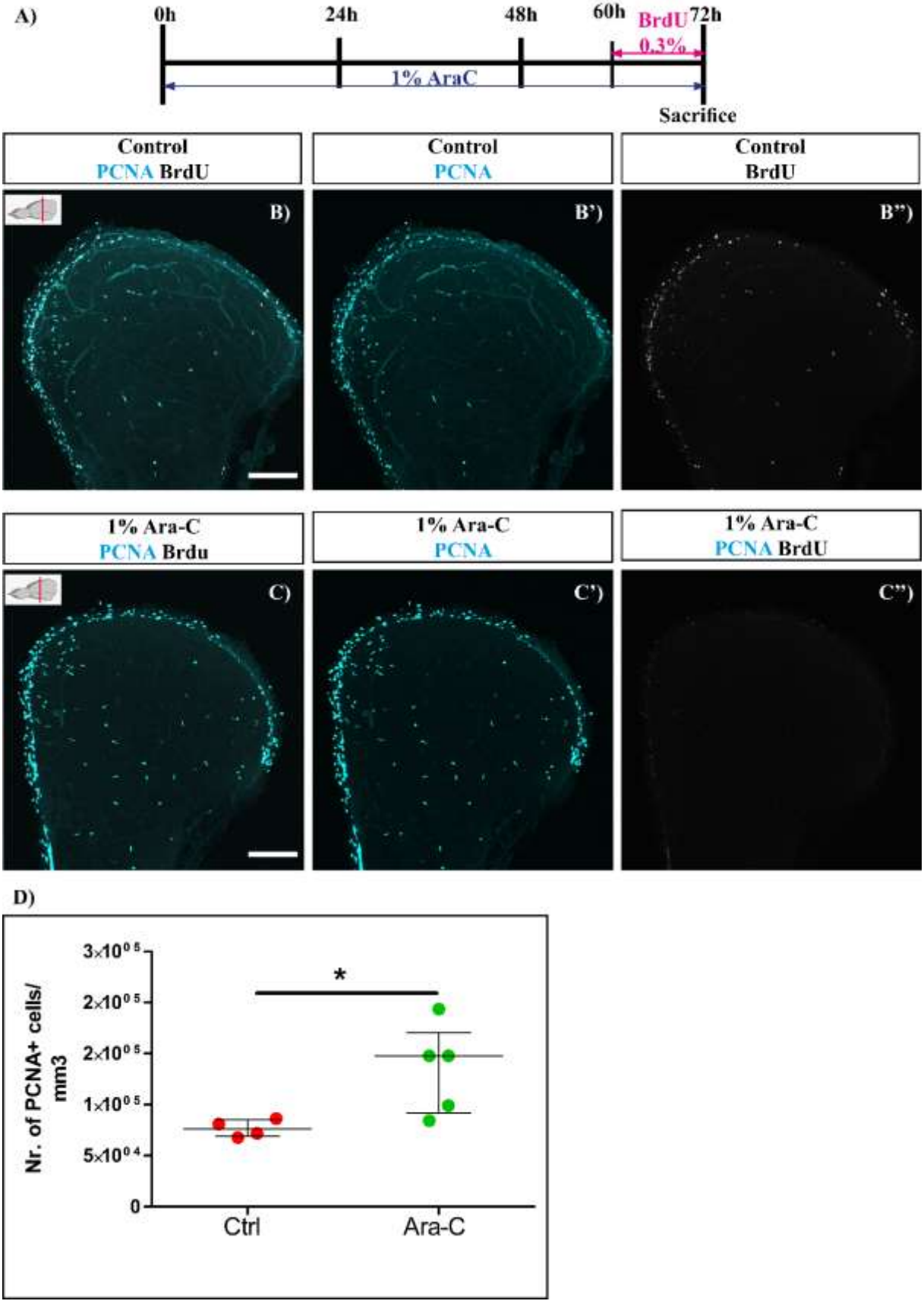

Figure 4.18. Ara-C inhibits the incorporation of BrdU in the DNA during the S-phase of the cell cycle in the neural stem/progenitor cells of the telencephalon of adult zebrafish and thereby their proliferation.

