



João Júlio Martins

Licenciatura em Biologia Celular e Molecular

**Ontogenesis of visual processing circuits
in the zebrafish: analysis of specific
neuronal subpopulations in development.**

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Doutora Ruth Diez del Corral, Fundação
Champalimaud

Júri:

Presidente: Professora Doutora Paula Maria Theriaga Mendes Bernardes
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Arguente: Doutora Joana Freire de Castiço Monteiro

Vogal: Doutora Ruth Diez del Corral



FACULDADE DE
CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro de 2018



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Resumo

A larva de zebrafish (*Danio rerio*) é um modelo ideal para a identificação de circuitos neuronais, por exemplo comportamentos associados à visão, como as respostas optomotora e optocinética. O uso de larvas que expressam o indicador de nível de cálcio GCaMP em subpopulações neuronais específicas, permite o registo da atividade desses neurónios e facilita a sua caracterização anatómica e funcional. Neste contexto, o nosso objetivo é caracterizar algumas destas subpopulações recorrendo à expressão de GFP usando o sistema Gal4-UAS, durante os primeiros 6 dias de desenvolvimento. Para examinar e caracterizar a expressão da GFP em larvas de zebrafish, aplicámos um protocolo de imunofluorescência e registámos imagens 3D obtidas por microscopia confocal. Esta informação reunida de vários indivíduos equivalentes e registada num cérebro de referência pode ser usada para estabelecer atlas anatómicos nestas fases iniciais de desenvolvimento que complementarão aqueles já desenvolvidos. A caracterização das linhas transgênicas permitirá localizar os grupos celulares funcionais com mais precisão. Este trabalho foca-se em linhas que expressam GFF ou GFP a partir de uma inserção próxima das regiões promotoras dos genes *olig2*, *chrna4* e *pcp4a*. Duas destas linhas (*olig2*: GFP e *chrna4*: GFF) representam um padrão de expressão semelhante ao gene original, ao contrário da linha *pcp4a*: GFF, ainda assim a sua caracterização é útil para complementar os mapas funcionais de atividade neuronal. Além disso, recorrendo a microscopia de Lightsheet conseguimos acompanhar a dinâmica de diferenciação neuronal e extensão de projeções durante as 20-48 horas pós fertilização de uma linha já caracterizada no laboratório (Chat: GFF).

Palavras-Chave: Zebrafish, *olig2*, *chrna4*, *pcp4a*, *pitx2c*, *chat*, Confocal, Light Sheet, Caracterização Anatômica.

Abstract

The Zebrafish (*Danio rerio*) larva is an ideal model for identification of neuronal circuits such as those associated with visually guided behaviors like the optomotor and the optokinetic responses. The use of larvae expressing the calcium level indicator GCaMP in particular neuronal subpopulations allows monitoring of activity of these neurons and facilitates their anatomical and functional characterization. Our goal, in this context, is to characterize some of these subpopulations taking advantage of the expression of GFP using the Gal4-UAS system during the first 6 days of development. To examine and characterize the GFP expression in zebrafish larvae, we have applied an immunofluorescence protocol and recorded anatomical stacks using confocal imaging. This information gathered from several equivalent individuals and registered to a reference brain may then be used to establish anatomical atlases at these early stages of development that will complement those already developed for the 6 dpf larvae. Characterization of the transgenic lines at the early stages will help us to locate the functional clusters identified at 6 dpf with more anatomical accuracy and precision. We will present our work focusing on zebrafish lines, generated using a BAC transgenic approach, that expresses GFF or GFP from an insertion next to the *olig2*, *chrna4* and *pcp4a* promoter regions. Two of these lines (*olig2*: GFP and *chrna4*: GFF) are shown to represent a similar pattern of expression has the original gene, whilst the *pcp4a*: GFF line does not, although its characterization is still of use to complement functional maps of neuron activity. In addition, we are using time lapse imaging with Lightsheet microscopy to follow the dynamics of neuronal differentiation and extension of projections during the 20-48 hours post fertilization period on an already characterized line(Chat:GFF).

Keywords: Zebrafish, *olig2*, *chrna4*, *pcp4a* *pitx2c*, *chat*, Confocal, Light sheet, Anatomical Characterization.

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Abbreviations List

ac - Anterior commissure
AP - Anteroposterior
cCer - Corpus cerebelli
Cer – Cerebellum
cHync – Caudal hypothalamus neural cluster
Di - Diencephalon
Dpf- Days post fertilization
EGr - Eminentia Granularis
EP – pigmented epithelium
GCL –Ganglion cell layer
GFP – Green fluorescent protein
Ha - Habenula
Hpf – Hours post fertilization
Hy – Hypothalamus
IC – Intertectal Commissure
inl- Inner nuclear layer
ipl – Inner plexiform layer
LcCer - Lobus caudallis cerebelli
Met – Metencephalon
mlf – Medial longitudinal fasciculus
MO- Medulla oblongata
NpR x - Neuropil Region x
OB – Olfactory bulb
Oc – Optic chiasm
Ocn IV – Oculomotor nucleus IV
OE – Olfactory epithelium
opl – Outer plexiform layer
Pa - Pallium
pc – Posterior commissure
Pi – Pineal Gland
Po - Preoptic area
poc - Post-optic commissure
Pr - Pretectum
PTub - Posterior tuberculum
Rho – Rhombencephalon
SC – Spinal Cord

SubPa - Sub-pallium
Teg - tegmentum
Tel - Telencephalon
TeO - Optic tectum
TeOn - TeO neuropil
TeOsp - TeO stratum periventriculare
Tha - Thalamus
ThaE - Thalamic eminence
Ts - Torus semicircularis
VagR - Vagal region
vcc – Ventral caudal cluster
vCer - Valvula cerebelli
vpni - Velocity to position neural integrator
vPTub - Ventral Posterior Tuberculum
vrc - Ventral rostral cluster
vsm - Velocity storage mechanism
vTha - Ventral thalamus

1-Introduction

1.1 The larval Zebrafish as a model system in neuroscience and developmental biology

The use of Zebrafish in research has grown exponentially in the past decades, becoming, in just a few years, one of the most used animal models, with substantial contributions to biomedicine and neuroscience. (Howe et al., 2013; Martins et al., 2016)

The zebrafish (*Danio rerio*) is a cyprinid fish found throughout Southern Asia, including northern and northeastern India, Bangladesh and Nepal, in rivers, small streams, other channels, and rice paddies. (Engeszer, Patterson, Rao, & Parichy, 2007) It shares 10.660 genes with human, mouse and chicken, with about 70% homology with human genes. (Howe et al., 2013)

They are easy to breed, amenable to genetic manipulations and transparent at embryonic and early larval stages. The larval brain has a size of <0.5 mm³ and contains about 100,000 neurons. (Marquart et al., 2017; Randlett et al., 2015) These advantages allow to have a high number of larvae with virtually all neurons accessible to multiphoton microscopy in vivo at once. (Del Bene & Wyart, 2012; Friedrich, Jacobson, & Zhu, 2010) For these and other reasons the larval stage zebrafish is a very popular model to address important questions in many different areas of systems and circuits neuroscience. (Feierstein, Portugues, & Orger, 2015; Friedrich et al., 2010; Randlett et al., 2015) Despite their compact nervous systems and their age of less than 1 week, these animals are capable of a diversity of behaviors. These include swimming in three dimensions, escape maneuvers, visually guided hunting, learning and sleep. (Randlett et al., 2015)

Typically, the behaviors that have been studied in larvae are innate, reflexive responses, for example the Optomotor Response (OMR) and the Optokinetic Response. (Orger & de Polavieja, 2017; Portugues & Engert, 2009; Severi et al., 2014)

Zebrafish offer an opportunity, among vertebrate model systems, to unravel the brain's vast complexity. The rapid pace of research in this area should bring a greater understanding of behavior ontogeny, and improved tools that will make zebrafish an appealing system for more studies in genetics, development and neuroscience. (Orger & de Polavieja, 2017; Randlett et al., 2015; Scott, 2009)

1.2 Embryonic development of the zebrafish

Development of the zebrafish is comprised of several distinct phases that include: zygote (0-0.75 hpf) (hours post fertilization); cleavage (0.75-2.25 hpf); blastula (2.25-5.25 hpf); gastrula (5.25-10 hpf); segmentation (10-24 hpf); Pharyngula (1-2 dpf)(days post fertilization); (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995)

The central nervous system (CNS), during the segmentation phase, starts to change from a flat sheet of ectodermal cells, the neural plate, to a neural tube that forms in a process called primary neurulation. This, in most vertebrates, involves the invagination of the neural plate until the lateral edges meet and

fuse at the dorsal midline, forming a hollow tube. In contrast, cell movements in the neural plate of zebrafish lead to the formation of a solid rod-like structure known as the neural keel at approximately 12 hpf. (Rubenstein & Rakic, 2013)

At 16 hpf ten swellings (or neuromeres) are present along the rostro-caudal axis of the neural tube (Kimmel et al., 1995). The seven most caudal neuromeres correspond to the rhombomeres, while the anterior three neuromeres correspond to the early telencephalon, diencephalon and midbrain. By the end of the segmentation stage, significant cell movements and constrictions in the wall of the neural tube have led to the formation of the major subdivisions of the brain. (Rubenstein & Rakic, 2013)

These neuromeres are restricted by gene expression profiles, leading to presumptive neural domains such as the forebrain, midbrain, and hindbrain. This early neural patterning involves interactions between retinoic acid (RA), FGF, and Wnt signals. Early Anteroposterior (AP) patterning also establishes secondary organizers in the diencephalon, and the Isthmic Organizer at the midbrain-hindbrain boundary. (Rubenstein & Rakic, 2013)

The forebrain is composed of the telencephalon and diencephalon while the midbrain is composed of the tectum dorsally and the tegmentum ventrally. The cerebellum is derived from the midbrain/hindbrain boundary and Rhombomere 1. (Kimmel et al., 1995)

The hindbrain at 20hpf consists of seven functionally distinct rhombomeres tapering caudally to the spinal cord. At 24 hpf the spinal cord is continuous with no obvious morphological distinctions along its rostro-caudal axis. (Kimmel et al., 1995)

1.2.1 Primary neurogenesis

During an initial phase, all cells in the neural tissue are actively proliferating, but from 14 hpf, some cells exit the cell cycle to differentiate as neurons in a process designated as primary neurogenesis. The first neuronal clusters appear in stereotyped positions between 14 and 24 hpf they give rise to long axons forming a simple scaffold of axon tracts and commissures connecting different areas of the brain (figure 1.1), the scaffold is composed of two longitudinal tracts (tract of the postoptic commissure = TPOC, medial longitudinal fascicle = MLF), three transversal tracts (supraoptic tract = SOT, dorsoventral diencephalic tract = DVDT, tract of the posterior commissure = TPC) and it also includes four commissures (anterior commissure = AC, postoptic commissure = POC, posterior commissure = PC, ventral commissure = VC). (Hjorth & Key, 2002; Rubenstein & Rakic, 2013)

The neuronal clusters that form the early scaffold are present in three areas, the telencephalon (dorso-rostral cluster), hypothalamus (ventral-rostral cluster) and tegmentum (ventral-caudal cluster) (Fig 1.1). (Hjorth & Key, 2002; Rubenstein & Rakic, 2013)

The first axons to navigate the brain emerge from the vcc, they grow caudally pioneering the medial longitudinal fasciculus (MLF), later neurons from this cluster project axons rostrally into the tract of the post-optic commissure (TPOC). (Chitnis' & Kuwadai, 1990; Ross, Parrett, & Easter, 1992).

By 18 hpf the first axons in the forebrain emerge from the vrc and grow caudally to pioneer the TPOC (Chitnis & Kuwada, 1990; Ross et al., 1992)

In the midbrain, at approximately 20 hpf, neurons of the nTPC project axons ventrally to pioneer the tract of the posterior commissure (TPC). (Hjorth & Key, 2002)

Cells of the nTPC also extend axons dorsally to establish the posterior commissure (PC) and contribute to the contralateral TPC. (Chitnis & Kuwada, 1990; Hjorth & Key, 2002; Wilson, Ross, Parrett, & Easter, 1990)

At 20 hpf the ventral commissure of the midbrain is formed by ventral growing axons from the vcc which cross the midline. The patterned neural plate described above serves as a basis for the establishment of the early neuronal scaffold, which supports the first embryonic and larval behaviors. (Rubenstein & Rakic, 2013)

This first wave of neurogenesis which converts neuroepithelial progenitors only into differentiated neurons is followed by a second phase starting at approximately 24 hours post fertilization (hpf). (Rubenstein & Rakic, 2013)

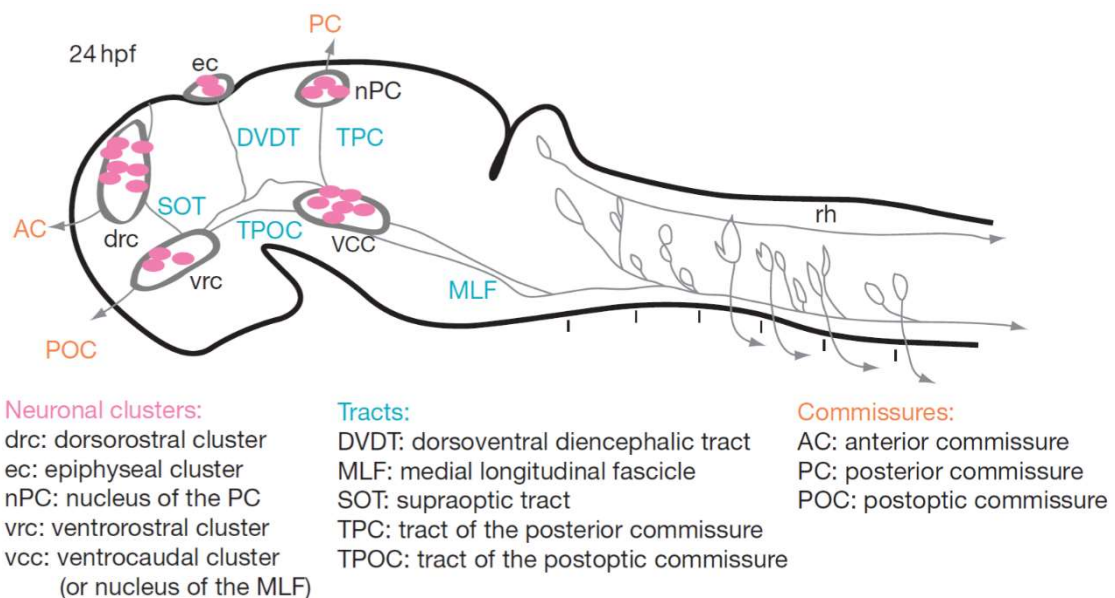


Figure 1.1: Tracts and neuronal clusters in the early zebrafish brain: Sagittal scheme of a 24 hpf brain, dorsal is to the left. Neuronal clusters (purple), tracts (blue), and commissures (red) are represented. Adapted from (Rubenstein & Rakic, 2013).

1.2.2 Secondary neurogenesis

Secondary neurogenesis first builds up on the primary scaffold increasing the number of neurons within each neuronal category. (Rubenstein & Rakic, 2013) In this second wave, both neurons and glia are produced as opposed to the early neurogenesis when only neurons are generated.

