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Formation of the posterior commissure in the developing zebrafish embryo

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Resumo

O sistema nervoso central está envolvido na sobrevivência e nas respostas comportamentais de animais a estímulos internos e externos. É composto por circuitos neuronais, constituídos por neurónios com identidades moleculares e conectividade específicas, estabelecidas durante o desenvolvimento embrionário. O estudo destes processos beneficia do uso de animais modelo, uma vez que a anatomia e as vias moleculares são altamente conservadas em vertebrados.

O peixe-zebra (*Danio rerio*) é um organismo modelo usado, por exemplo, em biologia do desenvolvimento e neurociências. Apresenta semelhanças genéticas com outros vertebrados e um cérebro de vertebrado estereotipado. O peixe-zebra permite a criação de linhas transgénicas, que expressam proteínas fluorescentes, ideais para o estudo do desenvolvimento de circuitos neuronais.

O desenvolvimento do sistema nervoso em vertebrados inclui processos coordenados no espaço e no tempo: indução neuronal, regionalização, neurogénese, migração e diferenciação celulares, axonogénese e sinaptogénese. Durante a fase de gástrula, as camadas germinativas primárias (endoderme, mesoderme e ectoderme) são formadas e os eixos embrionários principais são estabelecidos. Na ectoderme, o organizador induz tecido neuronal.

O eixo rostral-caudal subdivide o sistema nervoso em prosencéfalo, mesencéfalo, rombencéfalo e medula espinal. O prosencéfalo subdivide-se em prosencéfalo secundário e diencéfalo. O diencéfalo subdivide-se ainda em três prosómeros. O prosómero 1 (p1) origina estruturas pré-tectais, o prosómero 2 (p2) origina estruturas talâmicas e o prosómero 3 (p3) origina derivados pré-talâmicos.

Os circuitos neuronais ligam células dentro e entre regiões do cérebro, permitindo a comunicação entre zonas do sistema nervoso. A axonogénese é o processo pelo qual os axónios se desenvolvem, atingindo células-alvo, recorrendo a sinais de orientação, e dão origem aos circuitos neuronais. O cone de crescimento, na ponta dos axónios, controla a direção de projeção. Os axónios podem ser classificados como pioneiros, originando os tractos iniciais ou como seguidores, projetando-se ao longo destes tractos.

Os tractos podem ser longitudinais, conectando as regiões rostral e caudal ou comissurais/transversais, conectando as regiões esquerda e direita, cruzando a linha média dorsal ou ventralmente. Vários tractos e grupos de neurónios iniciais são conservadas em todos os vertebrados: comissura anterior, tracto da comissura posterior (TPC), comissura ventral, grupo longitudinal ventral (composto pelo fascículo longitudinal médio (MLF) e pelo tracto da comissura pós-óptica).

A comissura posterior é um trato axonal conservado nos vertebrados, que liga os hemisférios esquerdo e direito do cérebro, na região diencéfalo-mesencéfalo. Esta comissura parece formar-se como uma extensão do TPC. Estudos em amostras fixas determinaram o início da sua formação entre as 20 e 24 horas pós-fertilização (hpf). O grupo de neurónios ventral caudal (vcc) e o núcleo da comissura posterior contribuem com projeções para o TPC. Esta estrutura foi associada à integração binocular e comunicação inter-hemisférica, no peixe-zebra.

A linha transgénica Lx200 é expressa em várias subpopulações neuronais. O vcc e o TPC estão marcados nesta linha transgénica, desde o início do seu desenvolvimento. Isto torna a linha transgénica Lx200 uma excelente ferramenta para o estudo da formação do TPC e dos neurónios que para ele contribuem.

Imunohistoquímica foi utilizada para detetar a expressão de proteínas, utilizando anticorpos primários e secundários com marcadores fluorescentes, detetados durante a imagiologia. Através do uso do anticorpo anti-HuC/D, todos os neurónios presentes foram detetados, determinando-se que os neurónios marcados na linha transgénica Lx200 são um subgrupo do vcc, referido como Lx200-vcc.

A técnica de hibridação *in situ* permite caracterizar a identidade molecular de subpopulações neuronais,

avaliando a presença de RNAs específicos e, portanto, a expressão dos genes correspondentes. Para determinar com precisão a localização do vcc e do TPC usámos esta técnica, observando a expressão de mRNAs em amostras de Lx200, 1 dia pós-fertilização (dpf). Os marcadores examinados correspondem aos genes *her5*, *nkx2.2a*, *arxa*, *irx1b*, *olig3* e *pax6a*, que marcam apenas progenitores ou progenitores e neurónios.

Os resultados mostraram que as estruturas estão localizadas ventralmente, rostrais ao rombencéfalo. É provável que o TPC esteja localizado no diencéfalo, dentro do *pretectum* (derivado p1 dorsal). O Lx200-vcc pode ser considerado como tendo duas regiões, a mais rostral localizada no diencéfalo, provavelmente no p1 basal, e a mais caudal, provavelmente localizada no mesencéfalo.

A linha transgénica Lx200:GFF,UAS:Dendra2, possui um repórter irreversivelmente fotoconvertível, Dendra2, que permite selecionar e identificar células e as suas projeções.

Para determinar quais dos neurónios Lx200-vcc contribuem para o TPC, fotoconvertemos neurónios individuais, com base na proximidade do TPC. Pudemos concluir que: algumas células Lx200-vcc projetam ao longo do TPC, neurónios rostrais Lx200-vcc contribuem para o TPC e alguns dos neurónios Lx200-vcc projetam ao longo do MLF, provavelmente as células caudais. Os nossos dados são compatíveis com duas possíveis regiões de Lx200-vcc, uma rostral, projetando ao longo do TPC e uma caudal, contribuindo para o MLF.

A localização dos neurónios Lx200-vcc em fases larvares (3 e 4 dpf) foi determinada recorrendo a fotoconversão. As suas projeções apresentavam uma localização perto da linha média dorsal, na região p1-mesencéfalo. Os somas foram visíveis na região p1-mesencéfalo. Esta é uma localização semelhante à determinada a 1 dpf. O atlas cerebral de peixe-zebra, Mapzebrain, foi utilizado como referência para identificar a localização de somas de neurónios que cruzam a linha média na zona correspondente à comissura posterior. Estes dados confirmaram as nossas observações, a maioria dos neurónios identificados têm somas localizados na região p1-mesencéfalo.

Através de ensaios de imunohistoquímica, recorrendo ao anticorpo anti-tubulina-acetilada, marcador de axónios, foi possível determinar que os neurónios Lx200-vcc são pioneiros na formação do TPC.

Obtivemos imagens, recorrendo a microscopia *lightsheet* e confocal, da formação do TPC em embriões vivos da linha transgénica Lx200, que expressam o repórter fluorescente mNeonGreen. Tal permitiu uma análise qualitativa e quantitativa do processo.

A extensão dos primeiros axónios que projetam ao longo do TPC ocorreu entre as 20 e as 25 hpf. Após este período, as projeções ventrais-dorsais dos lados esquerdo e direito entraram em contacto, na região da linha média dorsal.

A extensão global das projeções resultou de uma combinação de eventos locais de extensão, retração e paragem. Eventos de retração tinham já sido reportados em neurónios pioneiros de outras estruturas.

A conexão entre projeções ocorreu entre os cones de crescimento ou entre o cone de crescimento e o axónio contralateral. A "hipótese do aperto de mão" parece estar a descrever um processo semelhante. Nesta hipótese, cones de crescimento de tipos de neurónios distintos interagem e prosseguem o crescimento em direções opostas, ao longo do axónio contralateral.

O software de análise de imagens, Imaris, foi usado para traçar e seguir o percurso das projeções axonais ao longo do tempo. Os comprimentos de projeções que antecederam a conexão foram semelhantes entre as amostras, as projeções seguiram caminhos semelhantes, sem curvar, para atingir a linha média. A velocidade foi semelhante entre os lados direito e esquerdo e entre as amostras, não sendo registado aumento ou diminuição da velocidade com o tempo.

Estudos futuros deverão focar-se nos mecanismos moleculares responsáveis pela formação do TPC, atualmente desconhecidos, recorrendo a estudos genéticos e do transcriptoma.
Em suma, obtivemos uma caracterização aprofundada do desenvolvimento inicial do TPC e dos seus neurónios pioneiros. Determinámos a localização em fases embrionárias e larvares e descrevemos qualitativa e quantitativamente o processo de formação desta estrutura.
O conhecimento aprofundado do processo de axonogénese em estruturas conservadas em vertebrados, como a comissura posterior, poderá ter impacto terapêutico e no diagnóstico. Tal deve-se ao facto de pequenos defeitos durante a formação de circuitos neuronais terem grandes impactos no desenvolvimento. Por exemplo, em humanos, problemas de conexão axonal foram associados a doenças neurológicas, como o autismo.

Palavras-chave: circuitos neuronais; formação da comissura; peixe zebra

Abstract

We aimed to study the development of the tract of the posterior commissure (TPC) in the early zebrafish embryo. Even though TPC anatomy has been described, its development has yet to be explored. The TPC is conserved in vertebrates and connects the right and left parts of the brain, in the diencephalon-mesencephalon region. In the zebrafish, it has been linked to interhemispheric communication and binocular integration. The Lx200 transgenic line, created in the laboratory, was used, since it expresses fluorescent proteins in neuronal subpopulations, including in the TPC and the ventral caudal cluster (vcc), whose neurons project along this tract.

We determined that the Lx200 transgenic line labels a subcluster of the vcc, the Lx200-vcc, with projections pioneering TPC development, at around 20 hpf. The location of the Lx200-vcc was determined as ventral, in the prosomere 1 (p1)-mesencephalon region, while proper TPC was located in the p1. *In situ* hybridization assays to observe the known expression pattern of genes allowed for this localization. At larval stages, the TPC location was similar, close to the dorsal midline in the p1 region, while somas were located in the p1-mesencephalon region, both were determined via photoconversion of the Dendra2 protein.

Dendra2 photoconversion also allowed for the determination of Lx200-vcc cells' contribution to the tracts. Rostral Lx200-vcc cells project along the TPC, but not to the medial-longitudinal fascicle (MLF), while caudal Lx200-vcc cells likely contribute to the MLF.

Whole mount *in vivo* lightsheet and confocal imaging allowed for a qualitative and quantitative description of the process of TPC formation. We utilised the Imaris image analysis software to trace and track the projections in time. TPC development occurred via non-turning, branching projections, in a ventral to dorsal direction. Connections occurred via the contact of opposing growth cones or by growth cone-axon connections.

We provide a detailed characterization of the Lx200-vcc neurons and of TPC development. Our work contributes to a deeper understanding of axonogenesis, a major step in central nervous system development, in a whole embryo context. Future work will focus on the molecular pathways responsible for TPC development.

Keywords: neuronal circuits; commissure formation; zebrafish

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Abbreviations List

AC - Anterior Commissure
ADt - Anterior Dorsal telencephalic neurons
ap - alar plate
BAC - Bacterial Artificial Chromosome
bp - basal plate
bp1 - basal prosomere 1
bp2 - basal prosomere 2
bp3 - basal prosomere 3
C - Caudal
CNS - Central Nervous System
D - Dorsal
d - diencephalon
dpf - days post fertilisation
drc - dorsal rostral cluster
DTmesV - Descending Tract of the mesencephalic nucleus of the trigeminal nerVe
DVDT - Dorsal Ventral Diencephalic Tract
E - Epiphysis
F - Forebrain
fp - floor-plate
HCRv3.0 - Hybridization Chain Reaction
hpf - hours post fertilisation
L - Left
M2 - early Migrated posterior tubercular area (preglomerular complex)
Mes - Mesencephalon
MLF - Medial Longitudinal Fascicle
mrb - mesencephalon-rhombencephalon boundary
nTPC - nucleus of the Tract of the Posterior Commissure
nTPOC - nucleus of the Tract of the Post Optic Commissure
nucMLF - nucleus of the Medial Longitudinal Fascicle
p1 - prosomere 1
p2 - prosomere 2
p3 - prosomere 3
PAG - PeriAqueductal Grey
PC - Posterior Commissure
Pt - Pretectum
Pth - Prethalamus
R - Rostral
Rh - Rhombencephalon
Ri - Right
SOT - SupraOptic Tract
Th - Thalamus
TPC - Tract of the Posterior Commissure
TPOC - Tract of the Post Optic Commissure

UAS - Upstream Activating Sequence
V - Ventral
VC - Ventral Commissure
vcc - ventral caudal cluster
Ve - prosencephalon Ventricle
VLT - Ventral Longitudinal Tract
vrc - ventral rostral cluster

1. Introduction

1.1. Zebrafish as a model organism in neuroscience and developmental biology

The central nervous system is involved in animals' survival and behavioural responses to internal and external stimuli. It is composed of neuronal circuits, constituted by neurons with specific molecular identities and connectivity, established during embryonic development. Structure and developmental molecular pathways are highly conserved in vertebrates, supporting the use of model organisms in the study of vertebrate neuronal circuit development[1].

The zebrafish (*Danio rerio*) is used as a model organism in distinct scientific fields, such as developmental biology, neuroscience, and cancer research, sharing genomic similarities with other vertebrates, such as mouse (*Mus musculus*), chicken (*Gallus gallus*) and human. Around 70% of human genes have at least one zebrafish orthologue[2].

Zebrafish is a teleost, cyprinid fish, from Southern Asia. Its biological characteristics make it a valuable model in research. It is easy to maintain, breed, and adapts well to different water conditions. Its transparent body and external embryonic development allow for accessibility during experimentation and imaging[3].

The zebrafish life cycle comprises several stages: embryonic, larval, juvenile, and adult. At 4 days post fertilisation (dpf), the larval form is very similar to the adult. At 7 dpf, the brain corresponds to a stereotypical vertebrate brain, including anatomical regions, neuronal circuits and cell classes and presents robust behaviours. At embryonic and larval stages, until 6 dpf, its small brain size[4] and lack of skull allow for whole-brain live imaging.

A large number of techniques and tools have been developed for zebrafish such as the use of transgenic lines that allow for the expression of fluorescent proteins, to further study neuronal circuit development. Notably, the Gal4-UAS system is a powerful genetic tool in the zebrafish model[5].

1.2. Early development of the zebrafish brain

Vertebrate nervous system development includes processes coordinated in time and space: neural induction, regionalization, neurogenesis, cell migration and differentiation, axonogenesis and synaptogenesis.

Zebrafish embryonic development is rapid and includes distinct phases[6]: zygote (0-0.75 hours post fertilisation (hpf)), cleavage (0.75-2.25 hpf), blastula (2.25-5.25 hpf), gastrula (5.25-10 hpf), segmentation of paraxial mesoderm into somites (10-24 hpf) and pharyngula (1-2 dpf)[6]. The first neurons are formed during gastrulation and, by 1 dpf, embryos have spontaneous contractions. Touch stimuli response develops at 2 dpf, and 7 dpf zebrafish larvae display innate behaviours. Hatching occurs spontaneously between 2 and 3 dpf[6].

1.2.1 Neural induction

During the gastrula stage, the primary germ layers (endoderm, mesoderm, and ectoderm) are formed, and the main embryonic axes are established[6].

Neural induction is the process of specification of neuroectoderm and involves the action of several tissues and signals on the ectoderm[7]. One of these tissues is known as the organiser, which induces neural tissue in the surrounding ectoderm[7].

Neural induction in the ectoderm happens via the activation of signalling pathways triggered by activators and inhibitors secreted by the presumptive mesoderm (e.g. BMP antagonists, Wnt and Fgfs) or intrinsic to the ectoderm (e.g. Sox)[7].
BMPs block neural induction, inducing an epidermal fate. In the pre-organizer region, which later becomes the shield organiser, Noggin and Chordin are produced, these are antagonists of BMP and act to establish the neural fate in the ectoderm, which results in the formation of the neural plate[7]. The inhibition of BMP is necessary for the formation of the neural plate, but not sufficient. Other molecules need to be present for the neural plate to be formed, such as Cerberus, secreted by the anterior endoderm or Fgfs, secreted by the blastoderm margin[7]. However, low levels of BMPs can maintain brain regions, such as the telencephalic fate in the neural plate developmental stage[7]. The neural tissue formed by Noggin and Chordin has anterior/prosencephalon character, to induce posterior identity, the organiser secretes Wnts, Fgfs and retinoic acid[8].

1.2.2. Neurulation

The neuroectoderm is part of the ectoderm, the embryonic germ outer layer. During its specification, the neuroectoderm becomes a pseudostratified thickened epithelial structure known as the neural plate[7]. At 12 hpf, during somitogenesis, primary neurulation, i.e., the formation of a neural tube from a plate, takes place. In most vertebrates, neurulation involves the invagination of the neural plate, with fusion of the lateral edges to create a hollow tube. However, in zebrafish, this process occurs differently and involves the formation of an intermediate structure, the neural keel, which fuses at the dorsal midline, forming the neural rod[7]. The neural rod then forms a neurocoele, which later gives rise to the brain ventricles and the central canal in the spinal cord.
Before neural tube formation, the regional morphogenesis process is initiated, subdividing the neural structures into domains along the rostral-caudal axis. At 16 hpf, the three rostral swellings develop, corresponding to telencephalon, presumptive diencephalon, and presumptive mesencephalon[6]. Additional caudal swellings are visible at 18 hpf, corresponding to the presumptive rhombomeres[6]. At the region of rhombomere 1, near the rhombencephalon-mesencephalon boundary, the cerebellum primordium develops at 19 hpf. At 19 hpf, the primordium of the central nervous system (CNS) has been delineated[6].

1.2.3. Regionalization

Early regionalization of the neural plate and the early neural tube involves heterogeneous expression of transcription factors along the rostral-caudal and dorsal-ventral axis. The rostral-caudal axis subdivides the nervous system into forebrain/prosencephalon, midbrain/mesencephalon, hindbrain/rhombencephalon and spinal cord[9] (**Fig.1.1**).
Further regionalization, relying on secondary organising centres, subdivides the major domains. For example, the prosencephalon is subdivided into the secondary prosencephalon and the diencephalon. The diencephalon is further subdivided into three prosomeres. Prosomere 1 (p1) gives rise to pretectal structures, prosomere 2 (p2) gives rise to thalamic structures and prosomere 3 (p3) gives rise to prethalamic derivatives[9]. The dorsal-ventral axis creates the alar/dorsal and basal/ventral regions[7]. The expression of transcription factors related to regionalization will contribute, in a combinatorial way, to the establishment of the identity of the neurons.