(A) Schematic representation of the experimental procedure used to test the effectiveness of Ara-C in inhibiting the proliferation of stem cells. (B, C) Representative confocal images of adult zebrafish telencephalon stained for PCNA+ (cyan) and BrdU+ after BrdU bath overnight (B) or after bath with Ara-C for 72 h and bath for BrdU the night before sacrifice (C). (C'') Please note the almost complete lack of BrdU staining in the telencephalon section of the animal that were swimming in Ara-C water. (D) Dot plot showing the density of PCNA+ cells in control and Ara-C treated animals. Data shown as median ± IQR. *p ≤ 0.05 (Mann-Whitney test). Single dots represent individual animals indicating biological replicates.

4.7.5 Cytosine arabinoside affects the generation of new neurons and the recovery of OKR gain after PSp injury

A very important question that arises from the above-mentioned findings is whether the inhibition of proliferation of neural stem cells suppresses the generation of neurons. To test this hypothesis, after injury of the right PSp nucleus animals were allowed to swim in water containing 1% Ara-C and 0.3 % BrdU from 3dpi-6dpi and sacrificed at 13dpi, which is a timepoint where the animals recover their OKR gain (Figure 4.19 A). Analysis of the density of newly generated neurons (BrdU+/HuC/D+ cells) in the PSp nucleus of Ara-C treated animals revealed a statistically significant decrease compared to control animals (Figure 4.19 D). Furthermore, the area where the injury was performed was devoid of any neurons at 13 dpi (Figure 4.19 C dashed area). These data suggest that the Ara-C treatment strongly reduces the generation of neurons after injury of the right PSp nucleus.

The next piece of the puzzle was to explore how the reduced neurogenesis affected the recovery of the OKR gain. To investigate this question, first the OKR gain of the animals was quantified before right PSp nucleus injury and at 2dpi, then the animals were let on to swim in Ara-C water from 3dpi-6dpi and their OKR gain was quantified again at 13dpi to assess the influence of reduced neurogenesis (Figure 4.20 A).

Analysis of the animals that recover their OKR gain during the CW stimulation at 13dpi, to the same levels as before injury, revealed a decrease in the Ara-C treated group compared to the control group (25% (1/4 of animals) vs. 75 % (3/4 animals)) (dots circled by black dashed circle Figure 4.20 B). No difference was observed in the OKR gain during CCW stimulation between control and treated animals, suggesting that the effect for the recovery of OKR gain during the CW stimulation is not due to side effects of Ara-C (Figure 4.20 C). These data indicate that the decreased

neurogenesis has a profound effect in the recovery of the OKR gain, implying it is a key factor in mediating functional recovery.