During secondary neurogenesis, some transient primary neuronal populations are also replaced. Finally, higher order circuits are established, permitting a finer modulation of larval behavior. Specifically, neuromodulatory monoaminergic circuits, neurohormones, and other regulatory systems involving excitatory and inhibitory neurotransmitters (glutamate, aspartate, GABA, glycine) are mostly established during this phase. (Rubenstein & Rakic, 2013)

The major signaling pathways, involved notably early on for the patterning of the neural plate, regulate at later stages the balance between proliferation and differentiation of neural progenitors. Newborn neurons migrate away from their birth location in the ventricular zone along distinct radial and tangential pathways to reach their final destination and integrate in the preexisting neural circuitry. (Rubenstein & Rakic, 2013)

1.3 Neurite growth

The first embryonic and larval behaviors are supported by an early neuronal scaffold, this primary scaffold, is composed of early born neurons. (Rubenstein & Rakic, 2013)

Outgrowth of axons during neuronal development is controlled by the growth cone at the tip of the axons. The growth cone is a highly motile structure at the distal tip of the axon, it has exceptionally differentiated cellular processes fulfilling several functions: it acts as sensor of environmental cues, signal transducer, and motility device.

To guide an axon towards the appropriate target, the growth cone must continuously sample the microenvironment for guidance cues and integrate this information rapidly into appropriate motility responses, this advance and targeting is mediated by specific extracellular cues which are diffusible, bound to cell membranes or the extracellular matrix, that trigger signaling cascades that eventually converge into the cytoskeleton. (Bagnard, 2007; Vidaki et al., 2017)

The axonal cytoskeleton is composed of three main filamentous polymers: neurofilaments, microtubules and actin microfilaments. Within the growth cone, actin filaments are one of the major cytoskeletal components. The peripheral domain (P-domain) is the most distal part of the growth cone, and highly dynamic, actin-rich structure. This domain possesses lamellipodia, which have actin filaments organized in a loose meshwork, from which many filopodia, with thick actin bundles, undergo elongation and retraction cycles allowing for the movement of the axon. (Bagnard, 2007)

The Actin filaments, that allow the elongation and retraction cycles, are helical polymers formed by addition of ATP-actin monomers, they are polarized structures characterized by a "pointed" and a "barbed" end. Attractive cues such as netrin-1 and nerve growth factor (NGF) stimulate axonal protein

synthesis of constituents of the cytoskeleton, whereas repulsive guidance cues like Sema3A and Slit2 induce local synthesis of proteins that promote the disassembly of the cytoskeleton. (Bagnard, 2007)

Actin-associated proteins in the neuronal growth cone help regulate actin polymerization and depolymerization. (Bagnard, 2007)

Actin nucleation/polymerization factors increase the number of free barbed ends increasing actin polymerization and actin depolymerizing factors when phosphorylated, bind to the rear end of actin filaments, generating actin fragments. Other proteins, such as gelsolin, stop actin polymerization by capping barbed ends inducing depolymerization. Modulation of actin polymerization by extracellular guidance cues via actin-associated proteins provides a mechanism to regulate the progress of growth cones. Besides this, there is also local synthesis of proteins in the growth cone that encode for cytoskeletal regulators, cell-adhesion molecules, guidance receptors and components of signaling pathways, which help with actin polymerization and depolymerization and other changes to the cytoskeleton. (Bagnard, 2007; Cioni, Koppers, & Holt, 2018)

The existence of axonally synthesized proteins at a precise developmental stage and in specific compartments implies the existence of mechanisms to coordinate and control axonal translation according to local demand. This is partly due to the stage specific changes in the axonal transcriptome. Spatial restriction of protein synthesis in specific cellular compartments is due to an evolutionarily conserved mechanism that allows asymmetric localization of RNA and that confers spatial and temporal control of protein expression. Incorrect processing and delivery of mRNA can cause developmental defects along with defects in the fundamental axonal behaviors. (Cioni et al., 2018; Holt & Schuman, 2013; Shigeoka et al., 2016)

Eukaryotic mRNAs contain 5' and 3' untranslated regions (UTRs), a modified base at the 5' end named "cap", and a stretch of adenosines at the 3' end named poly(A) tail to enable its translation. Regulation elements of mRNA transport and translation are found within the 3' and 5'UTRs. 3'UTRs regulate multiple aspects of mRNA metabolism, including nuclear export, cytoplasmic localization, translational efficiency and mRNA stability, while the 5'UTR is primarily involved in controlling mRNA translation. A localization element was first identified within the 3'UTR of b-actin and was named "zipcode" because it was necessary for delivering b-actin mRNA to the leading lamellae of fibroblasts and to dendrites. (Andreassi et al., 2017)

Some mRNAs are targeted specifically to axons and dendrites and even to the growth cone. While there are several "zip codes", there are many messages for which a clear consensus sequence in the UTR has not emerged. (Holt & Schuman, 2013)

1.4 Circuits involved in visually guided behaviors

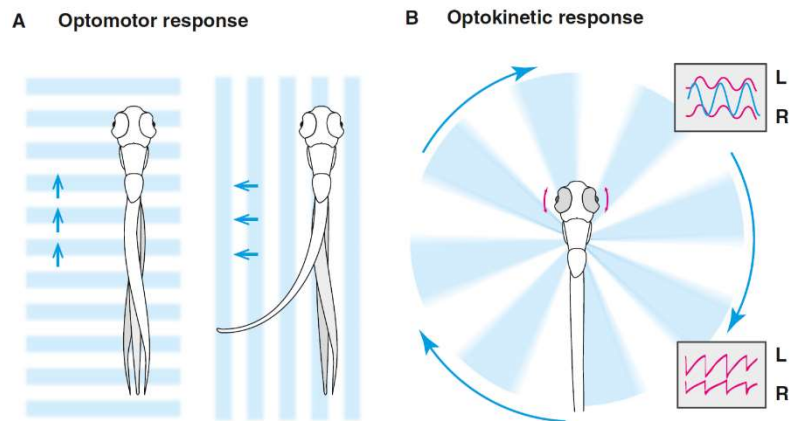


Figure 1.2: Optomotor and Optokinetic responses in zebrafish. A- Optomotor response (OMR). Zebrafish swim to follow translational whole-field motion, which serves to stabilize their position in moving water. B- Optokinetic response (OKR). Rotational motion induces horizontal eye movements. The fish alternates between slow tracking movements and fast reset saccades.

1.4.1 Optomotor Response Circuit

One innate sensory-driven locomotor behavior is the optomotor response (OMR), in which larvae respond to whole-field visual motion stimulus and will turn and swim in the direction of perceived motion (Fig 1.2). The OMR can be reliably evoked at 7 dpf and will persist throughout adulthood. Larvae will perform this behavior when swimming freely or when restrained. (Portugues & Engert, 2009)

Orger and colleagues used a combination of quantitative behavioral analysis, in vivo two photon imaging and targeted ablations to study the response properties of these neurons and their role in transducing visual information into motor output. (Portugues & Engert, 2009)

Olfactory inputs are processed in the forebrain and have been linked to a pathway leading to the Posterior Tuberculum (PT), the mesencephalic locomotor region (MLR), and to Reticulospinal cells (RS). The MLR, likely sends projections to the nucleus of the medial longitudinal fasciculus (nMLF). (Fig. 1.3)

Visual inputs from the retina are relayed to visual processing areas such as the pretectum, from where projections may be relayed to the nMLF. (Fig. 1.3)

Vestibular inputs may be relayed via the tangential nucleus located next to the ear with projections to the nMLF. Together all of these inputs could be integrated in the nMLF to direct locomotion. The spinal cord is activated by descending glutamatergic inputs from the RS cells driving locomotor output and receiving proprioceptive feedback. (Fig. 1.3) (Severi et al., 2014)

Within the spinal cord, there is a dorsal-ventral arrangement of activation with ventral spinal interneurons and motor neurons activated at slow swimming frequencies, and more dorsal recruitment as locomotor intensity must increase. (Severi et al., 2014)

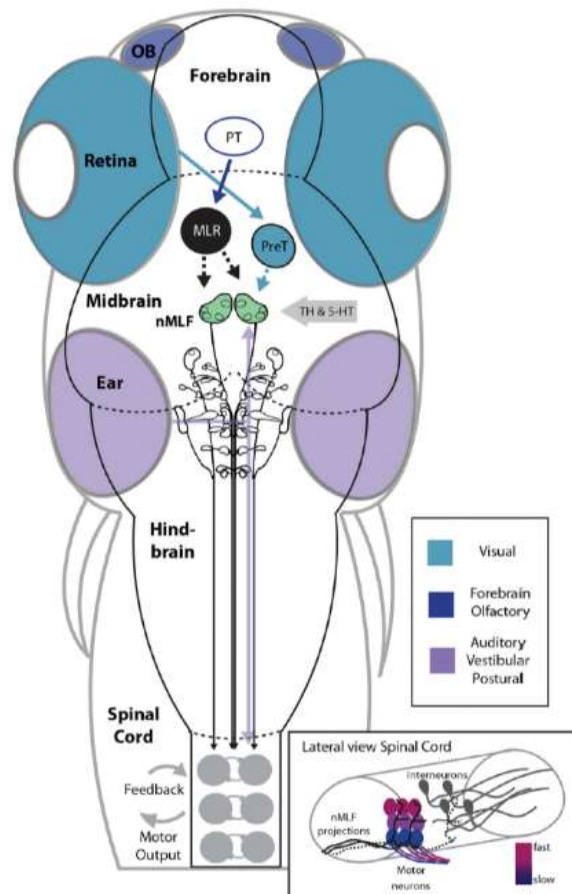


Figure 1.3: Schematic Model for the nMLF as a Center for Sensorimotor Processing and Locomotor Drive from (Severi et al., 2014).

1.4.2 Optokinetic Response Circuit

The Optokinetic Response (OKR) is one of the more widely studied behaviors, due to its reliability and to the fact that larvae will perform it even when immobilized.(Portugues & Engert, 2009) Neuronal responses during OKR are left-right symmetric and highly stereotyped across fish. (Portugues, Feierstein, Engert, & Orger, 2014)

The optokinetic response (OKR) is a reflexive behavior that reduces image motion on the retina, it is found in almost all vertebrates, and it consists in the rotational movement of the eyes in response to a whole-field rotational motion stimulus. (Portugues et al., 2014) These eye movements consist of two components: a smooth pursuit followed by a fast saccade to reset the eyes once the object has left the visual field (Fig 1.2). (Portugues & Engert, 2009)

On the OKR, the input from the pretectum is conveyed to extraocular motor neurons, directly or indirectly, via circuits including a velocity storage mechanism (vsm) and velocity to position neural integrator (vpni). The response takes place when the abducens nucleus (ABN) coordinates ipsiversive eye movements via the lateral rectus muscle (LR) and sends signals to the contralateral oculomotor nucleus (OMN) to drive contraversive movements of the stimulated eye via the medial rectus muscle (MR).

The cerebellum receives OKR-related sensory and motor signals through mossy fibers and, in particular, retinal image motion signals via climbing fibers from the contralateral inferior olive, which in turn receive direct input from the pretectum (Fig 1.4).

Portugues et al reveals that network activity in the vertebrate brain during a simple behavior is sparse and widely distributed, but shows a spatial organization that can be stereotyped across individuals. (Portugues et al. 2014) such that it is possible to reliably predict the activity in individual voxels from their anatomical position alone. This has important practical consequences, because these areas can now be targeted, for ablations, electrophysiological recordings, optogenetics, or photo activation. (Portugues et al., 2014; Ronneberger et al., 2012)

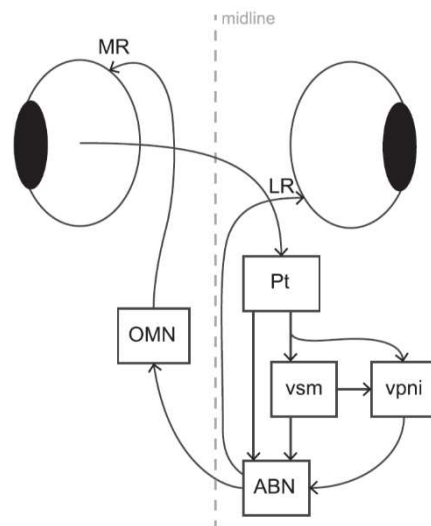


Figure 1.4: Schematic Model for the OKR Circuit adapted from Portugues et al., 2014

1.5 The use of transgenic lines to study neuronal subpopulations: tol2 mediated recombination and Gal 4 mediated reporter expression.

To understand how sensory information is transformed in a certain behavior, we have to fully understand the morphology, connectivity and function of clusters of neurons or even individual neurons. For this purpose genetic tools are increasingly being used to visualize, manipulate and monitor neurons in zebrafish, by easily activating transgene expression in specific subsets of neurons with a high degree of cell-type specificity. (Asakawa & Kawakami, 2008; Marquart et al., 2015; Scott et al., 2007) One of

these tools is the binary transgenic expression systems Gal4/UAS, this system is a powerful tool for dissecting neuronal circuitry and has provided the zebrafish community with many valuable lines. (Marquart et al., 2015)

The Gal4-UAS system was first used in drosophila were the expression of Gal4 drove the expression of the lacZ gene that was downstream of the UAS. Since then it has been shown that this system also works on zebrafish. (Asakawa & Kawakami, 2008)

Gal4 is a yeast transcriptional activator consisting of 881 amino acids (Asakawa & Kawakami, 2008). In cells where the Gal4 gene is expressed, the Gal4 protein targets its Upstream Activating Sequence (UAS), thus driving expression of any gene linked downstream to a UAS element. As a result, animals carrying both transgenes will have expression of any chosen gene attached to the UAS element. (Fig. 1.5) (Asakawa & Kawakami, 2008; Scott, 2009)

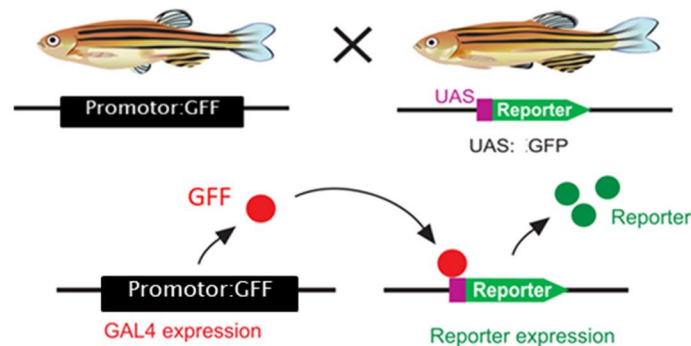


Figure 1.5: Representation of the Gal 4-UAS system, the driver line is using a variant of gal4 GFF adapted from Asakawa (2008).