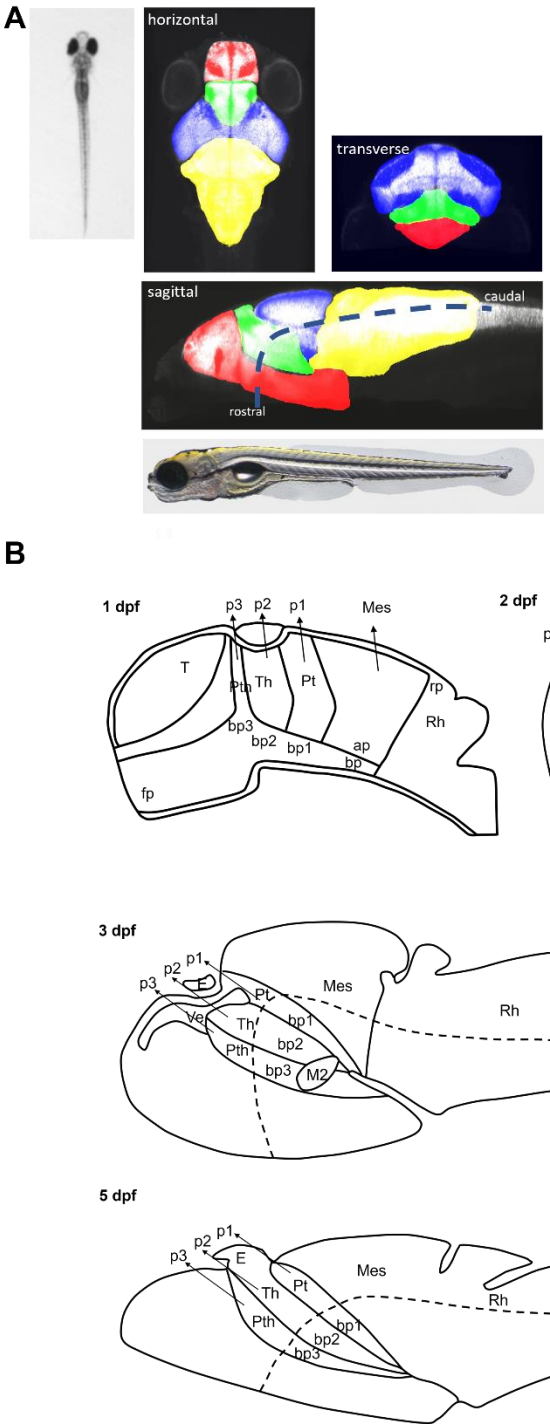

Figure 1.1: Prosomere Model for Vertebrate Brain Regionalization in Zebrafish. A. Larval zebrafish example images and zebrafish brain regionalization model. Horizontal, transverse, and sagittal views. Secondary prosencephalon: red. Diencephalon: green. Mesencephalon: blue. Rhombencephalon: yellow. **B.** Brain schematics in lateral/sagittal view for zebrafish at 2, 3 and 5 dpf, Wullimann and Puelles Model[9]. ap: alar plate, bp: basal plate, bp1-bp3: basal regions of prosomeres 1-3, E: epiphysis, M2: early migrated posterior tubercular area (preglomerular complex), Mes: mesencephalon, p1-p3: prosomeres 1-3, Pt: pretectum, Pth: prethalamus, Rh: rhombencephalon, Th: thalamus, Ve: prosencephalon ventricle. Adapted from Hauptmann *et al*., 2002[10] and Wullimann, 2022[9].

1.2.4. Neurogenesis

Neurogenesis refers to the generation of neurons, involving their exit from the cell cycle to give rise to postmitotic neurons. It is highly regulated in space and time[7].
During embryonic development, the molecular identities and connectivity of neurons are established. Undifferentiated neural progenitors initially divide, giving rise to 2 progenitors, creating a larger pool of progenitor cells. Depending on time and location, they can divide differently, giving rise to one progenitor and one neuron (asymmetric division) or 2 neurons. They also give rise to glial cells. The asymmetric cell fate has been linked to location asymmetry. Apical cells differentiate into neurons, having lower-notch activity, while basal cells remain as progenitors[7]. Neurogenesis is initiated during gastrulation, via the expression of proneural genes, which encode transcription factors, such as the basic helix-loop-helix class[7]. After cell cycle exit, early neurons are apt to migrate, interact with other cell types and create projections, depending on their identity.
In zebrafish, during primary neurogenesis cells exit the proliferating cell cycle and differentiate into neurons[7]. The first neuronal clusters are formed in the telencephalon, hypothalamus, and tegmentum regions, creating a primary scaffold of axonal tracts and commissures, connecting brain regions[1].
Secondary neurogenesis is characterised by an increasing number of neurons from each category, along with glia production. Transient primary neuronal populations are replaced, and higher-order circuits are developed, allowing for finely modulated behaviour.

1.2.5. Determination of neuronal identities

Neuronal progenitor cells give rise to diverse neuronal populations and glial cells, through processes of proliferation, diversification and terminal differentiation[11]. The establishment of neuronal identity is initiated during the regionalization of the proliferating progenitor domain. Once postmitotic, neurons continue to refine their identity under the influence of transcription factors and signalling pathways.
During the development of the central and peripheral nervous systems, progenitor cells and early post-mitotic cells express different transcription factors, resulting in the differentiation of various neuronal cell types (local signalling)[12]. Transcriptional changes, determinants of cell fate, are the result of inductive signals. Inductive signals are present in the nervous system at different developmental stages, in distinct regions. They affect transcription in progenitor cells and post-mitotic neurons in various ways. This means differentiation is position and time specific. Several transcription factors are necessary to establish each neuron class, this combination is referred to as the transcriptional code[12].

1.2.6. Formation of neuronal circuits in development: axonogenesis

Neuronal circuits connect cells within and across different brain regions, allowing for communication throughout the CNS. Axonogenesis is the process through which axons develop to reach target cells, guided by cues present in the microenvironment, creating the neuronal circuits.
The growth cone, at the tip of axons, controls their outgrowth. It is highly motile, senses environmental cues and transduces signals, being responsible for axonal guidance. The extracellular cues trigger signalling cascades, which reach the cytoskeleton[13]. Cytoskeleton changes, such as actin polymerization and depolymerization influence growth cone progress. The axonal cytoskeleton is made up of neurofilaments, microtubules and actin filaments. In the growth cone, the actin filaments are the main component, they are

organised into a loose mesh, from which filopodia elongate and retract, permitting the movement of the axon[13].

Neurons with developing axons can be classified as pioneers or followers[14]. Pioneer axons develop in the brain in an axon-free environment, projecting from discrete neuronal clusters, formed during primary neurogenesis. They rely on long and short-range guidance cues, sensed by their growth cones, to create axonal tracts. Follower axons grow along the pre-existing tracts (**Fig.1.2**).

Pioneer and follower axons have different growth cone characteristics (morphology, behaviour and actin dynamics)[15]. Pioneer neurons have slower diffusion of molecules in the growth cone, likely due to cytoskeletal differences, mediated by actin[16].

Pioneer axons slow down their growth at the midline region, a behaviour that followers do not display. When growing along a contralateral neuron, pioneer axons change their midline kinetics. Follower axons behave as pioneers when these are ablated. This means that exposure of the growth cone to midline cues and the presence of other axons impact axonal kinetics[17]. When pioneer neurons are ablated, the guidance of the follower axon is affected, delaying or resulting in incorrect routes, but it does not always prevent follower axon projection[18].

Although not the focus of the current study, dendritogenesis occurs through extension and branching, mostly after axonal growth. Primary dendrites extend from the cell body to create the dendritic arborization[19].

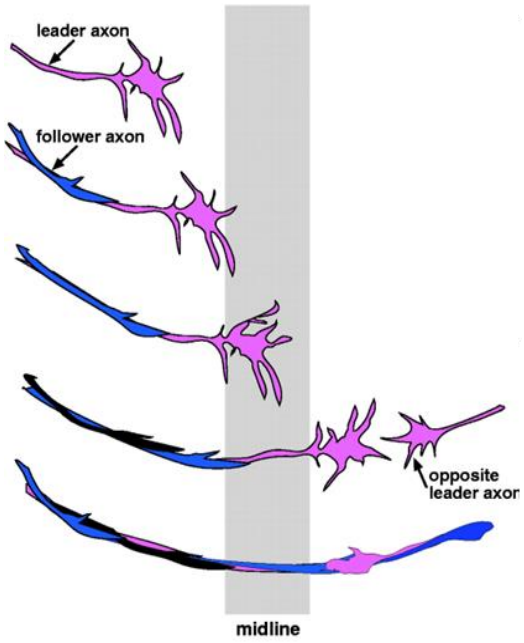

Figure 1.2: Schematics of the leader/pioneer and follower neurons. Schematic illustration showing a leader axon (pink) and follower axons (blue and black) projecting through the ventral midline, forming the post optic commissure, in zebrafish. Adapted from Bak and Fraser, 2003[17].

1.2.7. Early tracts of the vertebrate central nervous system

During CNS development, the early axon scaffold is formed, connecting brain regions through longitudinal and commissural/transverse tracts. Longitudinal tracts connect the rostral and caudal regions, while commissural/transverse tracts connect the left and right brain regions, crossing the midline ventrally or

dorsally. In anamniotes (fish and amphibians), the early scaffold is composed of commissural/transverse tracts: anterior commissure (AC), tract of the posterior commissure (TPC), ventral commissure (VC), supraoptic tract (SOT) and dorsoventral diencephalic tract (DVDT); and longitudinal tracts: tract of the post optic commissure (TPOC) and medial longitudinal fascicle (MLF)[1] (**Fig.1.3**). Several early scaffold structures are conserved in all vertebrates: AC, TPC, VC and ventral longitudinal tract (VLT) (made up of MLF and TPOC)[1].

The neurons that give rise to the early scaffold differentiate at specific positions and developmental stages. They are part of neuronal clusters located in different areas of the brain. In the zebrafish, the ventrocaudal cluster (vcc), the ventral rostral cluster (vrc)/nucleus of the TPOC (nTPOC) and the dorsal rostral cluster (drc)/anterior dorsal telencephalic (ADt) neurons are the clusters contributing to the early scaffold[20,21].

In zebrafish, early scaffold development begins with projections from the vcc, at around 16 hpf (contributing to the MLF) and is formed by 24 hpf. The established pathways are retained during development[22,23].

Commissures, part of the early axon scaffold, connect both sides of the brain and are essential for communication between areas, crossing the midline in different brain regions. While in anamniotes, the commissures form during early axon scaffold development, in amniotes most commissures are formed later in development. This variation in timing might be due to different interhemispheric communication needs[1].

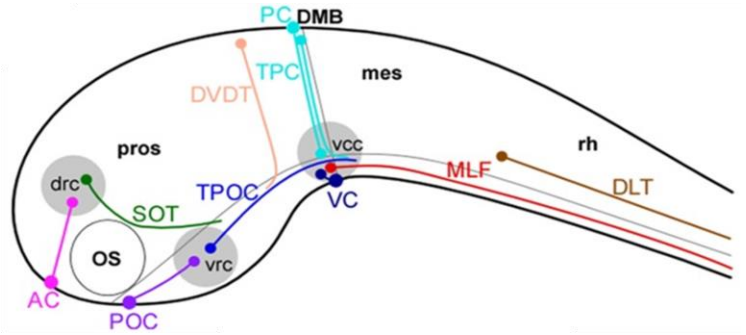

Figure 1.3: Schematics of the established early axon scaffold in the vertebrate brain of zebrafish at 24 hpf. Adapted from Ware *et al.*, 2015[1]. AC: anterior commissure, DLT: dorsolateral longitudinal tract, DMB: diencephalic-mesencephalic boundary, drc: dorsal rostral cluster, DVDT: dorsoventral diencephalic tract, mes: mesencephalon, MLF: medial longitudinal fascicle, os: optic stalk, PC: posterior commissure, POC: post-optic commissure, pro: prosencephalon, rh: rhombencephalon, SOT: supraoptic tract, TPC: tract of the posterior commissure, TPOC: tract of the post-optic commissure, VC: ventral commissure, vcc: ventral caudal cluster, vrc: ventral rostral cluster.

1.3. The posterior commissure and the tract of the posterior commissure

The posterior commissure (PC) is an axonal tract conserved in vertebrates that connects the left and right hemispheres of the brain, in the diencephalon-mesencephalon region. This commissure appears to form as an extension of the TPC. Studies done in fixed zebrafish samples described the TPC as beginning formation between 20 and 24 hpf[20,22]. Cell clusters contribute projections to the TPC, the vcc (projecting dorsally) and the nucleus of the posterior commissure (or nTPC)[22]. The nTPC is a broad category of neurons, located along the TPC that contribute to it, projecting dorsally or ventrally. However, as the TPC is a large tract, there might be axons from neurons of other clusters contributing to it. TPC projections have growth cones with complex morphology and many filopodia.

In zebrafish, the TPC has been proposed to be involved in interhemispheric communication important for binocular responses[24]. Direct retinal input to the zebrafish brain is monocular, to the contralateral side to the

eye, however, some pretectal neurons display binocular responses (i.e., to stimulus presented to both eyes). Thus, they must indirectly receive input from the ipsilateral eye. An example of the importance of excitatory and inhibitory signals crossing the midline is presented by Nauman *et al.*, 2016[24], in which both types of signals play a role in the response to different visual stimuli. The TPC is an anatomical candidate for these communication events, as it is located in the diencephalon-mesencephalon region.
The importance of the TPC in binocular integration and interhemispheric communication, both in neuron response and behaviour, has been described using laser ablation experiments. They found that its ablation disrupted binocular integration[24]. In rodents, it has been proposed to carry motion information[25]. In mammals, it has been described that the TPC is involved in consensual pupillary reflex[26]. Therefore, the TPC is of interest in both neuronal circuit formation and behavioural neuroscience.

1.4. The Lx200 transgenic line for labelling neuronal populations

One of the technical advantages of the use of the zebrafish model system is the ability to generate and use transgenic lines, allowing for the expression of reporter genes, such as those encoding fluorescent proteins, in neural subpopulations. The use of the Tol2 transposon allows for the insertion of a DNA fragment of interest (usually an artificial recombinant DNA molecule, flanked by Tol2-transposon sites) into the genomic DNA of germline cells so that it can later be transmitted to its descendants. This is achieved by injection of embryos at the 1-2 cell stage[5,27]. A common method used for the expression of reporter genes in specific cells is the Gal4-UAS system. This method uses two types of DNA insertions, one encoding the yeast transcriptional activators, Gal4 under the control of a gene regulatory sequence, and another encoding the selected reporter gene (i.e. GFP). The reporter gene is under the control of the Upstream Activating Sequence (UAS), which is activated directly by the Gal4 protein[5]. A variation of this method is the use of the GFF Gal4 variant, containing the Gal4 DNA binding domain and two VP16 short activation motifs[5]. This variant shows no toxicity, contrary to Gal4 and Gal4-VP16 which have been shown to result in severe developmental defects[28]. For generating Gal4 expressing lines, the recombinant DNA molecule includes either known gene regulatory regions or large pieces of genomic DNA that contain several genes and their gene regulatory sequences.
Depending on the gene regulatory region inserted adjacent to the *Gal4* gene, the expression of the reporter in the brain of transgenic animals can be pan-neuronal, expressing the reporter throughout the brain, or restricted, expressing it only in some neuronal subpopulations.
The Lx200 transgenic line, used in the current project, was generated in the Orger laboratory by Tol2-mediated BAC (bacterial artificial chromosome) transgenesis, where the GFF variant was cloned downstream of a large genomic sequence, presumably including several gene regulatory regions[29]. The expression patterns of this type of transgenic lines may also depend on regulatory regions in the vicinity of the site of insertion in the genome.
Although the regulatory region acting on the *Gal4* gene in this line is currently unknown, it displays expression in several interesting neuronal subpopulations during development[30]. Previous work from the laboratory characterised its expression at several developmental stages and in different brain regions. Several structures are labelled in this transgenic line: olfactory bulb, anterior commissure, pretectum, thalamus, hypothalamus, preoptic region, post-optic commissure, optic chiasm, optic tectum neuropil, stratum periventricular, tegmentum, medulla oblongata, clusters 1 and 2, vagus motor neurons and facial octavolateralis motor neurons. The vcc, whose projections contribute to the TPC, is also labelled early in

development[30]. This makes the Lx200 transgenic line an excellent tool for the study of TPC development and the neurons contributing to it. Assays previously done in the laboratory, observed the TPC structure and the Lx200-vcc neurons, which contribute to it in fixed samples (**Fig.1.4**).

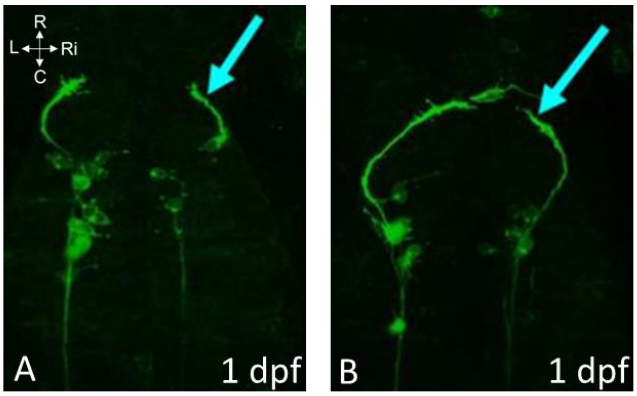

Figure 1.4: Posterior Commissure Formation in Lx200 fixed samples. Two examples of zebrafish embryos (1 dpf) from the transgenic line Tg(Lx200:GFF),UAS-GFP, showing GFP expression (Green) in a subset of neurons. **A:** less TPC extension. **B:** more TPC extension. Adapted from Ruth Diez del Corral, Orger Lab, Unpublished[31]. Arrows point to the forming posterior commissure. R: rostral, C: caudal, L: left, Ri: right.

1.5. Thesis Aims

The current study's first goal was characterising the Lx200-vcc. We started by establishing if the entire vcc was labelled in the Lx200 transgenic line. Then, we determined the location of the Lx200-vcc in the embryonic and larval zebrafish brain. We identified Lx200-vcc cells contributing to the TPC and assessed their pioneer nature. Lastly, we pursued a qualitative and quantitative analysis of TPC development, utilising whole embryo *in vivo* imaging. In sum, we aimed to study the development of the TPC, as this process is still unknown in zebrafish and other vertebrates, even if the anatomy and function of the TPC have been partially studied[1].

A deeper understanding of the axonogenesis of conserved structures in vertebrates, such as the posterior commissure, might have therapeutic and diagnostic implications. This is due to the fact that small defects during neuronal circuit formation impact development greatly. In humans, axon connectivity defects have been associated with neurological disorders, such as autism or congenital mirror movement disorder[32,33].

