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Developmental characterization of neuronal subpopulations involved in zebrafish visual processing

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RESUMO

O sistema nervoso é composto por diversos tipos de células neuronais e gliais organizadas de forma específica de modo a executarem corretamente funções imprescindíveis para o controlo de comportamentos básicos à sobrevivência e respostas comportamentais a estímulos internos e externos. Para tal, é necessária a aquisição de identidades celulares específicas que determinarão diversas características dos circuitos neuronais como: padrões de conectividade e produção de neurotransmissores e recetores, etc. Múltiplos processos imprescindíveis para um desenvolvimento morfológico e funcional adequado estão altamente cronometrados no espaço e no tempo: indução neural, regionalização, neurogénese, migração e diferenciação neural, axonogénese, sinaptogénese e remodelação sináptica.

Os processos moleculares e celulares envolvidos no desenvolvimento encontram-se altamente conservados dentro dos vertebrados, desde os anfíbios e peixes até às aves e aos mamíferos. Em particular, o peixe zebra tem desde os anos 80 revelado ser um modelo ideal para estudos de desenvolvimento embrionário e pós-natal, particularmente no desenvolvimento visual. O seu rápido desenvolvimento, transparência e morfologia canónica dos vertebrados que apresenta desde cedo no desenvolvimento são, aliados às novas ferramentas genéticas disponíveis, grandes vantagens deste modelo animal.

No início do desenvolvimento embrionário, durante a gastrulação (5-10 horas pós-fertilização, hpf), três folhetos embrionários são gerados: endoderme, mesoderme e ectoderme. Na ectoderme, o tecido neural é induzido pela influência de um grupo de células conhecido com o organizador. As células da futura placa neural convergem para a linha média e expandem-se ao longo do eixo rostro-caudal, através de movimentos de extensão convergente. Seguidamente, ocorre a neurulação onde a placa neural sofre rearranjos e cavita para formar o tubo neural.

Desde cedo no desenvolvimento que a regionalização é importante no controlo da diferenciação e aquisição de identidades neuronais, num processo gradual. Esta regionalização é guiada por gradientes de factores parácrinos que ativam vias de sinalização que influenciam a expressão de factores de transcrições específicos. Rapidamente depois da formação do tubo neural formam-se diversas constrições dividindo a sua parte anterior (futuro encéfalo) em diferentes segmentos: prosencéfalo (o mais rostral), mesencéfalo e rombencéfalo (o mais caudal). Esta regionalização serve de base para a produção do primeiro *scaffold* neural num processo intitulado de neurogénese primária. Os primeiros *clusters* neuronais são neurónios recém-formados derivados a partir dos *clusters* de progenitores neurais, que estendem os seus axónios longitudinal e transversalmente ligando-se entre si. No 1º dia após fertilização (dpf) destacam-se: os *clusters* dorso-rostral e ventro-rostral (prosencéfalo), o cluster ventro-caudal (mesencéfalo) e os mais dorsais *cluster* epifiseal e núcleo da comissura posterior. Numa região mais caudal, neurónios reticulo espinais estendem axónios para a espinal medula, cujos corpos celulares se localizam no mesencéfalo basal ou no centro de cada rombómero (rombencéfalo).

Antes do 2º dpf, a neurogénese secundária ocorre através do crescimento do número de neurónios tendo como suporte o *scaffold* primário anteriormente formado, reforçando as regiões anatómicas anteriormente visíveis. Neste estádio, o sistema nervoso central anterior apresenta uma organização anatómica canónica dos vertebrados, estando dividido em: prosencéfalo, (telencéfalo e diencéfalo), mesencéfalo (tecto óptico e tegmento) e rombencéfalo (cerebelo e medula oblongata).

Nos primeiros dias de desenvolvimento do peixe zebra, a visão é crucial para a sua sobrevivência, tendo um papel fulcral na alimentação, locomoção e reconhecimento do meio e também na modelação

e rearranjos nos circuitos neuronais. Na primeira semana, a larva é capaz de executar variados comportamentos como nadar em três dimensões, diversas manobras de fuga, predação e captura de presas, dormir e até aprender. Estes comportamentos estereotipados são baseados em estímulos visuais que são processados pela retina e enviados através de 10 *retinal arborization fields* para o resto do sistema nervoso central onde a informação será processada e integrada originando um *output* para uma resposta motora apropriada. Destes comportamentos que se iniciam no 3º dpf, quando as células ganglionares da retina alcançam e enervam os seus alvos, distinguem-se a resposta optomotora (OMR) e optocinética (OKR).

Face à movimentação de todo o ambiente, ou campo visual, o peixe responde movimentando-se no sentido contrário de modo a estabilizar a sua posição na água corrente, o qual é chamado de OMR. Por outro lado, face a um estímulo rotacional contínuo o peixe ajusta a direcção dos olhos, alternando entre movimentos oculares lentos e *saccades* mais rápidas, o qual é chamado de OKR. Nos últimos cinco anos foram propostos modelos para o funcionamento dos circuitos adjacentes, tendo sido identificados a área pretectal e o núcleo do fascículo medio-longitudinal (nMlf) como zonas envolvidas no processamento e integração de sinais nervosos.

Através de manipulação genética e com o avanço nas técnicas de imagiologia é agora possível ter uma perspectiva mais geral da formação de circuitos neurais. Contudo, apesar da sua vasta utilização como modelo para o estudo do desenvolvimento do sistema visual ainda existe uma grande necessidade de informação básica relativamente ao desenvolvimento dos circuitos neuronais durante os primeiros estádios do desenvolvimento. A identificação de populações neuronais e o seu papel em circuitos específicos, como no processamento visual, mantém-se crucial para aumentar o conhecimento acerca do desenvolvimento anatómico e funcional do sistema nervoso central no peixe zebra.

Uma metodologia para a identificação de populações neuronais é o uso de linhas transgénicas repórteres originadas para expressar GFP (*green fluorescente protein*) em populações e subpopulações específicas. Estas linhas podem conter um gene repórter (p. ex. GFP) diretamente controlado por sequências regulatórias de genes de interesse ou ter variantes de Gal4 (GFF) inseridas que em combinação com uma linha repórter UAS conduzirão à expressão de GFP nos locais apropriados.

Diversos projetos de larga escala têm permitido a geração de centenas de linhas transgénicas, já caracterizadas a nível celular, possibilitando o acesso a 70% do encéfalo larval do peixe zebra e a identificação de subpopulações dentro das diferentes subdivisões anatómicas. O mapeamento de subpopulações é tradicionalmente feito com a deteção múltipla de tipos celulares específicos utilizados como referência espacial anatómica (por ex. TH que marca o sistema catecholaminérgico), o que tem diversas limitações. A destacar, o atlas "*Atlas of Early Zebrafish Brain Development"* é a obra de referência do desenvolvimento neuronal embrionário e larval do peixe zebra, que intercala dados de expressão génica com encéfalos de referência anatómica, no 2º, 3º e 5º dpf.

Grandes esforços têm sido feitos para a compreensão anatómica do sistema nervoso central deste modelo animal através de *whole-brain imaging* de diversas linhas transgénicas e registo das imagens 3D numa mesma referência. Dois atlas 3D para o 6º dpf, *Z-brain* e *ZBB*, encontram-se disponíveis e abertos para consulta e também registo de informação 3D, contendo um canal comum com o sinal da expressão de uma proteína pan-neuronal (tERK). Apesar de representarem uma fonte aberta e poderosa de informação para o conhecimento neuroanatómico do peixe zebra, informação relativamente a estádios de desenvolvimento precoce ainda é escassa.

Diversas linhas GFP transgénicas têm sido usadas no laboratório de Mike Orger para o estudo do processamento e resposta de certas subpopulações neuronais a estímulos visuais de alteração da intensidade de luz e direcção de movimento. Com o objectivo de analisar a ontogenia de algumas destas

subpopulações durante o desenvolvimento, este estudo caracteriza a expressão de GFP de quatro linhas transgénicas, escolhidas pela sua expressão em estruturas anatómicas envolvidas no processamento visual, utilizando embriões e larvas de peixe zebra desde o 1º até ao 6º dpf.

Esta caracterização foi feita recorrendo a imuno-histoquímica das amostras fixadas em paraformaldeído das linhas transgénicas pitx2c:GFP, ChAT GFF, Gad1b GFF e Slc18a3b GFF. O anticorpo anti-GFP foi utilizado para detetar a expressão de GFP das linhas transgénicas em conjunto com um marcador pan-neuronal (anti-tERK) ou mais específico (anti-TH), para referência espacial. *Stacks* foram adquiridos com microscopia confocal gerando diversos exemplares de encéfalos 3D para cada estádio e linha.

A análise da expressão de GFP foi feita comparando as amostras com dados 3D relativos ao 6º dpf, Z-brain e ZBB, juntamente com imagens e representações anatómicas de encéfalos disponíveis no *Atlas of Early Zebrafish Brain Development* e outra bibliografia. Para todos os estádios, de cada linha, foram selecionadas amostras representativas, apresentadas como projecções em z dos *stacks* originais. A nomenclatura anatómica utilizada foi baseada na obra *Atlas of Early Zebrafish Brain Development.*

De um modo geral, expressão de GFP foi observada nos 6 dias de desenvolvimento em todas as linhas transgénicas utilizadas. Espacialmente, esta expressão revelou-se bem distribuída em todas as subdivisões anatómicas prosencéfalo, mesencéfalo e rombencéfalo, aumentando com o avançar do desenvolvimento. Expressão de GFP foi observada em regiões comuns às diferentes linhas, como: na área pretectal, tálamo e tubérculo posterior (diencéfalo), tecto óptico e tegmento (mesencéfalo) e medula oblongata (rombencéfalo). Como esperado, expressão de GFP foi observada em pelo menos uma estrutura anatómica envolvida nos circuitos adjacentes a OMR e OKR em cada uma das linhas transgénicas (por ex. área pretectal e nMlf).

Em suma, a caracterização da expressão de GFP de linhas transgénicas no desenvolvimento, é uma abordagem viável para a identificação anatómica de regiões de interesse envolvidas no processamento de estímulos visuais. Acreditamos que este estudo contribuiu para o conhecimento dos processos de aquisição de destinos neurais e desenvolvimento inicial dos circuitos neuronais, bem como para o delineamento e interpretação de futuras experiências de manipulação genética em estádios específicos do desenvolvimento.

Palavras-chave: Desenvolvimento; neurociências; processamento visual; peixe zebra; linhas transgénicas

ABSTRACT

The nervous system is composed of a number and variety of neuronal and glial cells appropriately connected to reliably perform its functions to control basic survival behaviours (i.e.: breathing) and responses to internal and external stimuli. This requires acquisition of specific cell identities that determine properties such as correct connectivity patterns and production of neurotransmitters and receptors. For an appropriate functional and morphological development multiple processes need to occur: neural induction, regionalization, neurogenesis, migration and neuronal differentiation, axogenesis, synaptogenesis and synaptic remodelling.

Since the 80s, the zebrafish has been used as a model to analyse the formation of neuronal circuits during embryonic and postnatal development. With the advances in the field of genetics and imaging it is now possible to address the remaining doubts and lack of knowledge regarding the establishment of neuronal circuits. Due to the array of genetic tools available, its rapid development, transparency and canonical vertebrate morphology, zebrafish is the ideal model to study the development of the central nervous system. Although it has been widely used to study the development of the visual apparatus, information about neuronal circuits involved in visual processing in early stages is still lacking in the bibliography.

With the aim of studying specific subpopulations of neurons involved in visual processing, in this project we characterized selected and established GFP-expressing reporter lines through development. The characterization was done on the zebrafish developing brain of embryos and larvae from the first day post fertilization (dpf) until the sixth dpf, using immunohistochemistry and image analysis of data acquired by confocal microscopy. Our analysis identified major anatomical structures and tracts expressing GFP through the prosencephalon, mesencephalon and rhombencephalon, by comparing our data with previous bibliography and 3D atlases on anatomical characterization of gene and protein expression. The characterization and analysis of the selected lines allowed us to examine the ontogenesis of the circuits. Furthermore, it provides a reference for the delineation of further studies involving manipulation of defined circuits at the appropriate time during development.

Keywords: development; neurosciences; visual processing; zebrafish; GFF lines

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ABBREVIATIONS LIST

AB – arborization fields

ABN - abducens nucleus

AC – amacrine cells

ac – anterior commissure

cCer – corpus cerebelli

Cer – cerebellum

Di – diencephalon

dnHy – hypothalamus difuse nucleus

dPTub – dorsal Posterior Tuberculum

drc – dorsorostral cluster

dTha – dorsal thalamus

ec - epiphyseal cluster

fomn - facial octavolateralis motor neurons

Ha - habenula

Hy – hypothalamus

LC – locus coeruleus

LR - lateral rectus muscle

Met – metencephalon

mlct – mediolongitudinal catecholaminergic tract

mlf - mediolongitudinal fasciculus

MO – medulla oblongata

Mye - myelenchepalon

nMlf – nucleus of the mlf

OB – olfactory bulb

oc – optic chiasm

OE - olfactory epithelium

OmNn – oculomotor nerve nucleus

P – pallium

pc - posterior comissure

Pi – pineal (epiphysis)

Po – preopctic region

poc – post-optic commissure

Pr – pretectum

Pro - prosencephalon

PTub – posterior tuberculum

RGC - retinal ganglion cells

Rho – rhombencephalon

SRa – superior raphe

SubP – sub-pallium

Teg – tegmentum

Tel – telencephalon

TeO – optic tectum

TeOmb – medial tectal band

TeOn – TeO neuropil

TeOsp – TeO stratum periventriculare

TG - trigeminal ganglion

TH - tyrosine hydroxylase

Tha - thalamus

Tpoc – tract of the postopctic comissure

tronuc – trochlear nucleus

Ts – torus semicircularis (Teg)

VagR – vagal region

VagusMN – vagus motor neurons

vcc - ventrocaudal cluster (nucMLF)

vCer – valvula cerebelli

vpni - velocity to position neural integrator

vPTub – ventral Posterior Tuberculum

vrc - ventrorostral cluster

vsm - velocity storage mechanism

vTha – ventral thalamus

1 INTRODUCTION

1.1 DEVELOPMENT OF THE ZEBRAFISH NERVOUS SYSTEM

One of the milestones of the development of the nervous system is the specification of a remarkable variety and number of neuronal and glial cells that, when appropriately connected, constitute the neuronal circuits responsible for all innate and non/innate behaviours. This cell type variety affects the expression of specific neurotransmitters and their receptors, synaptic modulators or presynaptic and post/synaptic connectivity patterns. From the specification of the neural progenitors to the formation of neuronal circuits, a large number of processes are highly coordinated in time and space, such as neural induction, regionalization, neurogenesis, migration, differentiation and the establishment of synaptic connections.

The basic steps and the molecular and cellular processes involved in the development of the nervous system are broadly conserved among vertebrates from amphibians and fish to birds and mammals and thus different vertebrate models have been selected for their study. The zebrafish has been used as a model system for developmental studies since the 80s because of the large clutches of over 100 embryos per mating pair, their very rapid and external development and the transparency of the embryos/larva. In addition, all the genetic and molecular tools and the wide variety of stable and reproducible transgenic lines available today makes it a great vertebrate model for developmental research purposes [1].

In zebrafish development, gastrulation starts at 5 hpf (hours post fertilization) and extends to 10 hpf, followed by the segmentation period (until 24 hpf), in which the paraxial mesoderm segments into somites (12 hpf) [2].During gastrulation, the three germ layers (endoderm, mesoderm and ectoderm) are specified along the vegetal-to-animal axis. Within the ectoderm, neural tissue is induced under the influence of a cell population known as the organizer (designated the Spemann organizer in the frog, Hensen's node in the chick or the node in mammals) [3].

Cells of the future neural plate converge to the midline and expand along the rostro-caudal axis (RC) by convergence and extension movements. Then neurulation occurs, the neural plate gives rise to a neural rod primordium that is rearranged and cavitates to originate the neural tube, contrasting with most vertebrates where the central lumen is formed by the folding of the epithelial sheet [2,3]. Shortly before the central cavity of the neural tube appears, several constrictions subdivide the neural structures into different segments along the RC axis. At 16 hpf, the first 3 swellings become prominent, giving rise to the two subdivisions of the prosencephalon (forebrain), telencephalon and diencephalon, and to the mesencephalon (midbrain). Later, at 18 hpf, 7 rhombomeres from the rhombencephalon (hindbrain) are already clearly visible and the primordium of the cerebellum starts to appear dorsally prominent in the region of the rhombomere 1, adjacent to hindbrain-midbrain boundary. Towards the end of segmentation, the diencephalon starts to expand, forming the primordial hypothalamus ventrally and the primordial epiphysis dorsally. At this stage the primordium of the central nervous system is already well delineated [2,3].

From the early stages of neural development, regionalization occurs along the rostro-caudal (from the prosencephalon to the spinal cord) and dorso-ventral (from the roof plate to floor plate) axes. This is important for both the spatio-temporal control of cell cycle exit and differentiation (which is regulated by Delta/Notch signalling) and for the acquisition of specific cells fates that allows the differentiation of many neuronal subtypes and glial cells. Regionalization is a progressive process in which the control of gene expression at the mRNA or posttranscriptional levels leads to the activation

of specific genetic programs, which differ depending on the location of the cells within the embryo. The organizer is involved in the early rostro-caudal regionalization of the neural tube, but the subsequent regionalization of the neural tube requires several secondary organizing centres acting through paracrine factor gradients, both along the rostro-caudal and the dorsal-ventral axis. The developing neural progenitors receive their identities by being exposed to these gradients: varying concentrations of different types of factors cause the transcription and activation of distinct transcription factors in the cells nuclei, depending on their position in the neural tube [3,4]. Subsequently, neurons are subject to additional extracellular and cell-cell communication signalling at different points in their differentiation program that will further refine their identities.

The patterned neural plate serves as a basis for the organization of the primary neuronal scaffold, the first circuits formed, required for the initial embryonic and larval behaviours. This primary neuronal scaffold (Figure 1.1A) is composed of large new-born neurons derived from the earliest clusters of neuronal progenitors, the proneural clusters, in a process known as primary neurogenesis. Axonogenesis from this early neurons starts between 14 and 24 hpf, giving rise to long extended axons. The main and most clear tract is the medial longitudinal fasciculus (mlf) located ventrally in the medial line from the midbrain, across the hindbrain to the spinal cord. It contains ipsilateral and contralateral axons of midbrain and hindbrain neurons projecting into the spinal cord [3].

At the 1 day post fertilization (dpf) stage, the embryonic brain is composed of several nuclei arranged in a number of clusters. From rostral to caudal, in the prosencephalon the dorso-rostral (drc), ventro-rostral (vrc) clusters and the more dorsal epiphyseal cluster (ec) and nucleus of the posterior commissure (npc) are already observed. Longitudinal and transverse axons extend between them, connecting the clusters with each other, with the spinal cord and with the peripheral nervous system (PNS). Caudally, there are the reticulospinal neurons extending to the spinal cord that are long projecting interneurons whose cells bodies are localized in the basal midbrain (mesencephalon), where the nucleus of the mlf (nMlf) is located (originated from the ventro-caudal cluster, vcc), or in the center of each rhombomere within the hindbrain (rhombencephalon). [3] (Figure 1.1.A)

Once neurons are post mitotic, they continue their process of acquisition of specific neuronal phenotypes through the action of multiple cues and concurrent signalling pathways. Electrical activity and environmental cues seem to have a remarkable role on developmental plasticity. These environmental cues include variations in temperature, availability of nutrients, water or illumination or even some behaviours and social experience. During brain and spinal cord development spontaneous electrical activity arises in embryonic neurons, prior or during synaptogenesis. Many studies have shown the versatile and important role of this early electrical activity not only on the refinement of prepatterned circuits but also on whole nervous system development, from neurogenesis and neuronal proliferation to axonal guidance and synaptogenesis [5].