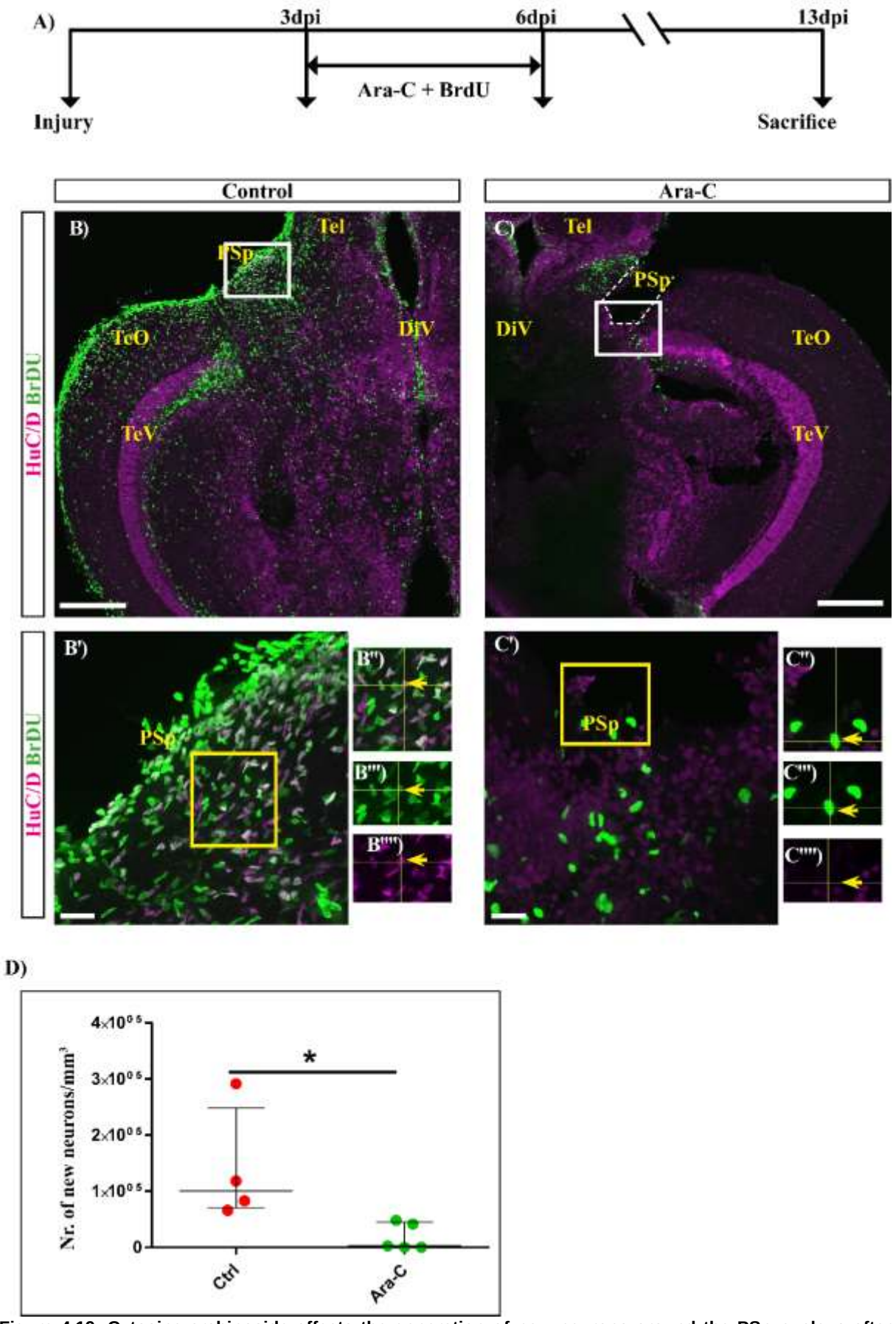

Figure 4.19. Cytosine arabinoside affects the generation of new neurons around the PSp nucleus after injury.

(A) Scheme showing the experimental outline to investigate the impact of Ara-C treatment in inhibiting the generation of new neurons after PSp injury. (B) Representative confocal image of an injured brain section 13dpi around the PSp nucleus. (B') Magnification of the white boxed area in B showing the neurogenesis around the PSp nucleus after injury. (B'', B''', B'''') Magnification of the boxed area in B', depicting an orthogonal projection of a cell positive for both BrdU and HuC/D. (C) Representative confocal image of an injured brain section 13dpi around the PSp nucleus which were treated with Ara-C. Note the dotted area, which is devoid of neurons. (C') Magnification of the white boxed area in C showing the neurogenesis around the PSp nucleus after injury. (C'', C''', C'''') Magnification of the boxed area in C', depicting an orthogonal projection of a cell positive for both BrdU and negative for HuC-D. Scale bars B,C-200µm, B',C'-20um. (D) Dot plot depicting the density of newly generated neurons (BrdU+ and HuC/D positive) around the PSp nucleus 13dpi in control and Ara-C treated animals. Data shown as median ± IQR. *$p \leq 0.05$ (Mann-Whitney test). Single dots represent individual animals indicating biological replicates. TeV-Tectal Ventricle, TeO-Tectum Opticum, Tel-Telencephalon, DiV-Diencephalic Ventricle, PSp- Parvocellular Superficial Pretectal nucleus.