2. Materials and Methods

2.1. Animals, Sample Selection and Transgenic Lines

2.1.1. Fish and Embryo/Larvae Maintenance

Zebrafish maintenance, welfare and breeding were provided by the Vivarium Platform at the Champalimaud Foundation. Fish were kept at 25ºC air temperature, 50-60% humidity, with a light/dark cycle (14h light/10h dark), with 200-300 lux of light intensity at the water surface, in 3.5L tanks. Feeding consisted of live feed (rotifers and artemia) and processed dry feed, the frequency of each feed type varied according to the fish's age. Maintenance conditions (temperature, pH, salinity, and dissolved gases) were monitored continuously and checked daily. Total ammonia, nitrite and nitrate levels were checked weekly[34].
For breeding, fish were kept in slanted breeding tanks, allowing for a shallow water environment, which increases breeding success[34]. Eggs were collected and kept in plates with E3 and incubated at 28.5ºC. Embryos selected for photoconversion assays were shielded from light during development, to avoid unintentional photoconversion. Non-developing embryos and debris were removed. At 24 hpf, the medium was changed to 0.2 mM PTU (1-phenyl 2-thiourea) in E3 to ensure no pigmentation developed in the samples. The current project was developed according to the CF Animal Welfare Body guidelines and in the context of a CF Ethics Committee-approved project.

2.1.2. Embryo/Larvae Selecting and Staging

Embryos from the Lx200:GFF,UAS:mNeonGreen,tub1a:mScarlet and Lx200:GFF,UAS:GFP lines were selected based on the presence of mNeonGreen or GFP and mScarlet signals, in the region of interest (diencephalon-mesencephalon). Selection was done under a fluorescence stereomicroscope (Zeiss SteREO Lumar.V12) or a zoomscope (Axio Zoom.V16). Selected embryos continued development, until live imaging or fixation at a specific developmental stage. Samples were staged to ensure the reproducibility and correct determination of the developmental time of TPC formation. Zebrafish embryonic development is asynchronous, even in same clutch samples, raised together, in the same conditions[35]. We cannot rely solely on developmental time. The staging was done according to Kimmel *et al*., 1995[6], after removing the chorion and before fixation. Key structures were used as references to stage the samples, along with the head-trunk angle. The head-trunk angle increases with the uncurling of the embryo, between 20 hpf and 70 hpf.

2.1.3. Transgenic lines

Alpha-tubulin:mScarlet[36] drives the expression of mScarlet in neurons and their progenitors and is used, in this project, as a pan-neuronal marker.
Tg(Lx200:GFF) line has been generated in the Orger laboratory by BAC-mediated transgenesis[29] and allows for the expression of transgenes under the control of UAS in neuronal subpopulations. There are several reporter genes associated with UAS[5] available in the laboratory:

- UAS:GFP, isolated by Champalimaud Foundation fish facility and molecular tools and transgenics platforms, from a double transgenic imported from Kawakami laboratory, National Institute of Genetics, Japan[37].
- UAS:mNeonGreen, generated by Champalimaud fish facility, using a plasmid created by the

molecular tools and transgenic platforms (ABP-FP-MNEONSB from Allele Biotechnology & Pharmaceuticals, Inc.). It encodes a brighter alternative to GFP.

- UAS:Dendra2[38], encodes a photoconvertible protein, which allows for irreversible labelling of cells.

2.2. *In situ* hybridization and immunohistochemistry

2.2.1. Sample preparation

At selected developmental stages, embryos/larvae were fixed with 4% paraformaldehyde (PFA) in PBS. Samples used in immunohistochemistry assays were fixed for 2 hours at room temperature, while samples for *in situ* hybridization protocols were fixed overnight at 4ºC. Before fixation, the embryos/larvae were euthanized in 4 g/L tricaine, followed by washing with PBS. Fixation was followed by washes with PBT (for immunohistochemistry) or PBTw (for *in situ* hybridization). Immunohistochemistry samples were kept at 4ºC in the PBT wash, with 0.05% azide for preservation, *in situ* hybridization samples were kept at -20ºC in a 100% methanol wash, after several dehydration steps.
Each probe/antibody was applied to 4 to 6 samples (**Table S1**). In some cases, the same sample was processed to detect two different mRNAs/proteins, when biologically relevant, this data is jointly presented. Samples were processed with anti-GFP to enhance the GFP reporter signal from the transgenic line.

2.2.2. *In situ* Hybridization

The *in situ* hybridization technique allows for the characterization of the molecular identity of neuronal subpopulations, assessing the presence of specific RNAs and thus the expression of the corresponding genes[39]. The hybridization chain reaction (HCRv3.0) approach to detect RNA relies on the hybridization of synthetic probes to a complementary RNA sequence, followed by amplification and fluorescent labelling by conditional nucleic acid self-assembly of a second DNA oligomer mix. The method uses split initiator oligomers, the amplification reaction only happens when 2 adjacent probes are bound to the region of interest. This technique has improved sensitivity and reduced noise[40]. The Molecular Instruments HCR protocol was used, which provided adaptations for embryos, 1 and 2 dpf. After fixation, samples were dehydrated and permeabilized with methanol washes, and stored in methanol at -20ºC before use (minimum overnight). Rehydration was done with a series of methanol/PBTw washes, increasing the PBTw concentration. Samples were incubated with probe hybridization buffer for 30 minutes, followed by overnight incubation with probe solution (probe hybridization buffer and selected probes). The embryos/larvae were washed with the hybridization probe wash buffer, followed by washes with 5x SSCT and pre-amplification with the amplification buffer for 30 minutes. Finally, the embryos/larvae were incubated overnight in the amplification buffer with amplification oligomer mix (previously heated and snap cooled) in the dark, at room temperature. Embryos/larvae were washed with 5x SSCT and stored at 4ºC protected from light before imaging or other protocols.
The RNA sequences used to design the probes for the *in situ* hybridization assays are listed below (**Table 2.1**) and were selected based on the goals of each assay. Probes were diluted 1:250 µL. Probes were designed and manufactured by Molecular Instruments.

Table 2.1: List of RNAs and their NCBI reference.

RNA	NCBI Reference Sequence
her5	NM_205577.3
nkx2.2a	NM_131422
arx1	NM_131384.1
irx1b	NM_131823
olig3	NM_001110393
pax6a	NM_131304

2.2.3. Immunohistochemistry

Whole-mount immunohistochemistry was used to detect protein expression, using antigen-specific primary antibodies and secondary antibodies with fluorescent tags, detected during imaging[41].
The immunohistochemistry protocol followed Randlett *et al*., 2015[4], with adaptations for younger samples. Fixation was followed by epitope retrieval, with quick rinses with Tris-HCl 0.15M, pH 9 and incubation with the same solution for 5 minutes at room temperature and 15 minutes at 70ºC. Samples were cooled on ice and washed with PBT. Samples were incubated in a blocking buffer (See *Solutions*) for 2 hours and incubated with the primary and secondary antibodies in the blocking buffer for one overnight each, at 4ºC with gentle rocking. PBT washes were done between primary and secondary antibody incubations. Finally, samples were rinsed and kept in PBT with 0.05% azide to be used in a subsequent protocol. Some samples previously subjected to the *in situ* hybridization protocol went through the immunohistochemistry protocol without the epitope retrieval steps.
Incubation of the samples was done with primary and secondary antibodies in the blocking buffer mix, all diluted 1:500 µL. The primary antibodies were selected based on the goals of the assay, while secondary antibodies were selected according to the primary antibodies used (**Table 2.2**).

2.2.4. Dyes

After *in situ* hybridization or immunohistochemistry protocols, samples were subjected to overnight incubation with nuclear dyes, bisbenzimide or DRAQ5, at a 1:1000 concentration in PBT (**Table 2.3**). These dyes label DNA, by intercalating in the A-T region, allowing for easier anatomical localization of the structures of interest. Samples were washed in PBT and stored at 4ºC before imaging.

Table 2.2: List of primary and secondary antibodies used, along with their manufacturer reference.

Antigen	Manufacturer Reference	Host (Isotype)	Fluorophore	RRID
Primary antibodies				
GFP	Abcam ab 13970	Chicken (IgY)	-	AB_300798
Acetylated tubulin	Sigma-T-7451clone 6-11B-1	Mouse (IgG2b)	-	AB_609894
HuC/D	Molecular probes A21271	Mouse (IgG2b)	-	AB_221448
Secondary antibodies				
Mouse-IgG (H+L)	Molecular probes A21052	Goat	Alexa 568 and Alexa 633	AB_143157 AB_2535719
Chicken-IgY (H+L)	ThermoFisher A11039	Goat	Alexa 488	AB_2534096
Rabbit-IgG (H+L)	ThermoFisher A-11011	Goat	Alexa 633	AB_2535719
Rabbit-IgG (H+L)	ThermoFisher A-21442	Chicken	Alexa 594	AB_2535860

Table 2.3: List of dyes used, along with their manufacturer reference.

Dye	Manufacturer Reference
Bisbenzimide H33258	Sigma B2883
DRAQ5	Thermo Scientific 62251

2.3. Sample mounting and Microscopy

2.3.1. Lightsheet imaging

Time-lapse images of live zebrafish were acquired on a Zeiss Z1 lightsheet, equipped with two LSFM 10x/0.2NA objectives for the emission and a W Plan-Apochromat 20x/1.0 objective for the detection. Excitation was achieved with two solid-state lasers, at 488 nm and 561 nm respectively. Detection was achieved with two Pco.edge 5.5 sCMOS cameras.

The imaging chamber was filled with 0.2 mM PTU in E3 medium, to guarantee development without the deposition of pigment, and 168 mg/mL tricaine, an anaesthetic, used to suppress movement. The chamber was kept at 28.5ºC using the microscope incubation system, allowing for sample survival and development

as close to natural as possible. Anatomical z-stacks were obtained every 15 or 5 minutes, depending on the sample and time of development. The voxel size used was 0.11×0.11×0.46 μm (x×y×z). One sample was recorded with a voxel size of 0.23×0.23×0.48 μm (x×y×z).
Live embryos were mounted on a glass capillary, creating an agarose rod, by using a Teflon plunger. All the samples imaged were 1 dpf, between the 22 hpf (26-somite) and 25 hpf (prim-6) developmental stages. The embryos were imaged in a horizontal position, keeping one eye on each side, centring the diencephalon-mesencephalon area in a 2 mm glass capillary (**Fig.2.1**).
Before mounting, samples were manually dechorionated, placed in 168 mg/mL tricaine in E3 and then in a drop of 0.8% low melting agarose in E3 with 168 mg/mL tricaine. The drop of agarose was kept in parafilm on a heating pad at 43ºC to ensure it remained melted. Samples were oriented with a needle and mounted with the Teflon plunger inside the glass capillary.

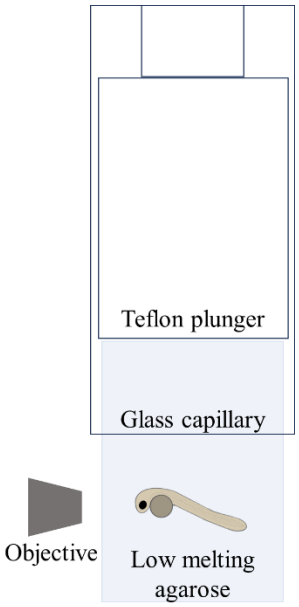

Figure 2.1: Scheme of the mounting protocol used for 1 dpf embryos for lightsheet imaging. The embryos were mounted in a horizontal position, keeping one eye on each side. The scheme is not to scale. Adapted from Viegas, 2019[42].

2.3.2. Confocal imaging

Confocal images were acquired using a ZEISS 980 inverted confocal microscope, equipped with an incubator to control temperature, humidity, and CO_2 levels. To observe the live samples, their chorions were manually removed. Live samples were mounted in round, glass-bottomed imaging plates (μ-Dish 35 mm) (with lids), using 0.8% or 1.5% low melting agarose in E3 with 168 mg/mL tricaine, for long or short imaging times, respectively. The samples were mounted in a dorsal or lateral view, depending on the region of interest for each assay (**Fig.2.2**). In long-time imaging assays, E3 medium was added to the mounting plates to ensure sample survival.
Fixed sample mounting was done in glass slides with coverslips, using 1.5% low melting agarose in PBS. Mounting was done either in a dorsal or lateral view (**Fig.2.2**), depending on the regions of interest in each assay. The laterally mounted samples had their yolks removed before mounting for better positioning.

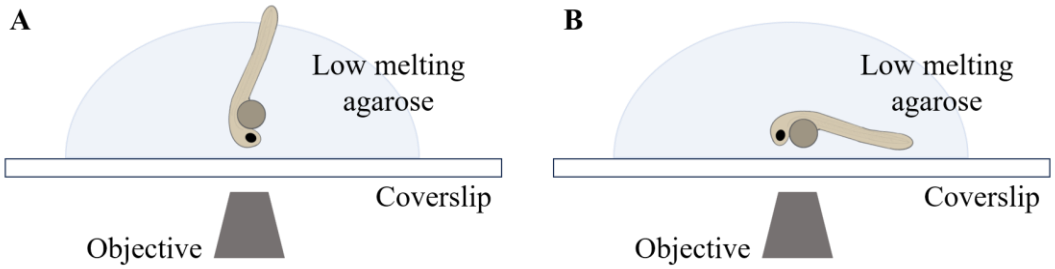

Figure 2.2: Scheme of the mounting protocol used for 1-5 dpf embryos for confocal imaging. A: Dorsal position, the dorsal region is in direct contact with the coverslip. **B:** Lateral position, the lateral region is in direct contact with the coverslip. The scheme is not to scale. Adapted from Viegas, 2019[42].

During confocal imaging, four lasers were used, Diode 405 nm (for bisbenzimide signal), DPSS 488 nm (for GFP and mNeonGreen), DPSS 561 nm (for Alexa 568 or Alexa 594) and HeNe 633 nm (for Alexa 633, Alexa 647 and DRAQ5). Two different lenses were used, a Pl-Apochromat 25x/N.A.0.8 multiimmersion objective and a PI-Apochromat 40x/N.A.1.2 multiimmersion objective. During imaging of live and fixed samples, water or oil (same refractive index as water) were used as the immersion medium. Using the ZEN software, the acquisition parameters (laser power, digital gain and offset) were adjusted for signal levels, avoiding saturation. All images acquired were 16-bit anatomical z-stack images. The voxel size used was 0.8×0.8×1 µm (x×y×z), with a pinhole diameter set to achieve a 2 µm optical section. Some images were acquired at different voxel sizes due to the needs of the assay, ranging between 0.1×0.1×0.48 µm and 0.7×0.7×0.39 µm (x×y×z). If needed, due to sample size, two imaging tiles with 10% overlap were acquired and stitched together, using the Fiji software.
To follow the development of the growth cone, live imaging was performed with a time interval between frames of either 5, 2.5 or 1.5 minutes, depending on the sample development stage. To ensure sample survival and development as close to natural as possible, the incubation was set to temperature and humidity of 28.5ºC.

2.3.3. Dendra2 Photoconversion

Photoconversion of Dendra2, the reporter in the Lx200:GFF,UAS:Dendra2 transgenic line, allowed to follow the cells in the cluster of interest during development. In its original state, the Dendra2 protein is fluorescent in the 488 nm wavelength and after the photoconversion protocol, it is fluorescent in the 568 nm wavelength. The cells to photoconvert were selected, in 1 dpf samples, based on their proximity to the TPC. The photoconversion protocol, performed in the confocal microscope, entailed hitting the selected cell with pulses of 405 nm light (20 times, with a dwell time of 6, pixel size of 0.1 µm and a 5-second interval between each light pulse). In some cases, a primed photoconversion assay was applied, allowing for confined photoconversion. This was done by hitting the cell with 488 nm light, followed by 638 nm light. During the photoconversion protocol, the cell was imaged to monitor photoconversion. A waiting period of 5-10 minutes before final imaging ensured that molecules diffused to the TPC, if there was a connection. The samples with photoconverted cells were kept and developed normally, being re-imaged at 3 and 4 dpf or 5 dpf, to determine if the same cells were present at later stages and where they were located.

2.4. Image and Data Analysis

Image analysis was performed using the open-source software Fiji[43]. Several of its tools (reslicing, orthogonal views and maximum intensity z-projections) were used to identify brain regions and expression patterns. Samples were compared with each other, between developmental stages and with the marker's known expression patterns. Representative samples were chosen for display and are shown in maximum intensity z-projections or in single planes for better observation of the expression patterns.

Imaris, a software that allows for the visualisation of a 3D rendering of the z-stacks obtained from each sample, was used for qualitative and quantitative analysis of TPC formation. Imaris was used to trace the axonal projections' path in time, allowing us to obtain quantitative data, such as structure length (**Video S1**). Tracings were assisted by the Imaris software but manually done, which can result in human error. However, they were done in sequence, using the same protocol, and corrected, if necessary, to mitigate errors. The obtained tracings and quantitative data were used to analyse several growth parameters: length, speed, and percentage of each movement type. Comparisons between each parameter in the left and right projections were done using paired student t-tests, in Excel. Comparisons were done regarding the final length before connection, the maximum extension speed, the minimum extension speed, the maximum retraction speed, the minimum retraction speed, and the percentage of retraction movement time points. The graphs presented were made using Excel.

2.5. Solutions

The solutions used during the current project are listed in **Table 2.4**.

Table 2.4: Solutions used and composition.

Solution	Composition
E3	5mM NaCl, 0.17mM KCl, 0.33mM CaCl2, 0.33mM MgS04, pH=7.2
PTU (0.2 mM)	PTU (0.2 mM) in E3
PBT	0.25% of Triton in PBS
PBTw	0.1% Tween 20 in PBS
Sodium azide	0.05% Sodium azide
4% PFA	4% PFA, 1× PBS
Blocking Buffer	1.25% PBT, 1% bovine serum albumin, 0.5% normal goat serum, 0.1% dimethyl sulfoxide, 0.05% sodium azide, and water
Primary and Secondary Antibody Mix	All primary and secondary antibodies diluted (1:500) in the Blocking buffer
5x SSCT	Tween 0.1% in 5x SSC

3. Results

3.1. Characterization of early neurons forming the tract of the posterior commissure

3.1.1. A subgroup of vcc neurons is labelled in the Lx200 line

The Lx200 transgenic line labels neurons located in the vcc region, at 1 dpf[20,30], with axons projecting to the TPC. It is unclear whether, in the Lx200 transgenic line, the entire vcc or only a subpopulation is labelled. To identify the vcc, we used an anti-HuC/D antibody, which labels the soma of most neurons in zebrafish[44]. Lx200 labelled cells are neurons, as they were labelled by anti-HuC/D (**Fig.3.1-A-D**). In the vcc, labelled by anti-HuC/D, at 1 dpf, two subpopulations were distinguishable, one also expressing Lx200 (referred to here as the Lx200-vcc) and one devoid of Lx200 (**Fig.3.1-A,D**). The Lx200-vcc is caudal to the TPC, located in a diencephalon-mesencephalon region. At 2 dpf, a larger number of neurons is observed in the corresponding position and only a small fraction expressed Lx200 (**Fig.3.1-E-G**).