To have a defined pattern of transgene expression, enhancer trapping can be used by linking a specific enhancer to Gal4, different and multiple genes can also be downstream of the UAS which allows for expression of a variety of genes and a variety of expression patterns. Having the Gal4 line separated from the UAS line allows for all types of combinations. Phenotypes can be analyzed in double transgenic offspring obtained by the cross of these fish. (Asakawa & Kawakami, 2008; K. Kawakami, Asakawa, Hibi, et al., 2016; Scott, 2009)

For this work Gal4FF, or GFF was used, GFF is a Gal4 variant that contains the DNA-binding domain of Gal4 and two short transcriptional activation motifs from VP16, which have a weaker transcriptional activity than VP16 but showed no obvious toxicity when compared to Gal4 and Gal4-VP16(Asakawa & Kawakami, 2008)

The Gal4-UAS system had not been applied effectively to vertebrate systems mainly due to the lack of an efficient transgenesis method. A more efficient transgenesis method based on the medaka fish Tol2 element was developed in zebrafish which allows the generation of vast numbers of transgenic lines and fish. (Asakawa & Kawakami, 2008; Koichi Kawakami et al., 2004; Scott, 2009)

The Tol2-mediated transgenesis has now become a standard approach to create transgenic zebrafish since its more efficient, between 50 and 70% of fish injected will become founder fish that transmit

transgenes to the offspring. (Asakawa & Kawakami, 2008; K. Kawakami, Asakawa, Muto, & Wada, 2016)

The system works by injecting tol2 transposase mRNA and a plasmid DNA with a transposon construct into one cell stage zebrafish eggs. The Tol2 construct is excised from the donor plasmid and integrated into the genome of the germ lineage during embryonic development, and the transposon insertions are transmitted to the next generation very efficiently as mentioned before. Since Tol2 has a surprisingly large cargo capacity (more than 50 kb) and can carry efficiently BAC inserts, which can carry up to 300kb of DNA fragments, into the zebrafish. (K. Kawakami, Asakawa, Muto, et al., 2016)

Two major advantages of Tol2-mediated BAC transgenesis over existing methods are increased efficiency of germline transmission and greater reliability of obtaining single-copy integrations in the genome. (K. Kawakami, Asakawa, Muto, et al., 2016)

Tol2-mediated BAC transgenesis in conjunction with the Gal4/UAS system allows for the answer of many biological questions and applications such as live imaging of cells and subcellular structures in complex tissues and organs such as the nervous system, targeted cell ablation and manipulation of neural circuits, analysis of gene function by targeted gene expression, remote control of neuronal activity by light, targeted inhibition of neuronal function by tetanus toxin light chain.(Asakawa & Kawakami, 2008; K. Kawakami, Asakawa, Muto, et al., 2016)

1.6 Anatomical characterization of the zebrafish brain and its circuits: Atlases

Developments in imaging technology and genetically encoded indicators that can reliably report neuronal firing and synaptic activity have created new possibilities for neural circuit research. To truly understand zebrafish behavior, we must first understand the connectivity of the neuronal circuits driving behavior. A first step will be to produce single-cell resolution, whole-brain maps of the functional signals underlying sensory and motor processing, but maps are of limited utility unless they intersect with detailed neuroanatomical information. (Randlett et al., 2015; Renninger & Orger, 2013; Turner, Bracewell, & Hawkins, 2014)

Zebrafish are a relatively new model system; as such, the zebrafish doesn't benefit from the extensive neuroanatomical descriptions that exist in other model species. This requires detailed neuroanatomical characterization of the zebrafish brain especially in very early developmental stages. (Turner et al., 2014)

This longstanding problem in zebrafish neuroscience has recently been addressed by the construction of digital atlases using confocal imaging of larvae with specific molecular markers, followed by three-dimensional image registration techniques: the Virtual Brain Explorer for Zebrafish (ViBE-Z), Z-Brain and the Zebrafish Brain Browser (ZBB). (Marquart et al., 2015; Randlett et al., 2015; Ronneberger et al., 2012) In these atlases, information of widely available transgenic lines are consolidated within a common framework. These atlases allow researchers to register their own datasets and take advantage of the information contained within. (Marquart et al., 2017)

High-resolution functional maps will eventually be registered with these anatomical maps, providing the identification of genetically targetable cell types involved in specific behaviors and allowing zebrafish researchers to guide studies of neural circuit structure and function, it allows for direct comparisons across experiments, and can reveal brain-wide functional connectivity patterns. As more labels and activity maps are accumulated, such analyses will become even more powerful and in cases where the position of neurons is highly stereotyped, the resolution can be near-cellular. (Marquart et al., 2017; Randlett et al., 2015; Renninger & Orger, 2013)

The atlases available:

The Z-brain atlas is focused on zebrafish at 6 dpf, since the often studied age range is of 5–7 dpf. The Z-Brain atlas contains information on gene expression, neural activity and neuroanatomical segmentation. The confocal stacks of the brain were registered to a template brain on the basis of expression of total ERK (tERK). (Randlett et al., 2015)

Z-Brain is an expandable, open source platform allowing the zebrafish community to rapidly expand the atlas by contributing with additional anatomical or functional stacks. (Randlett et al., 2015)

ZBB is a database of the expression patterns of 109 transgenic lines that have been registered into a common reference. The “brain browser” software facilitates the visualization of multiple lines and search using anatomic labels, or user defined 3-dimensional regions of interest (Marquart et al., 2015). The browser can be used to find enhancer trap lines that label specific neurons, or projection targets of neurons of interest.

In contrast to Z-brain, the Zebrafish Brain Browser (ZBB) atlas was constructed from live scans of fluorescent reporter genes in transgenic larvae limiting the range of available markers but avoids artifacts associated with fixation and staining and increases throughput. (Marquart et al., 2017)

Images from ZBB have been incorporated in ZBB and vice versa and it is, therefore possible to visualize the overlap of lines characterized for each project.

ANTs was used to register Z-Brain and ZBB to be co-aligned with acceptable precision and minimal perturbation of cell and tissue morphology since the two atlases are both based on confocal scans of 6 dpf larvae and share several transgenic markers. (Marquart et al., 2017)

ANTs provided superior image registration for live-scanned larvae, which allowed an improvement in precision of the registration and neuron morphology within the original ZBB atlas, this led to the creation of ZBB1.2 which is now the atlas used. (Marquart et al., 2017)

ViBE-Z’s high-quality data sets through fusion of multiple confocal stacks, it aligns 3D gene or protein expression patterns of different zebrafish larvae to a common reference based on a nuclear staining, and it stores the aligned patterns atlas of 2-, 3- and 4-day old zebrafish larval brains in the ViBE-Z database. It stands out since it is made with confocal stacks from the dorsal and ventral views of the brain and fused them with attenuation correction, resolving detailed structures deep inside brain using standard confocal microscope data. (Ronneberger et al., 2012)

Table 1.1: Summary of the atlases available and their main characteristics: Registration tool, stage of the fish registered and the main anatomical marker used.

Atlas	Days post fertilization	Registration Tool	Main Reference Marker	Reference
ZBB	6	CMTK	vglut2a:DsRed	(Marquart et al., 2015)
ZBrain	6	CMTK	tERK	(Randlett et al., 2015)
ViBE-Z	2,3,4	Landmark detector	Nuclear Staining	(Ronneberger et al., 2012)
ZBB1.2	6	ANTs	Common markers between ZBB and Zbrain	(Marquart et al., 2017)

1.7 Characterization of specific neuronal subpopulations in zebrafish brain development: the use of transgenic lines

The identification and characterization of neuronal populations and their involvement in specific circuits, such as those involved in visual processing, remains crucial to expand our knowledge on how the zebrafish brain is functionally organized.

One approach in the identification of distinct neuronal populations is the use of transgenic reporter fish lines designed to express fluorescent proteins in specific neuronal populations. These fish lines may contain reporter genes (such as GFP) directly under the control of the regulatory sequences of interesting genes or may drive the expression of Gal4 derivatives (such as GFF) that in combination with the appropriate UAS-reporter lines will drive reporter expression.

Several large-scale projects have allowed the generation of hundreds of transgenic lines which have already been imaged at the cellular level by whole brain imaging.

They involve the registration of individual 3D brain images into a reference brain. As a result, atlases have been created offering an open source database of gene expression of hundreds of transgenic lines and different antibodies together with anatomical labels, all registered into a common reference space however a similar characterization of zebrafish younger stages (1-5dpf) is still needed. Some data for the earlier developmental stages is available but this has not been further developed. (Mueller & Wullimann, n.d.; Ronneberger et al., 2012)

For the current project, we are collaborating with M. Orger laboratory in the characterization of the expression of several zebrafish lines recently generated, during embryonic and early larval development. These lines, in combination with the UAS-GCaMP6, are currently being examined in Michael Orger's

laboratory to determine the neuronal response of the GFF expressing neuronal subpopulations upon exposure of the fish to specific stimuli. Several interesting populations in the brain have been identified which respond to stimulus moving in specific directions (direction selective) and to abrupt changes in light intensity [S. Renninger and M. Orger, unpublished]. The lines studied in this work are:

The olig2: GFP line: Olig2 (Oligodendrocyte transcription factor 2) genes encode for basic helix–loop–helix (bHLH) transcription factors and are important for motor neuron and oligodendrocyte development. (McFarland, Topczewska, Weidinger, Dorsky, & Appel, 2008; Park, Mehta, Richardson, & Appel, 2002)

The Olig2: eGFP line contains a BAC insertion that includes the Olig2 genomic region where the eGFP has been inserted. (Shin, 2003)

The chrna4:GFP line: chrna4 (cholinergic receptor nicotinic alpha 4 subunit) is a subunit of the nicotinic acetylcholine receptor (nAChR), this line was created in Michael Orger's lab where the GFP variant of Gal4 was inserted into a bacterial artificial chromosome (BAC) containing the zebrafish genomic region including the regulatory region of the chrna4 gene. The GFP gene was specifically targeted to the starting ATG of the gene and may thus respond to the regulatory regions in a similar way as the gene of interest. The BAC was then integrated into the genome using the Tol2 transposon system. [S. Renninger and M. Orger, unpublished]

The pcp4: GFP line: pcp4a (Purkinje cell peptide 4) is a small polypeptide carrying an IQ motif: a 23-amino acid domain that binds EF-hand proteins and shows high affinity for calcium-poor calmodulin. (Mione, Lele, Kwong, Concha, & Clarke, 2006) This line was created in a very similar manner to the chrna4 using a BAC and the tol2 transposon system. [S. Renninger and M. Orger, unpublished]

The Chat: GFP line: This transgenic line genome harbors an insertion of a BAC containing the choline acetyl transferase genomic region with GFP cloned at the ChAT starting ATG. GFP expression was previously observed and characterized in M. Orger's lab. (Miguel & Madeira, 2017)

The characterization of GFP should provide useful information to understand acquisition of neuronal cell fates and the wiring mechanism during development. In addition, it will help in the design and interpretation of experiments where they can be used to manipulate gene expression in a cell specific manner at specific times in development.

We aim to characterize neuronal subpopulations expressed in lines involved in visual processing by the use of reporter lines that drive GFP expression and therefore contribute to a better understanding of the acquisition of neuronal cell fates and establishment of neuronal circuits. For that matter we will examine GFP expression in zebrafish embryos and larvae from 3 transgenic lines from the 1st to the 6th dpf, using immunofluorescence and confocal imaging. Label and identify the anatomical localization of GFP expressing cell populations by comparison with 3-D atlases and other studies on protein and gene expression. In addition, we are using time lapse imaging with Lightsheet microscopy to follow the dynamics of neuronal differentiation and extension of projections during the 20-48 hours post fertilization period of the Chat: GFP line.

2. Materials and Methods

2.1 Fish Husbandry

The zebrafish used were maintained by the vivarium platform at Champalimaud Research, where all aspects of fish housing were provided.

The rooms that accommodate the fish are ventilated through a centralized HVAC system and are kept at a controlled room temperature (25°C), with a 50%–60% humidity level and under a 14/10 hours light/dark cycle with a light intensity of 200–300lux at the water surface.

Temperature, pH, salinity (conductivity), and dissolved gases, are continuously monitored online and checked daily to be kept under the physiological range. (Martins et al., 2016)

2.2 Fish crosses and embryo collection

Static matings are currently done using tilted/slanted insert breeding tanks providing a shallow water environment that maximizes breeding behavior. (Martins et al., 2016)

Fish are placed in breeding tanks described above and on the next day embryos are collected and kept on E3+PTU (see Solutions), to prevent the development of pigmentation (Karlsson, von Hofsten, & Olsson, 2001), at a maximum density of 50 embryos/petri dish. After being collected in petri dishes embryos/larvae were raised at 28.5°C under a 14/10 hours light/dark cycle.

All animal experiments were done according to the CF Animal Welfare Body guidelines and under a CF Ethics Committee-approved project.

2.3 Transgenic Lines

The cell-specific lines *pcp4a*(*pcp4a*:GFF UAS:Lyn-mCherry; Renninger and Orger, unpublished work), *chrna4* (*chrna4*:GFF UAS:Lyn-mCherry; Renninger and Orger, unpublished work), and ChAT (*ChAT*:GFF;UASmCherry; Renninger and Orger, unpublished work) were created in the Orger lab using the *tol2* system(Asakawa & Kawakami, 2008). To generate the lines, the GFF variant of Gal4 was inserted into a bacterial artificial chromosome (BAC) containing the zebrafish genomic region including the respective gene of interest (*pcp4a*, *chrna4*, ChAT). The GFF gene was specifically targeted to the starting ATG of the gene and may thus respond to the regulatory regions in a similar way as the gene of interest. The BAC was then integrated into the genome using the Tol2 transposon system. The *Olig2*:eGFP line contains a BAC insertion that includes the *Olig2* genomic region where the eGFP has been inserted as described (Shin, 2003). This *Olig2*: eGFP line corresponds to allele *vu12* and was obtained from the European Zebrafish Resource Centre.

2.4 Whole body fluorescent immunostaining

The protocols used are modifications of those described in (Inoue & Wittbrodt, 2011; Marquart et al., 2017; Randlett et al., 2015) Zebrafish larvae are fixed in PFA 4%, triton 0,25% for at least 2 hours at room temperature or at 4°C overnight, they are rinsed 3x and washed 3x for 5min in PBT(see section

2.9), incubated in 150 mM Tris-HCl, pH 9, for 15 min at 70 °C (Inoue & Wittbrodt, 2011), rinsed 3x and washed 3x for 5min in PBT, permeabilized in 0.05% Trypsin-EDTA for 5 min on ice, washed in PBT, blocking occurs in a mix of PBT ,1% bovine serum albumin (BSA), 2% normal goat serum (NGS),1% dimethyl sulfoxide (DMSO), and then incubated for 3 overnights at 4 °C in a primary antibody mix containing PBT, 1% BSA, 1% DMSO and the primary antibodies at a 1:500 dilution after which the samples are rinsed 3x and washed 3x for 30min in PBT, incubation with secondary antibodies happens in similar conditions as the primary antibodies. For some samples besides the antibodies used, bisbenzimidazole at a 1:500 dilution was added with the primary antibodies, and the rest of the protocol proceeded normally.

2.5 Mounting protocols

For confocal imaging, the larvae are mounted directly to the cover slip with the dorsal side against the coverslip as straight as possible in a drop of 1,5%(w/v) low melting agarose in PBS, the larvae are then surrounded by a silicone grease well which is filled with PBS, the glass slide is then pressed against the coverslip which provides a watertight seal.(Turner et al., 2014).

For Lightsheet imaging, embryos with approximately 24hpf are placed in Bodipy-TR-methyl ester, a lipophilic dye that binds to inner membranes in the cells, for 1h the embryos were incubated in a 1:50 dilution. Embryos are then embedded in an agarose rod that can be rotated (Huisken, Swoger, Del Bene, Wittbrodt, & Stelzer, 2004). For embedding in 1.5% (w/v) agarose in E3, embryos were placed in liquid agarose, drawn into a glass capillary with Teflon-coated plungers and extruded after polymerization.

2.6 Confocal Microscopy

Images were acquired with the Zeiss LSM 710 which is an upright confocal laser-scanning microscope that allows for optical sectioning of thick fluorescent samples at high-resolution. The Objective used is a long distance (LD) LCI Plan-Apochromat 25x/0.8 multi immersion which is a highly performing lens for deep tissue imaging. Anatomical z-stacks were obtained at a voxel size of $0.55 \times 0.55 \times 1 \mu\text{m}$ ($x \times y \times z$). To cover the entire brain, we acquired two imaging tiles and stitched them together using the Pairwise Stitching plugin in FIJI (Preibisch, Saalfeld, & Tomancak, 2009). As imaging was done with the fish dorsal side up, image quality may be lower in the most ventral areas of the brain.

2.7 Characterization of the Lines

Anatomical characterization of GFP expression of the different lines was made by comparing the z-stacks obtained with reference brains and stacks from bibliography concerning the respective lines and the z-brain (Randlett et al., 2015) and ZBB1.2 atlases (Marquart et al., 2017). For the retina characterization we used a specific paper. (Avanesov & Malicki, 2010)

2.8 Light sheet Microscopy

The Zeiss Lightsheet Z.1 is a Lightsheet fluorescence microscope able to image optical sections of large samples at sub-cellular resolution and very fast rates, with very little excitation power.

The Z.1 Lightsheet possesses highly sensitive sCMOS camera (dual PCO.Edge cameras with up to 30 fps speed).