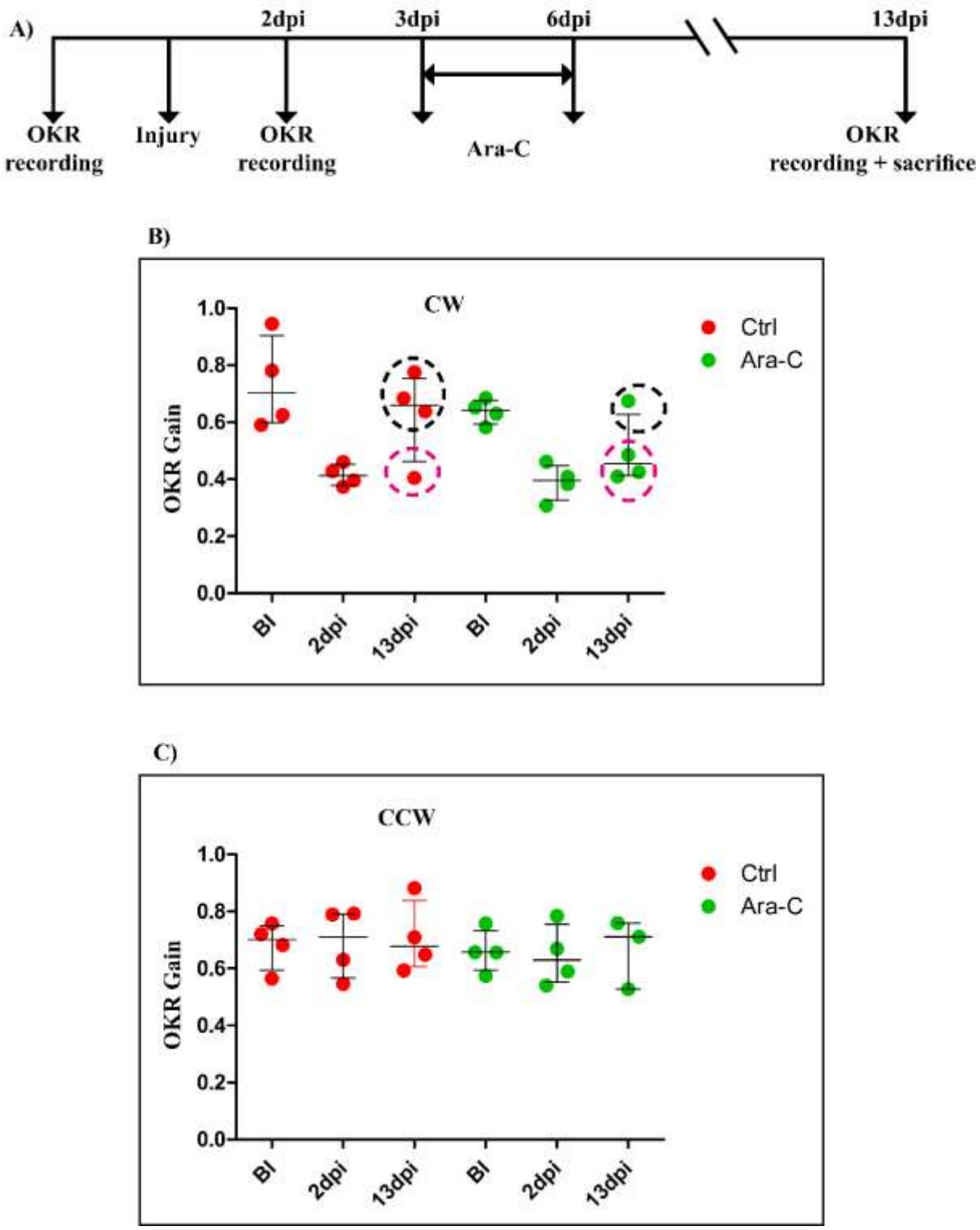

Figure 4.20. Inhibition of neurogenesis with Ara-C affects the recovery of the OKR gain after injury of PSp neurons.

(A) Scheme of the experimental protocol to assess the effect of reduced neurogenesis for the recovery of the OKR gain after PSp injury. (B) Dot plot depicting the OKR gain during the CW stimulation in control and Ara-C treated animals before injury (BI), 2dpi and 13dpi. Note that in contrast to the control animals, 3/4 of Ara-C treated animals fail to recover their OKR gain at 13dpi (dots circled by pink dashed circle. (C) Dot plot depicting the OKR gain during CCW stimulation in control and Ara-C treated animals before injury (BI), 2dpi and 13dpi. Data shown as median + IQR. Single dots represent individual animals indicating biological replicates.