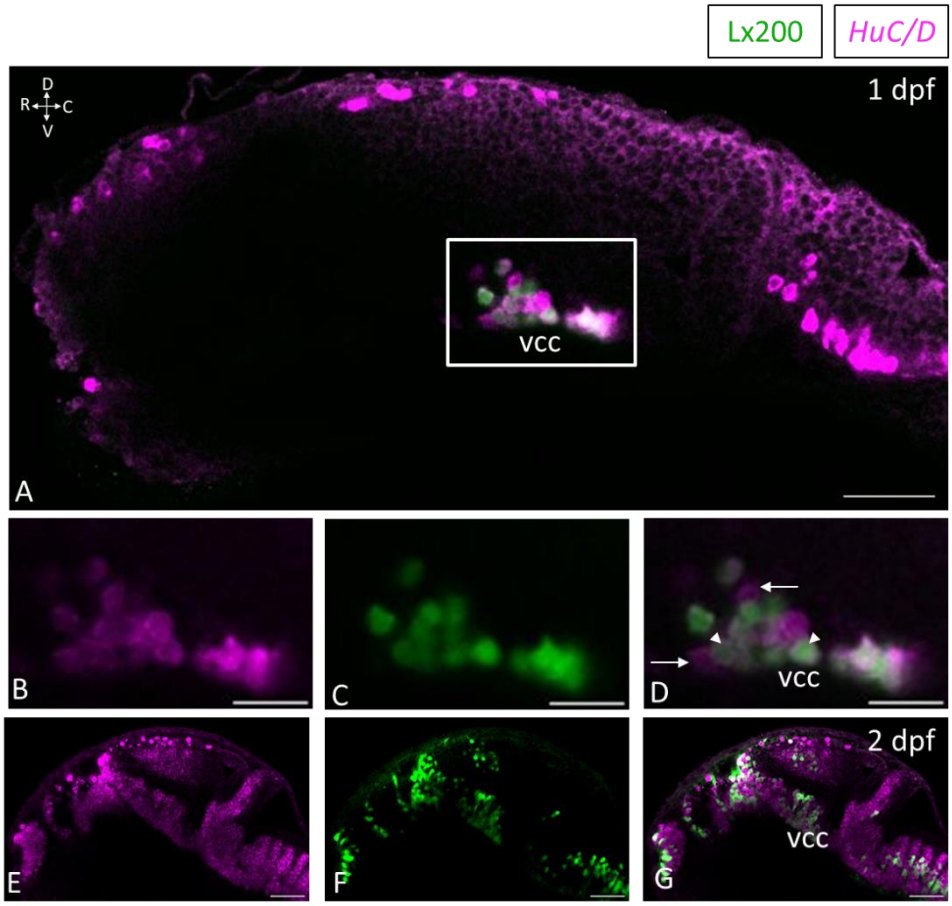

Figure 3.1: Cells labelled in Lx200:GFF;UAS:GFP 1 and 2 dpf embryos are a subpopulation of vcc neurons. A-D: Single plane from the Lx200 transgenic 1 dpf embryo, lateral view, **A:** Large field of view of the rostral part of the embryo. **B-D:** High magnification of the region of interest and **E-G:** Maximum intensity projection of a Lx200 transgenic 2 dpf embryo, lateral view. Images show GFP expression (anti-GFP, green) and HuC/D (anti-HuC/D, magenta). Scale bar indicates 25 µm (B-D) and 50 µm (A, E-G). 5 samples were analysed (1 dpf: 2 samples and 2 dpf: 3 samples). Arrows point to neurons labelled only by HuC and arrowheads point to neurons labelled by GFP and HuC/D. D: dorsal, V: ventral, R: rostral, C: caudal, vcc: ventral caudal cluster.

3.1.2. The Lx200-vcc cluster is present around prosomere 1 and mesencephalon ventral regions, while the TPC is located within prosomere 1

The vcc is located in the diencephalon-mesencephalon region[20]. However, its precise location, and that of the Lx200 subcluster, in the diencephalon, the mesencephalon or the rhombencephalon is unclear, since the cluster extends along the rostro-caudal axis. The TPC (including vcc neurons' projections) has been located near the diencephalon-mesencephalon boundary[21,45].

To determine more precisely the vcc location, we performed *in situ* hybridization assays, visualising the expression of mRNAs in 1 dpf Lx200 samples. The markers examined corresponded to *her5*, *nkx2.2a*, *arxa*, *irx1b*, *olig3* and *pax6a* genes. These genes label either only progenitors or progenitors and neurons.

In the embryo, the mesencephalon-rhombencephalon domain has a clear zone of delayed differentiation, the intervening zone, conserved in vertebrates. The *her5* gene delineates this zone during embryonic development (**Fig.3.2-A**)[46]. The probe designed for the *her5* gene labels the boundary between these brain regions.

Neither the Lx200-vcc neurons nor the TPC were part of the mesencephalon-rhombencephalon boundary (**Fig.3.2-B-G**). Both structures were rostral to the boundary. The TPC showed a large gap between its location and the rhombencephalon and is likely located in the diencephalon. Within the Lx200-vcc, a group of neurons is in close proximity to the TPC and distant from the *her5* domain and thus, presumably located in the diencephalon, while another is more caudal and closer to the *her5* stripe and may be located in the diencephalon but may include midbrain derivatives.

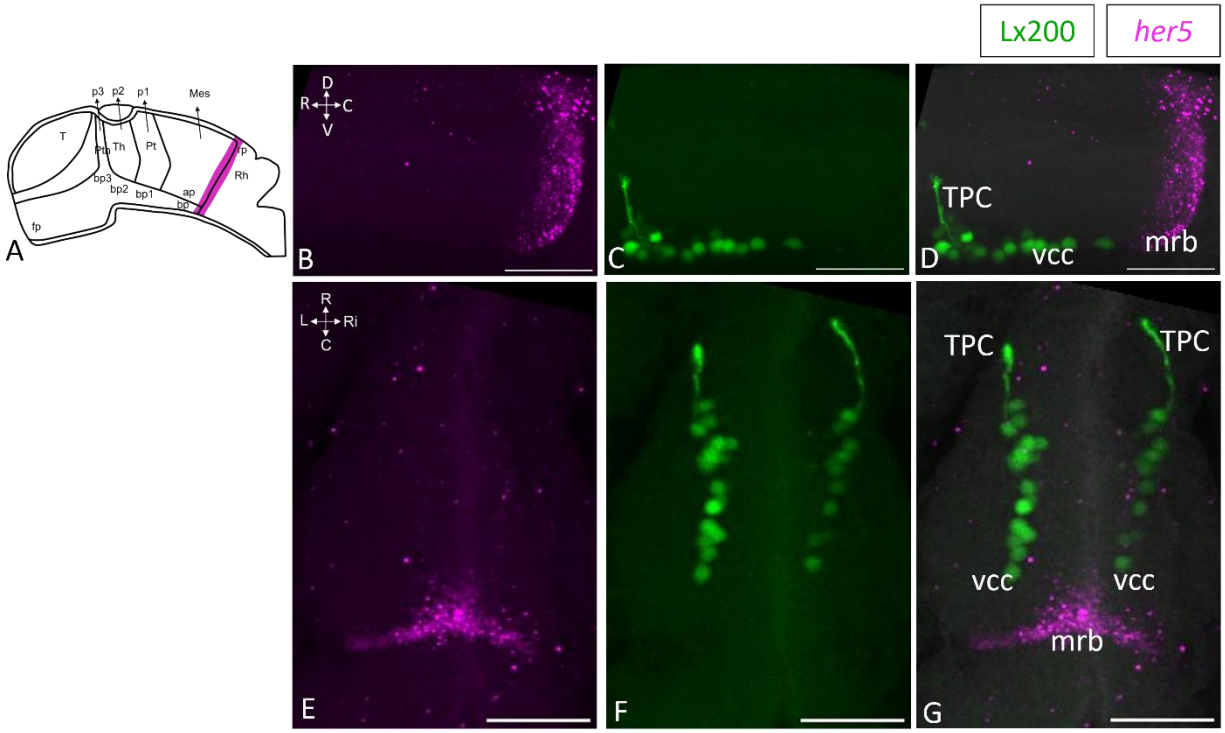

Figure 3.2: Lx200:GFF expression in 1 dpf embryos, with *her5* designed probe. A: Brain regions with *her5* expression. Adapted from Thisse *et al*., 2005[46]. ap: alar plate, bp: basal plate, bp1-bp3: basal region of prosomeres 1-3, Mes: mesencephalon, p1-p3: prosomeres 1-3, Pt: pretectum, Pth: prethalamus, Rh: rhombencephalon, Th: thalamus. **B-G:** Maximum intensity projections from the Lx200 transgenic line, B-D: lateral view and E-G: dorsal view. Images show GFP expression (anti-GFP, green) and *her5* expression (probe, magenta). Scale bar indicates 50 µm. 4 samples were analysed, all 1 dpf. D: dorsal, V: ventral, R: rostral, C: caudal, L: left, Ri: right, mrh: mesencephalon-rhombencephalon boundary, TPC: tract of the posterior commissure, vcc: ventral caudal cluster.

To ascertain the position of the Lx200-vcc neurons with respect to the dorsal-ventral axis, the *nkx2.2a* marker was used. At 1 dpf, *nkx2.2a* is expressed in progenitors in the hypothalamus, the diencephalon and the mesencephalon[45,47]. Within the diencephalon, it is expressed in alar and basal progenitors of prosomeres p2 and p3 located in proximity to zona limitans intrathalamica, and in basal progenitors of p1 (near the alar-basal boundary). Within the mesencephalon, it is expressed in part of the basal portion or tegmentum (**Fig.3.3-A**), close to the alar-basal boundary. In the rhombencephalon and spinal cord, *nkx2.2a* is expressed adjacent to the floorplate[48].

At 1 dpf, Lx200-vcc neurons are in close proximity to the most basal region of the *nkx2.2a* progenitor domain in the diencephalon-mesencephalon region (**Fig.3.3-B-D**), thus likely derived from progenitors expressing *nkx2.2a* or ventral to them. Characterization of different domains within the basal diencephalon and mesencephalon regions is currently missing at these early stages and no accurate markers can be used to ascertain the location of Lx200-vcc more precisely, within either basal p1 (bp1) or mesencephalon.

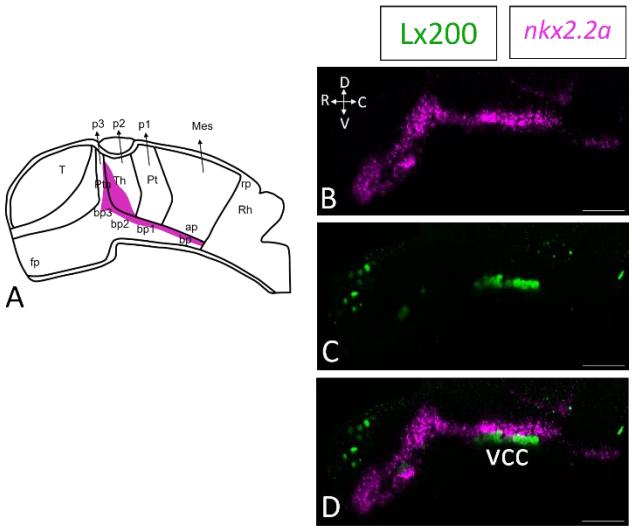

Figure 3.3: Lx200:GFF expression in 1 dpf embryos, with *nkx2.2a* designed probe. A: Brain regions with *nkx2.2a* expression. Adapted from Brozko *et al*., 2022[47]. ap: alar plate, bp: basal plate, bp1-bp3: basal region of prosomeres 1-3, Mes: mesencephalon, p1-p3: prosomeres 1-3, Pt: pretectum, Pth: prethalamus, Rh: rhombencephalon, Th: thalamus. **B-D:** Maximum intensity projections from the Lx200 transgenic line, lateral view. Images show GFP expression (anti-GFP, green) and *nkx2.2a* expression (probe, magenta). Scale bar indicates 50 µm. 4 samples were analysed (1 dpf: 3 samples and 2 dpf: 1 sample). D: dorsal, V: ventral, R: rostral, C: caudal, vcc: ventral caudal cluster.

arxa and *irx1b* are expressed in progenitor cells and neurons[49,50]. *arxa* is expressed in the prethalamus (alar p3 derivative) and the floor plate (along the rostral-caudal axis)[51], *irx1b* is expressed in the thalamus (alar p2 derivative), the alar region of mesencephalon and the rhombencephalon[49] (**Fig. 3.4-A**).

There is no overlap or proximity between those expression patterns and the Lx200-vcc neurons, (**Fig.3.4-B-E**), indicating that they are not located in p3 or the alar region of the p2, confirming that Lx200-vcc neurons are likely located in a ventral region of the mesencephalon or of prosomere p1, in the diencephalon. The ventral position of the cluster is confirmed by its proximity to the floor plate *arxa* expression pattern.

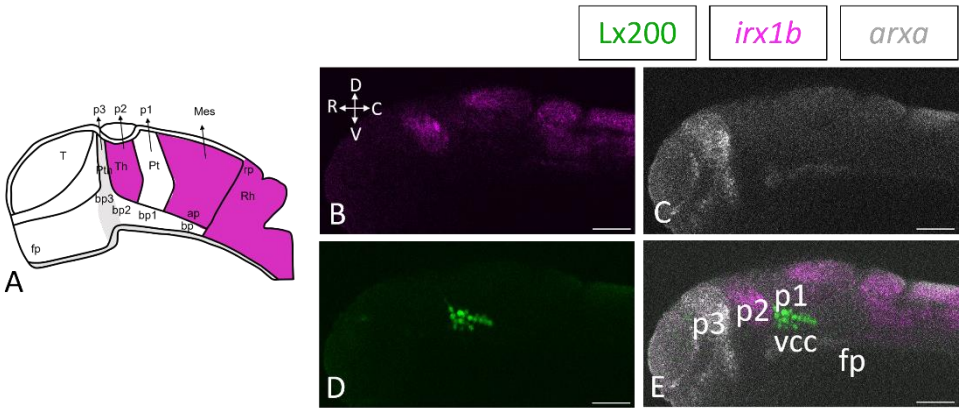

Figure 3.4: Lx200:GFF expression in 1 dpf embryos, with *irx1b* and *arxa* designed probes. A: Brain regions with *irx1b* and *arxa* expression. Adapted from Lecaudey *et al.*, 2005[49] and Rauch *et al.*, 2003[51]. ap: alar plate, bp: basal plate, bp1-bp3: basal region of prosomeres 1-3, Mes: mesencephalon, p1-p3: prosomeres 1-3, Pt: pretectum, Pth: prethalamus, Rh: rhombencephalon, Th: thalamus. **B-E:** Single plane from the Lx200 transgenic line, lateral view. Images show GFP expression (anti-GFP, green), *irx1b* expression (probe, magenta) and *arxa* expression (probe, grey). Scale bar indicates 50 µm. 4 samples were analysed, all 1 dpf. D: dorsal, V: ventral, R: rostral, C: caudal, fp: floor-plate, p1-p3: prosomeres 1-3, vcc: ventral caudal cluster.

olig3 is expressed in progenitor domains in the diencephalon and rhombencephalon from 1 dpf, particularly in the p2 and cerebellum[52] (**Fig.3.5-A**).
At 1 dpf, there is no overlap or proximity of the *olig3* expression with the Lx200-vcc or the TPC, which are located more caudally than the thalamic *olig3* expression pattern (**Fig.3.5-B-J**). The lack of proximity excludes the possibility of a p2 location for the cluster.

The *pax6a* gene is expressed in progenitor cells and neurons. At 1 dpf, it is present in the pretectum (alar p1 derivative)[47]. Its caudal border has been described as corresponding to the alar prosencephalon-mesencephalon boundary[53] (**Fig.3.6-A**). The TPC has been identified as being located one to two cell diameters inside the *pax6a* expression domain, in close proximity to the prosencephalon-mesencephalon boundary[21].
In the analysed samples there was a proximity of the rostral Lx200-vcc neurons to the *pax6a* expression signal (**Fig.3.6-E-G**). The most rostral region of Lx200-vcc is likely derived from progenitor cells near the *pax6a* expressing domain, such as the basal prosomere 1, as that is ventral to the pretectal pax6a expression pattern. The most caudal Lx200-vcc neurons were not in close proximity to the *pax6a* expression domain (**Fig.3.6-G**) and may be derived from the basal mesencephalon.
The early TPC projection labelled by the Lx200 line was located over the *pax6a* expressing progenitor region (**Fig.3.6-B-D,H-J**), indicating that it is located in the pretectum (alar p1 derivative).

In sum, a more precise location of Lx200-vcc neurons and TPC was determined. Results showed that the structures are ventrally located, rostral to the rhombencephalon. It is most likely that the TPC structure is located in the diencephalon, within the pretectum (alar p1 derivative). Lx200-vcc can be thought of as having two regions, the most rostral located in the diencephalon, likely basal p1 and the most caudal, likely part of the mesencephalon.