Secondary neurogenesis occurs during the embryonic and larval stages, partly overlapping, in time and space, with primary neurogenesis, building up on the primary scaffold by increasing the number of neurons in each neuronal category. Although some secondary neurogenesis starts before 2 dpf (pharyngula period: 1-2 dpf), we can state that massive overall secondary neurogenesis starts at 2 dpf. This process gives rise to the precursors of main anatomic structures within the initial primordial regions such as thalamic and hypothalamic regions from the diencephalon, developing tectum and the more ventral tegmentum from the mesencephalon, the cerebellum corpus and valvula cerebelli from the metencephalon and medulla oblongata from myelencephalon.[3,6] (Fig1.1B)

In the 2 dpf zebrafish brain, the three phases of neuronal development are represented and organized in layers: ventricular (proliferative), medial (recently determined neuronal cells) and peripheral (differentiated). From 3 dpf on, differentiation increases and there is a decreasing in proliferation although it is still observed at 5 dpf. This difference of the 2 dpf brain compared to later

stages may be due to the rates of both proliferation and cellular migration dynamics being much higher at this time period [6].

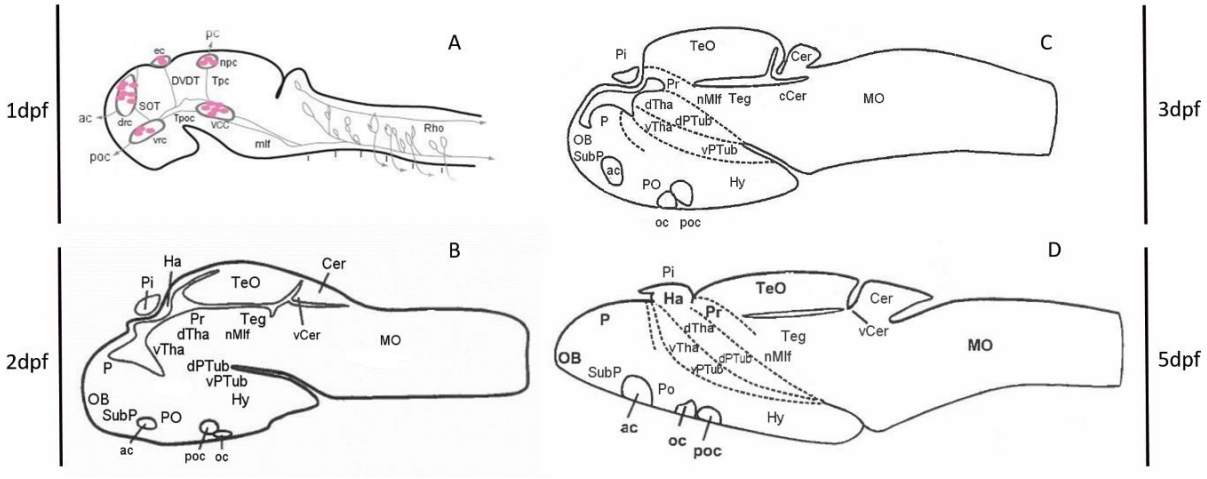

Fig. 1-1 Anatomic reference 1, 2, 3 and 5 dpf brains. Schematic representation of the major anatomical regions during development in four reference brains at stages 1 (A), 2 (B), 3 (C) and 5 dpf (D), adapted from Rubenstein and Rakic, 2013 (A) and Mueller and Wullimann, 2015 (B, C and D).

During the hatching period (48-72 hpf) larvae continue to grow in size at the same rate as earlier but morphogenesis of the organ rudiments slows down [2]. Since the hatching day, zebrafish shows a canonical vertebrate morphology, and within the first week its compact nervous system is already capable of performing a variety of visually guided actions and innate behaviours, which appear at 3 dpf when retinal ganglion cell axons reach their central targets. These largely innate and reflexive stereotyped behaviors include swimming in three dimensions, various escape manoeuvres, visually guided hunting, sleeping and even learning. [1,7]

1.2 DEVELOPMENT OF THE VISUAL SYSTEM IN ZEBRAFISH

In zebrafish, visual stimuli are initially processed by the retina, which contains a diversity of cell types and relays neuronal signals to the brain via 10 retinal ganglion cell arborization fields (AB), including the optic tectum, the largest of the AB [8]. Further, signal processing and integration of the information from these arborization fields involves other parts of the brain such as the pretectal area before an output is conveyed to the parts executing the visually guided behaviours.

The development of the zebrafish retina starts with the specification of the eye field, a region between the telencephalon and the diencephalon at 10 hpf that later forms the optic cup. At 11.5 hpf the optic lobes become increasingly evident as prominent thickenings of the anterior neural keel. At 13 hpf, the posterior part of the optic lobes starts to separate from the brain and as its morphogenesis advances, there is a turnaround in their AP axis, after which the ventral surface is facing the brain and the dorsal part is facing the exterior. Cells forming the outside surface will differentiate into the neural retina and the medial layer becomes thinner and subsequently differentiates as the retinal pigmented epithelium. In the next hours, both the invagination and the thickening become increasingly more prominent, transforming the optic lobe into the optic cup. [9,10]

Retinal ganglion cell (RGC) precursors are the first to become post-mitotic in a small patch of ventrally located cells, nasal to the optic nerve, between 27-28 hpf. At 34-36 hpf, the first retinal axons leave the eye through the optic stalk, invading the tectum at 46-48hpf and beginning to arborize in the tectum approximately at 60hpf. At 60 hpf, neurogenesis in the central retina is almost completed and 6

classes of neurons, stratified in layers, are identified: ganglion, amacrine, bipolar, horizontal, interplexiform, and photoreceptor cells (Fig1.2). At 72 hpf, retinal ganglion cell axons reach, cover and innervate the tectal neuropil. [10]

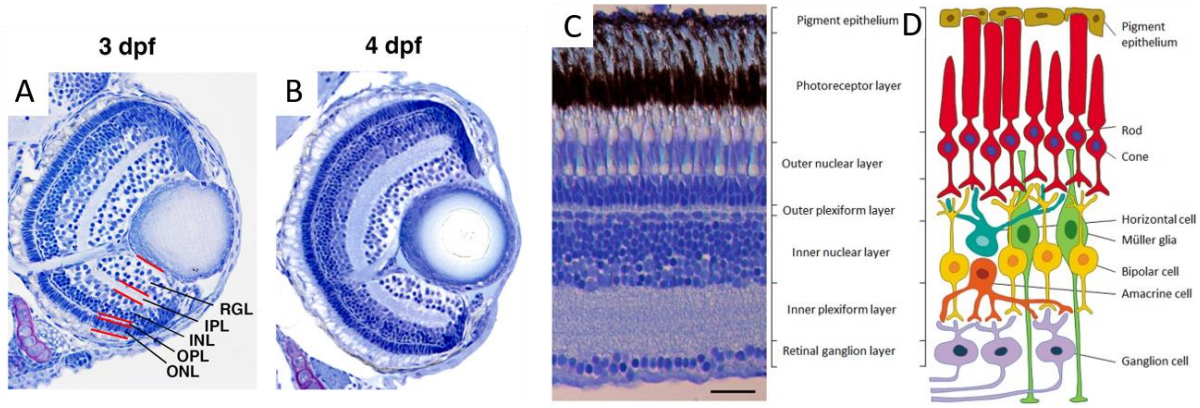

Fig. 1-2 – The retina's neural stratification. A,B: histological sections of the 3 and 4 dpf zebrafish retina, adapted from Okinawa Institute of Science and Technology Graduate University original photos. C: Microphotograph of a cross-section through the retina of an adult zebrafish, showing the different retinal layers; D: Diagram of the neural circuit of the retina, showing the six neuronal cell types and the two supporting cell types (Müller glia and retinal pigmented epithelium); C and D adapted from Gramage 2014S. Scale bar = 25µm. RGL: retinal ganglion layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer.

Within the tectum, RGC will connect to tectal neurons. Tectal neuron-dendrite growth and synaptogenesis begin around 72hpf continuing through approximately 7dpf. Although most of the tectal dendrites have a minimal number of synapses, at 7hpf half of the neurons already display responses similar to those of the circuits of the developed tectum. From 78 hpf to 7dpf topographic map and visual field sizes undergo very little refinement.[9] The connections between the topographic map of the visual field, present in the retina, and the tectum are assembled through retinal axon pathfinding, tectal cell dendrite extensions, and appropriate synapse formation. An initial arrangement of the map is thought to be due to chemical gradients that specify axonal arborization. The refinement of this initial map is characterized by the dynamics of the axon and dendrite arborization, with constant extension and retraction or elimination of nascent synapses, due to patterned synaptic activity and/or plasticity.

Note that, although there is a lot of information regarding retinal development there is still a lot do discover about the anatomical and functional development of the different structures and populations involved in visual processing within the zebrafish brain, as well as about the circuits themselves.

1.3 VISUALLY GUIDED BEHAVIOURS AND UNDERLYING CIRCUITS IN ZEBRAFISH

In the larval zebrafish, the first response properties (selectivity for motion/stationary and direction of stimuli) appear early in tectal development, at 72hpf, when axons of retinal ganglion cell have just covered and innervated the tectal neuropil. At this stage, zebrafish can already produce startle response and track eye movements, while visually guided hunting is only performed at 5 [9]. In earlier stages, vision is crucial for survival: feeding, recognition, avoiding predators and moving around their surroundings. Larvae show phototaxis by adjusting their swimming behaviour depending on temporal and spatial cues of light variation. Within these actions, we can specify two of the most studied responses: the optomotor response (OMR) and the optokinetic response (OKR). Fish respond to whole field motion pattern, which indicates the fish is moving relatively to fixed landmarks, by following the movement and stabilizing their position in the moving water (OMR). On the other hand, for continuous rotating stimuli fish adjust the direction of their eyes, alternating between slow eye movements to rapid saccades, which is called OKR. [1]

In recent years, efforts have been made to understand how the processing of sensorial inputs occurs in zebrafish. Based on the advances of imaging and the use of Genetically Encoded Calcium Indicators (GECI), it is now possible to have a global whole brain view of nervous system activity and function [1]. These activity maps are more informative when they intersect with precise anatomical information to identify an underlying circuit. Schematic models for the circuitry underlying optomotor response and the sensorimotor processing have been proposed [8,11].

Both OMR and OKR require detection of motion and directionality. This is partly achieved within the retina involving direction selective retinal ganglion cells but also in their projections to the contralateral arborization fields (ABs), where they contact neurons from the pretectal area.

On the matter of 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 (via internuclear neurons) to the contralateral oculomotor nucleus (OMN) to drive contraversive movements of the stimulated eye via the medial rectus muscle (MR).[11] (Fig. 1.3)

Regarding sensorimotor processing (OMR), information from the pretectal area may be relayed to the nucleus of the medio-longitudinal fasciculus (nMlf). Both visual and olfactory information are integrated on the nMlf that sends direct motor response to the spinal cord through descending glutamatergic inputs. Significant catecholaminergic and serotonergic projections are known to surround the nMlf that can provide a source of neuromodulation.[8] (Fig. 1.3)

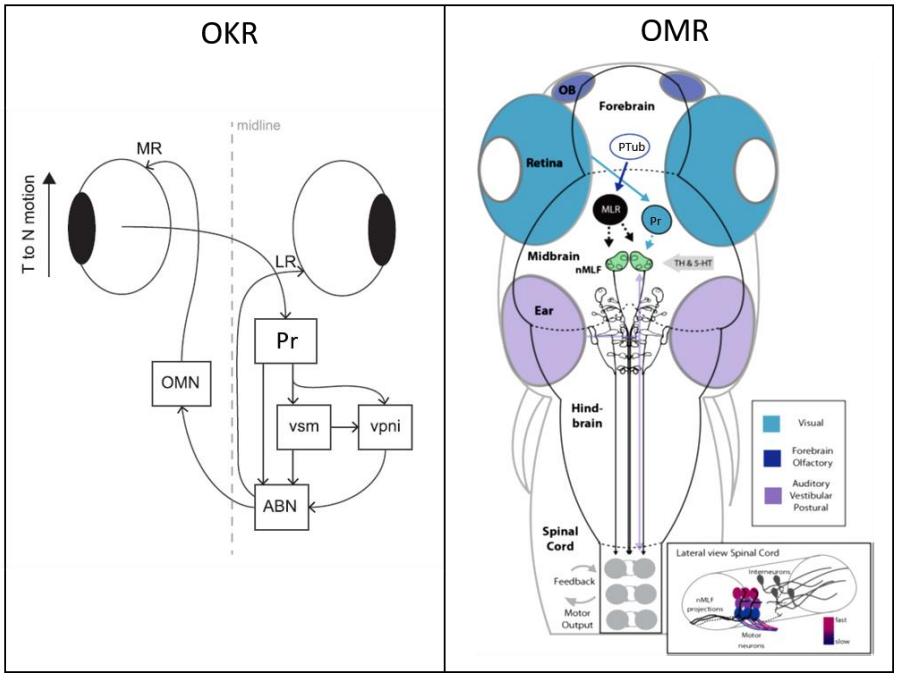

Fig. 1-3 Schematic model for the OKR and OMR circuits. Adapted from Portugues et al., 2014 and Severi et al., 2014, respectively. Abbreviations: 5-HT, serotonin; MLR, mesencephalic locomotor region; OB, olfactory bulb; Pr, pretectum; PTub, posterior tuberculum; TH, tyrosine hydroxylase.

It is worth mentioning that despite the knowledge of the circuitry underlying these behaviours, the development of its constituents downstream of the retina are still largely unknown.

1.4 *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. Interestingly, in combination with GECI, these latter can be used to assess the neuronal response to specific stimuli.

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, providing access to gene expression patterns in 70% of the larval brain and thus to numerous neuronal subpopulations that can be mapped to specific brain locations.

Mapping of neuronal populations traditionally relies on the simultaneous detection of cell types used as landmarks in anatomical annotation such as those identified by expression of neurotransmitters like GABA [12] or by expression of the tyrosine hydroxylase (TH), characteristic of the catecholaminergic neurons, already present at early stages [13,14]. However, this only provides partial information on the location of cells. Major efforts have been made recently to develop methods allowing a more comprehensive anatomical characterization of specific subpopulations in the 6 dpf zebrafish brain [7,15,16]. They involve the detection of a widely expressed protein (i.e. tERK) and the development of suitable algorithms that allow for the registration of individual 3D brain images into a reference brain. As a result, two atlases have been generated 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: Z-brain and ZBB[7,15].

However, although this constitutes a powerful and broadly accessible tool for the understanding of neuronal circuits for the 6 dpf, a similar characterization of zebrafish younger stages (1-5dpf) is still needed. Some data for the earlier developmental stages are available, including the major neuroanatomical expression atlas of important genetic and immunohistochemical markers in the early zebrafish [15], which offers an enormous quantity of 2-D visual data on gene expression patterns but is also compared with schematic anatomic reference brains from the 2nd to the 5th dpf. (Fig1.1B, C, D). In addition, 3-D maps of 2-4 dpf stages are included in the Virtual Brain Explorer (ViBE-Z) [17], but this has not been further developed.

For the current project, we are collaborating with M. Orger laboratory in the characterization of the expression of several GFF lines recently generated, during embryonic and early larval development. These lines, in combination with the UAS-GCaMP6, are currently being examined in M. Orger 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 characterization of the expression in development of both reporter lines and proteins, in particular related to circuits involved in visual processing, 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.

1.5 AIMS OF THIS THESIS

We aim to characterize neuronal subpopulations 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:

1. Examine GFP expression in zebrafish embryos and larvae from four transgenic reporter lines from the 1st to the 6th dpf, using immunohistochemistry and confocal imaging.
2. 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.

2 MATERIALS AND METHODS

2.1 ANIMALS AND TRANSGENIC LINES

The zebrafish used were maintained by the vivarium platform at Champalimaud Research, where all aspects of fish housing essential to animal welfare, including centralized life-support system maintenance, tank cleaning and disinfection, maintenance and optimization of water quality, feeding, live feed production and nursery care are provided. The fish are maintained at the facility at 25°C, 50%-60% humidity, 14h:10h light:dark cycle with 200-300lux ambient light intensity in 3.5 l tanks containing about with 35 fish per tank and fed on rotifers, artemia and dry food daily. Fish water pH, salinity and dissolved gases are kept in physiological conditions [18].

In order to further investigate the acquisition of neuronal cell fates we used several reporter lines that show expression in subpopulations of neurons that are under study in Michael Orger's lab for their involvement on visual stimulus processing. The three GFF lines have been generated in M. Orger´s laboratory and the other was previously generated in *Wolman et al.,* 2008 [19]. The lines examined have different DNA constructs including GFP or GFF (a Gal4 derivative) inserted in their genome:

- Pitx2c:GFP: previously generated [19] using an internal promoter of the pitx2 gene that specifically produces the pitx2c isoform together with the enhanced GFP (eGFP).
- Tg BAC(ChAT:GFF;UAS:GFP): previously generated in Mike Orger's laboratory (Renninger and Orger, unpublished) by transposon-mediated BAC (bacterial artificial chromosome) transgenesis. It harbours an insertion of a BAC containing the choline acetyl transferase genomic region with GFF cloned at the ChAT starting ATG.
- Tg BAC(Gad1b:GFF;UAS:GFP): previously generated in Mike Orger's laboratory (Renninger and Orger, unpublished) by transposon-mediated BAC transgenesis. It harbours an insertion of a BAC that includes the genomic region of Gad1b, which encodes the glutamate decarboxylase 1, with GFF cloned at the Gad1b starting ATG.
- Tg BAC(Slc18a3b:GFF;UAS:GFP): previously generated in Mike Orger's laboratory by transposon-mediated BAC transgenesis. It harbours an insertion of a BAC containing the solute carrier family 18 gene with GFF cloned at the Slc18a3b ATG.

The GFF lines were maintained in combination with UAS-GFP for their visualization [20], in other words, they were crossed with a UAS GFP line for the egg collection. Since zebra fish are photoperiodic in their breeding they produce embryos every morning after "sunrise", therefore breeding males and females were put together in the same tank but separated by plastic barrier a few hours before the light period end, the day before the embryos are needed. The following day at the beginning of the light period the barrier was removed to allow fertilization and egg collection in the following hour. The embryos collected were then staged in hours after fertilization[18]. For our experiments we collected eggs in the range of 24 hours to 6 days post fertilization (hpf and dpf respectively) but examination of the embryos also assisted in the staging (comparisons between them and with reference brains).

After being collected in petri dishes embryos/larvae were raised at 28°C in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH 7.2 RT), under 14/10 hours light/dark cycle. At 1 dpf, PTU (1-phenyl 2-thiourea) 1x was added every day to prevent the development of pigmentation.