5 Discussion

In order to employ the adult NSCs in repairing the adult mammalian brain, it is fundamental to first understand if and how the neurons integrate in an existing circuitry after brain injury in organisms with distinguished abilities to regenerate their nervous system. Toward this goal, first the technique to reliably quantify the OKR in the adult zebrafish brain was established and then the pretectal neurons, which are an essential part of the circuitry, were mapped. In the following sections I will discuss how the pretectal neurons were mapped and the importance of this finding for the OKR circuitry. I will then focus on the recovery of the OKR gain from the newly generated neurons at 13dpi and not from compensatory mechanisms in the contralesional hemisphere. I will continue the discussion by highlighting the importance of cell divisions after injury in order to mediate the recovery of behaviour. Finally, I will conclude by discussing how the age-dependent depletion of stem cells could affect the recovery of OKR gain during different stages and summarizing the main findings of the present work and arising questions.

5.1 PSp nucleus is part of the circuitry that enables the optokinetic reflex

By combining the anterograde labelling of the RGCs axons with BDA488 and retrograde labelling of pretectal neurons by placing DiI crystals into the abducens nucleus, cells stained with DiI and receiving retinal projections were found to be part of the PSp nucleus. Just recently Kramer and colleagues (Kramer et al., 2019) showed that the DS-RGCs responsible for the OKR in larval zebrafish terminate in AF5 and a small fraction terminate in AF6. It would be interesting to know if in adult zebrafish the AF5 is also responsible for mediating the OKR in adult zebrafish, however to achieve this, brain clearing would be necessary and DiI is not compatible with clearing treatments since it is a membrane-bound compound (Barbosa et al., 2016; Pan et al., 2016). Placement of DiI crystals into the PSp nucleus showed that the neurons of this nucleus project caudally towards the brainstem, where the abducens nuclei are located, however I was unable to see the axon terminals of the PSp neurons. It might be that at the axon terminals a dye leakage might have occurred and thus hamper the visualization of these structures (Lukas et al., 1998). The finding that the pretectal

nucleus projects toward the hindbrain is also supported by data in the larval zebrafish using the FuGIMA approach, which allows one to visualize the cytoplasmic processes of neurons activated during a specific task. Kramer and colleagues (Kramer et al., 2019) showed that some neurons located in the pretectum project toward the hindbrain.

A surprising finding from the retrograde labelling of pretectal neurons was the presence of a projection that projects in the contralateral pretectal area. This is in agreement with the results recently described by Yáñez and colleagues (Yáñez et al., 2018), who conducted a detailed study of pretectal afferent and efferent projections by using DiI. The functional significance of this projection is not known, and it would be very interesting to know if it is involved in the OKR circuitry.

Another point of proof, which further validated the connection of PSp nucleus in the OKR circuitry, is injury in of the left PSp nucleus, which resulted in a decrease of the OKR gain during the CW stimulation as expected. Since the performed injury was not specifically targeted to the PSp neurons, it might have killed not only the PSp neurons but also the nearby ones. However, with the combination of our tracing techniques, no other neurons except the PSp neurons were labelled; therefore even if the injury would kill other neurons this would probably not have an effect on the OKR performance.

In the future, transcriptomic analysis of PSp neurons would be essential to find molecular markers specific for this nucleus, which would allow the generation of transgenic animals to specifically track their connections, genetically activate and kill them (Chou et al., 2016), and test if their activation and depletion is sufficient to drive the OKR and interfere with its performance, respectively.

5.2 Role of contralesional hemisphere in mediating OKR recovery

Functional imaging in mammals has shown that the contralesional hemisphere plays an important role early after brain damage, since enhanced activity is observed (reviewed in (Caleo, 2015)). In mammals this has been interpreted as a transient vicariation of function from the contralesional hemisphere, until the relocalization of function in areas close to the injured areas in the ipsilesional hemisphere. The recovery of the OKR gain during the CW stimulation at 13dpi prompted the idea that the contralesional hemisphere could take over the lost function of the injured hemisphere,

however the contralesional hemisphere alone was not responsible for the observed recovery, suggesting that molecular and cellular events taking place in the ipsilesional hemisphere mediate the recovery of the OKR gain.