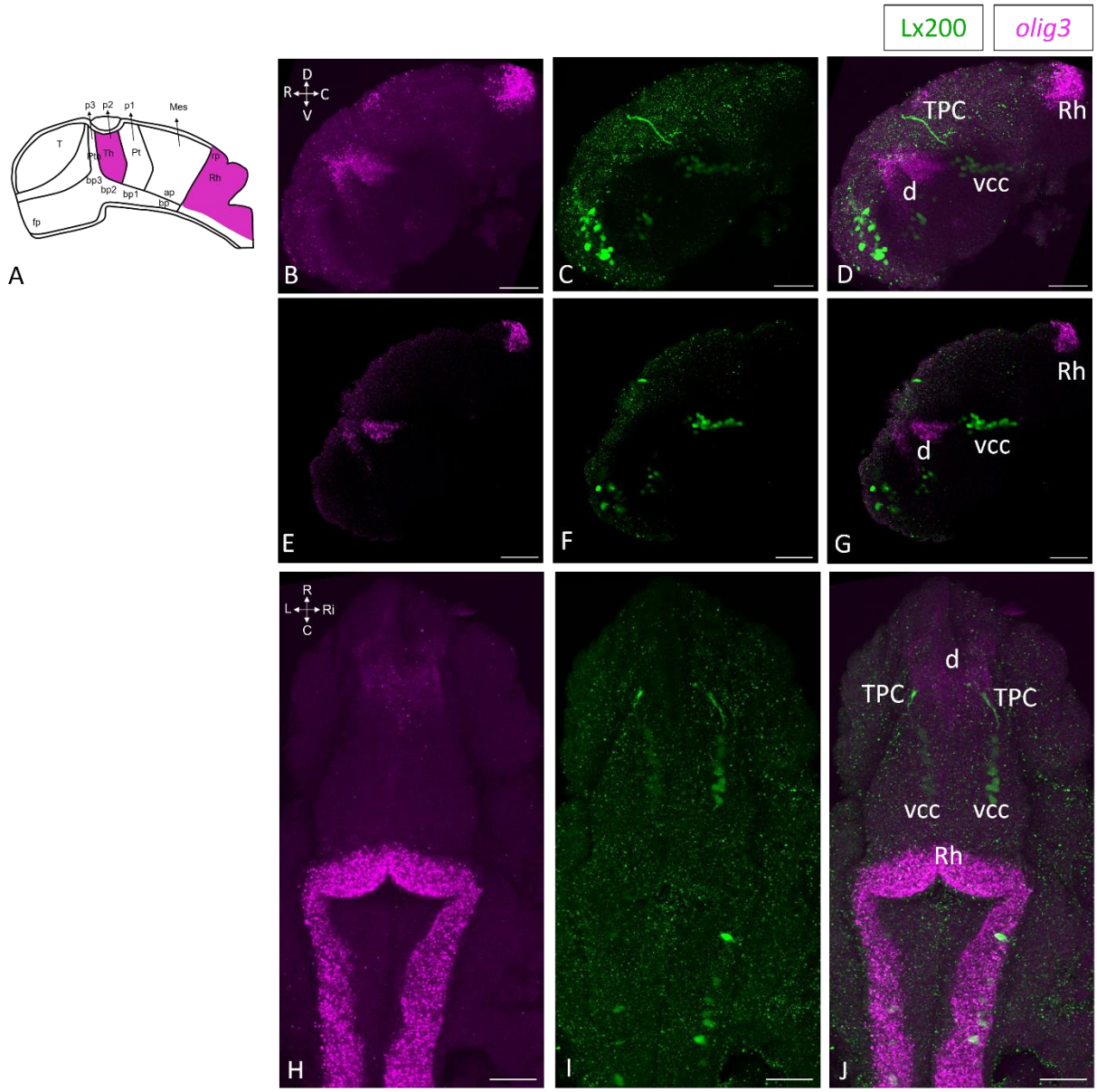

Figure 3.5: Lx200:GFF expression in 1 dpf embryos, with *olig3* designed probe. A: Brain regions with *olig3* expression. Adapted from Tiso *et al*., 2009[52]. ap: alar plate, bp: basal plate, bp1-bp3: basal region of prosomeres 1-3, Mes: mesencephalon, p1-p3: prosomeres 1-3, Pt: pretectum, Pth: prethalamus, Rh: rhombencephalon, Th: thalamus. **B-D:** Maximum intensity projections from the Lx200 transgenic line, lateral view; **E-G:** Single plane from the Lx200 transgenic line, lateral view; **H-J:** Maximum intensity projections from the Lx200 transgenic line, dorsal view. Images show GFP expression (anti-GFP, green) and *olig3* expression (probe, magenta). Scale bar indicates 50 µm. 6 samples were analysed, all 1 dpf. D: dorsal, V: ventral, R: rostral, C: caudal, L: left, Ri: right, d: diencephalon, Rh: rhombencephalon, TPC: tract of the posterior commissure, vcc: ventral caudal cluster.

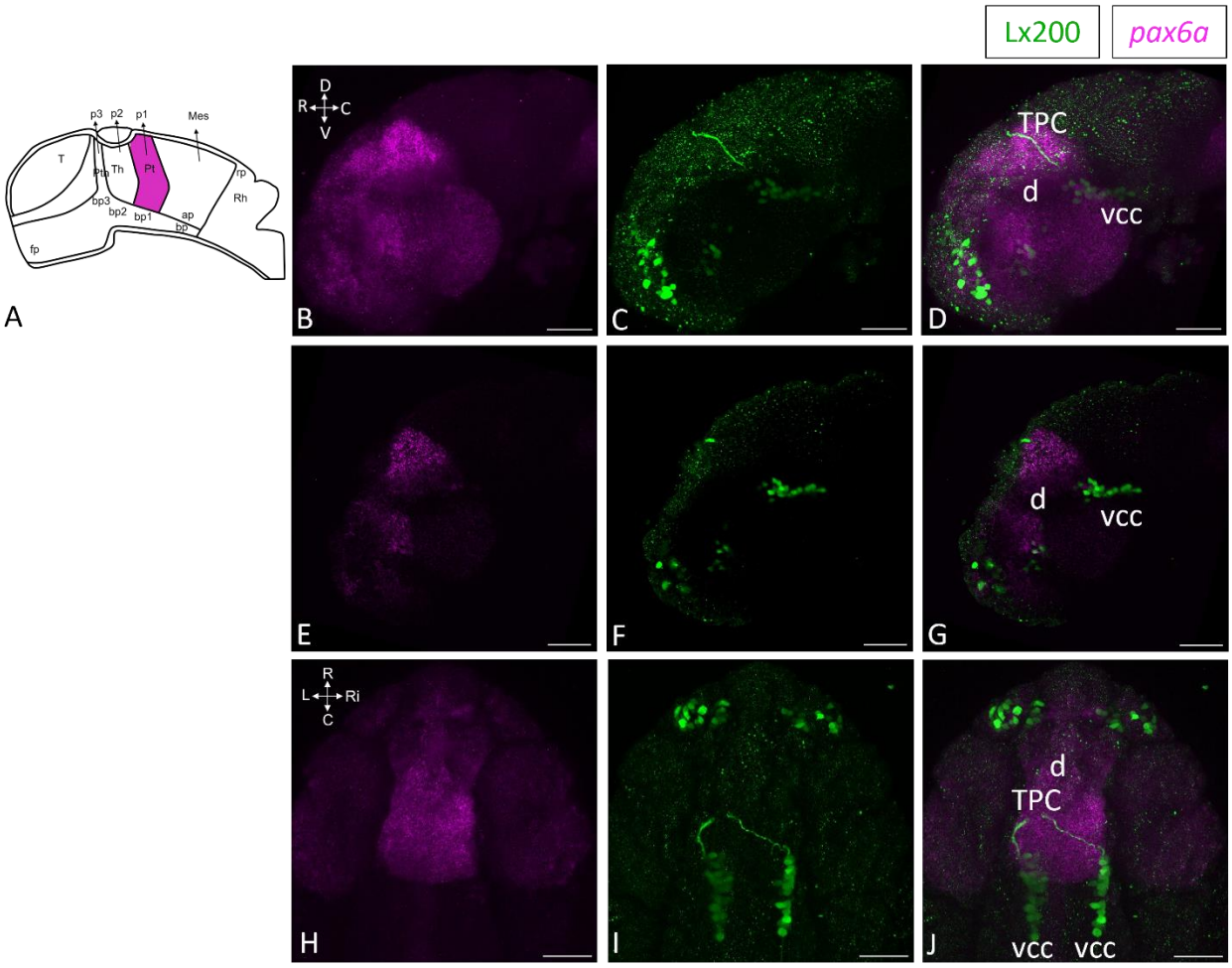

Figure 3.6: Lx200:GFF expression in 1 dpf embryos, with *pax6a* designed probe. A: Brain regions with *pax6a* expression. Adapted from Brozko *et al*., 2022[47]. ap: alar plate, bp: basal plate, bp1-bp3: basal region of prosomeres 1-3, Mes: mesencephalon, p1-p3: prosomeres 1-3, Pt: pretectum, Pth: prethalamus, Rh: rhombencephalon, Th: thalamus. **B-D:** Maximum intensity projections from the Lx200 transgenic line, lateral view; **E-G:** Single plane from the Lx200 transgenic line, lateral view, **H-J:** Maximum intensity projections from the Lx200 transgenic line, dorsal view. Images show GFP expression (anti-GFP, green) and *pax6a* expression (probe, magenta). Scale bar indicates 50 µm. 6 samples were analysed, all 1 dpf. D: dorsal, V: ventral, R: rostral, C: caudal, L: left, Ri: right, d: diencephalon, TPC: tract of the posterior commissure, vcc: ventral caudal cluster.

3.2. Assessing the identity of neurons derived from the early embryonic Lx200-vcc neurons

The Lx200-vcc neurons have been identified as contributing to the TPC, however, it is possible that not all neurons project to the TPC. We aimed to identify which cells are contributing to the TPC and their location at larval developmental stages. Photoconversion was used to identify the cells projecting to the TPC. In the current project, we relied on the availability of the reporter line UAS:Dendra2, combined with the Lx200:GFF transgenic line. Dendra2 is a photoconvertible protein that irreversibly changes its fluorescence emission spectrum from green (peak around 488 nm) to red (peak around 568 nm) when irradiated with blue light[54]. Photoconversion of this protein allowed for the labelling of specific neurons (including soma and projections) in the developing zebrafish brain, which were followed from embryonic to larval stages.

3.2.1. Assessing the contribution of Lx200-vcc neurons to the TPC

To determine which of the Lx200-vcc neurons contribute to the TPC, we selected individual neurons to be photoconverted, based on TPC proximity. Thirteen (out of seventeen) samples with photoconverted Lx200-vcc neurons showed photoconverted signal in the TPC (**Fig.3.7**). When only rostral cells were photoconverted (in five samples), the TPC showed photoconverted signal (**Fig. 3.7-A-C**), indicating that rostral Lx200-vcc neurons contribute to the TPC. Due to a lack of samples with only caudal Lx200-vcc cells photoconverted we are unable to determine if they contribute to the TPC.

Previous studies done in the laboratory showed that some Lx200-vcc neurons extend projections caudally, along the MLF[30]. Five of the seventeen samples with photoconverted neurons in the Lx200-vcc showed signal in the MLF, confirming that some of those neurons have projections along this tract. In four samples with only rostral cells photoconverted, there were no caudal projections with photoconverted signal (**Fig.3.7-A-C**), suggesting that rostral Lx200-vcc neurons do not contribute to the MLF. In one sample, caudal Lx200-vcc cells projected along the MLF.

The labelling of both the TPC and the MLF was seen in eight samples (**Fig.3.7-D-F**). However, this was observed when labelled cells were distributed throughout the cluster and we are unable to determine if individual cells are projecting along both tracts. Our data is compatible with two regions in the Lx200-vcc cluster, one rostral, projecting along the TPC and one caudal, projecting along the MLF.

3.2.2. Assessing the location of Lx200-vcc neurons at larval developmental stages

Four samples with cells photoconverted at 1 dpf were re-imaged at 3 and 4 dpf (**Fig.3.8**) to determine cell location at larval stages of development. During these stages, projections were visible close to the midline, while the photoconverted cells were in a rostral region, around the presumptive bp1 and mesencephalon region.

All 3 and 4 dpf samples showed the TPC with photoconverted signal, although dim (**Fig.3.8-A,D,G).** Contrary to previously observed structures, the TPC in photoconverted samples showed a non-straight pathway to the midline (**Fig.3.8-G**). Tracing with Imaris confirmed these observations (**Fig.S1**). This developmental difference might result from cell damage provoked by the photoconversion assay and subsequent imaging. To mitigate this effect, photoconversion at 3 and 4 dpf was carried out to attempt the visualisation of the normal TPC development in a photoconversion context. However, this visualization did not succeed, due to the large number of cells present at these developmental stages.

Four samples with cells photoconverted at 1 dpf, were re-imaged at 5 dpf. They lacked photoconverted signal in the TPC. Most likely, the photoconverted Dendra2 molecules, transported to the structure, decay and do not remain in this later developmental stage.

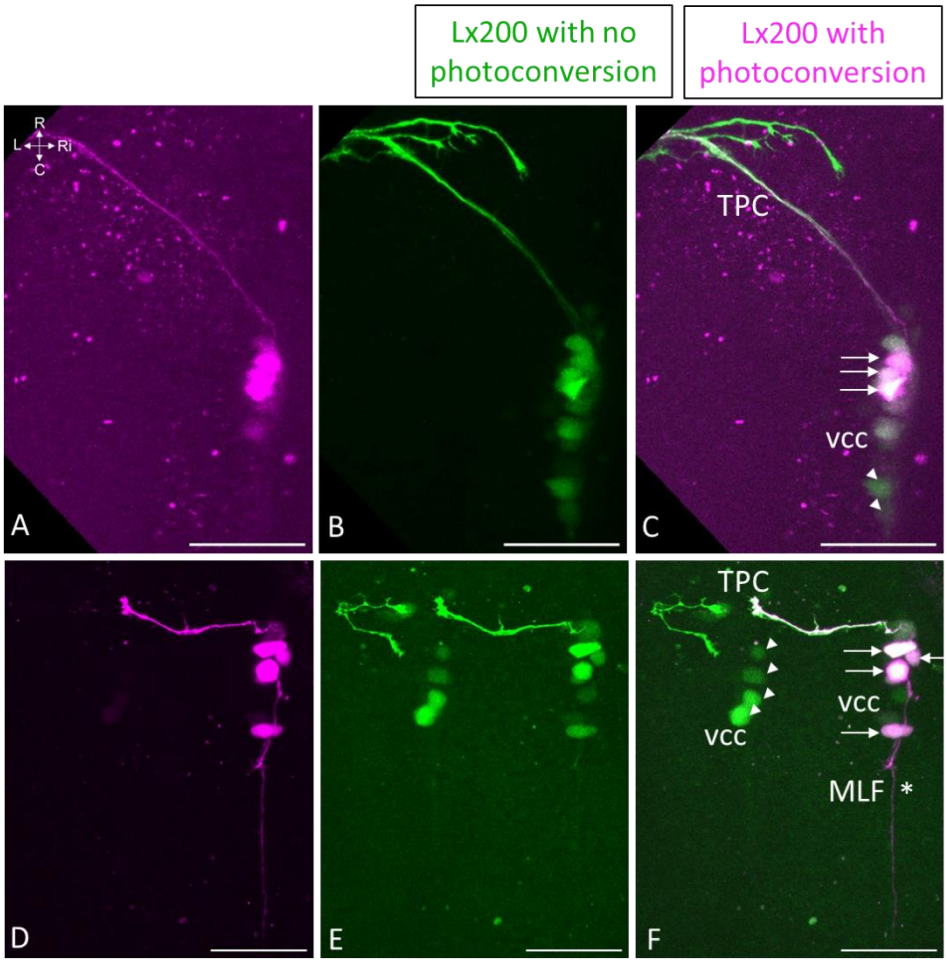

Figure 3.7: Lx200:GFF expression in 1 dpf embryos, with Dendra2 photoconversion. Maximum intensity projections from the Lx200 transgenic line dorsal view, after Dendra2 photoconversion, green (original Dendra2 signal) and magenta (photoconverted Dendra2 signal). Scale bar indicates 50 µm. Arrows point to neurons labelled by the original and the photoconverted Dendra2 signals, arrowheads point to neurons labelled only by the original Dendra2 signal, and the asterisk indicates the MLF labelled by the photoconverted Dendra2 signal. R: rostral, C: caudal, L: left, Ri: right, MLF: medial longitudinal fascicle, TPC: tract of the posterior commissure, vcc: ventral caudal cluster.

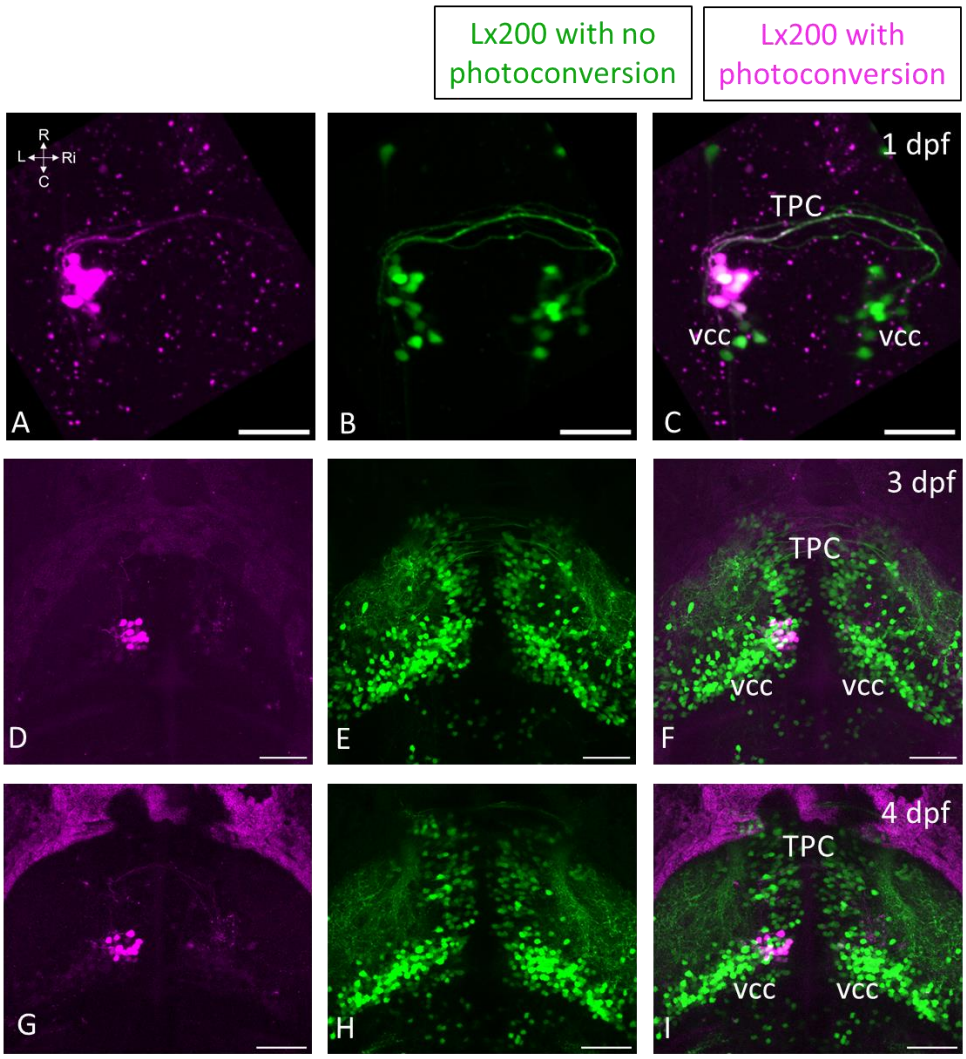

Figure 3.8: Lx200:GFF expression in 1, 3 and 4 dpf embryos, with Dendra2 photoconversion. Maximum intensity projections from the Lx200 transgenic line, dorsal view, after Dendra2 photoconversion, green (original Dendra2 signal) and magenta (photoconverted Dendra2 signal), **A-C:** 1 dpf, **D-F:** 3 dpf, **G-I:** 4 dpf. Scale bar indicates 50 µm. 4 samples were analysed. R: rostral, C: caudal, L: left, Ri: right, TPC: tract of the posterior commissure, vcc: ventral caudal cluster.