The chamber is filled with E3 +PTU (0,2 mM) + tricain to avoid pigmentation and anesthetize the embryo. The objective used was a Plan-Apochromat 10x/0.5 M27 75mm parfocal length 75mm, (FWD=3.7mm), (UV) VIS-IR the illumination lenses were 10x/0.2 Dry lenses.

2.9 Solutions

E3 (60x): 5mM NaCl, 0.17mM KCl, 0.33mM CaCl₂, 0.33mM MgSO₄, pH=7.2.

PBT- PBS 1x + Triton 0.25%

Bisbenzimidazole stock concentration: 10mg/ml

2.10 Antibodies and other counterstain reagents

Table 2.1: Table of the antibodies and stains used for all protocols.

antibody	Manufacturer Reference	Host/ Isotype
Bodipy TR methyl ester	Invitrogen C34556	-----
Anti-GFP antibody	Abcam ab13970	Chicken/ IgY
Goat anti-Chicken IgY (H+L) Secondary, Alexa fluor 488	ThermoFisher: A-11039	Goat / IgG
Anti-tERK antibody, p44/42 MAPK (Erk1/2)	Cell signalling: 4696	Mouse IgG1
Anti-Mouse IgG (H+L) polyclonal secondary Antibody, Alexa Fluor 568	ThermoFisher: A-11004	Goat / IgG
Bisbenzimidazole Hoechst 33258	Sigma B2883	Not applicable

3. Results

The Zebrafish (*Danio Rerio*) larva is an ideal model for identification of neuronal circuits such as those associated with visually guided behaviors like the optomotor and the optokinetic responses.

To characterize some of these subpopulations we are taking advantage of the expression of GFP, using the Gal4-UAS system, during the first 6 days of development.

The lines used are expressing GFF (Gal4 variant) and are currently being studied in the Vision to Action laboratory to identify neurons that are specifically active when the larvae are exposed to visual stimuli and/or performing motor behaviors. When ultimately combined with a UAS: GFP reporter line it is possible to characterize the GFP expression of the GFF lines.

In order to obtain the embryos of interest, we crossed adult zebrafish after selection of GFP expressing embryos, a group of embryos is fixed every 24 hours to obtain the stages presented in this work, after which we apply the Immunofluorescence protocol described in the methods section.

We then proceed to acquire z-stacks of the whole brain with confocal microscopy. Processing of the images was done using ImageJ.

In the following sections we present our observations of the confocal z-stacks corresponding to the different lines examined. We present for each line a description of the different clusters of cells, in the successive stages of development highlighting similarities and differences with the previous stage. This is summarized in a table and illustrated in the figures corresponding to the partial maximum intensity projections. At the end of the chapter for each line, an overall description of the GFP expression during development is presented.

Annotation was made based on existing literature, mainly based on Zbrain (Randlett et al., 2015) and ZBB (Marquart et al., 2015) for the later larval stages and (Mueller & Wullimann, n.d.) for the younger stages, the retina characterization was made using a specific paper (Avanesov & Malicki, 2010). We have tried to identify as many neuronal clusters and projections as possible and in the most specific way possible but their assignment is still tentative at this stage.

3.1 Olig2: eGFP - GFP expression

The Olig2: eGFP line contains a BAC insertion that includes the Olig2 genomic region where the eGFP has been inserted, this gene is expressed typically by motor neurons, oligodendrocytes and oligodendrocyte progenitor cells (OPCs). (Shin, 2003)

For the characterization of this line the olig2: eGFP line was incrossed, since eGFP is directly expressed under the regulatory region of the *olig2* gene, almost all embryos presented GFP being then used for characterization, this line expressed a very consistent pattern across embryos.

3.1.1 Olig2: eGFP 1dpf

In the first day of development GFP expression is observed in a few cells on the olfactory epithelium (Fig 3.1, A1). Expression of GFP in the Diencephalon is very general, with labelled cells spanning a broad region that may correspond to proliferating neuroepithelial cells. Some GFP expressing cells are also present in the hindbrain located in the mantle layer and thus most likely corresponding to early differentiating neurons (Fig 3.1, A1).

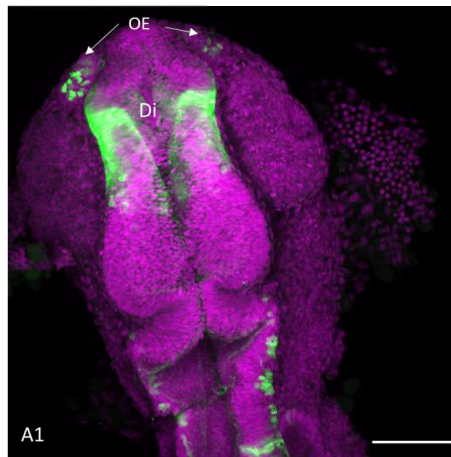


Figure 3.1: GFP expression in a 1dpf olig2: GFP zebrafish brain: A maximum intensity projections of a, 1dpf olig2: GFP, confocal z-stack is presented in panel A1. Immunofluorescence was performed with anti-GFP (green) and bisbenzimidide (magenta). Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: Di- Diencephalon; OE- Olfactory epithelium; Scale bar has a size of 100 microns.

3.1.2 Olig2: eGFP 2dpf

As the fish develops onto the second day post fertilization, the number of GFP expressing cells increases dramatically and we can start to distinguish within the Diencephalon the thalamus, ventral thalamus, thalamic eminence, posterior tuberculum and probably the preoptic area in a more ventral position (Fig 3.2, B2).

GFP+ cells can be identified in the optic tectum, in the corpus cerebellum and in the medulla oblongata (Fig 3.2, B2). A compact cluster of GFP+ cells is present at the eminentia granularis with projections to the midline of the embryo (Fig 3.2, B2).

Numerous scattered cells with a morphology of oligodendrocyte precursors can be observed throughout the midbrain and hindbrain. (Sidik & Talbot, 2015)

A band of GFP+ cells in the midline of the larva appears as a characteristic of this line, not to be confused with the expression of the spinal cord at the most caudal part of the embryo (Fig 3.2, B2).

Projections innervating the pectoral fin buds can be observed.

In the retina, cell in several layers are populated with GFP expressing cells (Fig 3.2, B3).

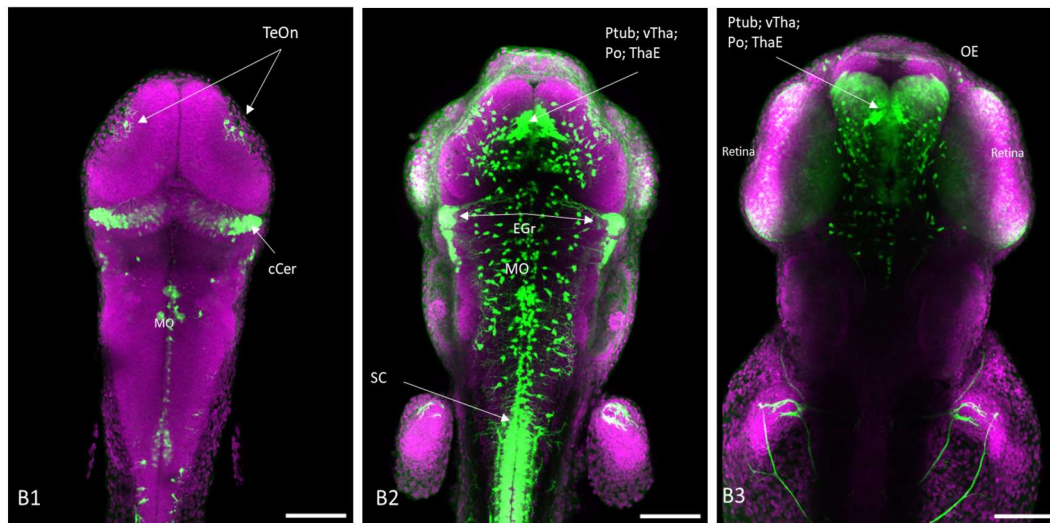


Figure 3.2: GFP expression in a 2dpf olig2: GFP zebrafish brain: From B1-B3 Partial maximum intensity projections of a, 2dpf olig2: GFP, confocal z-stack are presented, panels go from dorsal to ventral starting on panel B1. Immunofluorescence was performed with anti-GFP (green) and bisbenzamide (magenta). Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: cCer - corpus cerebelli; EGr - Eminentia Granularis; MO- Medulla oblongata; OE – Olfactory epithelium; Po - Preoptic area; Pr – Pretectum; PTub - Posterior tuberculum; SC – Spinal Cord; TeOn- TeO neuropil; ThaE - Thalamic eminance; vTha - Ventral thalamus; Scale bar has a size of 100 microns.

3.1.3 Olig2: eGFP 3dpf

Within the telencephalon there are sparse cells in the pallium and a cluster on the subpallium (Fig 3.3, C3, and C4).

GFP+ cells are present at this stage in the habenula, accompanied by an increase of cell bodies and dendrites in the optic tectum (Fig 3.3, C1, and C2).

A band of GFP expressing cells starts to appear at a location spanning several brain areas (the Posterior tuberculum, ventral thalamus and thalamic eminance) (Fig 3.3, C3).

In the cerebellum, the Olig2 specific clusters of cells accompany the growth of the cerebellum Fig 3.3, C2).

In the retina there are cells in the Inner Nuclear Layer and possibly expression in the Inner and outer Plexiform Layer and the Pigmented Epithelium (Fig 3.3, C4).

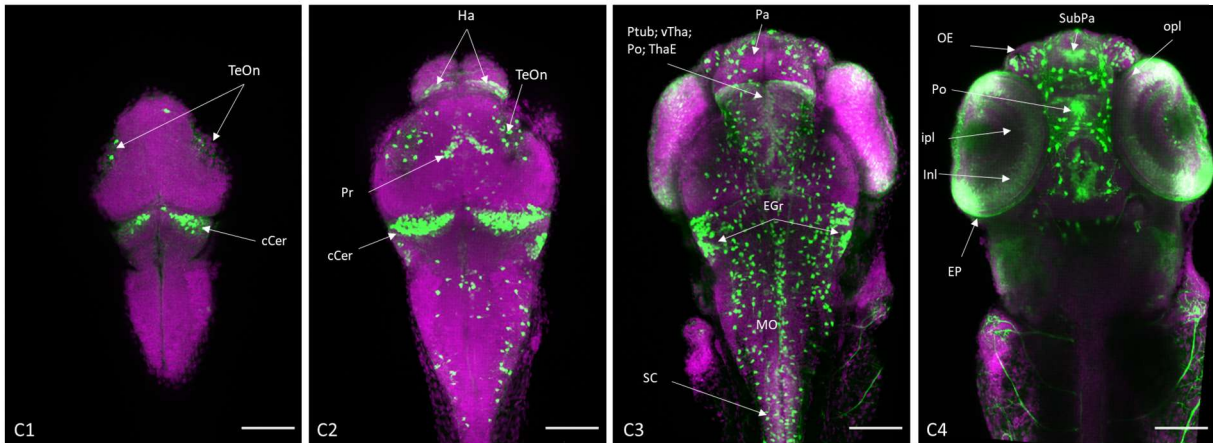


Figure 3.3: GFP expression in a 3dpf olig2: GFP zebrafish brain: From C1-C4 Partial maximum intensity projections of a, 3dpf olig2: GFP, confocal z-stack are presented, panels go from dorsal to ventral starting on panel C1. Immunofluorescence was performed with anti-GFP (green) and bisbenzimidazole (magenta). Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: cCer - corpus cerebelli; EGr - Eminentia Granularis; EP - pigmented epithelium; Ha - Habenula; inl - Inner nuclear layer; ipl - Inner plexiform layer; MO - Medulla oblongata; OE - Olfactory epithelium; Pa - Pallium; Po - Preoptic area; Pr - Pretectum; PTub - Posterior tuberculum; SC - Spinal Cord; SubPa - Sub-pallium; TeOn - TeO neuropil; ThaE - Thalamic eminence; vCer - Valvula cerebelli; vTha - Ventral thalamus; Scale bar has a size of 100 microns.

3.1.4 Olig2: eGFP at 4dpf, 5dpf and 6dpf

The expression patterns starting at 4 days post fertilization are very similar between each other and will be grouped together (Fig 3.4).

Expression in the habenula is maintained and in the MO expression is still abundant and disperse. (Fig 3.4, D2, E2, F2) The biggest differences from the third day stage are the increase in GFP expression on the whole brain specially in the amount of neuropil in the pallium and tectum neuropil (Fig 3.4, D2, F1,F2,F3), and the appearance of structures such as the neuropil region 2 on the vagal region (Fig 3.4, D2, E2).

The retina also seems to have more expression in all the layers described before (Fig 3.4, D3-F3, and D4-F4).

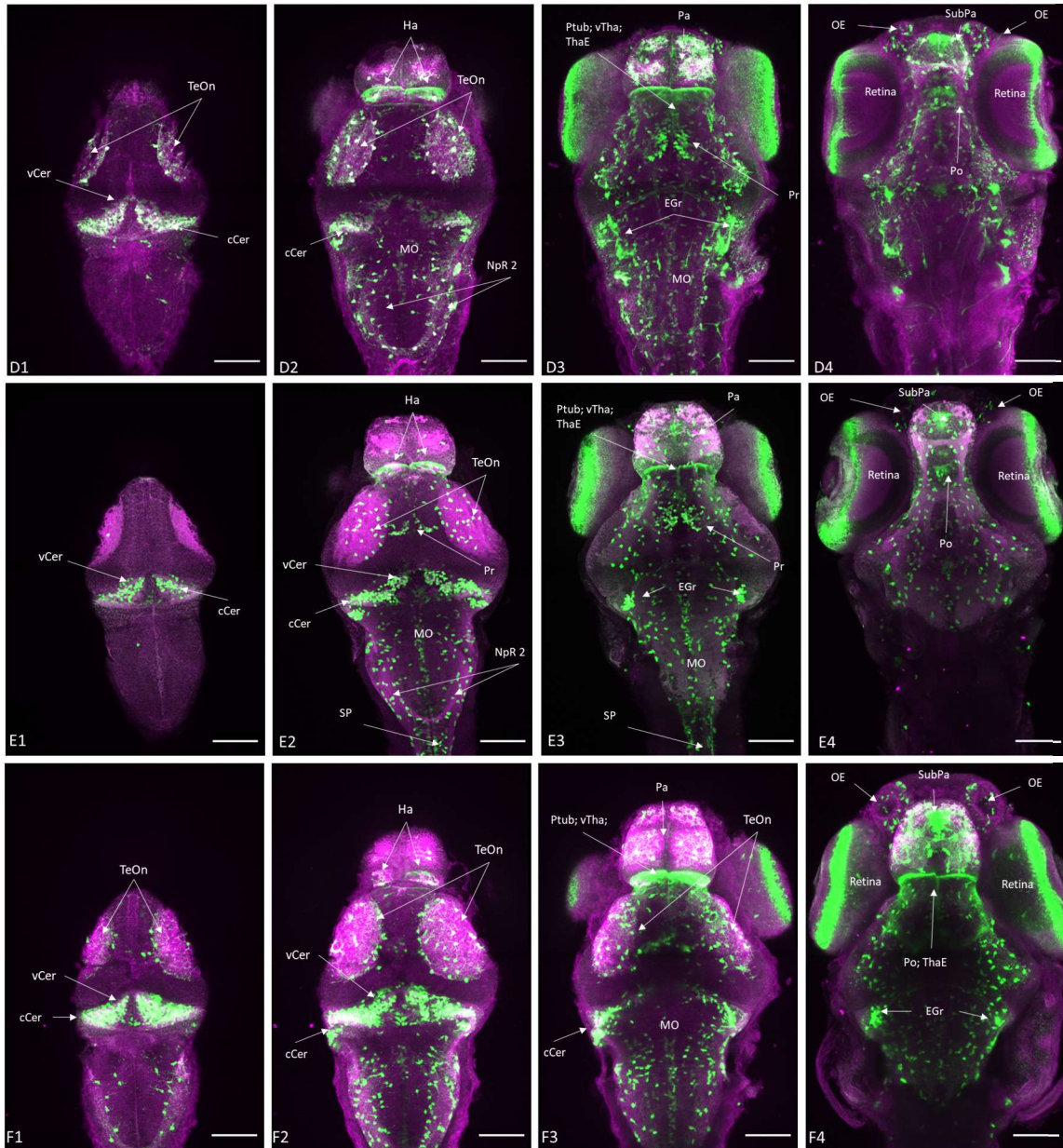


Figure 3.4 GFP expression in a 4dpf, 5dpf and 6dpf olig2: GFP zebrafish brains: From D1-D4 Partial maximum intensity projections of a, 4dpf olig2: GFP brain, confocal z-stack are presented, From E1-E4 Partial maximum intensity projections of a, 5dpf olig2: GFP brain, From F1-F4 Partial maximum intensity projections of a, 6dpf olig2: GFP brain. Panels go from dorsal to ventral from left to right. Immunofluorescence was performed with anti-GFP (green) and anti-tERK (magenta). Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: cCer - corpus cerebelli; EGr - Eminentia Granularis; Ha - Habenula; MO - Medulla oblongata; NpR 2 - Neuropil Region 2; OE - Olfactory epithelium; Pa - Pallium; Po - Preoptic area; Pr - Pretectum; Ptub - Posterior tuberculum; SubPa - Sub-pallium; TeOn - TeO neuropil; ThaE - Thalamic eminance; vCer - Valvula cerebelli; vTha - Ventral thalamus; Scale bar has a size of 100 microns.