The increased OKR gain during the CCW stimulation was unexpected. One possible interpretation of this is that the animals try to compensate for the reduced OKR during the CW stimulation. Indeed, 1 week after some animals recover the OKR gain during the CW stimulation, the OKR gain during the CCW stimulation is not statistically significant compared pre-lesion levels. It would be very interesting to uncover the mechanism that enables the adjustment of the CCW OKR gain. Do the neurons in the contralateral hemisphere increase the release and synthesis of the respective neurotransmitters, as spared dopaminergic neurons do in PD (Zigmond et al., 1990; Zigmond MJ, Acheson AL, Stachowiak MK, 1984) or are other mechanisms involved? Could it be that the increased OKR gain during CCW stimulation is due to the new, improperly integrated neurons in the uninjured pretectal neurons?

The cellular events that might happen in the ipsilesional hemisphere are the reorganization of the spared neural circuits and/or the generation of new neurons which functionally replace the lost ones. In the next sections I will discuss the impact of the newly generated neurons in mediating the functional recovery of the OKR gain. However, the reorganization of the neural circuits in the ipsilesional side after injury was not possible to study in this work due to time constraints.

5.3 Recovery of OKR gain at 13 days post-injury from newly generated neurons

Mechanical injury in the right PSp nucleus area elicits a proliferation response from the RG-like cells in the tectal ventricle in the ipsilesional and contralesional hemisphere, which do not proliferate in homeostatic conditions (Ito et al., 2010; Lindsey et al., 2019; Shimizu et al., 2018). Strikingly, while the number of proliferating cells in the ipsilesional tectal ventricle was bigger than in the contralesional hemisphere, the number of proliferating RG-like cells was similar between the two hemispheres. It is well known in zebrafish that injury in one part of the brain has, as a consequence, a diffused proliferation in all brain regions, however this proliferation lasts longer in the injured hemisphere. The importance of neural stem/progenitor cell proliferation in other brain

regions than the injury is unknown. It is not known if this activation of neural stem/progenitor cells results in neuronal progeny and also what the significance of the newly generated neurons in the non-injured areas is. Since, with this work, I suggest that the PSp nuclei are interconnected, it could be that injury in the right PSp nucleus affects the release of neurotransmitters from the left PSp nucleus and this further influences the proliferation of quiescent RG-like cells in the left tectal ventricle. The influence of neurotransmitters in inducing the proliferation of quiescent RG-like cells has been shown in the newt midbrain (Berg et al., 2011b) and in the zebrafish spinal cord (Reimer et al., 2013). It remains to be studied if the RG-like cells in the left tectal ventricle increase their proliferation to self-renew, increase in number to better support the adjacent PSp nucleus or if their proliferation results in neuronal output needed for the left PSp nucleus to better cope with the injury of the right PSp nucleus.

By labelling the neural stem/progenitor cells between 3 and 6 dpi in this thesis, it was shown that at 13 dpi around the injured PSp nucleus area new neurons are generated, and this coincides with the recovery of OKR gain during the CW stimulation. However, the precise time when these neurons are generated was not investigated in this study. It would also be very interesting to investigate their migration mechanism from the neural stem/progenitor cell niche towards the injury site. Also, the HuC/D marker that was used in this work to detect neurons generated after 3dpi does not distinguish between immature and mature neurons. Further studies in the future will be fundamental to explore if the newly generated neurons display features of mature neurons and show a correct regional identity.

Despite all the previously mentioned points that still need to be investigated, the above-mentioned results suggested that the new neurons generated might have an important role in mediating the recovery of the OKR gain.