3.3 Lx200-vcc neurons are pioneers in TPC formation

It is unknown whether the Lx200-vcc neuron's projections follow previous tracts (i.e., they are followers) or whether they are the first tracts present in the area (i.e., they are pioneers). Their pioneer vs. follower nature was determined via immunohistochemistry, using the antibody anti-acetylated tubulin that labels all axonal projections present. Tubulin is an important component of microtubules, partially responsible for structural integrity, function, and differentiation of neurons. Post-translational modifications, such as acetylation, result in different forms of tubulin, with different cellular distributions in neurons. Acetylated tubulin is mostly present in axons and not in dendrites, making it appropriate for the labelling of axonal structures[22,23,55–57].

Samples between 22 hpf (26-somite) and 31 hpf (prim-1) were examined. These are the developmental stages when the Lx200-vcc neurons are present with incipient projections extending towards the dorsal midline (**Fig.3.9-B,E)**. These projections contributing to the TPC were simultaneously labelled by the anti-acetylated tubulin antibody and in the Lx200 transgenic line (**Fig.3.9)**. No acetylated tubulin staining was observed in the region ahead of the TPC labelled with GFP, indicating that no axons exist in the area, previous to the Lx200-TPC axons.
Projections labelled only by anti-acetylated tubulin were found in other regions, as expected (**Fig.3.9-A,C,D,F**). Interestingly, the growth cone region of the TCP axon was not labelled by anti-acetylated tubulin. This is expected, as acetylated microtubules are stable and long-lived, they do not accumulate in the growth cone, a labile element where mostly tyrosinated tubulin is present[55–57].

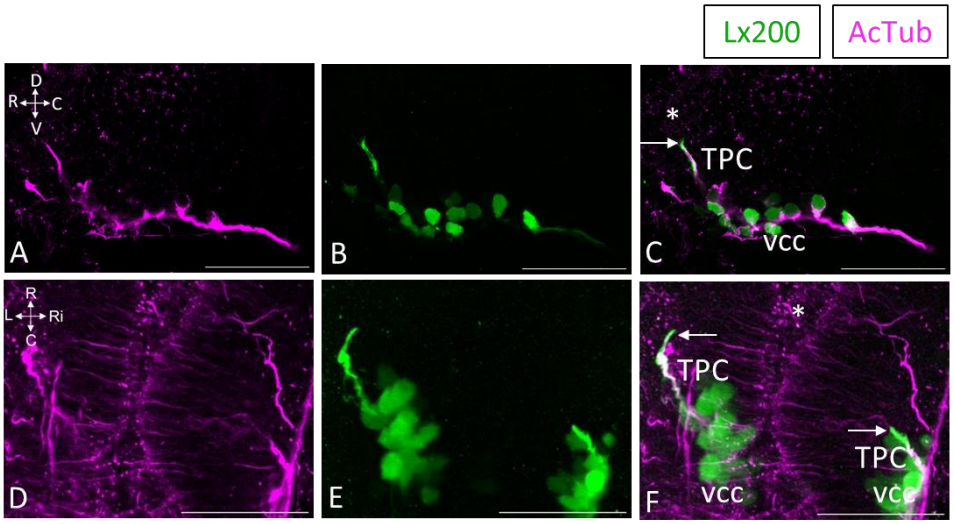

Figure 3.9: Lx200:GFF expression in 1 dpf embryos, with anti-acetylated tubulin antibody. Maximum intensity projections from the Lx200 transgenic line **A-C:** lateral view and **D-F:** dorsal view. Images show GFP expression (anti-GFP, green) and Acetylated tubulin expression (anti-AcTub, magenta). Scale bar indicates 50 µm. 9 samples were analysed, all 1 dpf. Arrows point to growth cones, axon tips. Asterisks show the region where the PC will form, devoid of axons at the time of Lx200 TPC extension. D: dorsal, V: ventral, R: rostral, C: caudal, L: left, Ri: right, TPC: tract of the posterior commissure, vcc: ventral caudal cluster.

3.4. TPC formation involves the branching of projections and occurs via extension and retraction events

We imaged TPC formation using Lx200 transgenic line live embryos expressing the fluorescent reporter, mNeonGreen. This allowed for qualitative and quantitative analysis of the process. Most imaging was performed with lightsheet microscopy, which allows for imaging at fast rates, with subcellular resolution, ideal for tracking cellular events. It creates little photodamage to samples, essential when live imaging.
Previous studies have determined that TPC formation occurs between 19.5 hpf (21-somite) and 25 hpf (prim-6). Our preliminary live imaging of Lx200 samples confirmed that TPC development occurred between 20 and 25 hpf, in our incubators. Previous live imaging trials in the lab used GFP as the reporter, which was not sufficiently bright. We used mNeonGreen as the reporter, which was much brighter[58] and allowed for cluster and TPC visualisation in live samples.

Five embryos were live imaged during TPC development, allowing for observation of the projections' development and the connection between projections from each side of the CNS. Imaging ranged from 80 to 220 minutes, starting as early as possible in axonal development. All five samples survived the process (fish #1 was imaged at 15-minute intervals and fish #2-5 at 5-minute intervals).

An observational analysis of the data, allowed for a qualitative description of TPC formation (**Fig.3.10**). A video of the timelapse was created for easier visualisation (**Video S2**). The extension of projections occurred from the ventral to the dorsal regions, from the Lx200-vcc neurons (**Fig.3.10-A**). Projections contributing to TPC formation, observed in the samples, were straight, reaching the midline region without turns or looping (**Fig.3.10-I)**. Ramifications from the main projections were visible, some contributing to TPC (**Fig.3.10-F**), while others were non-TPC related (not shown). Ramifications contacted with each other (**Fig.3.10-K**). Connections between projections of each side of the brain occurred via the growth cone in three of the analysed samples (**Fig.3.10-J**). In two samples, connection happened with one growth cone connecting to the contralateral projection (not shown). After the initial connection, projection extension continued, and other connections were formed (**Fig.3.10-K**). The details of the branching and connection differed from sample to sample and from right to left sides. Other non-TPC contributing projections were visible in the samples. The developing anterior commissure was visible in one sample.

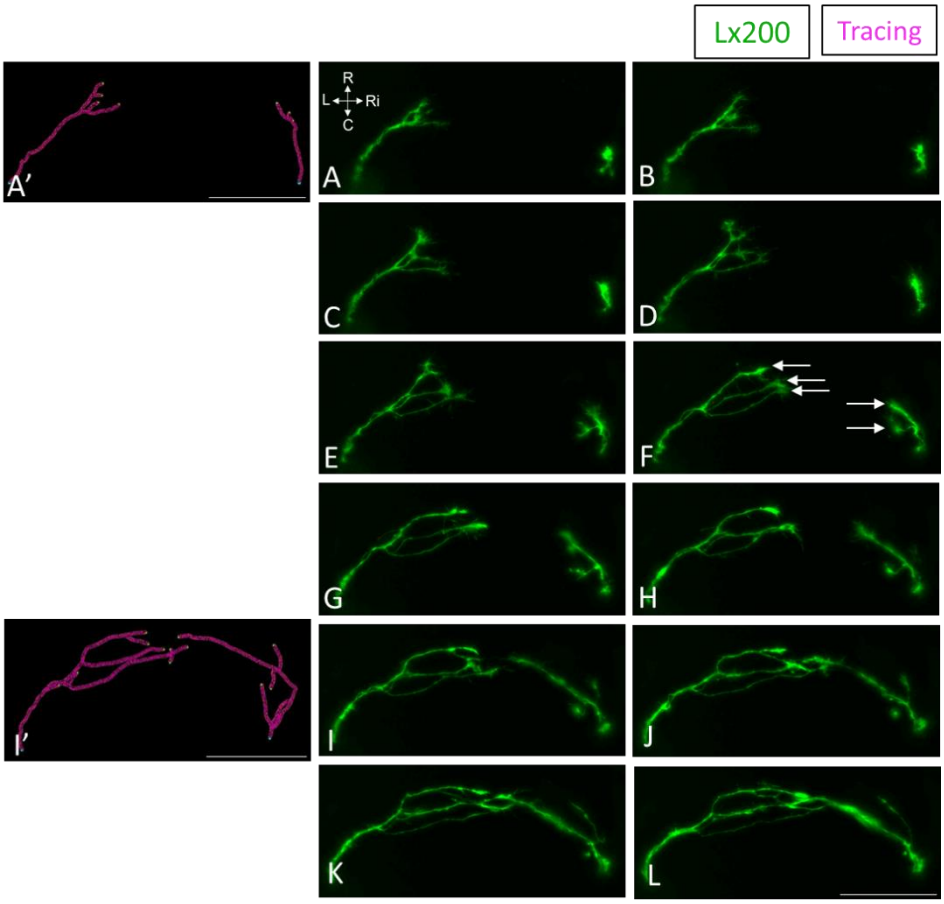

Figure 3.10: Time-lapse of TPC formation, Lx200:GFF expression in a 1 dpf embryo. Lightsheet Imaging. **A-L:** Maximum intensity projections from the Lx200 transgenic line, with mNeonGreen reporter. **A', I':** 3D rendered with Imaris, with corresponding tracing (magenta). Each frame with 15-minute interval (20 minutes between D and E). Scale bar indicates 50 µm. 5 samples were analysed, all 1 dpf. Arrows point to the main branching tips.

Lightsheet spatial resolution is lower than other imaging methods. For this reason, we used confocal microscopy to live image Lx200 samples at the same developmental time, to observe the projections contributing to the TPC and their growth cones in greater detail.
The observed projections showed no loops or turns but branched frequently. There were connections between axons coming from each side of the brain. Extension and retraction movements were visible (**Fig. 3.11-A**). Many filopodia were present at the growth cone and along the projection (**Fig. 3.11-B**).

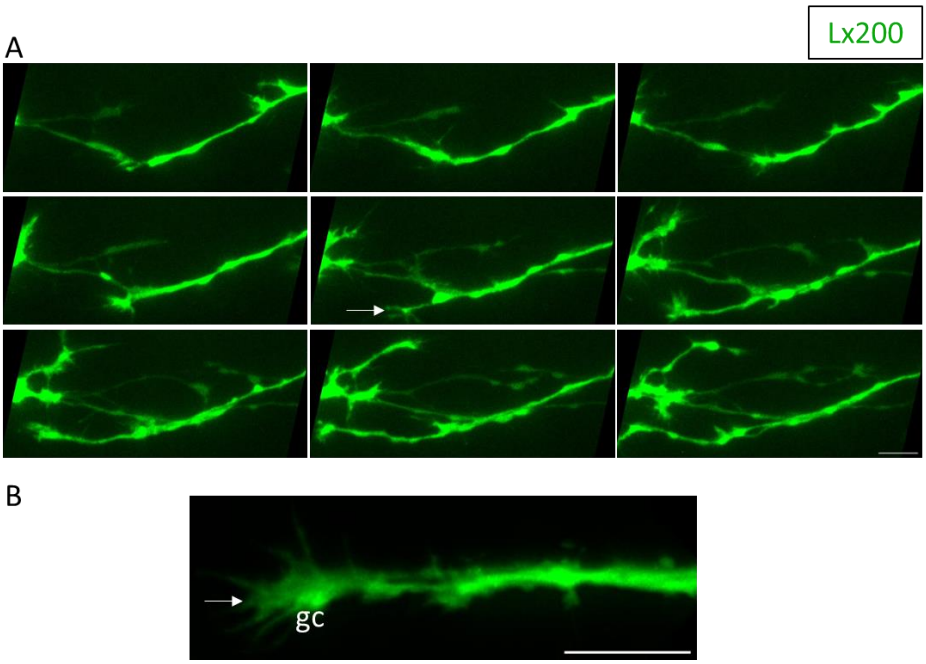

Figure 3.11: Time-lapse of TPC formation, Lx200:GFF expression in a 1 dpf embryo and growth cone detail. Confocal Imaging. Maximum intensity projections from the Lx200 transgenic line, with mNeonGreen reporter. **A:** Each frame with a 5-minute interval. Scale bar indicates 10 µm. 3 samples were analysed. Arrow points to an example tip/growth cone. **B:** Scale bar indicates 10 µm. Arrow points to growth cone filopodia. gc: growth cone.

The Imaris image analysis software was used to obtain quantitative data from the lightsheet data (fish #2 to fish #5, imaged at 5-minute intervals), regarding the extension process before connection. We first created a 3D rendering of each sample at each time point. We then performed software-assisted manual tracing of the projections and extracted length measurements of the branches (**Fig.3.10-A',I'**). These, in combination with the time intervals, were used to calculate the speed of projection.
For this quantitative work, we focussed on the main branch projecting towards the midline dorsally, selected based on observation during tracing (**Fig. 3.12**). Secondary branches were identified as branching from the main one and were not further considered here (**Fig. 3.12**). Quantitative data are presented for the four fish that were imaged every 5 minutes. During TPC development different pioneer neuron movement types were identified, based on observation and length changes. They either grew (extension), were shortened (retraction) or did not change (arrest).

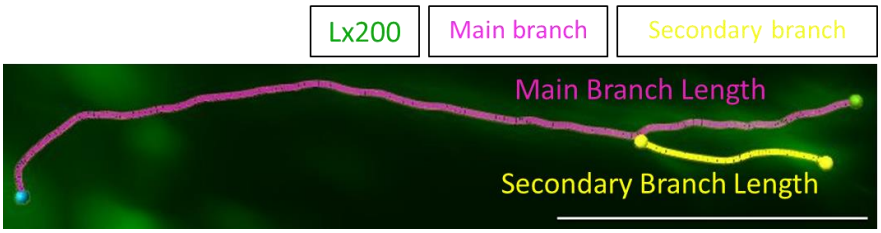

Figure 3.12: Main and secondary branches. Imaris software 3D rendering of projections from the Lx200 transgenic line, with mNeonGreen reporter. Main (magenta) and secondary (yellow) branches are highlighted, representing the measured length. Scale bar indicates 50 µm.

Length of left and right-side projections was plotted with the imaging time, for each sample (**Fig.3.13**). The final length of projection preceding the connection ranged from 106,2 µm to 217,1 µm. No significant differences were detected when comparing left and right-side final lengths (**Table S2**). However, given the small number of samples (n=4), we cannot rule out the possibility of a difference. The largest difference between left and right at the last time point was of 110,9 µm (sample #4) (**Fig. 3.13**). No side contributed with greater lengths to the TPC.

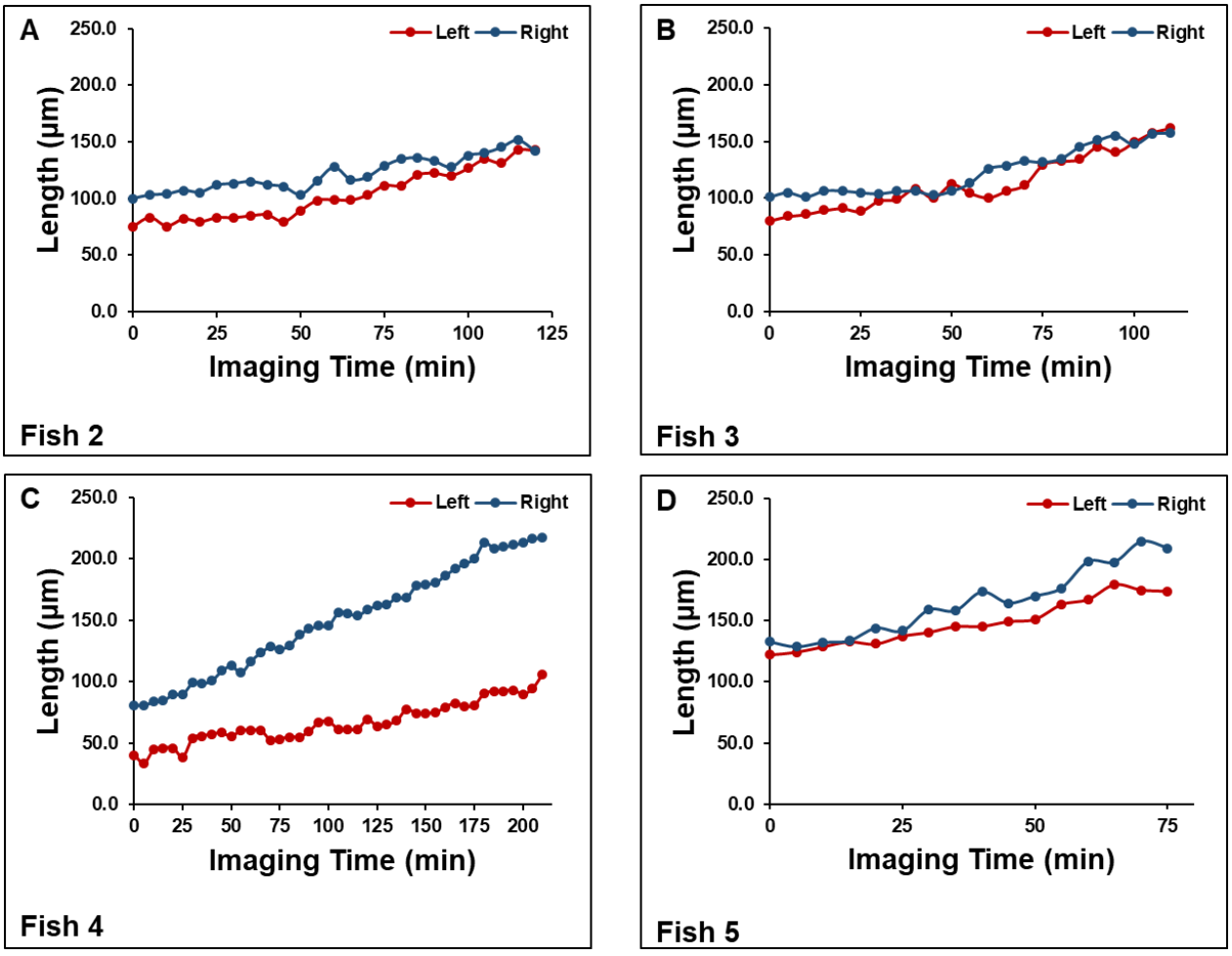

Figure 3.13: Branch Length (µm) at each imaging time (min). Left and right side lengths determined at each imaging time before connection. 4 samples were analysed. A: Fish #2, B: Fish #3, C: Fish #4, D: Fish #5.