3.1.5 Olig2: eGFP Expression summary

Table 3.1: Olig2: eGFP expression summary table: summary of all the areas where GFP is expressed in the 6 dpf larval brain from Olig2-GFP transgenic fish with indication of the stage when cells in the regions are first observed

Brain Regions	GFP expression Areas(Start of expression)	Abbreviations
Telencephalon	Pallium(3dpf); SubPallium(3dpf)	Pa; SubPa;
Diencephalon(1dpf)	Habenula(2dpf); Thalamic eminence(3dpf);Posterior Tuberculum(3dpf);Ventral Thalamus(3dpf);Preoptic area(2dpf); Pretectum(2dpf)	Ha; ThaE; PTub; vTha; Po;Pr
Mesencephalon	Optic Tectum Neuropil(2dpf);	TeO;
Rhombencephalon	Valvula cerebelli (3dpf); Corpus Cerebelli (2dpf); Medula Oblongata (1dpf); Neuropil Region 2 (4dpf); Eminentia Granularis (2dpf);	vCer; cCer; MO; NpR 2; EGr;
Retina	Pigmented Epithelium(2dpf); Outer Plexiform Layer(2dpf); Inner Nuclear Layer(2dpf);	pe; opl; inl;
Ganglia and Others	Olfactory Epithelium(1dpf);Spinal Cord(2dpf)	OE;

In this line, GFP+ cells can be found scattered throughout the brain from the 2 dpf, this most likely correspond to the oligodendrocyte progenitor cells as previously described (Shidik and Talbot, 2015). More spatially restricted expression is described below.

3.1.5.1 Olig2: eGFP expression in the telencephalon.

In the subpallium there is a cluster of cells which is more evident from 3dpf. There is no apparent expression in the olfactory bulb nor the pallium apart from the oligodendrocyte progenitor cells scattered throughout the brain.

3.1.5.2 Olig2: eGFP expression in the diencephalon.

A GFP expression domain in the habenula is observed from the 2 dpf stage which is enlarged as the larva brain grows. GFP is strongly expressed in a region that may include, the ventral thalamus, the posterior tuberculum, the thalamic eminence and the preoptic area more ventrally; from the 2 dpf stage, another distinctive “V” shape cluster in the pretectal area that may be extending to the mesencephalon, is observed from the 3dpf stage.

3.1.5.3 Olig2: eGFP expression in the mesencephalon.

GFP+ cells can be detected in the tectum neuropil and sparsely in the stratum periventriculare since the 2dpf stage. Some cells are found in a region corresponding to the pretectum as described in the diencephalon section above.

3.1.5.4 Olig2: eGFP expression in the rhombencephalon

Within the cerebellum, we find GFP+ cells in the Valvula cerebelli and also the corpus cerebelli more ventrally from the 2 dpf and a very distinctive cluster is present in the eminentia granularis on both sides of the larval brain with neurons projecting ventrally towards the midline.

Expression of GFP can only be detected starting in the 2dpf, at low levels in the medulla oblongata being more concentrated in a stripe along the ventral midline and in neuropil region 2.

3.1.5.5 Olig2: eGFP expression in the retina, ganglia and spinal cord.

From 1 dpf onwards, GFP is expressed in the olfactory epithelium. In the developing retina expression occurs in the Inner Nuclear Layer and possibly in the Inner, outer Plexiform Layer and the Pigmented Epithelium by 4 dpf.

A ventral domain in the spinal cord, corresponding to the motor neuron precursors is identified in the spinal cord.

3.2 Chrna4: GFF UAS: GFP - GFP expression

This specific line was created by inserting the GFF variant of Gal4 into a bacterial artificial chromosome (BAC) containing the regulatory region of the chrna4 gene. The BAC was then integrated into the genome using the Tol2 transposon system. [S. Renninger and M. Orger, unpublished].

For the characterization of this line the GFF driver line chrna4: GFF lyn-mCherry was crossed with the UAS: GFP reporter line, and the GFP expressing embryos (chrna4: GFF UAS: GFP) were selected for characterization, this line expressed a very consistent pattern across individuals.



Figure 3.5: GFP expression in a 1dpf *chrna4: GFF UAS: GFP* zebrafish brain: A maximum intensity projections of a, 1dpf *chrna4: GFF UAS: GFP*, confocal z-stack is presented in panel A1. Immunofluorescence was performed with anti-GFP (green) and bisbenzimidide (magenta). Annotations have been near the GFP expressing structures. Scale bar has a size of 100 microns.

3.2.1 *Chrna4: GFF UAS: GFP* 1dpf

For the first day of this line there is no expression on the CNS, the GFP seen is a structure central to the neural tube which persists in all the stages analyzed for this line and may correspond to neural crest cells in the pharyngeal arches (Fig 3.5, A1).

3.2.2 *Chrna4: GFF UAS: GFP* 2dpf

The first GFP+ domain in the CNS is at the lobus caudallis cerebellis (Fig 3.6, B1)

Ventrally the medulla oblongata presents two symmetrical unidentified clusters with contralateral projections between them GFP expression can also be observed in the early caudal hypothalamus cluster (Fig 3.6, B2). In the diencephalon, a structure of the primary scaffold begins to express GFP at this stage, it is composed of the ventral caudal cluster with the posterior commissure emerging from it and the mlf projecting from the vcc caudally (Fig 3.6, B3).

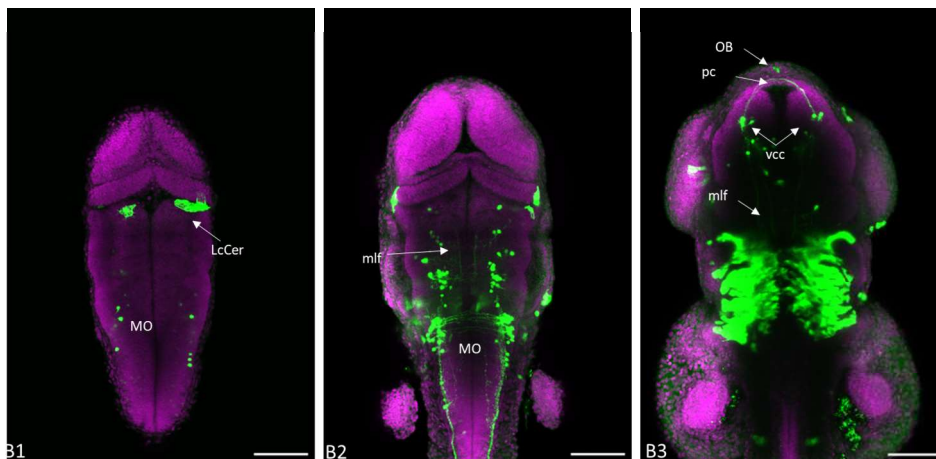


Figure 3.6: GFP expression in a 2dpf *chrna4: GFF UAS: GFP* zebrafish brain: From B1-B3 Partial maximum intensity projections of a, 2dpf *chrna4: GFF UAS: GFP*, confocal z-stack are presented, panels go from dorsal to ventral starting on panel B1. Immunofluorescence was performed with anti-GFP (green) and bisbenzimidide (magenta). Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: LcCer - Lobus caudallis cerebelli; mlf - Medial longitudinal fasciculus; MO - Medulla oblongata; OB - Olfactory bulb; pc - Posterior commissure; vcc - Ventral caudal cluster; Scale bar has a size of 100 microns.

3.2.3 Chrna4: GFF UAS: GFP 3dpf

At this stage, the previously mentioned scaffold is very well established with the posterior commissure, the vcc, the mlf and the now formed oculomotor nucleus clearly seen (Fig 3.7, C2). In the hindbrain, the MO clusters are spreading in the AP axis (Fig 3.7, C2).

In the mesencephalon, the caudal hypothalamus neural cluster is still acquiring a very identifiable structure (Fig 3.7, C3). In the eye there are elongated cells covering the lens and some retinal ganglion cells expressing GFP (Fig 3.7, C4).

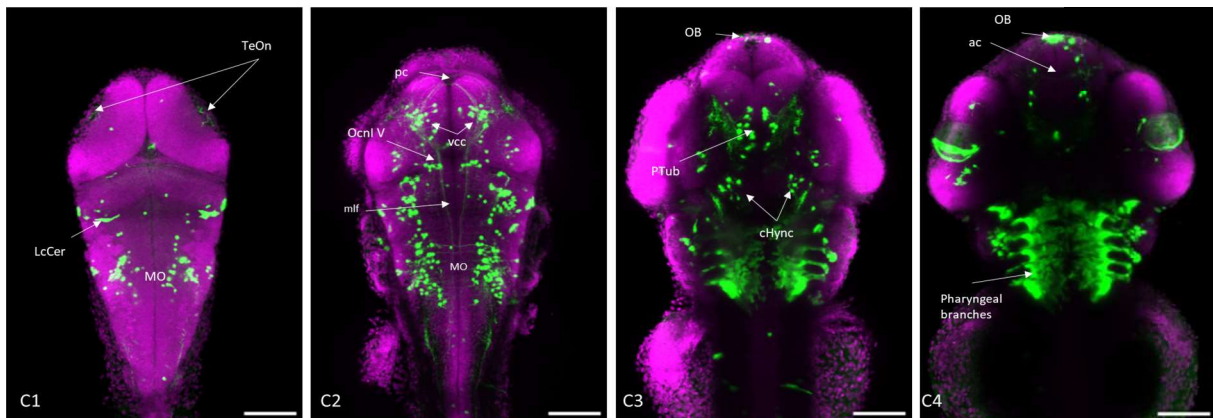


Figure 3.7: GFP expression in a 3dpf chrna4: GFF UAS: GFP zebrafish brain: From C1-C4 Partial maximum intensity projections of a, 3dpf chrna4: GFF UAS: GFP confocal z-stack are presented, panels go from dorsal to ventral starting on panel C1. Immunofluorescence was performed with anti-GFP (green) and bisbenzimidide (magenta). Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: ac - Anterior commissure; cHync - Caudal hypothalamus neural cluster; LcCer - Lobus caudallis cerebelli; mlf - Medial longitudinal fasciculus; MO - Medulla oblongata; OB - Olfactory bulb; Ocn IV - Oculomotor nucleus IV; pc - Posterior commissure; PTub - Posterior tuberculum; TeOn - TeO neuropil; vcc - Ventral caudal cluster; Scale bar has a size of 100 microns.

3.2.4 Chrna4: GFF UAS: GFP 4dpf

The valvula cerebelli in the cerebellum is now GFP+ (Fig 3.8, D1).

In the Diencephalon, the left habenula expresses GFP (Fig 3.8, D2). The posterior commissure starts to thicken, due to the differentiation of more neurons during the secondary neurogenesis process, (Fig 3.8, C2). GFP expression on the MO is now very broad beyond the first clusters described (Fig 3.8, D1, D2, and D3). New brain areas are now GFP+ such as two clusters in the subpallium, and the spinal cord (Fig 3.8, D3, and D4). The posterior tuberculum has a more defined and distinct structure (Fig 3.8, D4). In

the Retina there are GFP+ cell on different layers these being the inl (inner nuclear layer) with small projections to the ipl (inner plexiform layer) and some cells on the gcl (ganglion cell layer) (Fig 3.8,

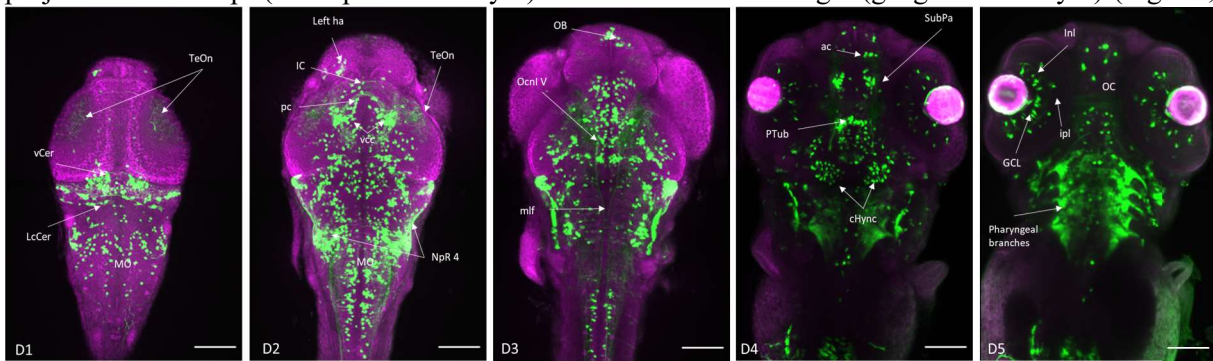


Figure 3.8: GFP expression in a 4dpf chrna4: GFF UAS: GFP zebrafish brain: From D1-D5 Partial maximum intensity projections of a, 4dpf chrna4: GFF UAS: GFP confocal z-stack are presented, panels go from dorsal to ventral starting on panel D1. Immunofluorescence was performed with anti-GFP (green) and anti-HuC (magenta). Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: ac - Anterior commissure; cHync - Caudal hypothalamus neural cluster; GCL - Ganglion cell layer; Ha - Habenula; IC - Intertectal Commissure; inl - Inner nuclear layer; ipl - Inner plexiform layer; LcCer - Lobus caudallis cerebelli; mlf - Medial longitudinal fasciculus; MO - Medulla oblongata; NpR 4 - Neuropil Region 4; OB - Olfactory bulb; Oc - Optic chiasm; Ocnl IV - Oculomotor nucleus IV; pc - Posterior commissure; PTub - Posterior tuberculum; SubPa - Sub-pallium; TeOn - TeO neuropil; vcc - Ventral caudal cluster; vCer - Valvula cerebelli; Scale bar has a size of 100 microns.

D5).

3.2.5 Chrna4: GFF UAS: GFP 5dpf and 6dpf

At this stage subpopulations of cells are more clearly defined from day 4, there are no new GFP+ clusters, overall there is no significant differences between day 5 and day 6 (Fig 3.9).