2.2 *SAMPLE PREPARATION AND IMMUNOHISTOCHEMISTRY*

The protocols used are modifications of those described in *Inoue et al.*, 2011; *Turner et al.*, 2014; *Randlett et al.*, 2015.[7,21,22]

Embryos and larvae, from 1 dpf to 6 dpf, were firstly screened for GFP expression of the reporter transgenes under a fluorescence dissecting microscope and the best and brightest specimens were selected and sacrificed with tricaine1.6 mg/ml. Then, they were fixed in 4% paraformaldehyde (PFA) during two hours at room temperature and washed in PBS with 0.25% Triton (PBT). As a fraction of the fluorescence of the transgenic lines is eliminated by fixation, samples were then processed directly for whole mount immunohistochemistry.

Whole larvae were subjected to epitope retrieval by treatment with Tris HCl 150 mM pH9 at 70 °C for 15 min and then permeabilized in 0.05% Trypsin-EDTA for 5 min on ice. After blocking in blocking buffer (PBT + 1% bovine serum albumin (BSA) + 2% normal goat serum (NGS) + 1% dimethyl sulfoxide (DMSO)), samples were incubated with primary antibody recognizing GFP (Rabbit or mouse) from the collection of antibodies available in the laboratory known to label specific cell populations in the 6 dpf larvae, together with a pan-neuronal antibody, tERK (mouse) or in some cases anti-TH (Rabbit). Both anti-tERK and anti-TH were used together with anti-GFP to have as reference the position of the all brain or some landmarks, as they label all the neural cells and the catecholaminergic system, respectively. Then, samples were washed in PBT and incubated with the appropriate secondary antibody coupled to fluorescent dyes like Alexa 488 (combined with anti-GFP) and 568 (combined with anti-tERK and anti-TH). All antibodies were diluted in 1/500 µL in PBT + 1% BSA + 1% DMSO, with the exception of the anti-TH (1/100 µL). (table2.1)

Table 2-1- Primary and secondary antibodies used and respective manufacturer's information and dilutions.

Antigen	**Manufacturer reference**	**Host / Isotype**	**RRID**	**Dilution (µL)**
GFP-Tag polyclonal antibody	Thermofisher: A-6455	Rabbit/ IgG	AB_221570	1/500
GFP-Tag monoclonal antibody (3E6)	ThermoFisher: A-11120	Mouse/ IgG2a	AB_221568	1/500
Anti-Tyrosine Hydroxylase polyclonal Antibody (TH)	Merckmillipore: AB152	Rabbit	-	1/100
Anti-tERK antibody, p44/42 MAPK (Erk1/2)	Cell signalling: 4696	Mouse IgG1	-	1/500
Anti-Rabbit IgG (H+L) polyclonal 2^{ary} Antibody, Alexa Fluor 488	ThermoFisher: AB_143165	Goat / IgG	AB_143165	1/500
Anti-Rabbit IgG (H+L) polyclonal 2^{ary} Antibody, Alexa Fluor 568	ThermoFisher: A-11011	Goat / IgG	AB_143157	1/500
Anti-Mouse IgG (H+L) polyclonal 2^{ary} Antibody, Alexa Fluor 488	ThermoFisher: A-11001	Goat / IgG	AB_2534069	1/500
Anti-Mouse IgG (H+L) polyclonal 2^{ary} Antibody, Alexa Fluor 568	ThermoFisher: A-11004	Goat / IgG	AB_2534072	1/500

After immunohistochemistry, samples were mounted on low-melting agarose (1.5% in PBS) directly on the slide, positioned the straightest way possible with their dorsal side closest to the coverslip with help of forceps and a plastic device built for this purpose. After being surrounded by a well of grease filled with PBS, the coverslip was added and the sample was ready to be imaged. [22]

2.3 CONFOCAL MICROSCOPY AND IMAGE ANALYSIS

For imaging the stained tissues we used an upright confocal laser point-scanning microscope, Zeiss LSM 710, a 25x multi- immersion objective (N.A. 0.8) in combination with the Argon Multi-line: 458 nm and DPSS: 561 nm lasers. The preparation was put on the microscope stage, fixed with modelling clay and a drop of glycerol was used as immersion medium. Using the ZEN 2010 software, focal planes were selected and the acquisition parameters (digital gain, offset and power) optimized for proper laser penetration and exposition. The whole brain was imaged at a voxel size of 1 × 1 × 2 μm (x × y × z) in a stack format. To cover the entire brain, two adjacent tiles were acquired and then stitched together.

All the image analysis was done with the open source software Fiji, where firstly the two tiles were stitched and the raw all-stack generated. To anatomically characterize GFP expression, the different samples from the different lines and stages were compared with anatomical reference brains and stacks and images from previous bibliography together with Z-brain [7] and zbb [15] atlases. Note that for the anatomical annotation on the 2 to the 5th dpf, and 6 dpf, Mueller and Wullimann 2015 and the zbb atlas [15] served as main references, respectively. Orthogonal views and 3D projections were also used to identify the anatomical regions through comparison. For presentation purposes the stacks of the chosen samples where made into z-projections, where the anatomical parts expressing GFP where clearly identified.

3 RESULTS

In order to characterize neuronal subpopulations that may be involved in visual processing and motor behaviour in the zebrafish larva throughout development, we selected three GFF lines generated in M. Orger laboratory :ChAT:GFF, Gad1b:GFF, Slc18a3b: GFF [S. Renninger, R. Tomas and M. Orger, unpublished]. These GFF expressing lines are currently being examined in the laboratory to identify neurons that are specifically active when the larvae are exposed to visual stimuli and/or performing motor behaviours. In addition, we selected a zebrafish line that has previously been described for the expression of GFP in a population of neurons involved in motor behaviour (Pitx2c:GFP).

We performed immunohistochemistry on larvae from 1st to the 6th dpf followed by confocal imaging and analysis. For immunostaining, anti-GFP antibodies were used in combination with either anti-tERK or anti-TH antibodies. The patterns obtained with the anti-tERK and anti-TH were used as reference for the identification of brain landmarks, as they reveal either all neurons or catecholaminergic neurons, respectively.

For each line and each developmental stage, the best representative samples are presented in the figures below as z-planes and z-maximum projections obtained from the original z-stacks (in Supplementary data). For the anatomical description of GFP expression (and TH expression when applicable), we follow a rostral to caudal order and separated structures from either the central (including the retina) or peripheral nervous system. The tentative assignment of the different structures is done by comparison of the original data with reference brains and gene and protein expression patterns reported in the literature and should be considered as an orientation for future work. Note that for the unknown anatomical structures, a name was created with the conjunction of the brain subdivision and a number given from rostral to caudal and dorsal to ventral (for ex. Rho1 – rhombencephalic cluster 1).

At the end of each descriptive section, the results are summarized as follows: (1) In a table: The GFP expression pattern for each line is summarized in a table of expression, where it is matched with anatomical terms and abbreviations. (2) In a written summary: A written subsection provides a general developmental overview focusing on the structures known to be involved in the OKR and OMR (in **bold**) and highlighting their most prominent features.

3.1 PITX2C: GFP

This transgenic line genome has GFP associated with the pitx2 gene, which encodes a homeodomain protein that plays a role in the terminal differentiation of cell phenotypes and involved in the development of the eye. In addition, expression of GFP in the nMlf and mlf, important in visually evoked motor behaviour, has been previously described in the 1st dpf stage [19].

3.1.1 1dpf

At 1 dpf, within the prosencephalon, the ventro-caudal cluster (vrc) and tract of the post-optic commissure, which connects the vrc to the ventro-caudal cluster (vcc), present GFP expression.

In the mesencephalon, there is a clear pattern of expression in the vcc and the caudal most part (the developing nMlf) that extends to the fasciculus itself.

Peripherally, on both sides, strong GFP expression is observed in the trigeminal ganglia (TG), which extend thick downward projections and thinner projections to the rostral-most part of the head.(Fig. 3.1.A_1)

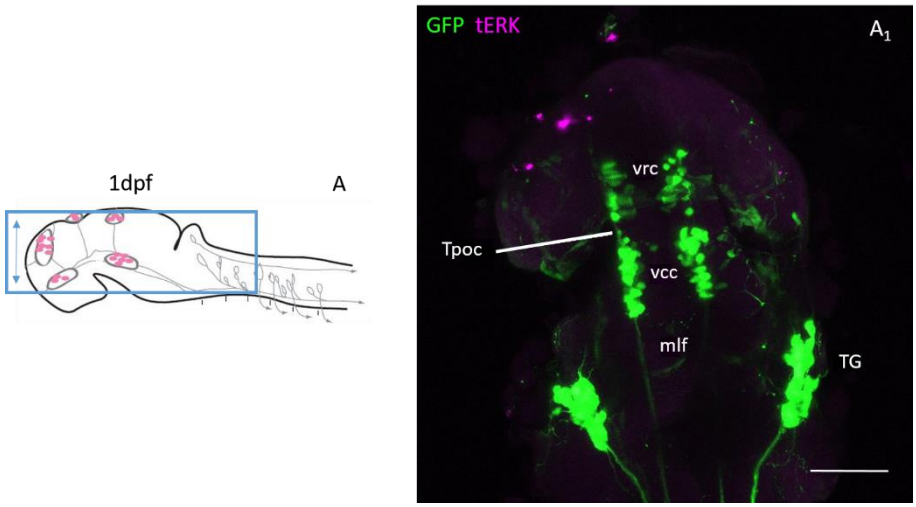

Fig. 3-1- GFP expression in 1 dpf Pitx2c: GFP zebrafish brain. A, sketch of 1dpf brain area shown in A_1, adapted from Rubenstein 2017. A_1, z-projection of Pitx2c: GFP 1dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bar indicates approximately 100 μm. Abbreviations: mlf, medio-longitudinal fasciculus; TG, trigeminal ganglia; Tpoc, tract of the post-optic commissure; vrc, ventrocaudal cluster; vrc, ventrorostral cluster.

3.1.2 2dpf

Within the diencephalon, strong GFP expression is observed in the ventral thalamus (vTha) (Fig. 3.2.B_3).

In the mesencephalon, GFP expression is observed dorsally in the developing optic tectum, in some disperse cells in the stratum periventriculare (TeOsp) and in the more ventral tegmentum (Fig. 3.2.$B_{1, 2}$).

GFP expression is observed in the mlf from the mesencephalon through the hindbrain, where it is almost absent (Fig. 3.2.B_{1-2}).

At the 2dpf stage a strong expression is observed in the peripheral trigeminal ganglia (TG) (Fig. 3.2.B2).

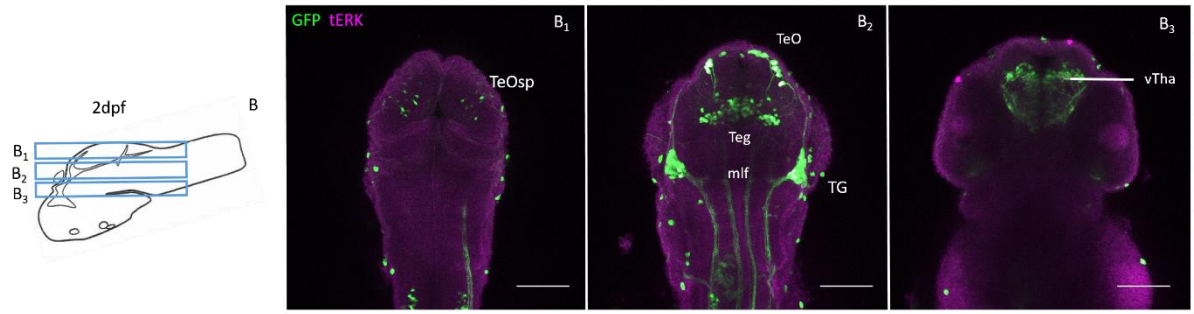

Fig. 3-2- GFP expression in 2 dpf Pitx2c: GFP zebrafish brain. B, sketch of 2dpf brain area shown in $B_{1\text{-}3}$. $B_{1\text{-}3}$, z-projection of Pitx2c: GFP 2dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 µm. Abbreviations: mlf; medio-longitudinal fasciculus; Teg, tegmentum; TeO, optic tectum; TeOsp, optic tectum stratum periventriculare; vTha, ventral thalamus.

3.1.3 3dpf

At the 3dpf stage, GFP expression is observed in the telencephalic pallium (P) and sub-pallium (SubP) (Fig. 3.3.$C_{3\text{-}4}$).

Within the diencephalon, a distinct GFP expression pattern is visible in the two radial groups of pretectal neurons, in the dorsal thalamus (dTha), posterior tuberculum (PTub) and the ventral posterior tuberculum (vPTub) (Fig. 3.3.$C_{3,\,4}$)

Dorsally in the mesencephalon, GFP expression is localized in the posterior commissure (pc), optic tectum neuropil (TeOn) and some cells of the stratum periventriculare (TeOsp), while ventrally it is also observed in the arrow-shaped tegmentum (where the nMlf is located) and the mlf, which extends to the hindbrain. (Fig. 3.3.$C_{1\text{-}3}$)

Caudally, GFP expression is localized in the cerebellum (Cer), and in two rostral clear groups of cells and the stripes of developing vagus motor neurons (vagus MN) of the medulla oblongata.

Trigeminal ganglia and their projections still show a clear GFP expression pattern as in the previous stages, as shown in Fig. 3.3.$C_{3\text{-}4}$.

Note that for this sample the anti-TH antibody was used. In panels $C_{1\text{-}4}$, TH expression is observed in the olfactory bulb (OB), in some medial pretectal cells, locus coeruleus (LC) and in several neurons of the vagal region.

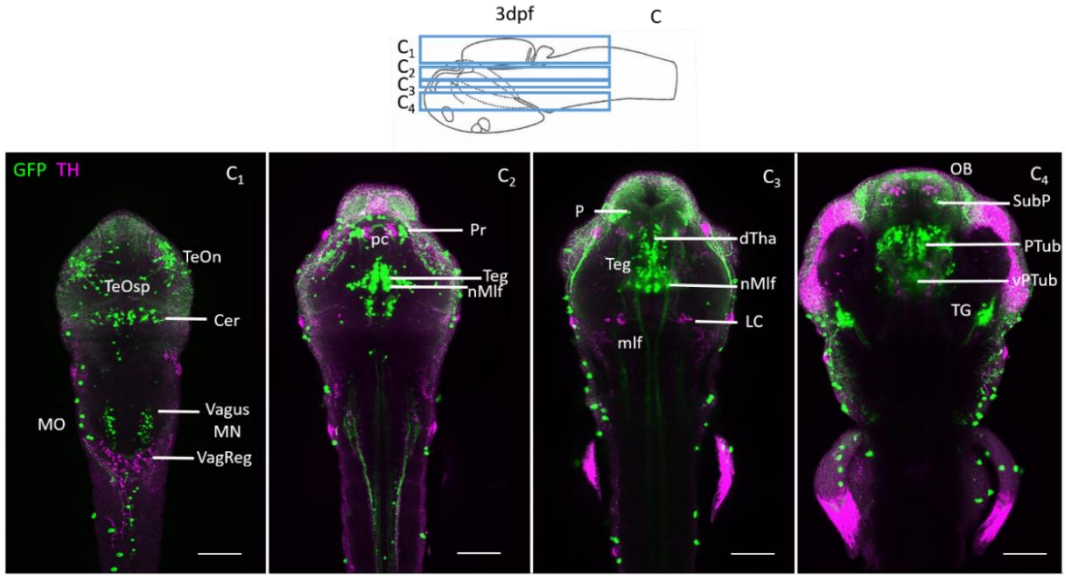

Fig. 3-3- GFP expression in 3 dpf Pitx2c: GFP zebrafish brain. C, sketch of 3dpf brain area shown in C_{1-4}. C_{1-4}, z-projection of Pitx2c: GFP 3dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-TH (magenta). The scale bars indicate approximately 100 μm. Abbreviations: Cer, cerebellum; dTha, dorsal thalamus; LC, locus coeruleus; mlf, medio-longitudinal fasciculus; MO, medulla oblongata; nMlf, nucleus of the mlf; OB, olfactory bulb; Teg, tegmentum; TeOsp, optic tectum stratum periventriculare; TeOn, optic tectum neuropil; P, pallium; pc, posterior commissure; Pr, pretectum; PTub, posterior tuberculum; SubP, sub-pallium; TG, trigeminal ganglia; Vagus MN, vagus motor neurons; Vag Reg, vagal region; vPTub, ventral posterior tuberculum.

3.1.4 4dpf

At 4 dpf, in the telencephalon, GFP expression is observed in the pallium (P) and sub-pallium (SubP), and the more ventral anterior commissure (ac) (Fig. 3.4.D_{2-4}).

Within the diencephalon, GFP expression is observed (from dorsal to ventral) in the posterior commissure (pc), some pretectal and thalamic cells (Pr and Tha), the posterior tuberculum (PTub), preoptic region (PO) and hypothalamus (Hy).

In the mesencephalon, tectal expression is observed in posterior commissure (pc) the neuropil (TeOn), some cells in the stratum periventriculare (TeOsp), the tegmentum (Teg, where is located the nMlf) and torus semicircularis (TS)(Fig. 3.4.D_{1-3}).

Caudally, there is an unclear GFP expression pattern through the medulla oblongata (MO) (Fig. 3.4.D_3).

Peripherally, trigeminal ganglia and their projections show a clear GFP expression (Fig. 3.4.D_{3-4}).

In this case the anti-TH antibody was used and its expression is observed through the olfactory bulb (OB), dopaminergic clusters within the hypothalamus (Hy) and many neurons of the vagal region.

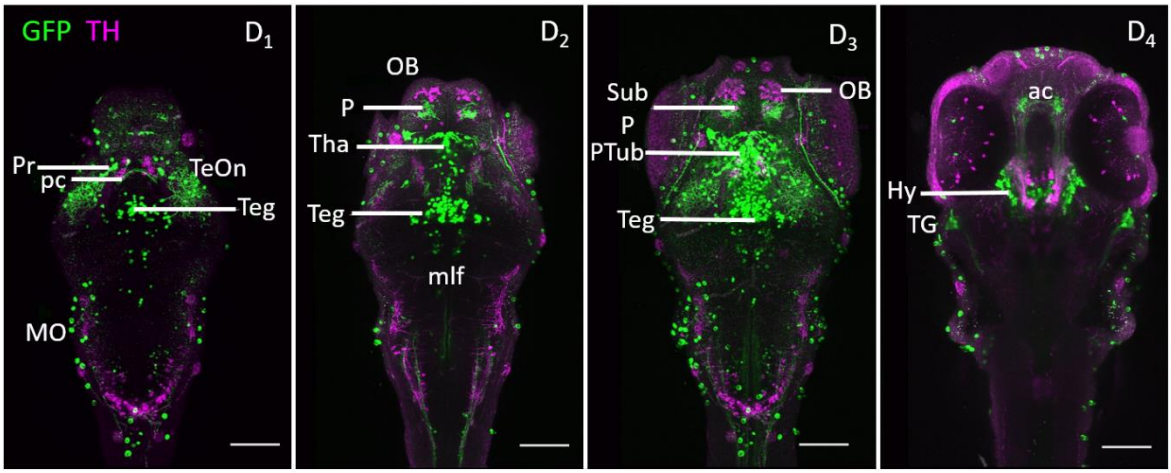

Fig. 3-4- GFP expression in 4 dpf Pitx2c: GFP zebrafish brain. D, sketch of 4dpf brain area shown in D1-4. D1-4, z-projection of Pitx2c: GFP 4dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-TH (magenta). The scale bars indicate approximately 100 μm. Abbreviations: ac, anterior commissure; Cer, cerebellum; Hy, hypothalamus; LC, locus coeruleus; mlf, medio-longitudinal fasciculus; MO, medulla oblongata; nMlf, nucleus of the mlf; OB, olfactory bulb; P, pallium; Teg, tegmentum; TeOn, optic tectum neuropil; Tha, thalamus; P, pallium; pc, posterior commissure; Pr, pretectum; PTub, posterior tuberculum; SubP, sub-pallium; TG, trigeminal ganglia.