5.4 Ara-C dependent inhibition of neural stem/progenitor cell proliferation inhibits the functional recovery of OKR gain

To rule out the significance of newly generated neurons in the recovery of OKR gain during the CW stimulation, I interfered with their production. Since most of the newly generated neurons were double positive for BrdU and HuC/D, this suggested that they are produced from cells that have proliferated during the BrdU pulse between 3 and

6dpi. It is well known that in adult zebrafish, after mechanical or excitotoxic injuries, neural stem/progenitor cells increase their proliferation in order to generate more neurons needed for the tissue recovery (Barbosa et al., 2015; Baumgart et al., 2012; Caldwell et al., 2019; Di Giaimo et al., 2018; Kaslin et al., 2017; Kishimoto et al., 2012; Kroehne et al., 2011; Lindsey et al., 2018; Shimizu et al., 2018; Skaggs et al., 2014), however none of the previous studies interfered with their proliferation response after injury.

Application of Ara-C 1% in the swimming water of adult zebrafish between 3dpi and 6dpi resulted in a drastic reduction in the number of newly generated neurons and in an injured area devoid of neurons. Moreover, 75% of animals treated with Ara-C failed to recover their OKR gain during the CW stimulation. These data strongly suggest that the newly generated neurons strongly influence the recovery of the OKR gains. The fact that Ara-C treatment does not hamper the recovery of 25% of animals might be due to genetic variances in the proteins that uptake Ara-C inside the cell, reduced levels of Ara-C activating enzymes (which convert Ara-C to Ara-CTP), increased levels of inactivating enzymes or increased cellular dCTP pools, that can compete with DNA incorporation of Ara-CTP (reviewed in (Lamba K, 2010).

In the adult zebrafish telencephalon pallial niche, one mode of the adult NSC to produce new neurons is to directly convert into neurons (Barbosa et al., 2015; Dray et al., 2015). However, this behaviour of stem cells has been observed only during homeostatic conditions (Barbosa et al., 2015; Dray et al., 2015) and after injury when the aryl hydrocarbon receptor pathway (AHR) pathway is activated (Di Giaimo et al., 2018). One way for the adult NSC to produce neurons when their proliferation is affected would be to directly differentiate into neurons. Our results suggest that this might not be the case for the cells in tectal niches, since the injured area is devoid of HuC/D+ cells*. In-vivo* imaging of stem cell niches in this area after injury of the PSp nucleus in Ara-C treated animals could shed better light on this hypothesis.

One mechanism by which the zebrafish regenerates its heart is by dedifferentiation and proliferation of spared cardiomyocytes. The lack of HuC/D+ cells in the injury area of animals treated with 1% Ara-C also excludes the possibility of dedifferentiation of spared neurons, however in case the spared neurons would dedifferentiate and proliferate this cannot be ruled out. *In-vivo* imaging of transgenic animals which express H2B-GFP (Dray et al., 2015; Hadjantonakis and Papaioannou, 2004) under

the promotor of a gene expressed in mature neurons would be crucial to determine if this happens as a regenerative response in the adult zebrafish brain.

Despite this work being the third study that performs injury in proximity to the optic tectum neural stem/progenitor cell niches (2 other studies done by (Lindsey et al., 2019; Shimizu et al., 2018)), the contribution of each niche (caudal PGZ vs RG-like cells in the tectal ventricle) is unclear. Although I show that new neurons are generated after injury in the PSp nucleus, the neural stem/progenitor cells that give rise to them remain to be identified. The location of RG-like cells closer to the PSp nucleus than the caudal PGZ niche and their proliferation after PSp nucleus injury makes them a good candidate. Lineage tracing experiments of tectal ventricle RG-like cells and neuroepithelial cells in caudal PGZ will be crucial to identify the origin of neurons generated after injury in the right PSp nucleus.

5.5 Why some animals do not recover their OKR gain after injury and what are the reasons for the different ratios of recovery between 3 month and 5 month old animals?

Within this work it was shown that despite the age of the animals when the injury is performed some animals fail to recover their OKR gain. The success of recovery is 30% (3 out of 10 animals) and 57% (4 out of 7 animals) when the injury is performed at 5- and 3-months old animals, respectively. One reason for the reduced ratio of recovery in 5-month-old animals might be the reduced neurogenesis with aging. Studies in the past have suggested a depletion of adult NSC pool with age in adult zebrafish telencephalon (Barbosa et al., 2015). Moreover, Edelmann and colleagues (Edelmann et al., 2013) showed that between 3 and 6 months old in animals there is reduced neurogenesis. Although these findings have not been directly tested in the neural/stem progenitor niches in the optic tectum, our results suggest that this might also happen with the stem cell/progenitor cells in this area. Rigorous studies in the future, such as testing the homeostatic and reactive neurogenesis in the optic tectum niches in 3- and 5-month-old zebrafish, would be decisive in ruling out the age-dependent depletion of neural stem cells and how this affects recovery.