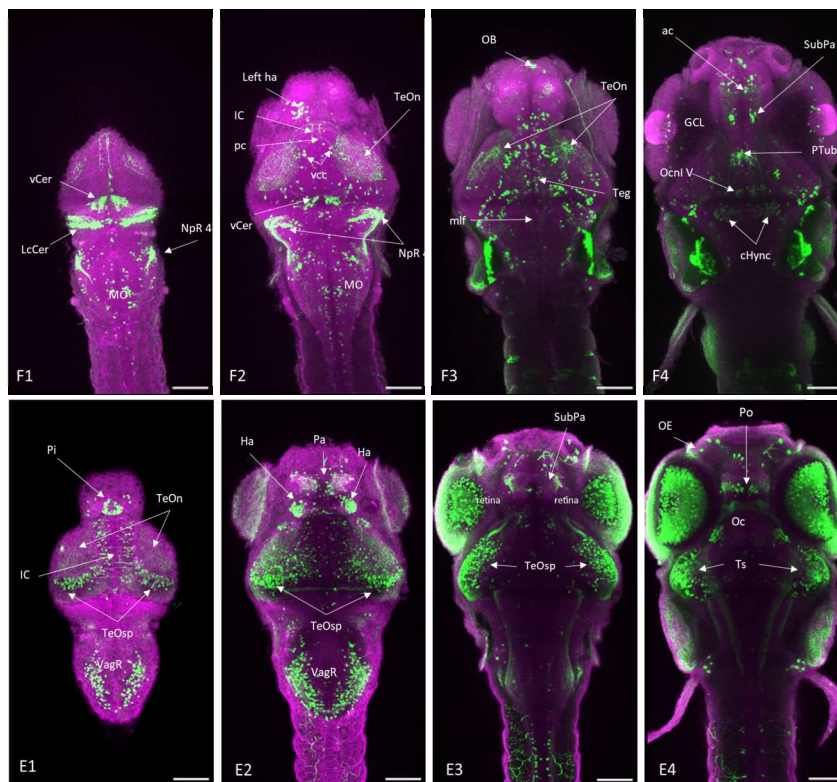


Figure 3.9: GFP expression in a 5dpf and 6dpf chrna4: GFF UAS: GFP zebrafish brain: From E1-E4 Partial maximum intensity projections of a, 5dpf chrna4: GFF UAS: GFP from F1-F4 Partial maximum intensity

projections of a, 6dpf *chrna4*: GFF UAS: GFP, panels go from dorsal to ventral starting from the panels on the left. Immunofluorescence was performed with anti-GFP (green) and anti-tERK (magenta). Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: *ac* - Anterior commissure; *cHync* – Caudal hypothalamus neural cluster; *GCL* –Ganglion cell layer; *Ha* – Habenula; *IC* – Intertectal Commissure; *inl*- Inner nuclear layer; *ipl* – Inner plexiform layer; *LcCer* - Lobus caudalis cerebelli; *mlf* – Medial longitudinal fasciculus; *MO*- Medulla oblongata; *NpR 4* - Neuropil Region 4; *OB* – Olfactory bulb; *Ocn IV* – Oculomotor nucleus IV; *pc* – Posterior commissure; *PTub* - Posterior tuberculum; *SubPa* - Sub-pallium; *TeOn* - TeO neuropil; *vcc* – Ventral caudal cluster; *vCer* - Valvula cerebelli; Scale bar has a size of 100 microns.

3.2.6 Chrna4: GFF UAS: GFP expression summary

Table 3.2: *Chrna4*: GFF UAS: GFP expression summary table: Summary of all the areas where GFP is expressed and the brain regions where expression appears and the day at which each subpopulation first appears.

Brain Regions	GFP expression Areas(time of expression)	Abbreviations
Telencephalon	Subpallium(3dpf); Olfactory Bulb(2dpf); Anterior Commissure(3dpf);	SubPa; OB; ac;
Diencephalon	Habenula(4dpf); Posterior Commissure(2dpf);Posterior tuberculum(3dpf);ventral caudal cluster(2dpf); Caudal Hypothalamus neural cluster(2dpf)	Ha; pc; PTub; vcc; cHync;
Mesencephalon	Optic Tectum Neuropil(3dpf); Tegmentum(4dpf); Medial longitudinal Fascicle(2dpf);; oculomotor nucleus IV(3dpf);	TeOn; Teg; mlf, Ocn IV;
Rhombencephalon	Valvula Cerebelli(4dpf); Lobus Caudalis Cerebelli(2dpf); Medulla Oblongata(3dpf); Neuropil region 4(4dpf);	vCer; LcCer; MO; NpR 4;
Retina	Inner Nuclear layer (4dpf); inner plexiform layer (4dpf); ganglion cell layer (3dpf).	Inl; ipl; GCL;
Ganglia and Others	Spinal cord(4dpf)	SC;

3.2.6.1 Chrna4: GFF UAS: GFP expression in the telencephalon.

In the telencephalon, GFP is only present in a very small amount of neuropil on the pallium. A few GFP+ cells are present in the olfactory bulb since the 3dpf stage. The anterior commissure expresses GFP and more caudally the subpallium possesses two small lateral clusters of expression.

3.2.6.2 Chrna4: GFF UAS: GFP expression in the diencephalon.

In the diencephalon, GFP is expressed in the left habenula only at the 4dpf stage, the vcc and posterior commissure between the optic tectum also expresses GFP. Ventrally a somewhat distinctive structure that may correspond to the posterior tuberculum also presents fluorescence at 3dpf.

In a more caudal and ventral region of the diencephalon, at 2dpf, the caudal hypothalamus neural cluster is GFP+ with a unique oval shape expression.

3.2.6.3 Chrna4: GFF UAS: GFP expression in the mesencephalon.

GFP+ cells can be detected in the optic tectum neuropil, the tegmentum is also GFP+ presenting a few intertectal commissures that can be seen very early in development but become very prominent at the 5 and 6dpf stage. GFP expression is found along the Medial longitudinal Fascicle and neighboring these structures is the GFP+ oculomotor nucleus IV (3dpf);

3.2.6.4 Chrna4: GFF UAS: GFP expression in the rhombencephalon

Expression of GFP can be detected at low levels in the medulla oblongata. The cerebellum possesses a higher concentration of GFP+ cells in the valvula cerebelli. Within the hindbrain, we find GFP+ cells in the Lobus Caudalis Cerebelli and in a region described in Zbrain as the neuropil region 4 at a later stage.

3.2.6.5 Chrna4: GFF UAS: GFP expression in the retina, ganglia and spinal cord.

In the retina expression occurs at the 3dpf stage in a few cells of the Inner Nuclear layer, retinal ganglion cell layer and expression throughout the inner plexiform layer. Sparse cells in the spinal cord also express GFP. No GFP+ ganglia were identified for this line.

3.3 pcp4a: GFF UAS: GFP - GFP Expression

This specific line was created by inserting the GFF variant of Gal4 into a bacterial artificial chromosome (BAC) containing the regulatory region of the pcp4a gene. The BAC was then integrated into the genome using the Tol2 transposon system. [S. Renninger and M. Orger, unpublished].

For the characterization of this line the GFF driver line pcp4a: GFF lyn-mCherry was crossed with the UAS: GFP reporter line, and the GFP expressing embryos (pcp4a: GFF UAS: GFP) were selected for characterization, this line similarly to the others expressed a very consistent pattern across individuals (Fig 1.5).

3.3.1 Pcp4a: GFF UAS: GFP 1dpf

Expression seems to be very variable among fish with only very few cell at different locations. The first cells expressing fluorescence are possibly from the pineal gland (Fig 3.10, A1).

In addition, some scattered GFP expressing cells are detected in the tail that may be part of the spinal cord and later start appearing closer to the brain (not shown).

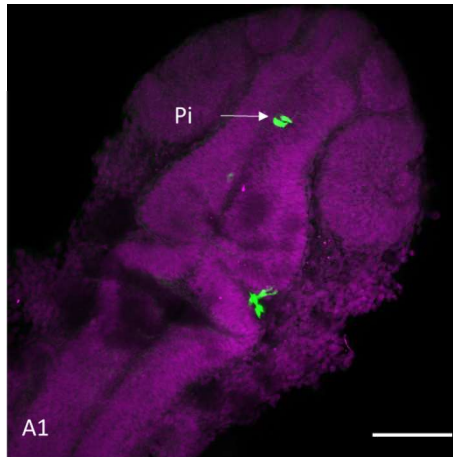


Figure 3.10 GFP expression in a *1dpf pcp4a: GFF UAS: GFP* zebrafish brain: *1dpf pcp4a: GFF UAS: GFP*. Immunofluorescence was performed with anti-GFP (green) and bisbenzimidide (magenta). Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: Pi – Pineal gland; Scale bar has a size of 100 microns.

3.3.2 Pcp4a: GFF UAS: GFP 2dpf

Expression of GFP is now confined to specific structures instead of being scattered, such as the optic tectum, pineal gland and torus semicircularis (Fig 3.11, B1, and B2).

Cells on the Pineal gland are detected and appear to project axons to the preoptic region more ventrally. The torus semicircularis also possesses clusters of cells projecting axons ventrally (Fig 3.11, B2).

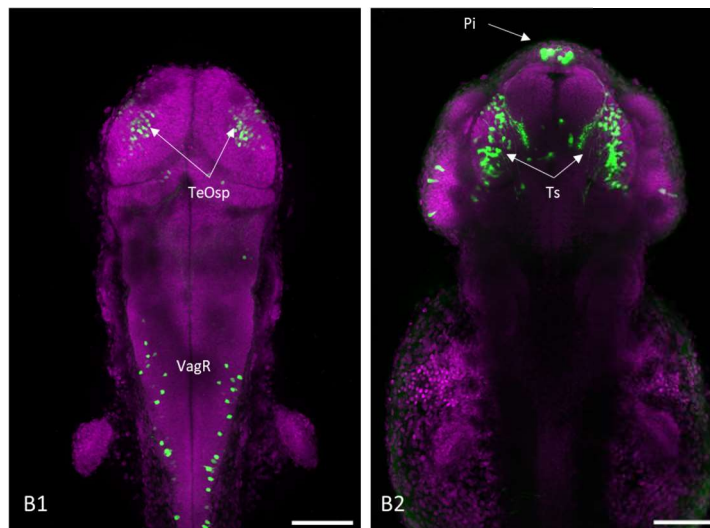


Figure 3.11: GFP expression in a *2dpf pcp4a: GFF UAS: GFP* zebrafish brain: From B1-B2 Partial maximum intensity projections of a *2dpf pcp4a: GFF UAS: GFP* confocal z-stack are presented, panels go from dorsal to ventral starting on panel B1. Immunofluorescence was performed with anti-GFP (green) and bisbenzimidide (magenta). Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: Pi – Pineal Gland; TeOsp - TeO stratum periventriculare; Ts - Torus semicircularis; VagR - Vagal region; Scale bar has a size of 100 microns.

3.3.3 Pcp4a: GFF UAS: GFP 3dpf

A dramatic increase in GFP expression is evident and restricted to specific structures.

In the diencephalon, the optic tectum both the neuropil and the stratum periventriculare area are fully covered in GFP+ cells (Fig 3.12, C1).

The projections on the torus semicircularis are thicker and longer (Fig 3.12, C3).

In the vagal region of the hindbrain, there is a more defined with an increase in GFP+ neuropil and cell bodies (Fig 3.12, C1, C2).

The retina also started to express GFP in the inner nuclear layer, inner plexiform layer and ganglion cell layer (Fig 3.12 C3, C4).

The optic chiasm is also GFP+ which is expected due to the amount of GFP expressed in the RGC in the retina (Fig 3.12, C4).

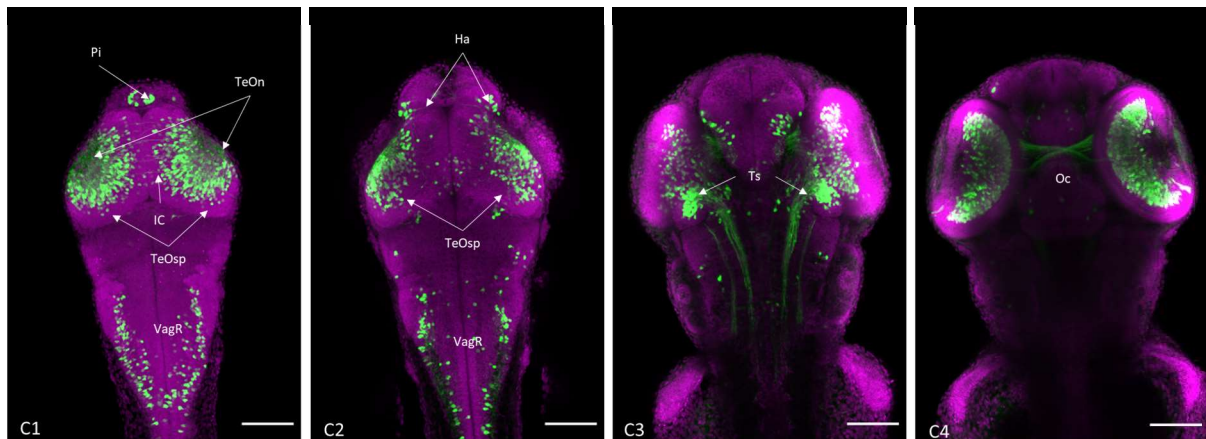


Figure 3.12: GFP expression in a 3dpf pcp4a: GFF UAS: GFP zebrafish brain: From C1-C4 Partial maximum intensity projections of a, 3dpf pcp4a: GFF UAS: GFP, confocal z-stack are presented, panels go from dorsal to ventral starting on panel C1. Immunofluorescence was performed with anti-GFP (green) and bisbenzimidide (magenta). Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: Ha – Habenula; IC – Intertectal Commissure; Oc – Optic chiasm; Pi – Pineal Gland; Po - Preoptic area; TeOn - TeO neuropil; TeOsp - TeO stratum periventriculare; Ts - Torus semicircularis; VagR - Vagal region; Scale bar has a size of 100 microns.

3.3.4 Pcp4a: GFF UAS: GFP 4dpf, 5dpf and 6dpf

From day 4, the expression patterns are very similar so the images have been grouped together (Fig 3.13).

At these later stages, intertectal commissures are very well established and visible (Fig 3.13, D1-F1).

In the Diencephalon the pineal gland and habenula have a very intense expression of GFP (Fig 3.13, D2, E1, and F2).

In the telencephalon the pallium and subpallium have some sparse GFP+ cells (Fig 3.13, D2-D4, E2-E4, and F2-F4). The same applies to the olfactory epithelium (Fig 3.11, D4-F4).

On the mesencephalon there are no new structures at this stage.

The fluorescence in the retina is very intense mainly due to the GCL (Fig 3.13, D3-F3, D4, and F4).

We also start to observe some expression on the spinal cord (Fig 3.13, E3).