3.1.5 5dpf

At 5dpf, there is some GFP expression in the pallium and sub pallial projections (Fig .3.5.E_{2-3}) that connect to the anterior commissure (Fig .3.5.D_4).

In the diencephalon, GFP expression is observed (from dorsal to ventral) in the lateral region of the pretectum (Pr) (Fig .3.5.E_2), dorsal thalamus (dTha), posterior tuberculum (PTub) and hypothalamus (Hy) (Fig .3.5.E_{3-4}).

In the mesencephalon, there is localized GFP expression in the posterior commissure (pc), optic tectum neuropil (TeOn) and in some neurons of the stratum periventriculare (TeOsp), while the more ventral tegmentum (Teg, where nMlf is located) is massively labelled with anti-GFP. (Fig .3.5.E_{1-3}). GFP expression can be also observed through the mlf.

Caudally, GFP expression can be observed in the cerebellum (Cer) and, within the medulla oblongata, in the anterior region and in the vagus motor neurons (vagusMN) (Fig .3.5.E_{2-3}).

In this case the anti-TH antibody was used and, as shown in panels $E_{1-4,}$ its expression can be observed in the olfactory bulb (OB), pretectal cells, dopaminergic clusters within the hypothalamus (Hy), vagal region, and mlct.

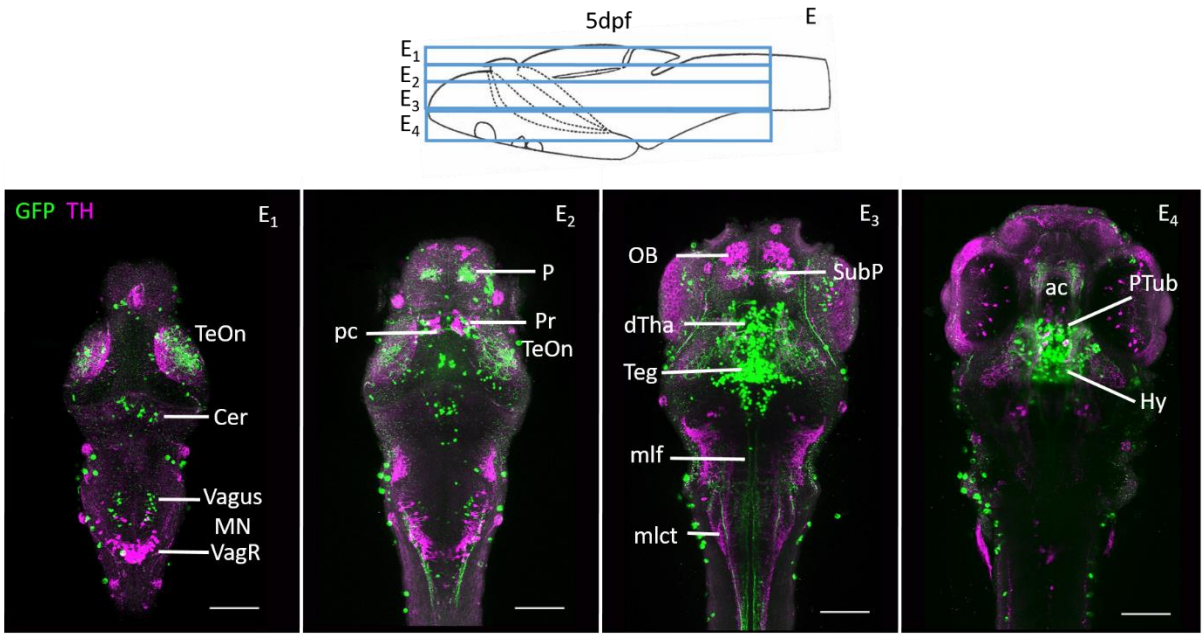

Fig. 3-5- GFP expression in 5 dpf Pitx2c: GFP zebrafish brain. E, sketch of 5dpf brain area shown in E_{1-4}. E_{1-4}, z-projection of Pitx2c: GFP 5dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-TH (magenta). The scale bars indicate approximately 100 µm. Abbreviations: ac, anterior commissure; Cer, cerebellum; dTha, dorsal thalamus; Hy, hypothalamus; LC, locus coeruleus; mlf, medio-longitudinal fasciculus; mlct, medio longitudinal cathecolaminergic tract; MO, medulla oblongata; nMlf, nucleus of the mlf; OB, olfactory bulb; P, pallium; pc, posterior commissure; Pr, pretectum; PTub, posterior tuberculum; SubP, sub pallium; Teg, tegmentum; TeOn, optic tectum neuropil; TG, trigeminal ganglia; Vagus MN, vagus motor neurons; Vag Reg, vagal region;

3.1.6 6dpf

At 6dpf, within the telencephalon GFP expression is observed in the pallium (P), sub-pallium (SubP) and the more ventral anterior commissure (ac). (Fig .3.6.F_{2-4})

In the diencephalon, two radial groups of pretectal cells (Pr), the thalamus (Tha), posterior tuberculum (PTub), and hypothalamic difuse nucleus (dnHy) show GFP expression.

In the mesencephalon, localized GFP expression is observed in the tectum neuropil (TeOn), in some cells of the stratum periventriculare (TeOsp) and in several distinct groups of the most ventral tegmentum (Teg): nucleus of the mlf, oculomotor nerve nucleus (OmNn) and trochlear nucleus (tronuc) (Fig .3.6.F_3).

Caudally, some cells in the cerebellum (Cer), anterior (superior raphe, SRa) and vagal regions of the medulla oblongata (OB) show GFP positive immunoreactivity (Fig .3.6.F_{1-2}).

In this stage (sample) the anti-TH was used instead of the anti-tERK, to visualize the olfactory bulb (OB), prectectal cells (Pr), dopaminergic clusters within the hypothalamus (Hy) and vagal region.

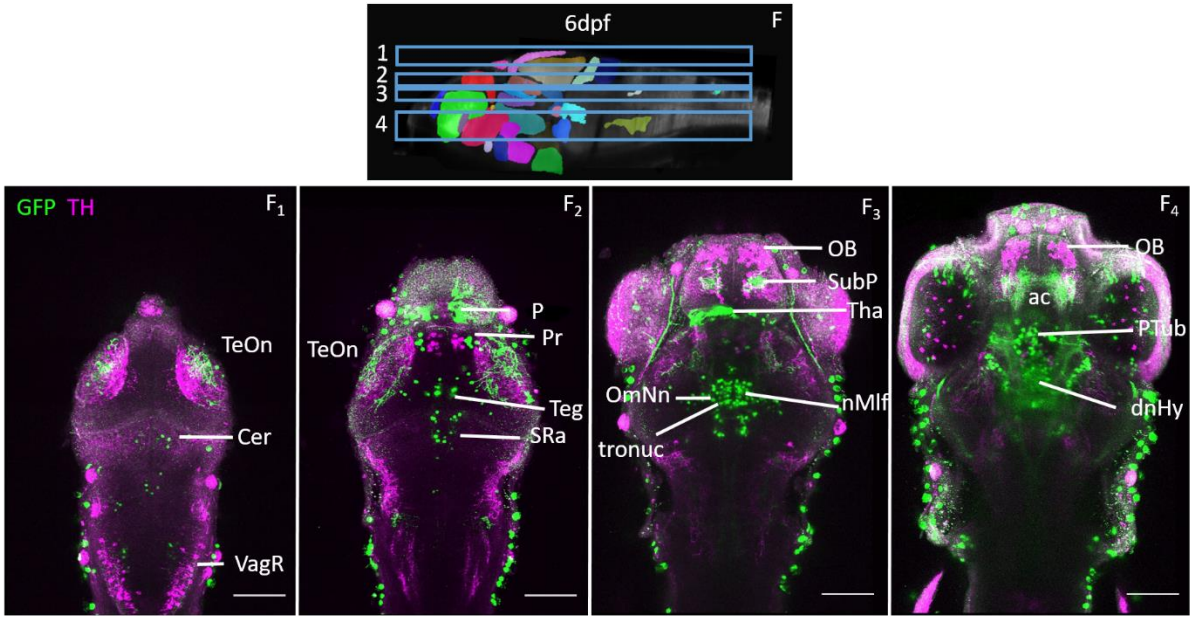

Fig. 3-6- GFP expression in 6 dpf Pitx2c: GFP zebrafish brain. F, sketch of 6dpf brain area shown in F_{1-4}. F_{1-4}, z-projection of Pitx2c: GFP 6dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-TH (magenta). The scale bars indicate approximately 100 μm. Abbreviations: ac, anterior commissure; Cer, cerebellum; dnHy, hypothalamus difuse nucleus; LC, locus coeruleus; mlf, medio-longitudinal fasciculus; mlct, medio longitudinal cathecolaminergic tract; MO, medulla oblongata; nMlf, nucleus of the mlf; OB, olfactory bulb; OmNn, oculomotor nerve nucleus; P, pallium; pc, posterior commissure; Pr, pretectum; PTub, posterior tuberculum; SubP, sub pallium; SRa, superior raphe; Teg, tegmentum; TeOn, optic tectum neuropil; Tha, Thalamus; TG, trigeminal ganglia; tronuc, trochlear nucleus; Vag R, vagal region;

3.1.7 Pitx2c:GFP: GFP expression summary

Table 3-1- Brain regions showing expression: summary of the GFP expression in the Pitx2c:GFP line providing a list of anatomical regions and its abbreviations, excluding the most primordial structures.

Brain subdivision	Expression	Abbreviations
Telencephalon	Pallium, sub-pallium and anterior commissure.	ac; SubP; SubP;
Diencephalon	Pretectum, thalamus, posterior tuberculum, preoptic region, hypothalamus.	Hy; PO; Pr; PTub; Tha;
Mesencephalon	Some neurons of the optic tectum neuropil and stratum periventriculare, posterior commissure, tegmentum (nucleus of the mlf, oculomotor nerve nucleus and trochlear nucleus)	nMlf; OmNn; pc; Teg; TeO; TeOn; TeOsp; tronuc;
Metencephalon	Cerebellum.	Cer;
Myelencephalon	Some neurons in the medulla oblongata in the superior raphe and the vagus motor neurons.	SRa; MO; VagusMn;
PNS	Trigeminal ganglia and its projections.	TG;
Retina	No specific pattern observed.	

3.1.8 Pitx2c: GFP overview

From early in development a characteristic GFP expression pattern is observed in the developing diencephalon (vrc) and mesencephalon (vcc) and mlf.

In the telencephalon, GFP expression is observed in the pallium and sub-pallium, while in the diencephalon, it is observed in radial **pretectal groups** and in the ventral-most diencephalic regions.

During development in the mesencephalon, GFP expression is observed in sparse tectal neurons and ventrally in a great area of the tegmentum (where the **nMlf** nucleus is clearly labelled, as the **oculomotor nerve nucleus**).

Caudally, GFP expression is observed in the cerebellum, while in the medulla oblongata it is localized only in some groups of anterior myelencephalic neurons and in the vagal region.

From the first day of development the peripheral trigeminal ganglia and their anterior and posterior projections show strong GFP expression.

3.2 ChAT GFF: UAS GFP

This transgenic line genome harbours an insertion of a BAC containing the choline acetyl transferase genomic region with GFF cloned at the ChAT starting ATG. GFP expression was previously observed in the 6 dpf within the pretectum, preoptic region and thalamus/posterior tuberculum in M. Orger's lab.

3.2.1 1dpf

At 1 day post-fertilization (dpf), within the prosencephalon, GFP is observed both in the post-optic commissure (poc) and the presumptive ventro-rostral cluster (vrc). Strong GFP expression is observed in the ventro-caudal cluster (vcc, within the mesencephalon), the medio-longitudinal fasciculus (mlf), and the tract of the posterior commissure (pc) that extends from the vcc (Fig. 3.7.A_1).

In the peripheral nervous system (PNS), some disperse cells of the olfactory placode are also strongly labelled (OE).

This pattern was very consistent between the different imaged fish (see Supplementary data).

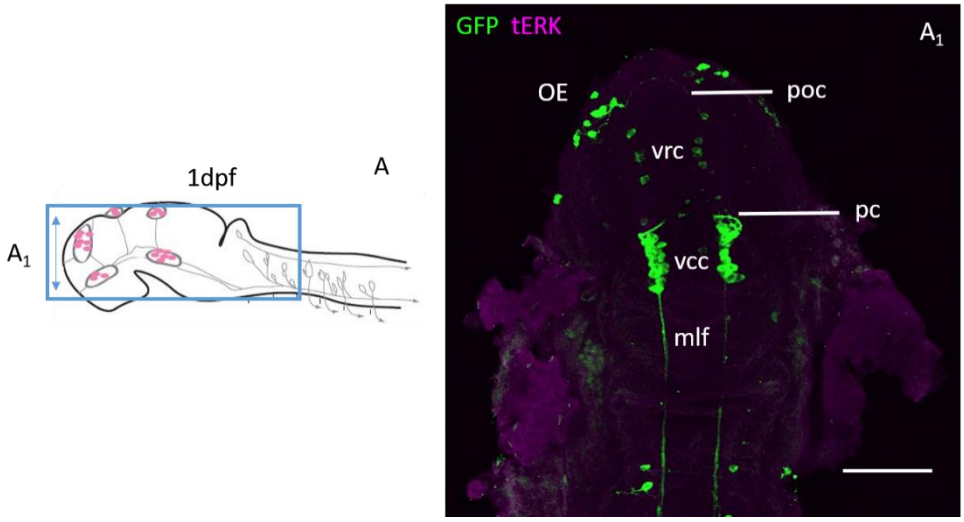

Fig. 3-7- GFP expression in 1 dpf ChAT GFF; UAS GFP zebrafish brain. A, sketch of 1dpf brain area shown in A1, adapted from Rubenstein 2017. A_1, z-projection of Chat GFF; UAS GFP 1dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bar indicates approximately 100 μm. Abbreviations: melf, medio-longitudinal fasciculus; OE, olfactory epithelium; pc, posterior commissure; poc, post optic commissure; vcc, ventro-caudal cluster; vrc, ventro-rostral cluster.

3.2.2 2dpf

At the 2 dpf stage, GFP expression is found in some groups of glomerular shaped cells located in the ventral prosencephalon, in the region corresponding to the olfactory bulb (OB) (Fig. 3.8.B_3).

Caudally, within the diencephalon, strong GFP expression is observed in cells distributed in a region that includes (from dorsal to ventral) the pretectum (Pr), ventral thalamus (vTha), and the posterior tuberculum (PTub), while the hypothalamus (located further ventrally) shows a dimmer signal close to the optic chiasm (oc) (Fig. 3.8.B_{2-3}).

The developing retina (Ret) and optic nerve also show a strong GFP signal (Fig, 3.8.B_3).

Within the mesencephalon there is a clear and strong expression pattern both in the optic tectum (TeO), posterior commissure (pc) and in the ventrally located tegmentum (Teg). The posterior commissure cells cluster (above the pc) is also clearly labelled with anti-GFP (Fig. 3.8.B_2).

In the most caudal rhombencephalon, there is a sparse GFP expression in some cells of the cerebellum (Cer) comparing with the broader and strong GFP expression in the medulla oblongata (MO), in the rhombencephalic neurons and its ipsi and contralateral projections. In the medial zone, the mlf shows weaker signal (Fig. 3.8.B_1).

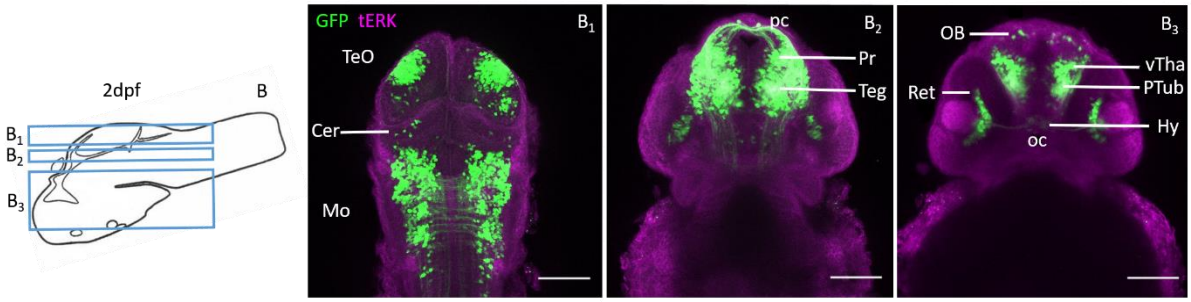

Fig. 3-8- GFP expression in 2 dpf ChAT GFF; UAS GFP zebrafish brain. B, sketch of 2dpf brain area shown in panels B_{1-3}, adapted from Mueller and Wullimann. B_{1-3}, from dorsal to ventral, z-projections of Chat GFF; UAS GFP 2dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 µm. Abbreviations: Cer, cerebellum; Hy, hypothalamus; MO, medulla oblongata; OB, olfactory bulb; oc, optic chiasm; pc, posterior commissure; PTub, posterior tuberculum; Ret, retina; Teg, tegmentum; TeO, optic tectum; vTha, ventral thalamus.

3.2.3 3dpf

At 3dpf stage, in the telencephalon GFP expression is only observed in the anterior commissure (ac) and the peripheral olfactory epithelium (OE) (Fig. 3.9.C_3).

The retina has a very clear expression pattern in the retinal ganglion cells (RGC) layer.

From dorsal to ventral, in the diencephalon, a clear GFP expression is observed in some pretectal cells (Pr), in the thalamus (Tha), ventral posterior tuberculum (vPTub) and some cells of the preoptic region (Po). GFP expression can be also be observed in the post optic commissure (poc) (Fig. 3.9.C_{1-3}).

Within the mesencephalon there is a strong and wide tectal expression, both in the optic tectum neuropil (TeOn) and stratum periventriculare (TeOsp), and in the tegmentum (Teg) (Fig. 3.9.C_1).

Caudally, the medulla oblongata shows a widespread GFP expression in different groups of cells.

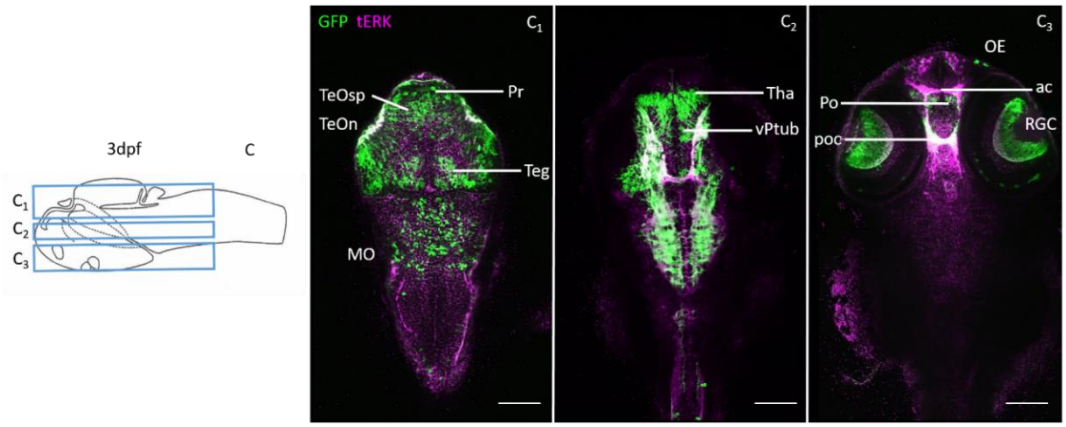

Fig. 3-9- GFP expression in 3 dpf ChAT GFF; UAS GFP zebrafish brain. C, sketch of 3dpf brain area shown in panels C_{1-3}, adapted from Mueller and Wullimann. C_{1-3}, from dorsal to ventral, z-projections of Chat GFF; UAS GFP 3dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 µm. Abbreviations: ac, anterior commissure; Cer, cerebellum; Hy, hypothalamus; MO, medulla oblongata; OE, olfactory epithelium; oc, optic chiasm; pc, posterior commissure; PTub, posterior tuberculum; RGC, retinal ganglion cells; Teg, tegmentum; TeOsp, optic tectum stratum periventriculare; TeOn, optic tectum neuropil; Tha, thalamus; vPTub, ventral posterior tuberculum.