Speed at each time point was similar between the right and left sides and between samples. Extension speed ranged between 0,1 µm/min and 4,3 µm/min, while retraction speed ranged between -0,1 µm/min and -2,2 µm/min (**Fig. 3.14**). There was no sustained increase or decrease in speed with time. When comparing the minimum and maximum, extension and retraction speeds of left and right sides no significant differences were found (**Table S2**). However, given the small number of samples (n=4), the possibility of difference cannot be ruled out.

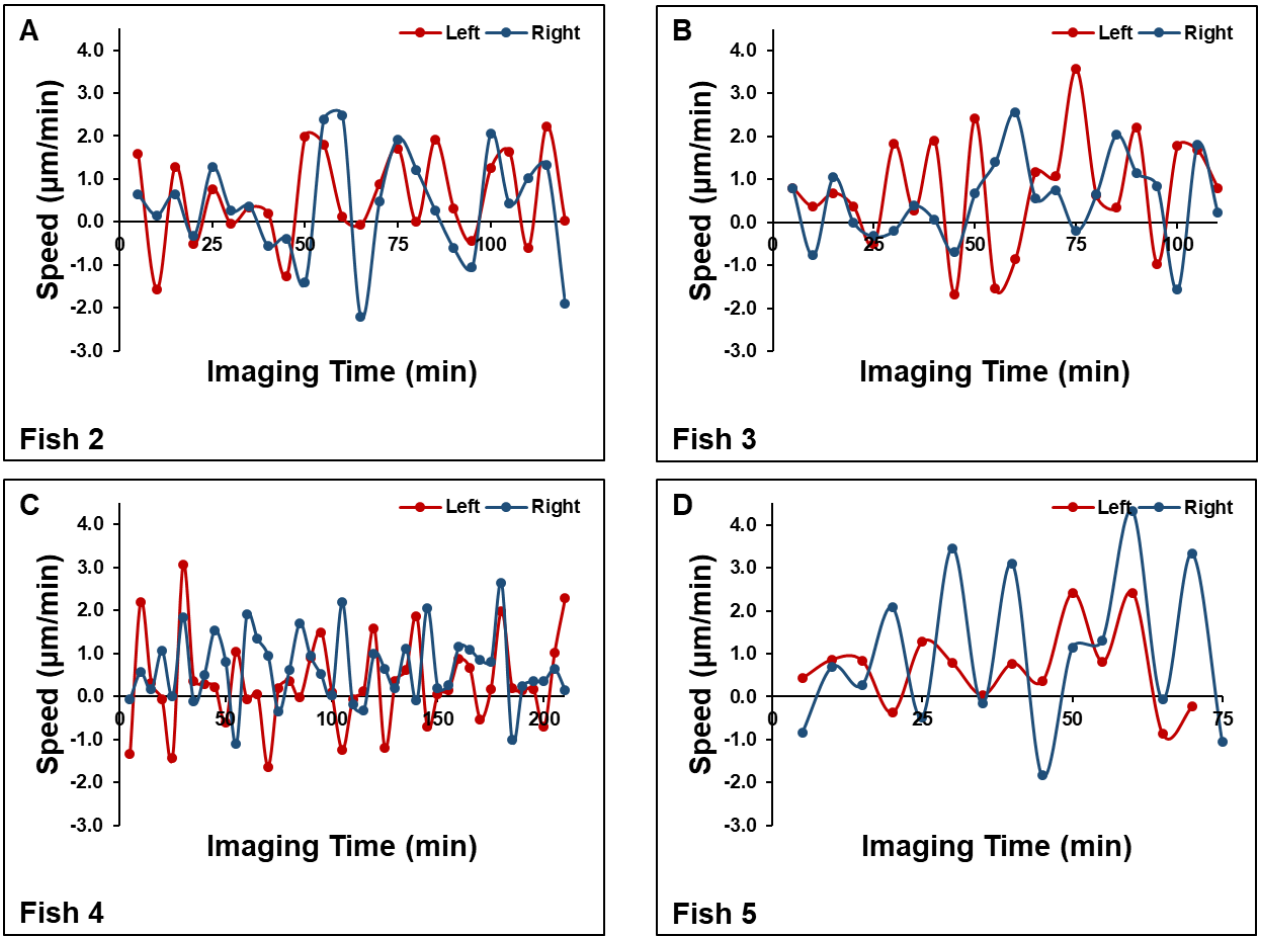

Figure 3.14: Speed (µm/min) at each imaging time (min). Left and right side speeds calculated based on length change between imaging times. 4 samples were analysed. A: Fish #2, B: Fish #3, C: Fish #4, D: Fish #5.

All analysed projections presented extension and retraction events. As projections are growing, the majority of movement events corresponded to extension (**Fig. 3.15**). Comparison of the fraction of retraction events between left and right sides yielded no significant differences (**Table S2**). Once again, due to the small number of samples (n=4), we cannot rule out the possibility of difference.

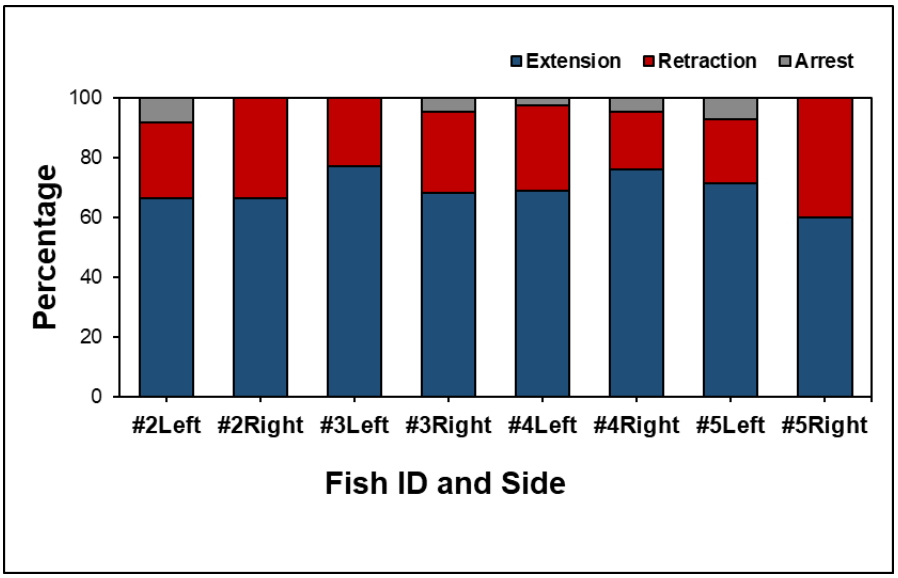

Figure 3.15: Movement Category Percentage. Left and right-side movement category percentages calculated based on the number of timepoints with extension, retraction, and arrest events. 4 samples were analysed (Fish #2-#5).

Our results show that extension and connection of the first axons extending along the TPC occurred between 20 and 25 hpf. After this period of time, the ventral to dorsal projections from the left and right sides of the embryo came in contact with each other, around the dorsal midline. The overall extension of the projections appears to result from a combination of local extension, retraction, and arrest events. In addition to the first event of connection of the main branch, secondary branches coming from either side of the brain also appear to connect through a similar mechanism, involving the growth cones.

4. Discussion

4.1 Location of early neurons forming the tract of the posterior commissure in the early embryo

The zebrafish Lx200 transgenic line was shown in our laboratory to drive gene expression in a group of neurons extending projections along the TPC and the PC proper, from 1 dpf. These neurons are located at the position of the vcc, generated during primary neurogenesis, around the ventral diencephalon-mesencephalon region. This is the first cluster in the zebrafish brain to undergo axonogenesis, beginning the development of the early scaffold, which serves as the basis for subsequent brain connectivity. We have shown that Lx200 labels a subgroup of vcc neurons.

Previous studies determined gene expression patterns in the diencephalic region[45,53,59]. We compared these expression patterns to the location of the Lx200-vcc neurons and the TPC, to provide a clearer location of these structures. Our analysis confirmed that the Lx200-vcc is located ventrally and away from the rhombencephalon. The lack of specific markers for the ventral parts of the diencephalon and the mesencephalon does not allow us to unequivocally determine whether they are located in prosomere 1 of the diencephalon or in the mesencephalon. Our results confirm that the TPC extends over the pretectum domain (p1 alar derivative, in the diencephalon), so it is likely that the corresponding soma, in the vcc, are located within this compartment.

Chitnis & Kuwada, 1990[22], one of the central studies of axonal development and location, described a ventral cluster, located in the anterior tegmentum, the most anterior region of the mesencephalon, near p1, contributing to the TPC. Our current results are in agreement with this study confirming a p1-mesencephalon ventral location of the Lx200-vcc neurons, contributing to the TPC.

The location of the Lx200-vcc neurons in the early zebrafish brain had been determined, however, the contribution of cells in the cluster to the TPC had not been established. Photoconversion of the Dendra2 protein, present in the Lx200-vcc neurons, allowed for the identification of neurons projecting to the TPC. Cells in the Lx200-vcc contribute to the TPC. Particularly, rostral cells in the cluster project along this tract. However, due to a lack of samples, we were unable conclude regarding the contribution of caudal cells to the TPC. Future work could help better define the function of the Lx200-vcc neurons in CNS early development.

In the laboratory, Lx200-vcc neurons were described as projecting caudally, along the MLF[30]. In the current work, photoconverted cells in the Lx200-vcc showed MLF projections. Rostral cells in the cluster showed no contribution to MLF. Likely, caudal cells project along the MLF. Even though both structures were labelled in the same samples, we cannot distinguish if individual neurons project along both tracts. It is possible that the rostral region of the cluster projects towards the TPC, while the caudal region contributes to the MLF. The MLF is part of the early scaffold in the zebrafish brain. It connects the mesencephalon to the spinal cord. There are reports of vcc neurons contributing to MLF[20], strengthening the possibility of caudal Lx200-vcc neurons contributing to the structure.

The knowledge of which cells in the cluster contribute to each structure could inform future work focussed on neuronal circuit development, allowing for a more oriented study and photoconversion targeting of the cluster.

4.2 Location of the Lx200-vcc neurons at larval stages

During larval development, photoconverted cells that contribute to the TPC and were part of vcc (at 1 dpf) were visible rostrally, likely in the diencephalon-mesencephalon area. It seems the cells did not migrate significantly and that the labelled region, at later stages, might correspond to the vcc location, at earlier stages of development.

Refining the photoconversion protocol to allow for the photoconversion of TPC-contributing cells, at larval stages, can provide more information regarding their location. It would also allow for the visualisation of close-to-natural TPC development and structure in a photoconversion context.

Several known subpopulations develop around this area and may include Lx200-vcc cells or be adjacent to them, such as the pretectum[24], the nucleus of the MLF (nucMLF)[60] and periaqueductal grey (PAG)[61]. Different markers and techniques can be applied to determine the Lx200-vcc location regarding these subpopulations. The nucMLF is a cluster of cells projecting along the MLF, which has been shown to project along the PC and can be identified via reticulospinal injections[60]. The PAG corresponds to two bilaterally paired clusters in the diencephalon, which express *relaxin 3a*, meaning that a marker targeting this gene can be used to label these cells. Ablation studies found that these neurons contribute greatly to axonal projections, for example, in the diencephalon[61], as do the Lx200-vcc neurons.

The behavioural function of the Lx200-vcc was not explored in the current study. In future work, it could be determined, assessing whether they have similar functions to those of closely located neuronal subpopulations. For example, pretectal neurons are involved in binocular whole field motion, nucMLF neurons contribute to bout swimming and PAG neurons are described as being involved in behavioural control, during stress and fear.

Having gained insight into the location of the Lx200-vcc neurons and the TPC at later developmental stages, it was important to compare our findings to those of previous studies. There are several zebrafish brain atlases available, which compile various studies' data. Mapzebrain[62] (available at: https://mapzebrain.org/home) includes a large collection of individual neuron traces. We were interested in identifying which of them cross the midline at the TPC region and might correspond to the Lx200-vcc neurons. 44 neurons were identified (**Table S3**). The somas of these neurons were located using the available anatomical regions. Most were present in the pretectum (dorsal p1) (19 neurons), others were located in the pretectum (dorsal p1)-mesencephalon boundary (2 neurons) and tegmentum (mesencephalon) (4 neurons). Neurons were also located in other brain regions, including the prosencephalon and rhombencephalon.

At embryonic stages our data located Lx200-vcc neurons, contributing to TPC, in a p1-mesencephalon region, confirming a similar location (yet less precise) at larval stages. The Lx200 neurons may represent the most abundant type of neuron crossing the posterior commissure located in the p1-mesencephalon region (**Table S3**). In the future, examining their connection to other brain regions might allow for a better assessment of their correspondence to the Lx200-vcc neurons.

4.3. Formation of the TPC

Pioneer neurons are responsible for creating the early axonal scaffold, which guides follower neurons. Their growth cones receive guidance cues, which influence pathfinding. Our results show that the first projections extending along the posterior commissure are derived from the TPC, extending from the vcc, and not from other neurons that are known to contribute to the PC. A previous study had determined that pioneer neurons in the prosencephalon did not project along the borders of gene expression domains[59]. The same was visible in the current study, the TPC was not located along the border of the analysed gene expression domains.

The identification of the Lx200-vcc neurons as pioneers of the TPC encouraged the use of this line to study TPC formation, using live imaging. This approach overcomes the limitations of studies using fixed samples[20,22], or cell culture[63]. Whole embryo *in vivo* imaging allows for a development as close to natural as possible, maintaining the microenvironment influencing development, while permitting a temporal characterization done in the same samples, otherwise not available.

However, it should be noted that natural CNS development, in particular of the TPC, might have been affected by the imaging methods. Temperature and humidity conditions were controlled, remaining equal to those of laboratory development. Nevertheless, imaging required agarose embedding and exposure to light, which might interfere with development. *In vivo* imaging was mainly done using lightsheet microscopy, a less photodamaging alternative, which reduced the impact on the samples. When comparing observations done in live imaging to fixed samples, TPC development was similar, confirming that the measures taken to mitigate imaging effects were successful. Yet, the photoconversion assays and subsequent confocal imaging, repeated every 24 hours, until 4 dpf, showed an impact on development - the TPC structure was different from the non-turning observed in fixed and other live samples.

The projections contributing to the TPC and crossing the posterior commissure were ventral to dorsal, as previously described[22]. The projections showed ramifications, but no drastic turning or loops. The observational lack of loops or turning was confirmed by the measured lengths of projections. Lengths preceding the connection, between projections from each side of the brain, were similar between samples since all projections took similar non-turning, non-looping paths to reach the midline. Branching and the lack of turning seem to be common characteristics in axonal pioneer projections. This was also described in retinotectal projections, with authors hypothesising that branching could contribute to pathfinding[64]. The growth cone, which plays a major role in pathfinding, showed many filopodia, a characteristic seen in axons of other neurons and model organisms[13].

During the connection between axons of both sides of the brain, the growth cones seemed to connect and interact. After this first connection, the projections continued, and other connections were established. The “handshake hypothesis” seems to be describing a similar process. In the "handshake” process, the growth cones of thalamocortical and corticothalamic mice axons interact and then grow in opposite directions, along each other[65]. This same interaction may be occurring between Lx200-vcc axons from each side of the brain, contributing to the establishment of the posterior commissure, at the dorsal midline.

Even though it was possible to visualise TPC development, we were unable to determine if a single axon pioneered the structure or if several were projecting together. From observation alone, it seems likely that a single neuron is projecting and branching. However, we cannot exclude the possibility that several axons project together and are not distinguishable due to spatial resolution. Studies in other structures have proposed a single pioneer axon, such as the DVDT[66], which strengthens the possibility that the TPC might be formed by a single axon.

During TPC development characterization, we identified three movement types: extension, retraction, and arrest. The overall extension to reach the dorsal midline was mostly made up of local extension movements, but all the analysed samples showed retraction movements in their main branches. Retraction movements were expected to be present in these pioneering axons, as a similar study, focussed on TPOC development, found that 90% of pioneer neurons retracted when near the ventral midline[17].
Extension speeds ranged between 0,1 µm/min and 4.3 µm/min, while retraction speed varied between -0,1 µm/min and -2,2 µm/min. The observed speed seems to be typical of pioneer neurons, as other studies determined similar values. A study of TPOC projections in zebrafish found that pioneer neurons projected at 85±5 µm/hour (1.42 µm/min), away from the midline and 35±5 µm/hour (0.59 µm/min)[17], at the midline. Both values are in the range of observed speeds in the current study. Retinal ganglion cell axons were described as maintaining speed steady during development[64], just as TPC projection speed did not change with time. Similar to TPC projections, retinal ganglion axons showed arrested movement events, but a lower value for maximum speed of 65 µm/hour (1.08 µm/min). In *Drosophila*, the TSM1 axon, growing in the wing, showed a growth rate of 120 µm in 9 to 12 hours (0.22 to 0.16 µm/min)[67], this value is similar to that of minimum speeds observed in the TPC axons in zebrafish. It seems that zebrafish axons show similar projection speeds, validating the registered speeds in Lx200-vcc projections, while *Drosophila* has a slower projection growth rate.

4.4 Future perspectives

Our work has provided large amounts of data on the extension of neurites in development. We have started the main branch analysis. Future work will focus on furthering this data analysis, focussing on branching, number of tips or straightness parameters. Along with exploring secondary branch characteristics.
Our data on axon extension are limited to the period before closure, as tracing the growth cone path became impossible after the first connection between axons on each side of the brain. It was not possible to compare pioneer neuron behaviour before and after connection. This can be solved by following just the growth cone. Transgenic lines expressing fluorescent proteins targeted to the growth cone are being developed in the laboratory using the kif5c560 construct. It contains the kif5c protein, an axonal growth targeting domain[68], which has allowed for labelling of the growth cone, in previous contexts.
Currently, the molecular mechanisms that influence axonogenesis responsible for TPC formation are unknown. This is the clear next step in comprehending TPC development. Previous research has used genetic screening and analysis to determine the genetic pathways guiding axonal projections[59]. Transcriptomic assays can determine the mRNA and proteins produced in the growth cone of the pioneer neurons. Ablation studies can be used to understand neuronal influence on contralateral axons, by ablating single axons or the entire cluster and characterising the contralateral projections.