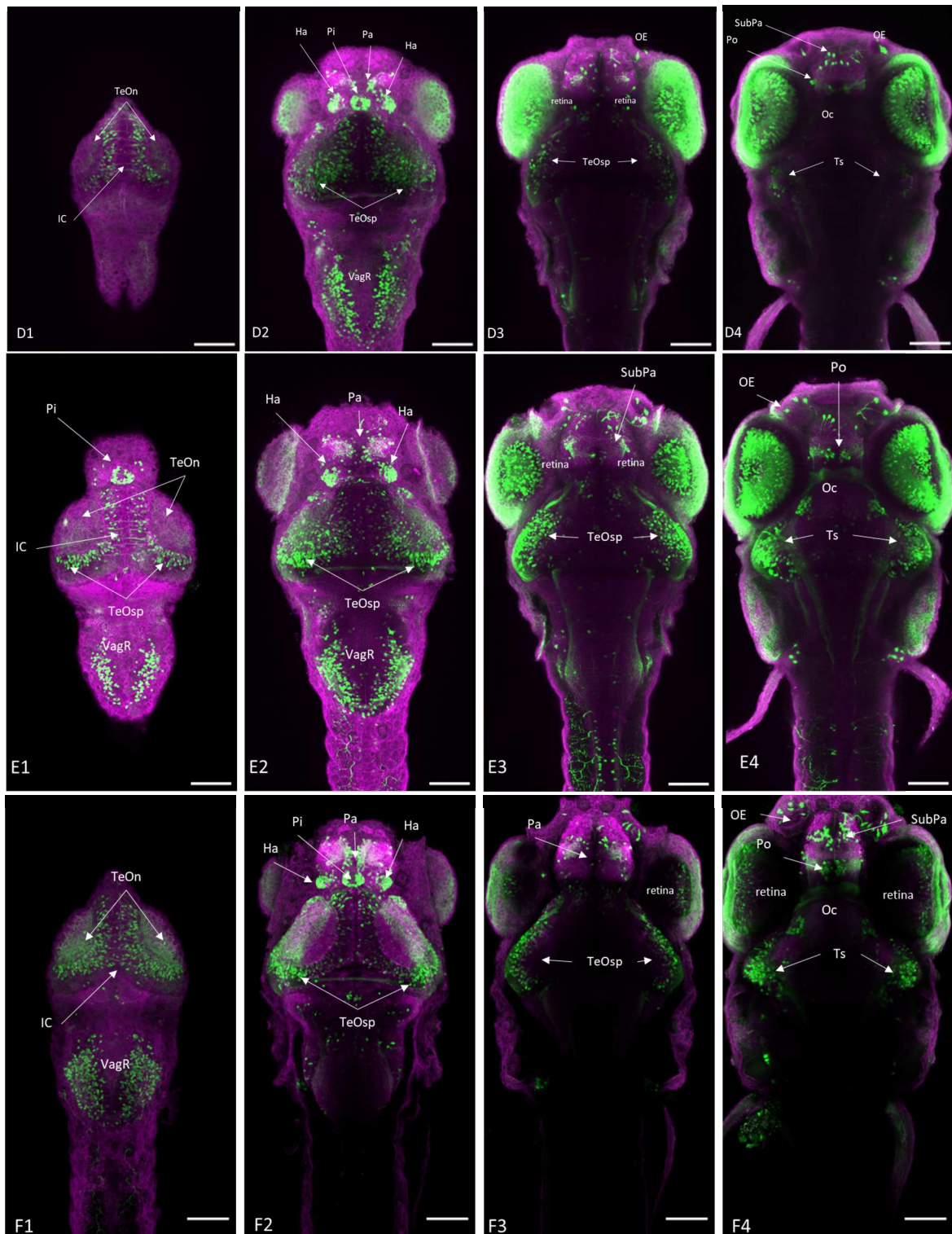


Figure 3.13: GFP expression in a 4dpf, 5dpf and 6dpf *pcp4a: GFF UAS: GFP* zebrafish brain: From D1-D4 Partial maximum intensity projections of a, 4dpf *pcp4a: GFF UAS: GFP*. From E1-E4 Partial maximum intensity projections of a, 5dpf *pcp4a: GFF UAS: GFP*. From F1-F4 Partial maximum intensity projections of a, 6dpf *pcp4a: GFF UAS: GFP*, confocal z-stack are presented. Panels go from dorsal to ventral starting on the left panels. Immunofluorescence was performed with anti-GFP (green) and anti-tERK (magenta). Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: Ha – Habenula; IC – Intertectal Commissure; Oc – Optic chiasm; OE – Olfactory epithelium; Pa – Pallium; Pi – Pineal Gland; Po - Preoptic area; SubPa - Sub-pallium; TeOn - TeO neuropil; TeOsp - TeO stratum periventriculare; Ts - Torus semicircularis; VagR - Vagal region; Scale bar has a size of 100 microns.

3.3.5 Pcp4a: GFF UAS: GFP expression summary

Table 3.3: *pcp4a*: GFF UAS: GFP expression summary table: Summary of all the areas where GFP is expressed in the 6 dpf larval brain from *pcp4-GFF*; UAS-GFP transgenic fish with indication of the stage when cells in the regions are first observed.

Brain Regions	GFP expression Areas(time of expression)	Abbreviations
Telencephalon	Pallium(4dpf); Subpallium(4dpf);	Pa; SubPa;
Diencephalon	Optic chiasma(3dpf); Preoptic Area(3dpf);Habenula(3dpf);Pineal Gland(3dpf)	Oc; Po; Ha; Pi;
Mesencephalon	Torus Semicircularis(2dpf);Tectum Stratum Periventriculare(2dpf);	Ts; TeOsp;
Rhombencephalon	Vagal region (3dpf); Medulla oblongata (1dpf);	VagR; MO;
Retina	Inner Nuclear layer(3dpf); inner plexiform layer(3dpf); ganglion cell layer(3dpf)	Inl; ipl; GCL
Ganglia and Others	Olfactory Epithelium(3dpf)	OE

3.3.5.1 Pcp4a: GFF UAS: GFP expression in the telencephalon.

In the telencephalon, GFP is only present in a small amount in the pallium and subpallium in small subpopulations, expression on these areas occurs rather late in development at 4dpf.

3.3.5.2 Pcp4a: GFF UAS: GFP expression in the diencephalon.

In the diencephalon GFP is expressed in its majority at the 3dpf stage with all the structures expressing GFP consistently over the next 3 days of development. GFP is located in the most outer region on both sides of the habenula, the pineal gland also presents GFP+ cells, ventral to those regions the preoptic area and optic chiasm express some GFP.

3.3.5.3 Pcp4a: GFF UAS: GFP expression in the mesencephalon.

GFP+ cells can be detected in the tectum stratum periventriculare and optic tectum neuropil which is also presenting expression in intertectal commissures

The Torus semicircularis since the 2dpf stage is expressing fluorescence developing thick and large projections, throughout development, travelling in a ventral and caudal direction.

3.3.5.4 Pcp4a: GFF UAS: GFP expression in the rhombencephalon

Expression of GFP can be detected in the medulla oblongata very early (2dpf) but only more specifically in the vagal region at 3-4dpf stage, this region presents GFP+ cell bodies in a more dorsal region with neuropil right below them,, they also project axons rostrally and ventral, that upon reaching the mesencephalon climb and move contralaterally.

3.3.5.5 Pcp4a: GFF UAS: GFP expression in the retina, ganglia and spinal cord.

On the retina expression occurs in the Inner Nuclear layer, inner plexiform layer and ganglion cell layer similarly to the chrna4 line at approximately the same time point (3dpf).

The olfactory epithelium has a few GFP+ cells. There is some expression in the spinal cord.

3.4 Live Imaging of early embryos

The confocal characterization of the GFP expression shown above is limited to the analysis of images of fixed tissue; however additional and more dynamic information can be extracted by time lapse imaging of the embryos. We initiated this type of experiments by imaging live embryos using Lightsheet microscopy. For this, we used two lines previously described in fixed samples in the laboratory.

Firstly we used the pitx2c: GFP line, since it had a known expression of GFP, to determine if this technique was appropriate for the study of specific subpopulation dynamics during the 20-48hpf period (Fig 3.14).

For this the line pitx2c:GFP line was crossed with HuC:GFF UAS: Lyn-mCherry in order to obtain embryos with the already described pitx2c GFP expression pattern and mCherry being expressed under the control of a pan neuronal marker(Hu) to observe the general anatomy and contrast with the GFP.

The embryos were mounted (Methods 2.5) and imaging was done for 18h with z-stacks being obtained every 10 minutes. Movie available in a supplementary data folder (S.3.14- pitx2c: GFP HuC: GFF UAS: Lyn-mCherry).

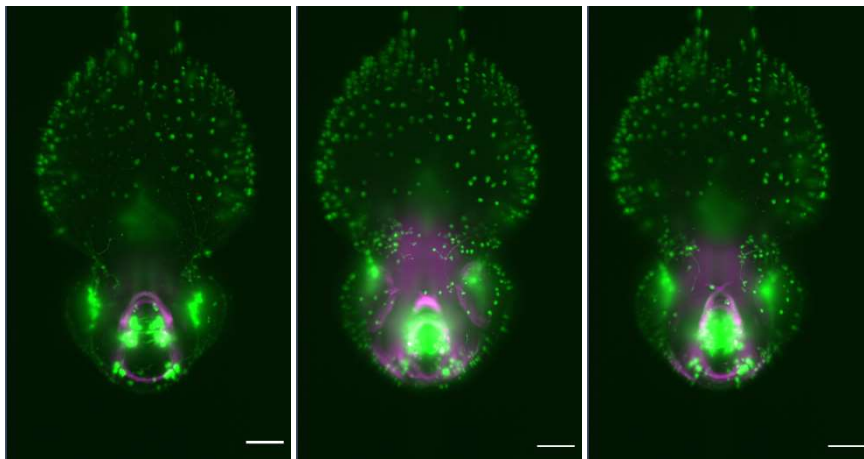


Figure 3.14: Lightsheet ventral projections of a pitx2c: GFP HuC: GFF UAS: Lyn-mCherry zebrafish embryo: Maximum intensity projections of 3 time points of a 1dpf embryo over an 18h period. Endogenous GFP expression is represented in green, and lyn-mCherry in magenta. Scale bar has a size of 100 microns.

The embryo presents a pattern very similar to what has been described before (Miguel & Madeira, 2017), being the main defining characteristics the GFP expression in Trigeminal ganglia projecting to the spinal cord and the mlf. The pc and poc and MO are expressing mCherry and help identify the larval brain structures.

There are several rapid moving cell like bodies on the surface of the fish, these have not been described but are possible keratinocytes present in the skin of the fish.(Shi et al., 2010) (Fig 3.14) Growth cones of several neurons throughout the fish are very visible with this technique. The transparency of the fish

is due to its PTU treatment again allows us to image very deeply, and the advantages that the Lightsheet microscopy brings in contrast to confocal microscopy allow to analyze the cell dynamics and reduce the phototoxicity of the lasers used. These preliminary results indicated that this technique could be used to follow the growth of axons and circuit formation.

We thus, decided to focus on a specific commissure, the posterior commissure, which is very dorsal and clearly visible in the transgenic line Chat: GFF characterized beforehand in fixed samples. (Miguel & Madeira, 2017) This transgenic line genome harbors an insertion of a BAC containing the choline acetyl transferase genomic region with GFF cloned at the Chat starting ATG. The embryos selected for the experiments are the offspring of an incross of the Chat: GFF UAS: GFP line.

To contrast with the GFP signal and give indication of the anatomy of the embryo, we used Bodipy-TR-methyl ester (Fig 3.15, A-C 24hpf). However, a decrease of the Bodipy signal was observed through the course of the imaging probably due to the slow dilution of the dye into the chamber medium (Fig 3.15, A 24hpf-44hpf), z-stacks were acquired from the embryos oriented at different angles with respect to the light sheet's objective to have different points of view of the structure of interest. Between 22 and 24 hpf the embryo expresses GFP in the poc, vcc, vrc, mlf and OE. These regions have been annotated in figure 3.15.

During the time lapse it is possible to observe the development of each individual subpopulation of GFP expressing cells, during a 20 hour period (Fig 3.15) Movie available in a supplementary data folder. (S.3.15- Chat: GFF UAS: GFP)

This is a very good example and we can clearly see the primary and secondary neurogenesis occur in the 20h period. (Fig 3.15, A-C 24hpf-44hpf)

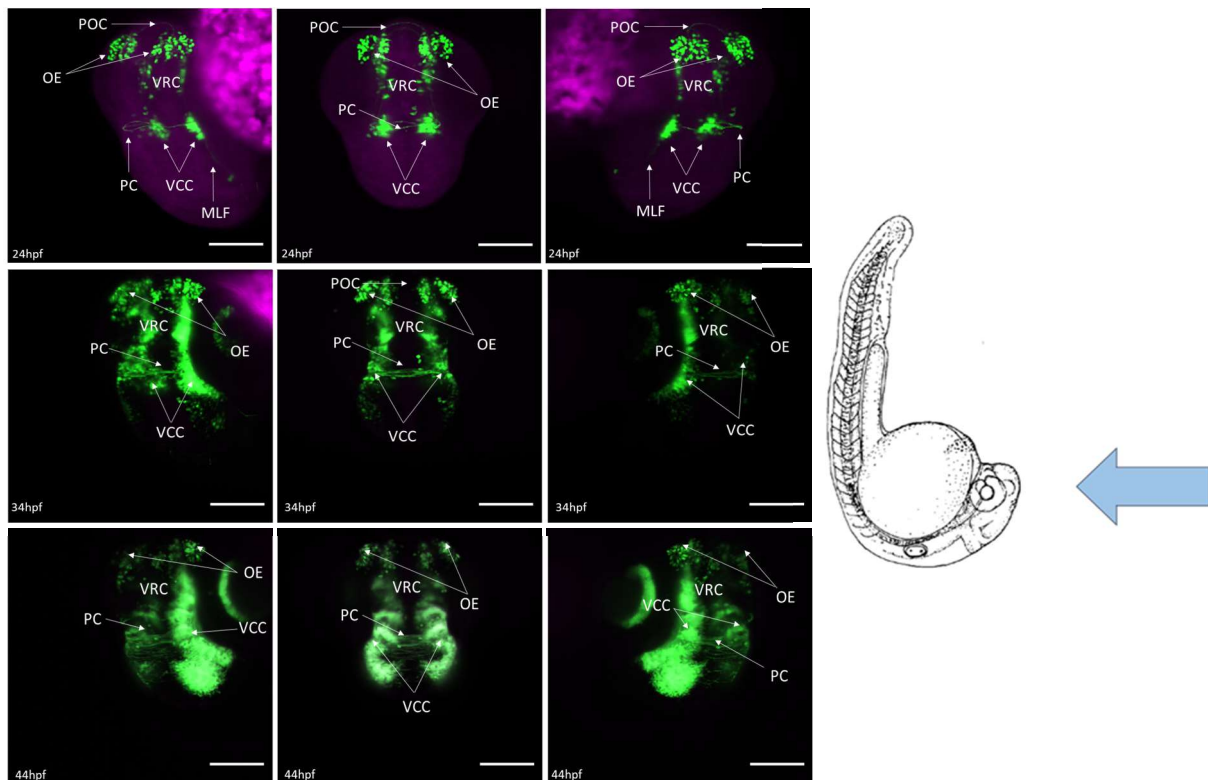


Figure 3.15: Lightsheet projections of a *Chat: GFF UAS: GFP* zebrafish brain over time: Maximum intensity projections of 3 different angles of a 1dpf embryo brain over a 20h period. Panels A, B and C show 3 different angles at the time point indicated, in hpf, in each figure. The sketch on the right (adapted from kimmel et al. 1995) represents the position of the objective for angle B, the other two angles A and C are 40° rotations of the embryo to the left and right respectively. Endogenous GFP expression is represented in green, and Bodipy-TR-methyl ester in magenta. Annotations have been made using abbreviations of the brain areas expressing GFP. Abbreviations: OE – Olfactory epithelium; pc – Posterior commissure; poc - Post-optic commissure; vcc – Ventral caudal cluster; vrc - Ventral rostral cluster; Scale bar has a size of 100 microns.

Focusing on the posterior commissures after 10 hours an increase in the number of axons that were projected to the posterior commissure is observed and at 44hpf there is a great difference in thickness compared to the start of the experiment figures. Interestingly, we observed cells migrating following the projections of commissure suggesting that commissures may serve as a “highway” for cell migration. Future experiments will aim at imaging with higher spatial resolution and to track individual projections to extract parameters such as directionality or speed.

In other areas it is also possible to study the growth of individual axons but a more localized GFP expression in the growth cone could help.