3.2.4 4dpf

Rostrally at 4dpf, GFP expression observed in some cells in the telencephalic olfactory bulb (OB) and anterior commissure (ac) and in the peripheral olfactory epithelium (OE) (Fig. 3.10.D_{2-4}).

Dorsally on the diencephalon, strong GFP expression is observed in the pretectum (not shown), dorsal and ventral thalamus (Tha) (Fig. 3.10.D_{2-3}), while the posterior tuberculum (PTub) and preoptic region (PO) show a dimmer expression (Fig. 3.10.D_{3-4}). Ventrally, the post optic commissure and a large group of cells in the hypothalamus (Hy) also show GFP expression (Fig. 3.10.D_4).

The retina still shows a remarkable GFP expression in the RGC layer, shown in Fig. 3.10.D_4.

Within the mesencephalon, GFP expression is observed in the posterior commissure (pc), optic tectum (neuropil and stratum periventriculare), and the ventral tegmentum (Teg), especially in the torus semicircularis (TS). (Fig. 3.10.D_{1-3})

Caudally, in the medulla oblongata several groups of rhombencephalic neurons are labelled from the rostral to the caudal-most region, namely Rho-R1 in a medial position and Rho-R2, in bilateral dorsal positions (Fig. 3.10.D_{1-2}). In Fig. 3.10.D_3, ventral rhombencephalic neurons and its axons projecting either ipsilaterally or contralaterally into the mlf or the llf are shown.

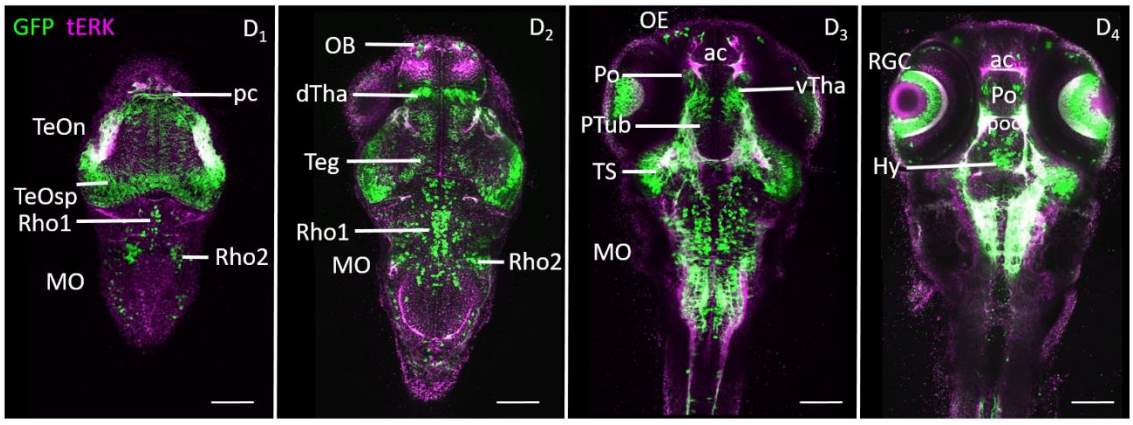

Fig. 3-10- GFP expression in 4 dpf ChAT GFF; UAS GFP zebrafish brain. D_{1-4}, from dorsal to ventral, z-projections of Chat GFF; UAS GFP 4dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 µm. Abbreviations: ac, anterior commissure; Cer, cerebellum; Hy, hypothalamus; MO, medulla oblongata; OB, olfactory bulb; OE, olfactory epithelium; oc, optic chiasm; pc, posterior commissure; PO, preoptic area; PTub, posterior tuberculum; Rho1,2, rhombencephalic clusters 1 and 2; RGC, retinal ganglion cells; Teg, tegmentum; TeOn, optic tectum neuropil; TS, torus semicircularis; vTha, ventral thalamus.

3.2.5 5dpf

Five days post fertilization, the telencephalon presents localized expression in some cells of the olfactory bulb (OB) (Fig. 3.11.E_{2-3}). Ventrally, the anterior commissure and some cells of the peripheral olfactory epithelium show GFP expression. (Fig. 3.11.E_4)

In the diencephalon, from dorsal to ventral, GFP expression is observed in the dorsal and ventral regions of the thalamus (Tha) and posterior tuberculum (PTub), in some cells of the hypothalamus (Hy) and preoptic region (PO). Both the post-optic commissure (poc) and optic chiasm (oc) present GFP expression. The retina's inner layer, of retinal ganglion cells (RGC), strongly expresses GFP. (Fig. 3.11.E_4)

Within the mesencephalon, GFP expression is observed in the lateral-most part of optic tectum's neuropil and stratum periventriculare (TeOn and TeOsp), and torus semicircularis (TS), within the tegmentum. (Fig. 3.11.E_{1-2})

Caudally, GFP is widely expressed through the medulla oblongata, namely in two distinct subpopulations of rhombencephalic neurons (Rho1 and Rho2) and in the vagus motor neurons (VagusMN). (Fig. 3.11.E_{1-2})

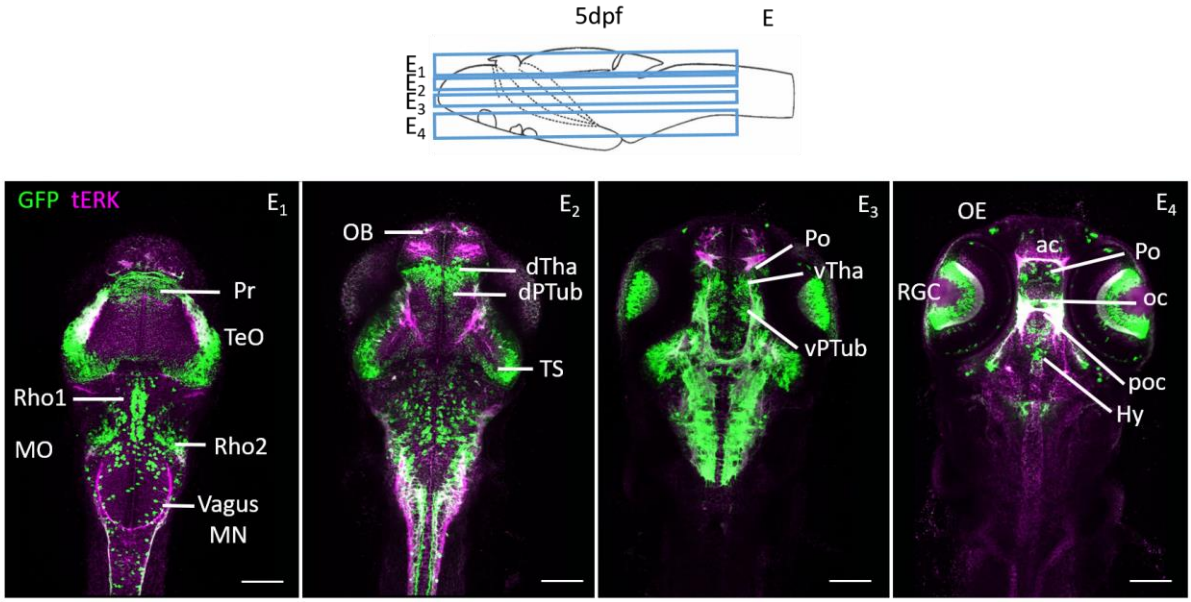

Fig. 3-11- GFP expression in 5 dpf ChAT GFF; UAS GFP zebrafish brain. E, sketch of 5dpf brain area shown in panels E_{1-4}, adapted from Mueller and Wullimann. E_{1-4}, from dorsal to ventral, z-projections of Chat GFF; UAS GFP 5dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 μm. Abbreviations: ac, anterior commissure; Cer, cerebellum; Hy, hypothalamus; MO, medulla oblongata; OB, olfactory bulb; OE, olfactory epithelium; oc, optic chiasm; pc, posterior commissure; PO, preoptic area; Rho1,2, rhombencephalic clusters 1 and 2; RGC, retinal ganglion cells; Teg, tegmentum; TeOn, optic tectum neuropil; TS, torus semicircularis; vPTub, ventral posterior tuberculum; vTha, ventral thalamus; Vagus MN, vagus motor neurons.

3.2.6 6dpf

In the 6dpf telencephalon there is no clear GFP-expression, with the exception of some tracts in the sub-pallial (not visible in z-projections) and anterior commissure (ac) regions.

Within the diencephalon, strong GFP expression is observed, from dorsal to ventral, in the pretectum (Pr), thalamus (Tha), posterior tuberculum (PTub) and hypothalamus (Hy), where four group of immunoreactive cells are clearly visible (Fig. 3.12.F_{2-4}). Some isolated cells in the preoptic region (Po) and both the optic chiasm (oc) and post-optic commissure (poc) also express GFP (Fig. 3.12.F_{4-5}).

In the retina some retinal ganglion cells (RGC) strongly express GFP as is visible in the last z-projection (Fig. 3.12.F_5).

In the mesencephalon, a wide tectal GFP expression is visible in the neuropil (TeOn), stratum periventriculare (TeOsp) and medial tectal band (TeOmb). Ventrally, within the tegmentum, the nucleus of the mlf, trochlear nucleus (tronuc) and torus semicircularis (TS) all show GFP expression (Fig. 3.12.F_{1-3}).

Caudally, in the medulla oblongata (MO), GFP is widely expressed in many rhombencephalic neurons, especially in Rho1 and Rho 2 and the facial octavolateralis motor neurons (fomn). (Fig. 3.12.F_{1-3}).

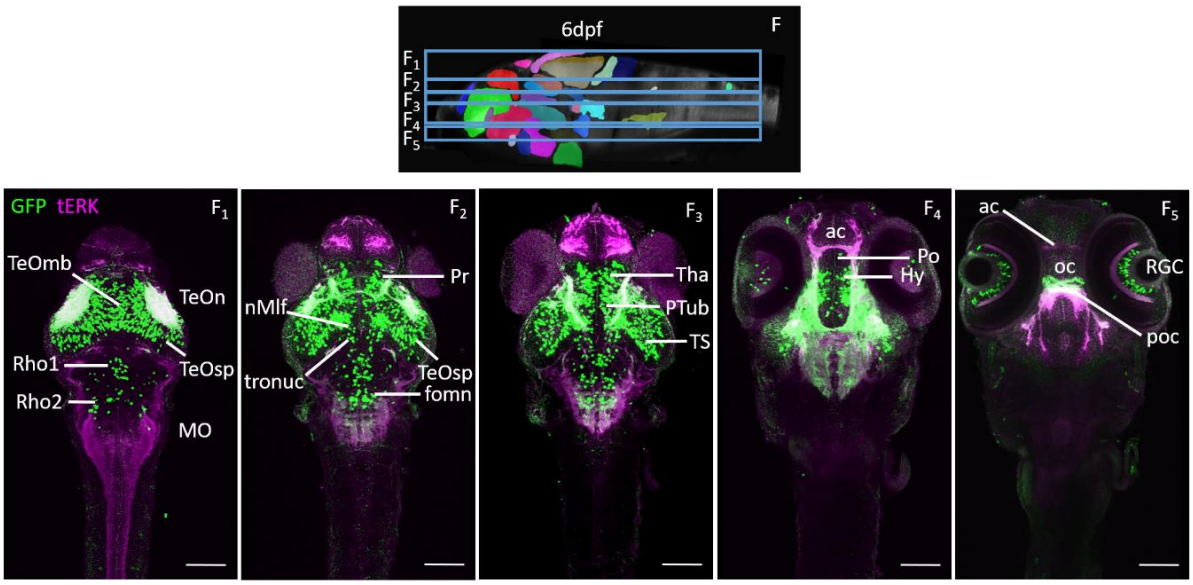

Fig. 3-12- GFP expression in 6 dpf ChAT GFF; UAS GFP zebrafish brain. F, sketch of 6dpf brain area shown in panels $F_{1\text{-}5}$, adapted from zbb atlas. $F_{1\text{-}5}$, from dorsal to ventral, z-projections of Chat GFF; UAS GFP 6dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 µm. Abbreviations: ac, anterior commissure; Cer, cerebellum; fomn, facial octavolateralis motor neurons; Hy, hypothalamus; MO, medulla oblongata; nMlf, nucleus of the mlf; OB, olfactory bulb; OE, olfactory epithelium; oc, optic chiasm; pc, posterior commissure; PO, preoptic area; Rho1,2, rhombencephalic clusters 1 and 2; RGC, retinal ganglion cells; Teg, tegmentum; TeOn, optic tectum neuropil; Tha, thalamus; trocnuc, trochlear nucleus; TS, torus semicircularis; vPTub, ventral posterior tuberculum; Vagus MN, vagus motor neurons.

3.2.7 ChAt GFF; UAS GFP: GFP expression summary

Table 3-2- Brain regions showing GFP expression during development: summary of the GFP expression in the ChAt GFF UAS GFP line providing a list of anatomical regions and its abbreviations, excluding the primordial structures.

Anatomic region	Expression	Abbreviations
Telencephalon	Olfactory bulb and anterior commissure.	ac; OB;
Diencephalon	Pretectum, thalamus, posterior tuberculum, hypothalamus, some cells in the preoptic region, post-optic commissure and optic chiasm.	Hy; oc; PO; poc; Pr; PTub; Tha;
Mesencephalon	Optic tectum neuropil and stratum periventriculare, posterior commissure, tegmentum (nucleus of the mlf, trochlear nucleus and torus semicircularis)	nMlf; pc; Teg; TeO; TeOn; TeOsp; tronuc; Ts;
Metencephalon	Not observed.	
Myelencephalon	Many neurons in the medulla oblongata including, newly identified clusters 1 and 2, vagus motor neurons and facial octavolateralis motor neurons.	fomn; MO; Rho1; Rho2; VagusMn;
Retina	Retinal ganglion cells	RGC;
PNS	Olfactory epithelium.	OE;

3.2.8 ChAt GFF; UAS GFP: GFP expression overview

The ChAT GFF UAS GFP line shows GFP expression early in development (1dpf) in the developing prosencephalon and mesencephalon, including the developing **nucleus of the mlf (nMlf)** and its projections, with a very clear expression pattern.

Later in development, telencephalic GFP expression is reduced until it is almost absent, only weakly observed in the olfactory bulb. On the contrary, in the diencephalon the number of GFP expressing cells increases, as they are clearly widespread through the major anatomical regions (including the **pretectal area**).

Within the retina, GFP expression increases within the RGC layer from the 2 to the 5 dpf, although at 6dpf GFP expression is observed in just spaced RGCs.

In the mesencephalon, both the dorsal optic tectum and the ventral tegmentum (where the **nMlf** is located) show strong positive GFP immunoreactivity until the sixth dpf.

Caudally, in the rhombencephalon, GFP expression becomes increasingly widespread through the medulla oblongata and especially localized in the clusters Rho1 and Rho2.

The peripheral olfactory epithelium also shows GFP expression from the first day of development but at 6dpf has become localized in sparse groups of cells.

3.3 *GAD1B GFF: UAS GFP*

This transgenic line harbours an insertion of a BAC that includes the genomic region of Gad1b, which encodes the glutamate decarboxylase 1, involved on the synthesis of the neurotransmitter GABA, with GFF cloned at the Gad1b starting ATG.

3.3.1 1dpf

At 1dpf, GFP expression is rostrally observed in the future olfactory bulb, within the telencephalon (Tel), and ventro-rostral cluster (vrc), within the primordial diencephalon. Caudally, there is GFP expression spread through the mesencephalon (Mes) and primordial rhombencephalon (Rho). (Fig. 3.13.A_1).

In the developing retina (mostly proliferating precursors at this stage) there are positive immune-reactive progenitor cells as in the olfactory placode, developing olfactory organ (OE).

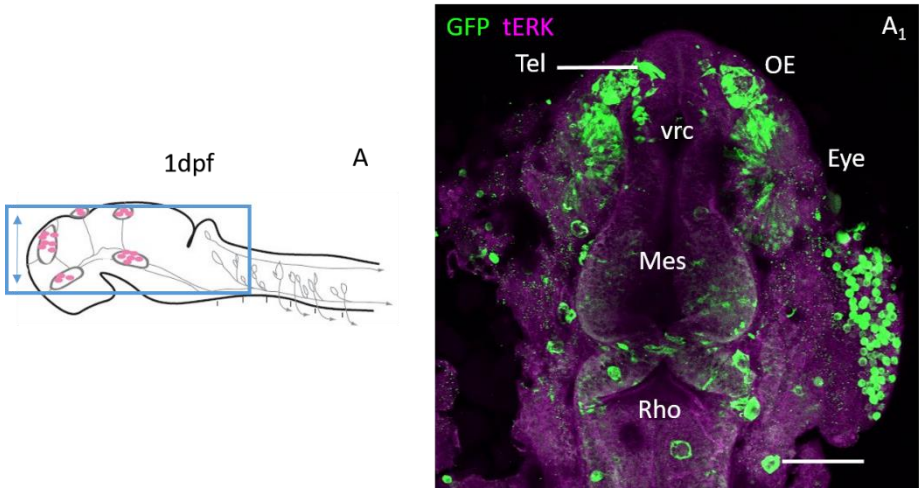

Fig. 3-13- GFP expression in 1 dpf Gad1b GFF; UAS GFP zebrafish brain. A, sketch of 1dpf brain area shown in A_1, adapted from Rubenstein 2017. A_1, z-projection of Gad1b GFF; UAS GFP 1dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bar indicates approximately 100 μm. Abbreviations: telencephalon (Tel), ventro-rostral cluster (vrc), mesencephalon (Mes), rhombencephalon (Rho), olfactory epithelium (OE).

3.3.2 2dpf

At 2dpf, within the developing telencephalon, the olfactory bulb (OB) and the sub-pallium (SubP) show a clear GFP expression (Fig. 3.14.B_4).

Within the diencephalon, from dorsal to ventral, there is a strong GFP expression in the pretectum (Pr), ventral thalamus (vTha), posterior tuberculum (PTub) and preoptic region (PO) (Fig2.B_{2-4}). Dorsally, the pineal gland (Pi) also shows a very clear GFP expression (Fig. 3.14.B_{1-2}).

In the developing retina we can observe a strong and broad GFP expression presumably including the proliferating precursors (Fig. 3.14.$B_{3, 4}$).

Within the mesencephalon, GFP expression occurs in the posterior commissure (pc), optic tectum (TeO) and in many neurons bilaterally located in the tegmentum (Teg).

From the mesencephalon through the myelencephalon, the mlf shows a clear expression pattern together with the dorsal disperse groups of cells in the medulla oblongata (MO).