Though the number of animals that recover the OKR gain during the CW stimulation doubles in 3-month-old animals, it never reaches 100%. This might be due to different

injury size between animals. Animals with bigger injury sizes might not be able to regenerate all the lost neuronal subtypes and consequently recover their OKR gain after injury. Two studies published recently (Caldwell et al., 2019; Kaslin et al., 2017), showed that the adult zebrafish does not possess the capacity to generate all the lost neuronal subtypes and the only neuronal subtypes that can be regenerated are those produced during constitutive neurogenesis. It remains to be studied if these findings also apply to the neural stem/progenitor niches in the adult zebrafish optic tectum, however my results suggest so. A detailed analysis of the neuronal subtypes present at the pretectal nucleus, and of neurons generated by the neural stem/progenitor niches during homeostatic and reactive neurogenesis would be helpful to dissect this hypothesis.

5.6 Summary and conclusions

The present work shows for the first time that after traumatic brain injury in the adult zebrafish brain, where the neurons that mediate the OKR are lost, new neurons that functionally replace the lost ones are generated. It remains to be elucidated how the newly generated neurons integrate in pre-existing circuitry in order to mediate the observed functional recovery. Do they innervate the same neurons as the previous lost neurons, or do they have a role in making the circuitry more plastic? If the newly generated neurons innervate the same targets as the lost neurons, then what are the molecules that guide their axons toward those areas? With the existing molecular and genetic tools available currently in zebrafish it was not possible to answer these questions in the present work.

The intriguing finding that not all the animals recover the OKR gain during the CW stimulation after injury opens new possibilities to tease out the molecular mechanisms that allow and restrict brain regeneration in the vertebrate brain. Moreover, the above-mentioned results highlight once more the impact of aging-dependent adult NSC depletion. Although there are still a lot of questions that need to be elucidated, this work provides for the first time a vertebrate model where the molecular mechanisms which enable and restrict functional brain regeneration can be studied.

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7 List of publications

Di Giaimo, R*., Durovic, T*., Barquin, P., **Kociaj, A.,** Lepko, T., Aschenbroich, S., Breunig, C. T., Irmler, M., Cernilogar, F. M., Schotta, G., Barbosa, J. S., Trümbach, D., Baumgart, E. V., Neuner, A. M., Beckers, J., Wurst, W., Stricker, S. H., Ninkovic, J (2018). The Aryl Hydrocarbon Receptor Pathway Defines the Time Frame for Restorative Neurogenesis. Cell Reports 25(12), 3241-3251.

Obermann, J., Wagner, F., **Kociaj, A.,** Zambusi, A., Ninkovic, J., Hauck, S. M., Chapouton, P. (2019). The Surface Proteome of Adult Neural Stem Cells in Zebrafish Unveils Long-Range Cell-Cell Connections and Age-Related Changes in Responsiveness to IGF. Stem Cell Reports 12(2), 258-273.

* Equal contribution

8 Affidavit

Eidesstattliche Versicherung/Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation “The Role of Neural Stem/Progenitor Cells in Mediating the Functional Recovery of Adult Zebrafish Brain After Traumatic Injury„ selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation “The Role of Neural Stem/Progenitor Cells in Mediating the Functional Recovery of Adult Zebrafish Brain After Traumatic Injury„ is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den 11.12.2019 Anita Kociaj

9 List of contributions

The following people listed below provided help with experiments or helped with the analysis of data.
Dr. Johanna Miriam Schuller build the setup to elicit the OKR in adult zebrafish and to record the eye movements of adult zebrafish during OKR stimulation.

Alexander Knorr wrote the MATLAB scripts to track the adult zebrafish eye movements during OKR stimulation and to quantify the OKR gain.

Anita Kociaj Prof. Dr. Jovica Ninkovic