4. Discussion

4.1 GFP expression in transgenic lines and “host” gene expression patterns

In this study, several GFP expressing transgenic zebrafish lines, were chosen because current studies in the Orger laboratory indicated that they label neuronal subpopulations involved in visual processing (Renninger and Orger, unpublished). These lines were characterized for the first six days of development through immunofluorescence and confocal imaging.

The GFP expressing cells were identified by comparison with available atlases (ZBB1.2, Zbrain) and other more specific studies.(Avanesov & Malicki, 2010; Marquart et al., 2017; Mueller & Wullmann, n.d.; Randlett et al., 2015)

Throughout the project there were some limitations and challenges, that can be mitigated in future experiments.

Although the GFP expression described was consistent across samples for each day and line, minor differences still exist. This may be due to variability in the stage of development, as larvae were fixed on a day post fertilization basis and there may be several hours' difference within and between clutches. Samples may present different levels of GFP expression at the time of fixation and there are always little differences in circuitry assembly and position of neurons in different individuals that should be taken into account. IN addition, immunofluorescence protocols have been performed on different days with some varying conditions in immunofluorescence protocols. Furthermore, the analysis was based on visually comparing the GFP expression of our data with anatomical reference brains 3D atlases and a few other so, anatomical annotation is only tentative. The use of in situ hybridization or immunohistochemistry co-labelling with other structure specific antibodies would help with the characterization of the line.

The work done here is important to start fill the gap of information regarding anatomical information and gene and protein expression in earlier developmental stages.

Registration of the different samples for the same stage of each line into a reference brain would allow to anatomically label the GFP expression areas (at the population level) thus reaching a higher level of certainty.

Registration would definitely address most of these problems, but it is of utmost importance to be aware of them when performing some experiments and analyzing some results until the lines are properly registered and characterized.

Several algorithms exist to register the confocal stacks into a reference brain like the CMTK or ANTs, with ANTs being now used in the lab, since it has been shown to supersede previous ones as shown for the construction of the ZBB1.2 atlas. In this work we have used two markers for future registration of the images, one is the pan neuronal marker, tErk,, this is a good anatomy marker for the later stages of

larval development (4, 5 and 6dpf) and for the younger stages we have been using bisbenzimidazole, a nuclear staining, which is easier and cheaper to use, but has a penetration problem that impairs imaging of deeper areas of the brain and in addition it does not stain neuropil.

The lines analyzed include BAC insertions of genomic fragments that contain GFF or GFP in the gene of interest. It is thus possible that the GFF or GFP are expressed in the same cells as the corresponding genes. In the following section we discuss the coincidence and difference between the reporters and the endogenous gene.

4.1.1 OLIG2: eGFP

Olig2 genes, which encode basic helix–loop–helix (bHLH) transcription factors, are important for motor neuron and oligodendrocyte development. (McFarland et al., 2008; Park et al., 2002) The olig2 transgenic line, Tg(olig2:egfp), expresses a soluble form of EGFP, which occupies the cytoplasm, reveals both cell bodies and processes allowing the identification of cells by morphology. ((McFarland et al., 2008; Park et al., 2002; Shin, 2003; Sidik & Talbot, 2015; Zannino & Appel, 2009)

This line since its creation has been confirmed to accurately mark olig2 expression in motor neurons, oligodendrocytes and oligodendrocyte progenitor cells (OPCs), and since used for different studies involving these types of cells. (Shin, 2003)

Cerebellar cells of zebrafish embryos have been confirmed to prominently express olig2 RNA and EGFP driven by olig2 regulatory sequences although presenting a delay. (McFarland et al., 2008)

In the zebrafish hindbrain, the olig2 gene is expressed in the ventral medial ventricular zone of rhombomeres 5 and 6 at 30 hpf. and by 48 hpf olig2 was expressed in rhombomeres 2 and 7 and the midbrain/Rhombomere 1 region (Esain, Postlethwait, Charnay, & Ghislain, 2010), this RNA expression confirmed the GFP expression later observed using the olig2:GFP line, we also observed similar results at approximately the same time period.

Another study focused on the spinal cord and hindbrain confirming once again the olig2: GFP line is an accurate indicator of hindbrain olig2 expression. (Zannino & Appel, 2009)

Olig2-positive neural plate cells develop as primary motor neurons (pMN) and oligodendrocytes, indicating that these cell types arise from a common precursor population. (Esain et al., 2010; Park et al., 2002) Olig2/Ngn2 ratio in progenitor cells serves as a gate for timing proper gene expression during the development of pMN cells: high levels of Olig2 maintains the pMN state, thereby holding cells in reserve for oligodendrocyte generation, whereas high levels of Ngn2 favors the conversion of pMN cells into post-mitotic motor neurons. (Lee, Lee, Ruiz, & Pfaff, 2005)

The line used has already been confirmed that it expresses GFP in accordance to olig2 gene expression, already being used for many studies related to motor neurons oligodendrocytes, especially in the spinal cord but there is a lack of characterization of this line in young larval stages and the whole brain

4.1.2 CHRNA4: GFF UAS: GFP

Acetylcholine plays an important role in the regulation of nervous system development and function. Dysfunctions in cholinergic signaling have been shown to result in structural damage with subsequent effects on behavior. (Ackerman, Nakkula, Zirger, Beattie, & Boyd, 2009)

In vertebrates, there are two main classes of cholinergic receptors: muscarinic and nicotinic acetylcholine receptors (nAChR), the latter are ligand-gated ion channels that are responsible for rapid neurotransmission and expressed in many regions of the brain (neuronal nAChR), neuromuscular junction (muscle nAChR), and autonomic ganglia). *Chrna4* is one of the subunits that compose these channels. (Ackerman et al., 2009)

Chrna4 expression analysis by in situ hybridization has been previously done at 24, 48, 72, and 96 hpf. (Ackerman et al., 2009)

In 24 hpf embryos (Fig 3.5), *chrna4* transcript has been detected in a subset of neurons in rhombomeres 4–7 and expression in cranial neural crest cells in the pharyngeal arch. Limited expression was observed in forebrain and midbrain structures. Comparing to our results at the 1 dpf stage, we only found coincident GFP expression, in what seems to be the neural crest cells of the pharyngeal arches which corresponds in part to the *chrna4* transcript at that time. (Fig 3.5)

At 48 hpf, significant bilateral *Chrna4* expression has been seen in both midbrain and hindbrain (Fig. 5F, G) consistent with the nucleus of the medial longitudinal fascicle and reticulospinal neurons, with no expression detected in the spinal cord. For the 2dpf stage we found GFP expression similar to what was described, additionally we have expression more rostrally in the posterior commissure and ventral caudal cluster. (Fig 3.6)

At 72 hpf and 96 hpf, *Chrna4* continues to be highly expressed in specific midbrain and hindbrain areas. Which goes in accordance with what we describe, although for these stages we detected GFP expression in the optic tectum opposed to the lack of *chrna4* RNA in that area.

These results of the previously described *in situ* hybridization seem to go in accordance to the GFP expression pattern on the *chrna4*: GFF line characterized, but maybe a more detailed RNA expression pattern study would be useful in confirming this result.

4.1.3 PCP4A: GFF UAS: GFP

Purkinje cell peptide 4 (Pcp4) is a small polypeptide carrying an IQ motif: a 23-amino acid domain that binds EF-hand proteins and shows high affinity for calcium-poor calmodulin (Mione et al., 2006)

In situ hybridization technique has been previously used by to analyze the expression of *pcp4a* in the zebrafish brain. (Mione et al., 2006) The expression patterns coincide in some areas at the same stage, but overall the expression patterns of the *pcp4* transcript and the GFP is different, so we conclude that the line we are using does not correspond to the real regulatory region of the *pcp4a* gene in zebrafish. Expression of *pcp4a* transcript appears in the tegmentum, some restricted cells in the optic tectum, and

cells at the mid-hindbrain boundary and large cells at the ventral side of the torus longitudinalis (TL) express *pcp4a*, comparing with our results only the optic tectum expresses GFP.

Pcp4a transcripts are expressed in a group of cells found in the lateral torus semicircularis, but it does not appear to be projecting axons caudally as seen in our results. In addition, large cells in the nucleus of the medial longitudinal fascicle (nMLF) express *pcp4a* transcript on contrary to our GFP expression pattern.

At 48 hpf transcript expression was found on the pineal gland and their projections, these results match with ours, but we possibly have expression on this area starting about 24 hours earlier. GFP expression also seems to match the transcript expression in the habenula but not in the eminentia thalami.

Low levels of expression of *pcp4a* transcript were also detected in posterior, the hypothalamic region where the caudal hypothalamus expresses the highest levels, whereas on our results we don't see any clear expression on these areas of the diencephalon.

The expression of *pcp4a* transcript was detected at 22 hpf in two bilateral cellular groups located in the lateral region of the hindbrain possibly being reticulospinal neurons and 8 hours later the presumptive reticulospinal neurons are located in a more medial position and persist until at least the 5dpf stage. The *pcp4a*: GFF UAS: GFP line also has expression in the hindbrain, but not with the same characteristics of the transcript expression described before.

Expression of *pcp4a* can be detected at low levels in the Purkinje cells of the cerebellum whereas in this work GFP was not seen to be expressed on the cerebellum. From 48 hpf *pcp4a* is expressed in the ganglion cell layer of the developing retina. The expression is initially confined to the medial sector and then spreads to the rest of the ganglion cell layer by 4 dpf (Fig. 6B). Sparse cells in the inner nuclear layer also express *pcp4a* (Fig. 6B).

We found expression on the same areas of the retina at the same stage (2dpf), but by the 4dpf stage expression of GFP appears to be superior.

Other issues that we have to take in account for all the lines studied involve the possible inaccurate expression of GFP since when compared to other mRNAs and other proteins may have a different life time than that of most mRNAs and thus may not reflect ongoing transcription of the GFF or GFP genes. This is relevant when comparing our data with gene expression of the "host" gene using in situ hybridization, because GFP can last longer in cells. Besides temporal differences in the expression of GFP the expression pattern may be different as we will discuss later

Overall the expression does not seem to correspond with the endogenous *pcp4a* gene expression.

This mismatch might be explained by the nature of the construction of the line: the BAC, which includes a genomic region of zebrafish genome with part of the gene encoding *pcp4a* as well as other genes, is introduced randomly in the zebrafish genome. This particular BAC may not contain all the regulatory regions of the *pcp4a* gene. Depending on the region in which it was inserted, the GFP expression can be driven by regulatory regions of other genes near the construct, or even by enhancers inside the construct

that may change their interactions with the GFF. This may lead to an overlap between the *pcp4a* expression patterns with that of other genes.

Although some lines created with this type of strategy, like the *pcp4a* line, do not exactly match the original gene expression they are still of importance since they express GFF or GFP in areas associated with visual responses. With the characterization we can now observe exactly what cells are responding to stimuli and what they are, registration steps are still needed, and structures can still be further identified with more detail, and even near cellular resolution with registration to a reference brain.

4.2 Live Imaging with Lightsheet Microscopy

The Lightsheet allows us to image *in vivo*, it brings several advantages compared to confocal microscopy mainly reducing phototoxicity to the embryo, which means we can image at a high resolution for longer and, if needed, even with increased laser power, due to the illumination of a single plane at a time in the sample.

Several improvements of the imaging can be made, such as the use calibration of the Lightsheet with beads to accurately do a 3D reconstruction of the different view angles. This type of data would also enable us to quantify and describe the growth of axons and compare the growth of different types of neurons with what has been described so far (Simpson, Kita, Scott, & Goodhill, 2013) not do any specific growth cone and axon extension analyses, but studies already exist doing his describing the extension of neurons. (Simpson et al., 2013)

In addition, other general labels for reference should be assayed to use as counterstains as we could detect dilution of Bodipy-Methyl ester in the chamber medium in the course of the experiments. Other more a permanent markers such as membrane bound molecules may be suitable. In addition, transgenic zebrafish lines driving novel and better fluorescent proteins such as mOrange (Shcherbakova, Hink, Joosen, Gadella, & Verkhusha, 2012) and mScarlett (Howe et al., 2013) under the pan neural alpha tubulin regulatory region are being developed at the moment (Orger laboratory and Champalimaud Research Molecular and transgenic tools platform). The use of such a line would allow us to have a permanent expression of a pan neuronal marker providing information on the overall anatomy and contrast for the GFP. Plans are also being made to create lines with mNeon green (Hostettler et al., 2017) under UAS regulation as an alternative to the GFP used, in order to have a brighter protein that may allow us to see expression sooner since the fluorescence of the embryo only appeared in the 22-24hpf period which causes problems in studying structures that develop at earlier stages.

Nonetheless, we were able to see how the GFP expressing subpopulations of cells develop, focusing on the posterior commissure PC which will be the focus of future work.

The Lightsheet microscope allows us to further study the development of certain structures and we aim to develop tools to better visualize GFP expression on the growth cones, to better track axon growth.

A more classical solution for this problem would be to create a reporter line that leads to the expression of an actin-GFP fusion protein. However, all described actin fusions are functionally impaired (Riedl et al., 2008), a more suitable option would be Life act-GFP, this is a C-terminal GFP fusion protein made with the first 17 amino acids of the yeast protein Abp140 which are sufficient to bind to actin interfering less with cellular processes. (Riedl et al., 2008)

But actin is present not only on the growth cone but the whole cell and proteins localized to the axonal or dendritic terminal would be more appropriate. One possibility would be the generation of a fusion protein with a more growth cone specific protein such as Gap43, but this requires the use at least two structural components: a membrane attachment domain and a recognition sequence for (Liu, Chapman, & Storm, 1991). Other proteins, such as ATE1 (J. Wang et al., 2017) or DSCR1 (W. Wang et al., 2016), would most likely have similar problems.

To overcome these challenges, we may put our attention to what transports most of these proteins' mRNAs to the growth cone itself, one protein that carries this function is the zipcode-binding protein 1 (ZBP1)

ZBP1 was originally discovered as a trans-acting factor for the “zipcode” in the 3' untranslated region (UTR) of the *b-actin* mRNA, being important for its localization and translational regulation. (Chao et al., 2010)

Besides *b-actin*, *Gap 43* and *ATE 1* mRNAs also contain these “zipcode” sequences, and it is known that the corresponding proteins are then translated locally in the growth cone (J. Wang et al., 2017). This would allow us to create a GFP mRNA that contains this “zipcode” sequence within its 3'UTR region making it recognizable by ZBP1, transported to the growth cone and being locally translated, possibly giving a very prominent expression of GFP in the very tip of the growth cone.

These reporter mRNAs can compete with endogenous mRNAs (Donnelly et al., 2013), but optimization of this sequence or the use of a homologous but less active 43-nt segment could help overcome this problem.

The development of these tools, would allow us to better track the development of specific neurons and their projections, in vivo, and with better optimization and standardization of the process, especially the use of a permanently expressed pan neuronal markers, a live atlas could be developed in order, to study the development of specific structures in such early stages, since confocal microscopy brings several disadvantages such as the long preparation time needed, and the difficulty of mounting such young embryos.

All of this work represents a step forward in the characterization of new transgenic zebrafish lines during early developmental stages (1-6dpf), even though a lot of work is still needed to have a better comprehension of the zebrafish brain. The confocal stacks for the Olig2: GFP, chrna4: GFF and pcp4a: GFF lines still require registration to a common reference in order to assign the location of labelled cells and to compare their positions to other neuronal populations more accurately. The characterization

provided by this work, although tentative, can be used as a starting point to the understanding of the development of the circuits underlying specific visually guided behaviors studied in the Vision to Action Lab (M. Orger, Champalimaud Research). The live imaging experiments also proved very useful in the study of the ontogenesis of specific brain structures, such as the posterior commissure. We also aim to use tools to better label neurites and growth cones using both available constructs and other we will specifically design for this purpose. This would allow to better track neuronal circuit formation and connections, more specifically in subpopulations of neurons and circuits associated to vision in the zebrafish larva.

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