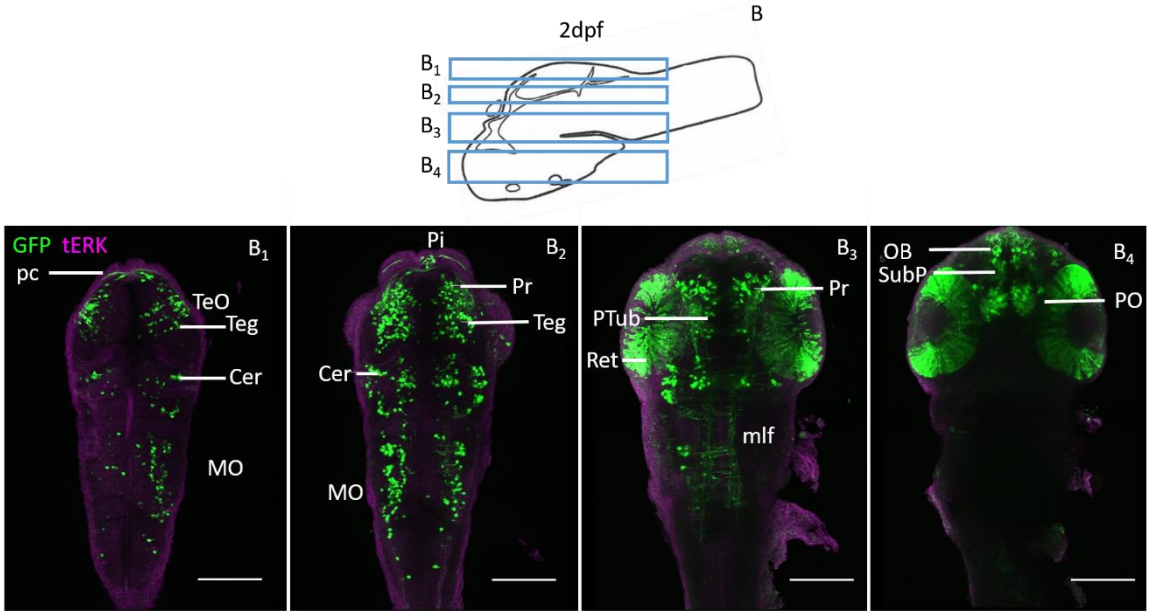

Fig. 3-14- GFP expression in 2 dpf Gad1b GFF; UAS GFP zebrafish brain. B, sketch of 2dpf brain area shown in panels B_{1-4}. B_{1-4}, from dorsal to ventral, z-projections of Gad1b GFF; UAS GFP 2dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 µm. Abbreviations: cerebellum (Cer), olfactory bulb (OB) and the sub-pallium (SubP), pretectum (Pr), ventral thalamus (vTha), posterior tuberculum (PTub), preoptic region (PO), pineal gland (Pi), posterior commissure (pc), optic tectum (TeO), tegmentum (Teg), medulla oblongata (MO), retina (Ret).

3.3.3 3dpf

In the 3dpf telencephalon, GFP expression is wider and stronger in the olfactory bulb (OB) than in the more ventral subpallium (SubP).

In the diencephalon there is GFP expression in some cells of the pretectum (Pr), in the ventral thalamus (vTha), posterior tuberculum (PTub), preoptic region (PO), hypothalamus (Hy) and in the more dorsal pineal gland (Pi) (Fig. 3.15.C_{2-4}). The posterior commissure (pc) also shows strong GFP expression (Fig. 3.15.C_2).

Within the retina, GFP expression is clearly observed in the amacrine cells (AC) (Fig. 3.15.C_4).

In the mesencephalon, widespread GFP expression occurs in the tectal stratum periventriculare and neuropil, as in the tegmentum (Teg). GFP expression is also observed through the mlf. (Fig. 3.15.C_{1-2})

Caudally, within the metencephalon and myelencephalon respectively, GFP expression is observed in the cerebellum (Cer), valvula and corpus cerebelli, and medulla oblongata (MO), where several widespread groups of cells show positive GFP immunoreactivity. (Fig. 3.15.C_{1-2}).

Note that for this sample the anti-TH was used and its expression can be observed in the locus coeruleus (LC), mlct, and some cells in the preoptic region area show positive immunoreactivity.

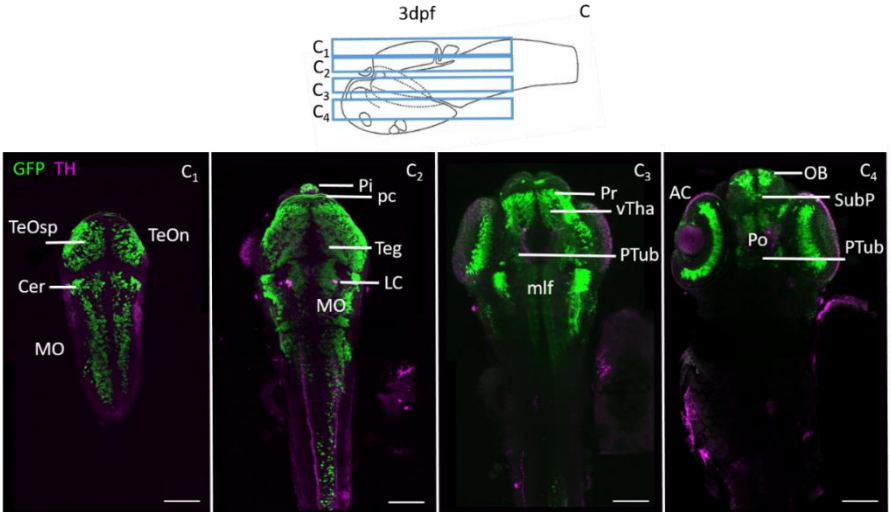

Fig. 3-15- GFP expression in 3 dpf Gad1b GFF; UAS GFP zebrafish brain. C, sketch of 3dpf brain area shown in panels C_{1-4}, adapted from Mueller and Wulliman 2015. C_{1-4}, from dorsal to ventral, z-projections of Gad1b GFF; UAS GFP 3dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-TH (magenta). The scale bars indicate approximately 100 µm. Abbreviations: anterior commissure (ac), cerebellum (Cer), olfactory bulb (OB) and the sub-pallium (SubP), pretectum (Pr), ventral thalamus (vTha), posterior tuberculum (PTub), preoptic region (PO), pineal gland (Pi), ventral thalamus (vTha), posterior commissure (pc), optic tectum (TeO), tegmentum (Teg), medulla oblongata (MO), retina (Ret), hypothalamus (Hy).

3.3.4 4dpf

At 4dpf stage, within the telencephalon, strong GFP expression is observed in the olfactory bulb (OB) and more ventral sub-pallium (SubP) (Fig. 3.16.D_{2-4})

Dorsally in the diencephalon, the pineal gland (Pi) shows some GFP positive immunoreactive cells (Fig. 3.16.D1). GFP expression is also clearly observed in the ventral thalamus (vTha), posterior tuberculum (PTub), preoptic region (PO) and hypothalamus (Hy).

At this stage GFP expression is still observed in the amacrine cells layer. (Fig. 3.16.D_{2-4})

In the mesencephalon, GFP expression is observed in the tectal neuropil and stratum periventriculare (TeOn and TeOsp), and torus semicircularis (TS, within the tegmentum).

Caudally, compact GFP expression is observed in the cerebellum (Cer), especially in the valvula cerebelli (anterior and medial cerebellar region, vCer), while in the medulla oblongata GFP expression is more widespread, from the anterior rhombencephalic neurons to the most caudal vagal region (Fig. 3.16.D_2). Many different rhombencephalic neurons that project to the mlf and llf are also labelled (Fig. 3.16.D_3).

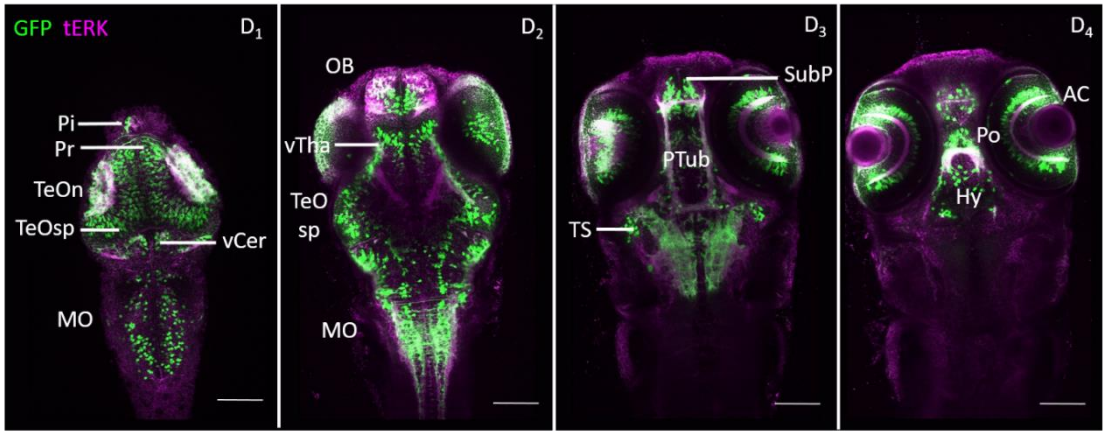

Fig. 3-16 - GFP expression in 4 dpf Gad1b GFF; UAS GFP zebrafish brain. D, sketch of 4dpf brain area shown in panels D_{1-4}, adapted from Mueller and Wullimann 2015. D_{1-4}, from dorsal to ventral, z-projections of Gad1b GFF; UAS GFP 4dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 μm. Abbreviations: anterior commissure (ac), cerebellum (Cer), olfactory bulb (OB) and the sub-pallium (SubP), pretectum (Pr), ventral thalamus (vTha), posterior tuberculum (PTub), preoptic region (PO), pineal gland (Pi), ventral thalamus (vTha), posterior commissure (pc), optic tectum (TeO), tegmentum (Teg), medulla oblongata (MO), retina (Ret), hypothalamus (Hy).

3.3.5 5dpf

At 5 dpf, a strong telencephalic GFP expression is observed in the olfactory bulb (OB), sub-pallium (SubP) and the more ventral anterior commissure (ac).

Within the diencephalon, GFP expression is distributed, from dorsal to ventral, in the pineal gland (Pi), in the pretectum (Pr), dorsal thalamus (dTha), ventral thalamus (vTha) and ventral posterior tuberculum (vPTub) (Fig. 3.17.E_{1-3}). Ventrally, some positive GFP immunoreactive cells can be obserbed in the preoptic (PO) and hypothalamic (Hy) regions (Fig5.E_4). GFP expression is also observed in the post-optic commissure (poc).

In the retina, the amacrine cells layer is still strongly expressing GFP.

Within the mesencephalon, GFP expression is observed through the optic tectum neuropil (TeOn), stratum periventriculare (TeOsp), and torus semicircularis (TS), within the tegmentum (Teg).Caudally, wide GPF expression is observed in the cerebellum (valvula cerebelli, vCer, and corpus cerebeli, cCer), and through the medulla oblongata (MO), from the most anterior region to the vagus motor neurons (vagusMN) (Fig. 3.17.E_1).

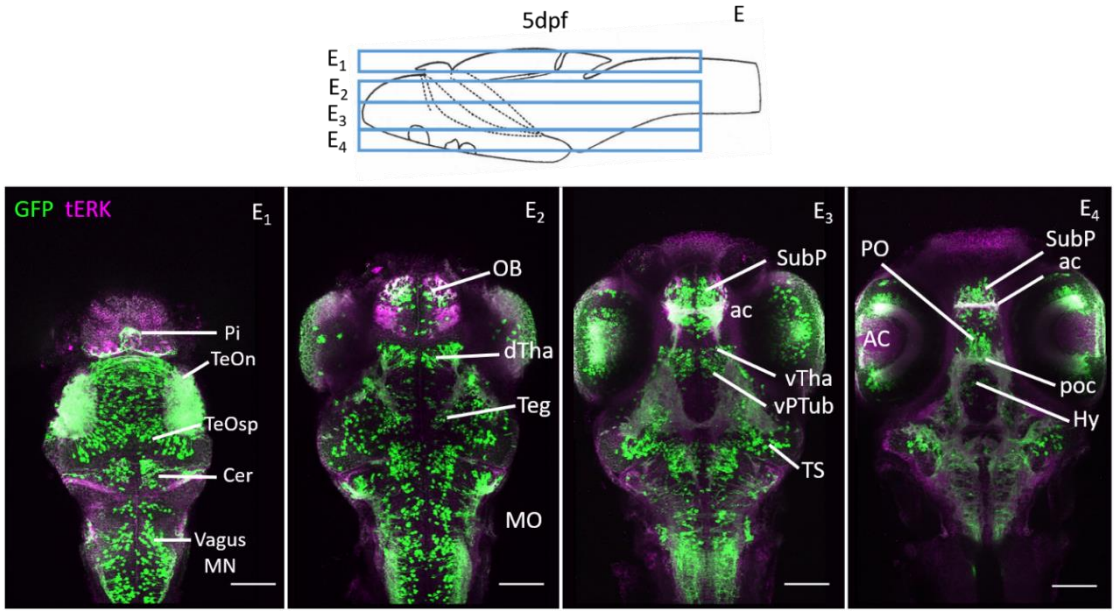

Fig. 3-17- GFP expression in 5 dpf Gad1b GFF; UAS GFP zebrafish brain. E, sketch of 5dpf brain area shown in panels E_{1-4}, adapted from Mueller and Wullimann 2015. E_{1-4}, from dorsal to ventral, z-projections of Gad1b GFF; UAS GFP 5dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 μm. Abbreviations: anterior commissure (ac), cerebellum (Cer), olfactory bulb (OB) and the sub-pallium (SubP), pretectum (Pr), ventral thalamus (vTha), posterior tuberculum (PTub), preoptic region (PO), pineal gland (Pi), ventral thalamus (vTha), posterior commissure (pc), optic tectum (TeO), tegmentum (Teg), medulla oblongata (MO), retina (Ret), hypothalamus (Hy).

3.3.6 6dpf

At 6dpf, within the telencephalon, GFP expression is observed in the olfactory bulb (OB) and sub-pallium (SubP) and the more ventral anterior commissure (ac) (Fig. 3.18.F_{3-5}).

In the diencephalon, GFP expression is observed, from dorsal to ventral, in the pretectum (Pr), thalamus (Tha), ventral posterior tuberculum (vPTub), preoptic region (PO), post-optic commissure (poc) and hypothalamus (Hy). Dorsally, the peripheral pineal gland (Pi) also shows well-marked GFP expression (Fig. 3.18.F_1).

Within the retina, strong GFP expression is observed in the amacrine cells layer, shown in Fig3.18.F_5.

Within the mesencephalon, GFP expression is observed in the optic tectum (neuropil, TeOn, and stratum periventriculare, TeOsp), and within the tegmentum: nucleus of the mlf (nMlf), trochlear nucleus (trocnuc) and torus semicircularis (TS) (Fig. 3.18.F_{2-4}).

Caudally, GFP expression is observed in the cerebellum (Cer), especially in valvula cerebelli (vCer), and through the medulla oblongata (MO) until the vagal region and the vagus motor neurons (vagusMN). (Fig. 3.18.F_{1-3}).

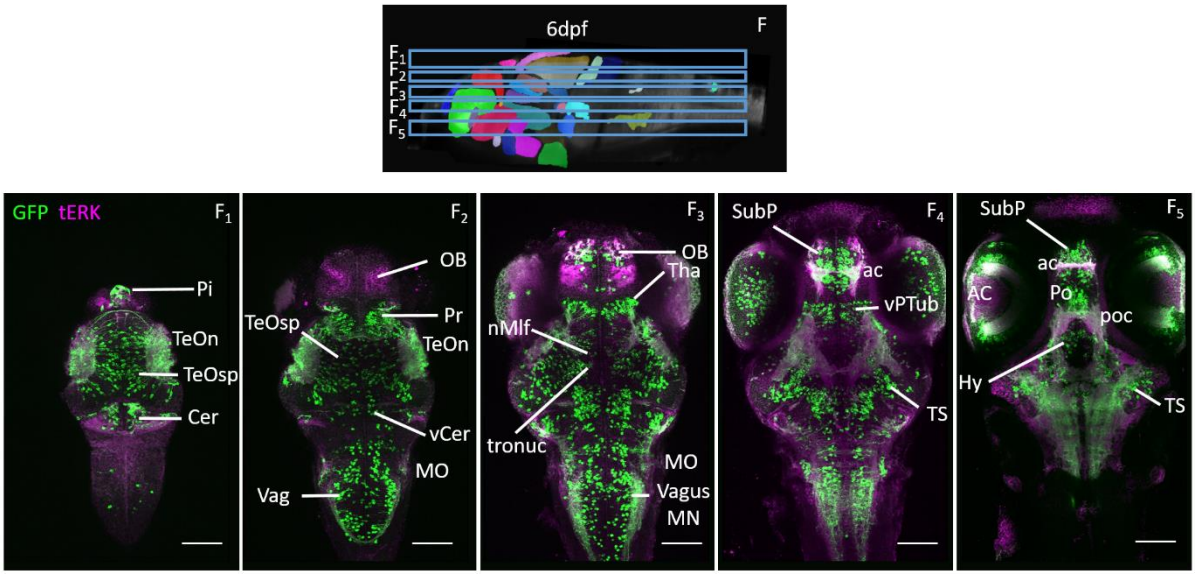

Fig. 3-18- GFP expression in 6 dpf Gad1b GFF; UAS GFP zebrafish brain. F, sketch of 6dpf brain area shown in panels F_{1-5}, adapted from Mueller and Wullimann 2015. $F_{1-5,}$ from dorsal to ventral, z-projections of Gad1b GFF; UAS GFP 6dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 µm. Abbreviations: anterior commissure (ac), cerebellum (Cer), olfactory bulb (OB) and the sub-pallium (SubP), pretectum (Pr), ventral thalamus (vTha), posterior tuberculum (PTub), preoptic region (PO), pineal gland (Pi), ventral thalamus (vTha), posterior commissure (pc), optic tectum (TeO), tegmentum (Teg), medulla oblongata (MO), retina (Ret), hypothalamus (Hy).

3.3.7 Gad1b GFF; UAS GFP: GFP expression summary

Table 3-3- Brain regions showing GFP expression during development: summary of the GFP expression in the Gad1b GFF UAS GFP line providing a list of anatomical regions and its abbreviations, excluding primordial structures.

Anatomical regions	Expression	Abbreviations
Telencephalon	Olfactory bulb, sub-pallium and anterior commissure.	ac; OB; SubP;
Diencephalon	Pineal gland, pretectum, thalamus, posterior tuberculum, some hypothalamic cells, preoptic region, post-optic commissure and optic chiasm.	Hy; oc; Pi; PO; poc; Pr; PTub; Tha;
Mesencephalon	Optic tectum neuropil and stratum periventriculare, posterior commissure, tegmentum (nucleus of the mlf, trochlear nucleus and torus semicircularis).	nMlf; pc; Teg; TeO; TeOn; TeOsp; tronuc; Ts;
Metencephalon	Cerebellum, especially valvula cerebelli.	Cer; vCer;
Myelencephalon	Many neurons in the medulla oblongata, in the vagal region and especially vagus motor neurons.	MO; VagR; VagusMn;
Retina	Amacrine cells layer.	AC.
PNS	Not observed after 1 dpf.	