4.5 Conclusions

In the current study, we set out to elucidate the process through which the TPC forms, along with characterising the Lx200 cluster, contributing to this structure.

The Lx200-vcc cluster was identified as a subcluster of the vcc, located in the p1-mesencephalon region at 1 dpf (the time of TPC development). We were able to localize the TPC itself in the pretectal region (alar derivative of p1). The Lx200-vcc neurons were identified as projecting along both the TPC and the MLF. Rostral cells project only along the TPC, and it seems likely that caudal cells project along the MLF. At larval stages, the location of the Lx200-vcc neurons was very similar to the one described at embryonic stages, but less precise.

We concluded that the Lx200-vcc projections are pioneers of the TPC. TPC formation occurred through the extension of projections, from the ventral region to the dorsal midline, between 20 and 25 hpf. The growth process resulted from extension, retraction, and arrest events. Projections presented branches and were non-turning, connecting to contralateral axons, mostly via their growth cones. The speed of projections did not change with time and the length of projections was similar between samples and sides.

Deepening the current knowledge regarding TPC formation, both regarding how the process occurs and which neurons contribute to it, is of great importance, as the TPC is part of the early scaffold and is conserved in vertebrates. The development of the early scaffold is an essential step in axonogenesis, creating the path that will be followed by later projecting neurons. Even small defects in axonogenesis can have great impacts on development. In humans, axon connectivity issues have been linked to neurodevelopmental disorders, such as autism[32]. A deeper comprehension of the axonogenesis process might have an impact on our understanding of these disorders.

All in all, we were able to fulfil our goals, contributing to the efforts of characterising central nervous system development in the zebrafish.

Bibliography

1. Ware, M., Dupé, V. & Schubert, F. R. Evolutionary Conservation of the Early Axon Scaffold in the Vertebrate Brain: Evolution of the Initial Nerve Connections. Dev. Dyn. 244, 1202–1214 (2015).
2. Howe, K. et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503 (2013).
3. Rahman Khan, F. & Sulaiman Alhewairini, S. Zebrafish (Danio rerio) as a Model Organism. in Current Trends in Cancer Management (eds. Streba, L., Ionut Gheonea, D. & Schenker, M.) (IntechOpen, 2019). doi:10.5772/intechopen.81517.
4. Randlett, O. et al. Whole-brain activity mapping onto a zebrafish brain atlas. Nat. Methods 12, 1039–1046 (2015).
5. Asakawa, K. et al. Genetic dissection of neural circuits by Tol2 transposon-mediated Gal4 gene and enhancer trapping in zebrafish. Proc. Natl. Acad. Sci. 105, 1255–1260 (2008).
6. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310 (1995).
7. Schmidt, R., Strähle, U. & Scholpp, S. Neurogenesis in zebrafish – from embryo to adult. Neural Develop. 8, 3 (2013).
8. Appel, B. Zebrafish neural induction and patterning.
9. Wullimann, M. F. The Neuromeric/Prosomeric Model in Teleost Fish Neurobiology. Brain. Behav. Evol. 97, 336–360 (2022).
10. Hauptmann, G., Söll, I. & Gerster, T. The early embryonic zebrafish forebrain is subdivided into molecularly distinct transverse and longitudinal domains. Brain Res. Bull. 57, 371–375 (2002).
11. Raj, B. et al. Emergence of Neuronal Diversity during Vertebrate Brain Development. Neuron 108, 1058-1074.e6 (2020).
12. Purves, D. et al. Neuroscience. (Sinauer Associates, 2017).
13. Axon growth and guidance. (Springer Science + Business Media ; Landes Bioscience, 2007).
14. Goodman, C. S. & Shatz, C. J. Developmental mechanisms that generate precise patterns of neuronal connectivity. Cell 72, 77–98 (1993).
15. Chedotal, A. & Richards, L. J. Wiring the Brain: The Biology of Neuronal Guidance. Cold Spring Harb. Perspect. Biol. 2, a001917–a001917 (2010).
16. Kulkarni, R. P., Bak-Maier, M. & Fraser, S. E. Differences in protein mobility between pioneer versus follower growth cones. Proc. Natl. Acad. Sci. 104, 1207–1212 (2007).
17. Bak, M. & Fraser, S. E. Axon fasciculation and differences in midline kinetics between pioneer and follower axons within commissural fascicles. Development 130, 4999–5008 (2003).
18. Pike, S. H., Melancon, E. F. & Eisen, J. S. Pathfinding by zebrafish motoneurons in the absence of normal pioneer axons. Development 114, 825–831 (1992).
19. McAllister, A. K. Cellular and Molecular Mechanisms of Dendrite Growth. Cereb. Cortex 10, 963–973 (2000).
20. Ross, L., Parrett, T. & Easter, S. Axonogenesis and morphogenesis in the embryonic zebrafish brain. J. Neurosci. 12, 467–482 (1992).
21. Macdonald, R. et al. Regulatory gene expression boundaries demarcate sites of neuronal differentiation in the embryonic zebrafish forebrain. Neuron 13, 1039–1053 (1994).
22. Chitnis, A. & Kuwada, J. Axonogenesis in the brain of zebrafish embryos. J. Neurosci. 10, 1892–1905 (1990).

23. Wilson, S. W., Ross, L. S., Parrett, T. & Easter, S. S. The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, Brachydanio rerio.
24. Naumann, E. A. et al. From Whole-Brain Data to Functional Circuit Models: The Zebrafish Optomotor Response. Cell 167, 947-960.e20 (2016).
25. Giolli, R. A., Blanks, R. H. I. & Torigoe, Y. Pretectal and brain stem projections of the medial terminal nucleus of the accessory optic system of the rabbit and rat as studied by anterograde and retrograde neuronal tracing methods. J. Comp. Neurol. 227, 228–251 (1984).
26. McDougal, D. H. & Gamlin, P. D. Autonomic Control of the Eye. in Comprehensive Physiology (ed. Terjung, R.) 439–473 (Wiley, 2014). doi:10.1002/cphy.c140014.
27. Suster, M. L., Abe, G., Schouw, A. & Kawakami, K. Transposon-mediated BAC transgenesis in zebrafish. Nat. Protoc. 6, 1998–2021 (2011).
28. Halpern, M. E. et al. Gal4/UAS Transgenic Tools and Their Application to Zebrafish. Zebrafish 5, 97–110 (2008).
29. Renninger, S. & Orger Lab. (Unplublished).
30. Esteves, Bernardo. Developmental characterization of neuronal subpopulations involved in zebrafish visual processing (Master's dissertation). (2017).
31. Diez del Corral, R. & Orger Lab. (Unpublished).
32. McFadden, K. & Minshew, N. J. Evidence for dysregulation of axonal growth and guidance in the etiology of ASD. Front. Hum. Neurosci. 7, 671 (2013).
33. Marsh, A. P. L. et al. DCC mutation update: Congenital mirror movements, isolated agenesis of the corpus callosum, and developmental split brain syndrome. Hum. Mutat. 39, 23–39 (2018).
34. Martins, S. et al. Toward an Integrated Zebrafish Health Management Program Supporting Cancer and Neuroscience Research. Zebrafish 13, S-47-S-55 (2016).
35. Streisinger, G., Walker, C., Dower, N., Knauber, D. & Singer, F. Production of clones of homozygous diploid zebra fish (Brachydanio rerio). Nature 291, 293–296 (1981).
36. Velez, M. (2018).
37. Takeuchi, M. et al. Establishment of Gal4 transgenic zebrafish lines for analysis of development of cerebellar neural circuitry. Dev. Biol. 397, 1–17 (2015).
38. Taniguchi, A., Kimura, Y., Mori, I., Nonaka, S. & Higashijima, S. Axially-confined in vivo single-cell labeling by primed conversion using blue and red lasers with conventional confocal microscopes. Dev. Growth Differ. 59, 741–748 (2017).
39. Thisse, C. & Thisse, B. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat. Protoc. 3, 59–69 (2008).
40. Choi, H. M. T. et al. Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. Development 145, dev165753 (2018).
41. Hammond-Weinberger, D. R. & ZeRuth, G. T. Whole Mount Immunohistochemistry in Zebrafish Embryos and Larvae. J. Vis. Exp. 60575 (2020) doi:10.3791/60575.
42. Viegas, M. B. Establishment of Neural Circuits During Zebrafish Development: Characterization and Optimization of Imaging Conditions.
43. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).
44. Kim, C.-H. et al. Zebrafish elav/HuC homologue as a very early neuronal marker. (1996).
45. Lauter, G., Söll, I. & Hauptmann, G. Molecular characterization of prosomeric and intraprosomeric subdivisions of the embryonic zebrafish diencephalon. J. Comp. Neurol. 521, 1093–1118 (2013).

46. ZFIN Publication: Thisse et al., 2005. https://zfin.org/ZDB-PUB-051025-1#abstract.
47. Brożko, N. et al. Genoarchitecture of the Early Postmitotic Pretectum and the Role of Wnt Signaling in Shaping Pretectal Neurochemical Anatomy in Zebrafish. Front. Neuroanat. 16, 838567 (2022).
48. Yang, L., Rastegar, S. & Strähle, U. Regulatory interactions specifying Kolmer-Agduhr interneurons. Development 137, 2713–2722 (2010).
49. Lecaudey, V., Anselme, I., Dildrop, R., Rüther, U. & Schneider-Maunoury, S. Expression of the zebrafish Iroquois genes during early nervous system formation and patterning. J. Comp. Neurol. 492, 289–302 (2005).
50. Colombo, E. et al. Inactivation of Arx , the Murine Ortholog of the X-Linked Lissencephaly with Ambiguous Genitalia Gene, Leads to Severe Disorganization of the Ventral Telencephalon with Impaired Neuronal Migration and Differentiation. J. Neurosci. 27, 4786–4798 (2007).
51. ZFIN Image: Rauch et al., 2003, Fig. 2. https://zfin.org/ZDB-IMAGE-060607-271.
52. Tiso, N. et al. Differential expression and regulation of olig genes in zebrafish. J. Comp. Neurol. NA-NA (2009) doi:10.1002/cne.22054.
53. Hauptmann, G. & Gerster, T. Regulatory gene expression patterns reveal transverse and longitudinal subdivisions of the embryonic zebra®sh forebrain. Mech. Dev. (2000).
54. Kalyviotis, K., Qin, H. & Pantazis, P. PhOTO zebrafish and primed conversion: advancing the mechanistic view of development and disease. in Behavioral and Neural Genetics of Zebrafish 309–322 (Elsevier, 2020). doi:10.1016/B978-0-12-817528-6.00019-X.
55. Robson, S. J. & Burgoyne, R. D. Differential localisation of tyrosinated, detyrosinated, and acetylated alpha-tubulins in neurites and growth cones of dorsal root ganglion neurons. Cell Motil. Cytoskeleton 12, 273–282 (1989).
56. Baas, P. W., Rao, A. N., Matamoros, A. J. & Leo, L. Stability properties of neuronal microtubules: Stability Properties of Neuronal Microtubules. Cytoskeleton 73, 442–460 (2016).
57. Moutin, M., Bosc, C., Peris, L. & Andrieux, A. Tubulin post-translational modifications control neuronal development and functions. Dev. Neurobiol. 81, 253–272 (2021).
58. Hostettler, L. et al. The Bright Fluorescent Protein mNeonGreen Facilitates Protein Expression Analysis In Vivo. G3 GenesGenomesGenetics 7, 607–615 (2017).
59. Hjorth, J. T. & Key, B. Are Pioneer Axons Guided by Regulatory Gene Expression Domains in the Zebrafish Forebrain? High-Resolution Analysis of the Patterning of the Zebrafish Brain during Axon Tract Formation. Dev. Biol. 229, 271–286 (2001).
60. Thiele, T. R., Donovan, J. C. & Baier, H. Descending Control of Swim Posture by a Midbrain Nucleus in Zebrafish. Neuron 83, 679–691 (2014).
61. Spikol, E. D., Cheng, J., Macurak, M., Subedi, A. & Halpern, M. E. Genetically defined nucleus incertus neurons differ in connectivity and function. http://biorxiv.org/lookup/doi/10.1101/2022.04.07.487414 (2022) doi:10.1101/2022.04.07.487414.
62. Kunst, M. et al. A Cellular-Resolution Atlas of the Larval Zebrafish Brain. Neuron 103, 21-38.e5 (2019).
63. Chen, Z. et al. Primary Neuron Culture for Nerve Growth and Axon Guidance Studies in Zebrafish (Danio rerio). PLoS ONE 8, e57539 (2013).
64. Simpson, H. D., Kita, E. M., Scott, E. K. & Goodhill, G. J. A quantitative analysis of branching, growth cone turning, and directed growth in zebrafish retinotectal axon guidance. J. Comp. Neurol. 521, 1409–1429 (2013).
65. Molnár, Z. & Blakemore, C. Lack of regional specificity for connections formed between thalamus and cortex in coculture. Nature 351, 475–477 (1991).

66. Wilson, S. W. & Easter, S. S. A pioneering growth cone in the embryonic zebrafish brain. Proc. Natl. Acad. Sci. U. S. A. 88, 2293–2296 (1991).
67. Clarke, A. et al. Dynamic morphogenesis of a pioneer axon in Drosophila and its regulation by Abl tyrosine kinase. Mol. Biol. Cell 31, 452–465 (2020).
68. Randlett, O., Poggi, L., Zolessi, F. R. & Harris, W. A. The Oriented Emergence of Axons from Retinal Ganglion Cells Is Directed by Laminin Contact In Vivo. Neuron 70, 266–280 (2011).

Supplementary Material

Table S1: Assays and number of samples used in each.

Assay	Number of Samples
Anti-HuC/D antibody labelling	5 (2 samples 1 dpf and 3 samples 2 dpf)
her5 expression	4
nkx2.2a expression	4 (3 samples 1 dpf and 1 sample 2 dpf)
arxa expression	4
irx1b expression	4
olig3 expression	6
pax6a expression	6
Dendra2 Photoconversion (all analysed)	17
Dendra2 Photoconversion (1, 3 and 4 dpf)	4
Anti-AcTub antibody labelling	9
Live Imaging - Lightsheet Microscopy	5
Live Imaging - Confocal Microscopy	3

Table S2: Mean, Standard Error and P-values for each paired student t-test. Statistical analysis data regarding mean, standard error, and p-values for each of the paired student t-test done, comparing left and right-side quantitative data. All non-significative differences. However, given the small number of samples (n=4), we cannot rule out the possibility of differences.

Tested Data (Left vs. Right)	Mean Left	Mean Right	Standard Error Left	Standard Error Right	P-value
Final length before connection	146,3 µm	166,2 µm	14,83 µm	14,79 µm	0,23
Minimum extension speed	0,1 µm/min	0,1 µm/min	0,06 µm/min	0,06 µm/min	0,88
Maximum extension speed	2,8 µm/min	3,0 µm/min	0,31 µm/min	0,44 µm/min	0,79
Minimum retraction speed	0,2 µm/min	0,2 µm/min	0,11 µm/min	0,06 µm/min	0,81
Maximum retraction speed	1,4µm/min	1,7µm/min	0,19 µm/min	0,23 µm/min	0,53
Fraction of retraction time points	0,2	0,3	0,02	0,04	0,42

Table S3: Mapzebrain - number and neuron identity number per brain region of the TPC projecting neurons. Number of neurons and neuron identity number per brain region, regarding the neurons identified as projecting axons through the midline in the TPC region of interest (ROI), using the Mapzebrain zebrafish brain atlas.

Brain Region		Nº of neurons	Neuron Identity Number
Prosencephalon	Hypothalamus	1	9604
	Eminentia Thalami	1	12766
	Prethalamus (alar p3 derivative)	1	13897
	Prethalamus-Thalamus Boundary	1	12383
	Thalamus (alar p2 derivative)	8	9804; 11517; 11889; 13840; 13842; 13927; 13935; 13992
	Thalamus-Pretectum Boundary	1	10525
	Pretectum (alar p1 derivative)	19	10571; 10789; 10953; 10957; 11406; 11771; 11774; 11794; 12335; 12347;12384; 12513; 12715; 13860; 13877; 13903; 14082; 14179; 14413
	Total	32	-
Prosencephalon-Mesencephalon Boundary	Pretectum and Tegmentum	2	12238; 12351
Mesencephalon	Tegmentum	4	11955; 12357; 12361; 12389
Rhombencephalon	Cerebellum	4	9576; 9579; 10324; 11270
	Medulla Oblongata	2	11985; 12371
	Total	6	-

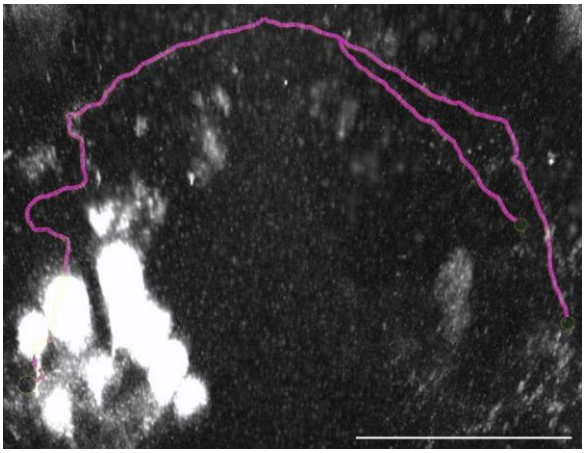

Figure S1: Tracing of the TPC at 4 dpf, post-photoconversion. Confocal Imaging. 3D rendering and tracing, done with Imaris Software. Scale corresponds to 50 µm.

Video S1: Imaris Tracing Tutorial, with audio narration.

https://drive.google.com/file/d/1ZOuK6MFOPocURYAT4bEfezdAPTe91aIw/view?usp=sharing

Video S2: Time-lapse of TPC formation, Lx200:GFF expression in a 1 dpf embryo. Lightsheet Imaging. Maximum intensity projections from the Lx200 transgenic line, with mNeonGreen reporter. A frame is taken every 5 minutes. Scale bar indicates 50 µm.

https://drive.google.com/file/d/11Ody01zW48EVzoUmDLJVIVBJUiHGk9oL/view?usp=sharing