3.3.8 Gad1b GFF; UAS GFP: GFP expression overview

Early in development, GFP expression is spread through the major divisions of the brain, from the developing telencephalon to the more caudal rhombencephalon.

Through development, within the telencephalon, GFP expression is observed both in the optic bulb and sub-pallium, while in the diencephalon strong GFP expression is widespread through the major anatomical regions (including the **pretectal area** and the pineal gland).

Within the retina, GFP expression increases from 1 until 6dpf, within the amacrine cells layer (identified from 3 dpf).

In the mesencephalon, GFP expression is observed through the dorsal optic tectum and the ventral-most tegmentum (where the **nucMLF** is located) until 6 dpf.

Caudally, in the rhombencephalon, GFP expression remains widespread through the cerebellum and medulla oblongata.

The peripheral (developing) olfactory epithelium only shows strong GFP expression on the first day post fertilization.

3.4 SLC18A3B GFF: UAS GFP

Transgenic line harbouring an insertion of a BAC in the genome, containing the solute carrier family 18 gene, which encodes a vesicular acetylcholine transporter, and GFF cloned in its starting ATG codon. GFP expression was expected to be similar to cholinergic neurons distribution.

3.4.1 1dpf

At 1dpf, within the prosencephalon there are strong positive GFP immunoreactive cells spread through both the developing diencephalon and telencephalon (future OB), and the two olfactory placodes (developing olfactory epithelium).

In the mesencephalon, GFP expression is strongly observed in the ventro-caudal cluster (vcc) and, in a more dimmed fashion, through the medio-longitudinal fasciculus (mlf). The developing eye (progenitor cells) and the epiphyseal cluster (ec) also express GFP at this stage. (Fig. 3.19.A_1)

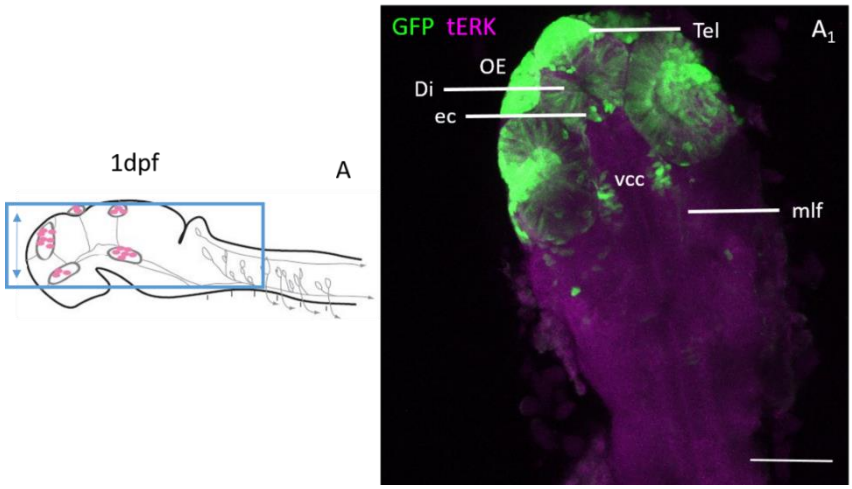

Fig. 3-19- GFP expression in 1 dpf Slc18a3b GFF; UAS GFP zebrafish brain. A, sketch of 1dpf brain area shown in A_1, adapted from Rubenstein 2017. A_1, z-projection of Slc18a3b GFF; UAS GFP 1dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bar indicates approximately 100 µm. Abbreviations: diencephalon (Di), telencephalon (Te), olfactory placodes (OE), medio-longitudinal fasciculus (mlf), the epiphyseal cluster (ec), ventrocaudal cluster (vcc).

3.4.2 2dpf

At 2dpf, GFP expression is observed through the telencephalon, and more precisely in the developing olfactory bulb (OB) (Fig. 3.20.B_3). Anteriorly, the peripheral olfactory epithelium (OE) also expresses GFP.

Within the retina GFP is observed in the proliferative cells in a nonspecific pattern.

GFP expression is very clearly observed throughout the diencephalon and pineal gland (Pi) in Fig. 3.20.B_1.

In Fig. 3.20.B_2, GFP expression is observed in the mesencephalon (Mes), including the developing ventro-caudal cluster,) and ascending posterior commissure (pc).

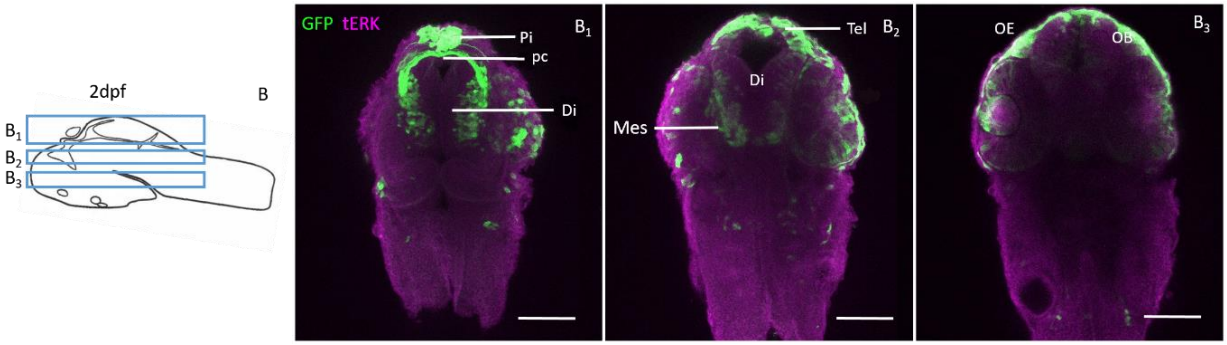

Fig. 3-20- GFP expression in 2dpf Slc18a3b GFF; UAS GFP zebrafish brain. B, sketch of 2dpf brain area shown in B_{1-3}, adapted from Mueller and Wullimann 2015. B1-3, z-projections of Slc18a3b GFF; UAS GFP 2dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 µm. Abbreviations: olfactory bulb (OB), mesencephalon (Mes), posterior commissure (pc), olfactory epithelium (OE), diencephalon (Di), pineal gland (Pi).

3.4.3 3dpf

Anteriorly, within the telencephalon GFP expression is only observed in the sub-pallium (SubP).

In the diencephalon, the pineal gland (Pi), the pretectum (Pr), dorsal and ventral thalamus (Tha) and posterior tuberculum (PTub) show a clear GFP expression (Fig. 3.21.C_{1-3}). In C_4, GFP expression is observed in the preoptic region (PO) and the hypothalamus (Hy) in some groups of positive immunoreactive cells.

In the retina there is a clear pattern of GFP expression in both the RGC and AC layers (Fig. 3.21.C_4).

Within the mesencephalon, GFP expression is observed in the optic tectum neuropil (TeOn), posterior commissure (pc), tegmentum (Teg) and the more ventral torus semicircularis (TS) (Fig. 3.21.C_{1-2}).

Caudally, there are some positive immune-reactive cells spread through the medulla oblongata (MO), but not in a very distinct pattern (Fig. 3.21.C_{1-2}).

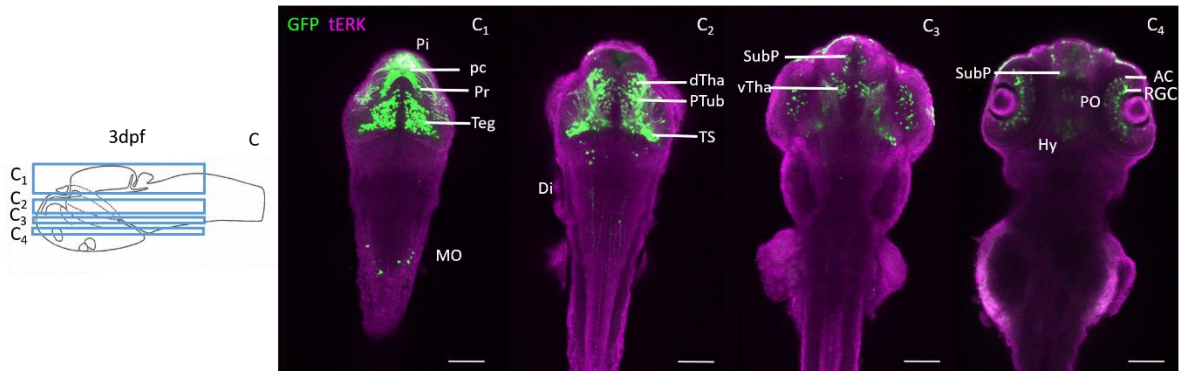

Fig. 3-21- GFP expression in 3dpf Slc18a3b GFF; UAS GFP zebrafish brain. C, sketch of 1dpf brain area shown in C_{1-4}, adapted from Mueller and Wullimann 2015. C1-4, z-projections of Slc18a3b GFF; UAS GFP 3dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 µm. Abbreviations: sub-pallium (SubP), pineal gland (Pi), pretectum (Pr), dorsal and ventral thalamus (Tha), posterior tuberculum (PTub), preoptic region (PO), hypothalamus (Hy), retinal ganglion and amacrine cells layers (RGC and AC), optic tectum neuropil (TeOn), posterior commissure (pc), tegmentum (Teg),torus semicircularis (TS), medulla oblongata (MO).

3.4.4 4dpf

In the telencephalon, there is a strong GFP expression in the optic bulb (OB) and sub-pallium (SubP) (Fig. 3.22.D_3).

Within the diencephalon there is GFP expression in the pineal gland (Pi) and the habenula (Ha), pretectum (Pr), thalamus (Tha), posterior tuberculum (PTub) and some groups of hypothalamic cells (Fig. 3.22.D_{1-4}).

The retina expresses GFP in the RGC and AC layers (Fig. 3.22.D_4).

Within the mesencephalon GFP expression is observed in the optic tectum (TeO), posterior commissure (pc), tegmentum (Teg) and torus semicircularis (TS) (Fig. 3.22.D_{1-4}).

Caudally, within the rhombencephalon GFP expression is observed through the full extension of the mlf (data not shown), in the cerebellum (Cer) and some disperse groups of cells in the medulla oblongata (MO). (Fig. 3.22.D_{1-3}).

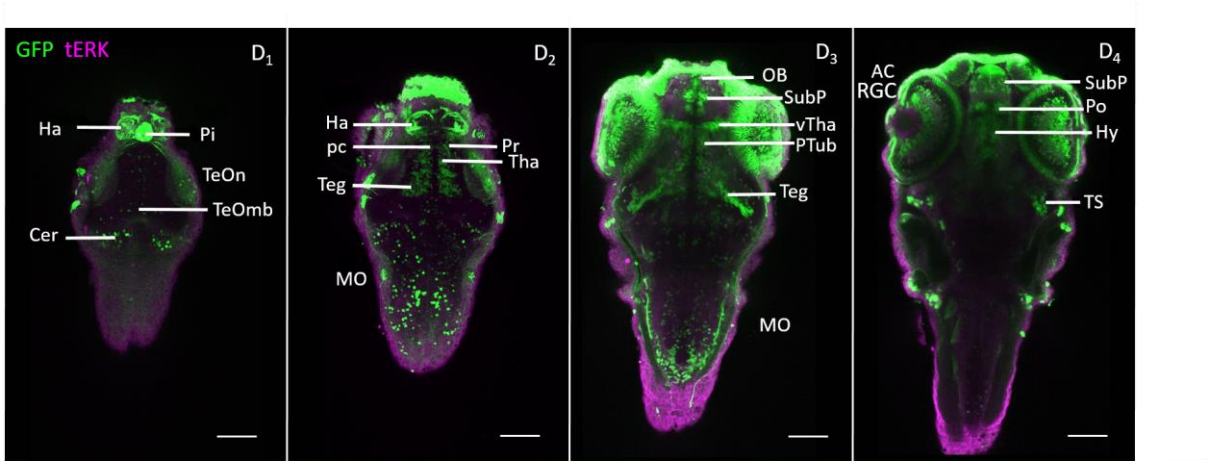

Fig. 3-22- GFP expression in 4 dpf Slc18a3b GFF; UAS GFP zebrafish brain. D_{1-4}, z-projections of Slc18a3b GFF; UAS GFP 4dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 µm. Abbreviations: optic bulb (OB) and sub-pallium (SubP), pineal gland (Pi), habenula (Ha), pretectum (Pr), thalamus (Tha), posterior tuberculum (PTub), hypothalamic cells (Hy), retinal ganglion and amacrine cells layers (RGC and AC), optic tectum (TeO), posterior commissure (pc), tegmentum (Teg), torus semicircularis (TS), cerebellum (Cer) and medulla oblongata (MO).

3.4.5 5dpf

At 5dpf, GFP expression can be observed, within the telencephalon, in the olfactory bulb (OB) and sub-pallium (SubP) (Fig. 3.23.E_3).

In the dorsal diencephalon, GFP is expressed in the pineal gland (Pi), habenula (Ha), thalamus (Tha), posterior tuberculum (PTub), preoptic region (PO) and hypothalamus (Hy). (Fig. 3.23.E_{1-4}).

At this stage the retina still expresses GFP in both retinal ganglion cells (RGC) and amacrine cells (AC) layers (Fig. 3.23.E_1).

Within the mesencephalon, GFP expression is observed in some cells of the tectal stratum periventricular (TeOsp) and in the medial tectal band (TeOmb), the posterior commissure (pc) and tegmentum (Teg) (Fig. 3.23.E_{1-3}).

Caudally, GFP expression is present bilaterally in the corpus cerebelli, within the cerebellum (Cer) and in more groups of rhombencephalic neurons, namely in the vagal region (VagR) and the vagus motor neurons (vagusMN).

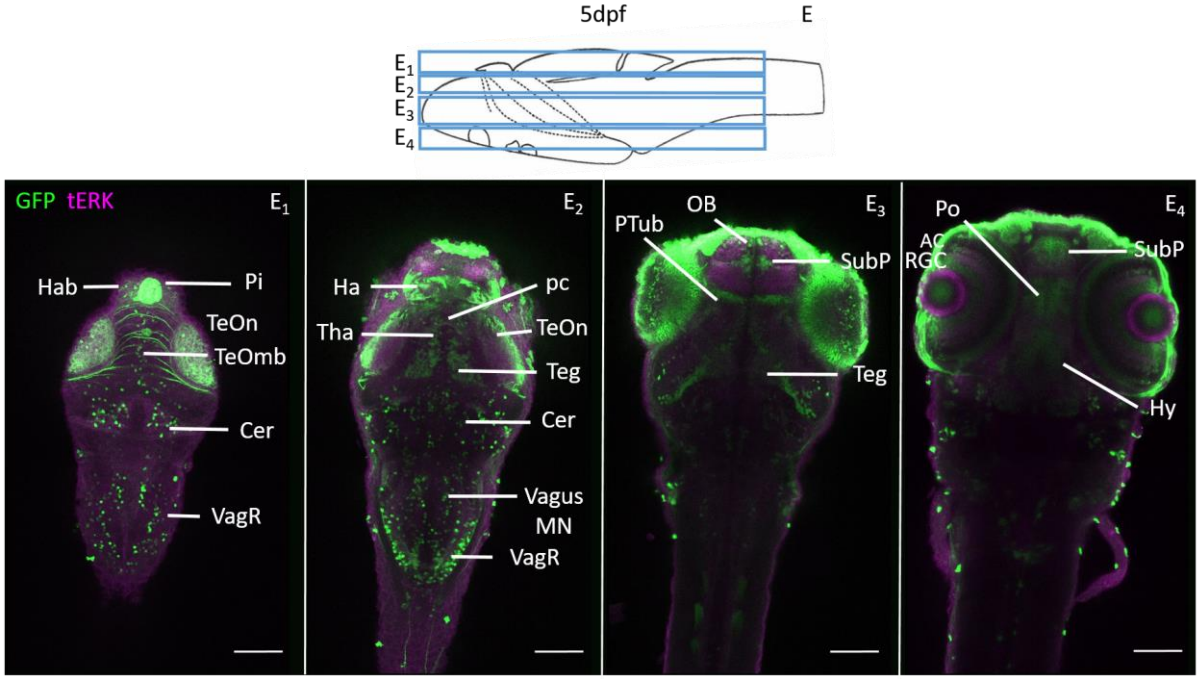

Fig. 3-23- GFP expression in 5dpf Slc18a3b GFF; UAS GFP zebrafish brain. E, sketch of 5dpf brain area shown in E_{1-4}, adapted from Mueller and Wullimann 2015. E_{1-4}, z-projections of Slc18a3b GFF; UAS GFP 5dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 µm. Abbreviations: olfactory bulb (OB), sub-pallium (SubP), the pineal gland (Pi), habenula (Ha), thalamus (Tha), posterior tuberculum (PTub), preoptic region (PO), hypothalamus (Hy), retinal ganglion and amacrine cells layers (RGC and AC), tectal stratum periventricular (TeOsp), the medial tectal band (TeOmb), posterior commissure (pc), tegmentum (Teg), cerebellum (Cer), vagal region (VagR), vagus motor neurons (vagusMN).

3.4.6 6dpf

At 6dpf, both the olfactory bulb (OB) and the sub-pallium (SubP) clearly express GFP, within the telencephalon (Fig. 3.24.F_{3-4})

In the diencephalon, the pineal gland (Pi) and habenula (Ha) still strongly express GFP, as the pretectum (Pr), thalamus (Tha) and posterior tuberculum (PTub). (Fig. 3.24.F_{1-3})

Within the mesencephalon, there is a locally GFP tectal expression in a few sparse cells in the stratum periventriculare (TeOsp) and medial band (TeOmb), in the neuropil (TeOn), posterior commissure (pc), and also in the tegmentum (Teg) and torus semicircularis (TS).

In the rhombencephalon, some cerebellar cells and neurons in the medulla oblongata, especially of the vagal region (and VagusMN) show GFP expression (Fig. 3.24.F_1).

Caudally, the musculature around the spinal cord is also labelled (Fig. 3.24.$F_{2,3}$).

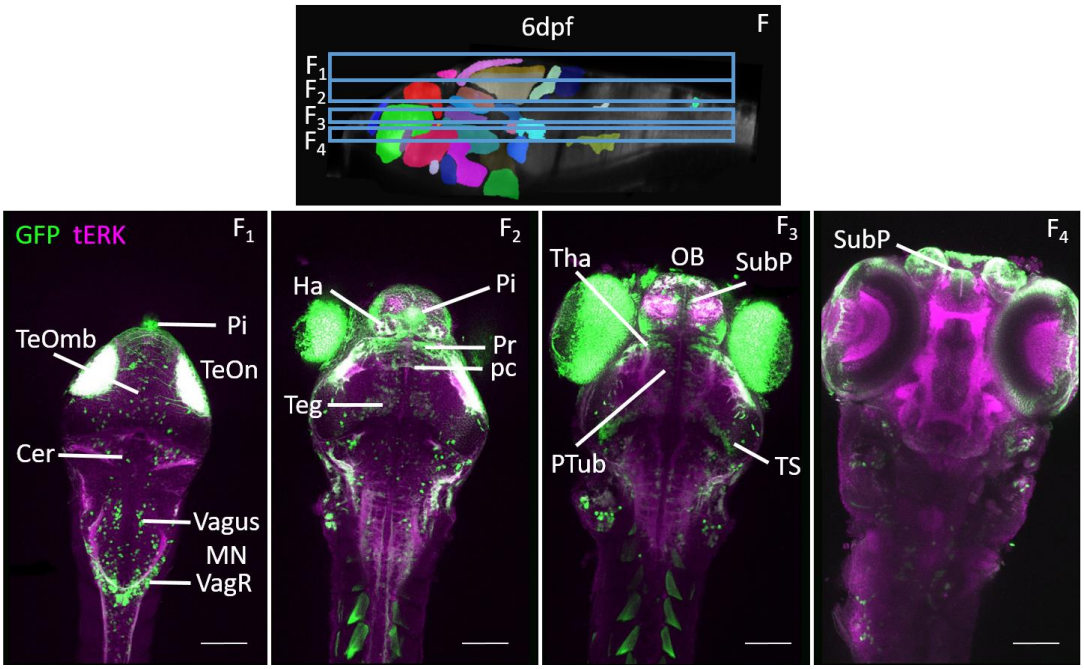

Fig. 3-24- GFP expression in 6dpf Slc18a3b GFF; UAS GFP zebrafish brain. F, sketch of 6dpf brain area shown in F_{1-4}, adapted from the zbb Atlas. F_{1-4}, z-projections of Slc18a3b GFF; UAS GFP 6dpf zebrafish brain after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). The scale bars indicate approximately 100 µm. Abbreviations: olfactory bulb (OB), sub-pallium (SubP), pineal gland (Pi), habenula (Ha), pretectum (Pr), thalamus (Tha), posterior tuberculum (PTub), stratum periventriculare (TeOsp), tectal medial band (TeOmb), tectal neuropil (TeOn), posterior commissure (pc), tegmentum (Teg), torus semicircularis (TS), vagal region (VagR) and vagus motor neurons (VagusMN).

3.4.7 Slc18a3b GFF; UAS GFP: GFP expression summary

Table 3-4- Table 3.4 - Brain regions showing GFP expression during development: summary of the GFP expression in the Slc18a3b GFF UAS GFP line, providing a list of anatomical regions and its abbreviations, excluding the primordial structures.

Anatomical region	Expression	Abbreviations
Telencephalon	Olfactory bulb, sub-pallium and anterior commissure.	ac; OB; SubP;
Diencephalon	Pineal gland, habenula; pretectum, thalamus, posterior tuberculum, preoptic region and hypothalamus.	Ha; Hy; oc; Pi; PO; poc; Pr; PTub; Tha;
Mesencephalon	Optic tectum neuropil and stratum periventriculare, medial band, posterior commissure, tegmentum (particularly torus semicircularis).	pc; Teg; TeO; TeOn; TeOsp; TeOmb; Ts;
Metencephalon	Cerebellum.	Cer;
Myelencephalon	Many neurons in the medulla oblongata, in the vagal region and particularly vagus motor neurons.	MO; VagR; VagusMn;
Retina	Retinal ganglion and amacrine cells.	AM; RGC.

3.4.8 Slc18a3b GFF; UAS GFP: GFP expression overview

In the first 2 days of development, GFP expression is observed in the developing telencephalon, diencephalon, and the mesencephalon.

Through development, GFP expression is widespread through both the olfactory bulb and sub-pallium, within the telencephalon, while in the diencephalon GFP expression is observed in the major anatomical regions, including in some groups of **pretectal cells**. Strong GFP expression is also observed in a characteristic fashion in the pineal gland and the habenula.

In the retina both the retinal ganglion and amacrine cells layers show a clear GFP signal.

Within the mesencephalon, during development GFP expression remains strongly present in the ventral tegmentum (where the **nMlf** is) but dorsally, on 6 dpf, is confined to some groups of cells of the optic tectum.

Caudally, in the rhombencephalon GFP expression is observed in the cerebellum and medulla oblongata, while in the latter it becomes increasingly localized in the vagal region through in development.

4 DISCUSSION

In this study, four GFP expressing transgenic zebrafish lines, chosen by their known expression in anatomical structures and neuronal subpopulations involved in visual processing, were characterized in the first six days of development through immunohistochemistry and confocal imaging. The GFP expression was then analyzed by identification of the anatomical localization of GFP expressing cells populations by comparison with 3-D information available in atlases and other studies on protein and gene expression. In addition, our analysis also identified some unknown structures that showed GFP expression during development.

In a general note, the GFF lines characterized in this study presented a spatially well spread GFP expression through the 3 brain subdivisions: prosencephalon, mesencephalon and rhombencephalon. All of them showed a solid GFP expression in several common areas like the tectum (mesencephalon), major anatomical diencephalic regions and medulla oblongata during the 6 days of development. They also showed a clear GFP expression in at least one retinal layer: RGC (Chat GFF), AC (Gad1b GFF) and both RGC and AC layers (Slc18a3b). No difference in pattern was observed along the dorso-ventral axis, GFP expression was spread from the dorsal (ie: optic tectum) to the ventral-most regions (ie: hypothalamus).

GFP expression was observed in the main axonal tracts and commissures, both in the telencephalon (anterior commissure) and diencephalon (post-optic commissure), and in the mesencephalon (posterior commissure). Likewise, we found that the medial longitudinal fascicle (mlf) largely presented GFP expression, its medial location being apparent comparing with the medial longitudinal catecholaminergic tract (mlct) in cases in which the anti-TH antibody was used. This was previously observed by *Kastenhuber et al.,* 2010 [13] until the 3dpf stage. As observed, both on Gad1b and ChAt GFF lines (Results section and Supplementary data), anti-TH expression did not overlap with GFP expression. Although some pretectal subpopulations are really close together, co-localization is not suggested by our data. From this, it is valid to assume that none of the subpopulations expressing GFP in both lines are dopaminergic nor noradrenergic (catecholaminergic system).

Generally, we can state that the GFP distribution observed in these GFF lines is mostly broader when compared to other Gal4 lines previously studied at 6dpf [15,16], which have more localized GFP expression within one or two subdivisions of the brain and in distinct circuits or populations (like the Pitx2c: GFP). And, as expected, all of the transgenic lines showed GFP expression in at least one anatomical structure or subpopulation involved in either the OMR or OKR.

Overall, temporal GFP expression was consistent through development in all transgenic lines, being always observed from 1dpf onwards (becoming broader with time). From our data we could only assume that the majority of the cells labelled had already some degree of differentiation/commitment but proliferative cells were also labelled, presumably, throughout the brain and developing retina at 1 and 2 dpf stages. (See Future perspectives)

4.1 PROBLEMS AND LIMITATIONS TO THE INTERPRETATION

One limitation to be taken in account is the variability between samples. Data shown in the Results section only represents a chosen sample representing each stage of each transgenic line. Although the GFP expression described was generally observed in all collected samples, there are always little differences in circuitry assembly and position of neurons in different individuals that should

be taken into account. One way of addressing this limitation is registering the different samples for the same stage of each line into a reference brain, thus mitigating some of the variability between fish. It is also important to refer that the samples were fixed in stages of days post fertilization and not hours post fertilization and, consequently, some discrepancies in development are to be expected within the same dpf stage.

The experimental setup was based on immunostaining the different staged fish with an anti-GFP antibody and imaging them on the confocal, but the anti-GFP was only used as a way of enhancing the signal of the reporter GFP. Consequently, other issues have to be taken into account: GFP has different properties when compared to mRNAs and other proteins. In particular, it has a life time that is usually bigger 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 (see below). Thus, when using GFP, the expression data may be elongated in terms of time, when compared to endogenous expression during development. Another factor worth mentioning is when we have GFP driven by a certain gene, the expression pattern is not necessarily the same as observed for that gene or the resulting protein. Although an effort was made to select the best imaging settings (laser power, gain), it remains possible that some differences in the levels of expression in different regions may not be detected with GFP.

On the other hand, low levels of GFP in regions where GFF expression is expected may result from silencing of the UAS-GFP.

Regarding the variability between antibodies, the most used anti-GFP (Rabbit) showed some divergent patterns in comparison with the anti-GFP (mouse). The expression of the latter was observed to be more localized and restricted, with slight anatomical differences. Indeed these antibodies are generated separately and may have slightly different characteristics. Alternatively, these discrepancies can also be due to variability in the stage of development or some particularities of immunohistochemistry experiments. (in Supplementary data)

Furthermore, the analysis was based on comparing the GFP expression of our data with anatomical reference brains and previous findings on gene and protein expression. Although orthogonal views and 3D projections of several samples were used for each stage and line, anatomical annotation is tentative. Once again, 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.

One approach to confirm the GFP expression and the related anatomical structures and neuronal subpopulations would be to perform more immunohistochemistry with antibodies specific to the proteins associated with the genes of interest. If antibodies against pitx2c, GABA, ChAt and slc18a3b are available, a next step would be co-labelling the transgenic fish with anti-GFP and the appropriate antibody. Furthermore it would be useful to perform, for example, immunohistochemistry with antibodies against several neurotransmitters in every stage of development, to compare the GFP expression with other well-known populations and circuits (as we did in some cases with the anti-TH to label the catecholaminergic system).

Another fact to be worth reinforced is the lack of information on previous bibliography regarding anatomical information and gene and protein expression in earlier developmental stages. Further, most of the consulted bibliography it was purely used for the purpose of visual comparisons and anatomy identification from other systems, thus it is not partially mentioned in the Bibliography section.

4.2 GFP EXPRESSION IN TRANSGENIC LINES AND "HOST" GENE EXPRESSION PATTERNS

The GFF transgenic lines were previously constructed by targeting one specific gene or genomic region as the driver of the GFP expression (GFF/UAS system), expecting GFP to recapitulate the expression pattern of the endogenous/"host" gene. The GFF was inserted in the initiation codon of the gene of interest, included in the BAC. Although is likely that the GFF is regulated by the regulatory regions of this gene, other genes are also contained in the BAC and genic relationships can change when the BAC is inserted randomly in the genome of the fish. Furthermore, the insert can be almost anywhere within the genome, thus the GFF may be regulated by other endogenous enhancers/regulatory regions. Below we compare the GFP expression from the lines with the available data on the expression of the endogenous genes.

4.2.1 Pitx2c: GFP

This transgenic line has the GFP associated with the gene codifying for the pitx2c isoform. From early in development 1 and 2 dpf, we observed GFP expression distributed through the diencephalon and the region of the nMlf, mlf and trigeminal ganglia. This expression pattern matches previous observations at 1dpf by Wolman, *et al.* 2008 [19], using this transgenic line.

However, in *Essner, et al.* 2000 [23], *in situ* hybridization showed *pitx2c* expression asymmetrically distributed in the right dorsal diencephalon between 1 and 2dpf, and area which was predicted to represent the future habenula. In our results no expression was observed specifically in the habenula, but some dorsal diencephalic regions (ie: the preoptic area and thalamus) did show symmetric GFP expression. This discrepancy can be due to the ease in detecting GFP, when comparing with gene expression via *in situ* hybridization. Additionally, the acquisition parameters used could lead to overexposing the GFP signal and thus lessening the asymmetry of expression.

4.2.2 ChAt GFF; UAS GFP

In the ChAt GFF line, the transcriptional activator GFF is inserted at the initiation ATG codon of the acetylcholine transferase genomic region. This line was expected to recapitulate the expression of the enzyme, however previous observations in the lab already suggested a discordant GFP pattern of expression.

According to previous bibliography, at 2dpf, *chat* gene (presumably isoform *a*) expression was observed within both the diencephalon and rhombencephalon [24]. These findings coincide partially with GFP expression of our data, observed within rhombencephalic and diencephalic regions, but not with the telencephalic and tectal GFP expression we also observed.

Chat gene (presumably isoform *a*) expression was previously assed by *in situ* hybridization from 2 to 5 dpf by *Arenzana et al.*, 2005 [25], showing expression through the optic tectum, tegmentum, preoptic area, medulla oblongata and retinal amacrine cells. Although GFP expression observed in our data overlaps with some anatomical structures described before, *chat* expression in *Arenzana et al.*, 2005 was only observed from 5 dpf in the optic tectum, and from 60 hpf in the tegmentum, preoptic area and medulla oblongata, respectively. In addition, our results show GFP expression in the retinal ganglion cell layer while in previous bibliography *chat* expression is only observed in the amacrine cells[25].

At 4dpf stage, unlike our results, *chat a* (choline O-acetyltransferase a) gene expression was previously observed by *Hong et al.*, 2013 in a very conspicuous pattern [26], only in some tectal cells, the

tegmentum and distinct groups of neurons in the medulla oblongata [Supplementary data], coinciding with GFP expression observed at 6 dpf in the chat: Gal4 line by *Forster et al., 2017* [16].

Altogether, these findings lead us to believe that the GFP expression pattern observed in the ChAt GFF; UAS GFP line does not reflect the acetylcholine transferase pattern of expression. This can be explained by the nature of the construction of the line: the BAC, including a genomic region of zebrafish chromosome 13 with part of the gene encoding ChAT as well as other seven known genes, is introduced randomly in the zebrafish genome. This particular BAC harbours the ChAt genomic region in its margin, thus it may not contain all the regulatory regions of the *chat* gene. Depending on the region in which it was inserted, the GFP expression can be driven by any 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 acetylcholine transferase expression pattern with that of other genes.

4.2.3 Gad1b GFF; UAS GFP

In the Gad1b GFF line, the GFF is inserted in the starting ATG of the *Gad1b* gene that encodes for one of the glutamate decarboxylases involved in the synthesis of the neurotransmitter GABA. GFP expression was expected to at least partly reflect GABAergic distribution patterns.

The expression of the neurotransmitter GABA was previously studied in 2 and 3 dpf stages in *Mueller et al.*, 2007 [12]. Like our results suggest, at 2 and 3dpf, GABAergic cells were found already widely distributed in the zebrafish brain: in the telencephalon (optic bulb and sub-pallium), diencephalon (habenula, pineal, pretectum, thalamus, posterior tuberculum, preoptic region and hypothalamus), mesencephalon (optic tectum, tegmentum: region of the nMlf and torus semicircularis) and rhombencephalon (cerebellum and medulla oblongata).

Although the majority of the GFP expression pattern observed in the Gad1b GFF 2dpf samples coincides with the previous findings, we observed GFP expression in the developing retina already at the 1 and 2dpf, among the proliferative progenitor cells, which will later differentiate in amacrine cells (as observed in our data and *Mueller, et al.* 2007 at 3dpf). This can be explained by the construction of the line: the GFF is associated with the *Gad1b* gene that encodes glutamate decarboxylase and, although it is involved in the synthesis of GABA, the pattern of expression is not necessarily the same as the neurotransmitter, being expressed earlier in development. On the other hand, it can also be explained by the fact that using GFP in characterization of expression can lead to overexpression patterns that do not fully coincide with the protein expression patterns, as the GFP is more stable. Again, the limitations of the staging of the samples should be taken into account.

In the peripheral olfactory epithelium GFP expression was not observed from the 2dpf onwards, as in *Mueller, et al. 2007*, however at 1dpf our data showed strong GFP labelling in the presumptive olfactory placodes. This can be just a transient expression that does not go on during development. However, more 1dpf brain data is necessary for comparison with our data before drawing concrete conclusions. Furthermore, compared our data with Gad1b gene transcript from previous bibliography confirming that it is likely the Gad1b GFF line reflects the pattern of expression of the endogenous gene[24,27].

In summary, from the comparison between the previous findings and our own data, we can conclude that the GFP expression observed in the Gad1b GFF line likely reproduces a GABAergic cells (and Gad1b) pattern of expression

4.2.4 Slc18a3b GFF; UAS GFP

In the Slc18a3b-GFF line, GFF is inserted in the ATG starting codon of the solute carrier family 18 member 3 gene, coding the vesicular acetylcholine transporter b, which is responsible for making acetylcholine available for secretion.

In *Hong, et al.* 2013 [26], the expression of the gene *vachtb* (previous name for *slc18a3b*) was observed by *in situ* hybridization in 3 and 5dpf larvae. At 3dpf, *vachtb* expression was found in some tectal cells, distinct subpopulations in the hindbrain and asymmetrically distributed in the right habenula. In turn, in 3dpf Slc18a3b fish, GFP expression was observed in the sub pallium, pineal gland, tectum and tegmentum, some cells in the hindbrain, but not in the habenula. Our results on 3dpf fish thus do not match the findings by Hong, *et al. 2013*.

At 5dpf, *in situ* hybridization of *vachtb* revealed localized expression in the tegmentum, expression in some tectal cells, diencephalon and medial region of the hindbrain, and asymmetric expression in the right habenula. On the other hand, at 5dpf we found symmetric GFP expression in the habenula, through the sub pallium and optic bulb, tectum and tegmentum, major diencephalic anatomical subdivisions and in several rhombencephalic subpopulations distinct from the ones labelled in Hong, *et al. 2013*.

Although, GFP was observed in some anatomical regions that coincide with those that have been shown to have *vachtb* expression, we can conclude that the GFP expression of the Slc18a3b GFF line does not recapitulate the expression pattern of this gene. Again, this can be due to the way the line was constructed, i.e. the GFP expression can be driven by other regulatory regions within the BAC construct or in the region were the BAC was inserted in the genome. In addition, the immunohistochemistry experiments done in this particular transgenic line resulted in really bright auto fluorescence which could induce us to interpret a GFP expression broader than the actual reported expression pattern (i.e. in the olfactory bulb and habenula).

4.3 FUTURE PERSPECTIVES

Concerning the limitations in the utility of the general anatomical labelling, one future aim is to analyse specific subpopulations or subgroups of neuronal cells within the large groups of cells labelled in each transgenic line in more detail. To address this matter, besides the aforementioned co-labelling with other antibodies or performing *in situ* hybridization against mRNAs of interest, several methods should be considered like microinjections with UAS GFP (or UAS LynmCherry), without crossing the GFF line with the UAS GFP.

An alternative method would be to use the photo-conversion properties of the fluorophore Kaede by crossing the GFF line with carriers of the UAS:Kaede[28]. This photo-convertible fluorophore emits either green fluorescence (without conversion) or red fluorescence (after conversion with UV light). Another possible approach is using the highly variegated *UAS:mGFP* contained within the *Brn3c:Gal4*, *UAS:mGFP* (BGUG) transgene by crossing the GFF line with the BGUG transgenic line. This would result in the labelling of a small subset of GFF expressing cells.[29]

Furthermore, it would be interesting to cross our spatial and temporal data on GFP expression of the lines with information concerning neuronal differentiation state. To further address this matter it would be useful to study expression of neurogenic genes (i.e. Notch- a, deltaA), through *in situ* hybridization, or by performing an immunostaining against proliferating markers like proliferating cell nuclear antigen (PCNA), bromodeoxyuridine (BrdU) or Hu-proteins (marker for early neuronal differentiation). [6,30]

The most pressing future aim is the registration of the different samples into a reference brain. In the majority of the samples we used an anti-tERK that can function as a common channel to register the GFP channel of the different brains into an average representation. Thus, confocal imaging of more samples of the same (and possibly other) transgenic lines would be a very useful step towards registration. In some of the samples, successful registration was already performed using the Advanced Normalization Tools (ANTs)[15,31], further reinforcing the viability and power of this ongoing work.

4.4 CONCLUSION

The use of transgenic GFP and GFF lines proved to be a successful approach to characterize neuronal subpopulations involved in visual processing through the first six days of development. Despite the limitations inherent to the construction of the lines and consequently of the interpretation of the observed GFP expression patterns, we believe we could identify and anatomically characterize, with some confidence, many interesting GFP expressing neuronal populations. Furthermore, we consider that our work may be used to contribute to a better understanding on the acquisition of neuronal cell fates and connections, particularly in circuits involved in visual processing, during early stages of development. In addition, we believe that it can be used as a reference to better design future experiments requiring the manipulation of specific subpopulations and single neurons with the appropriate UAS constructs.

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