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Multiple circuits controlling the versatility of locomotion

Molecular and connectivity principles in zebrafish

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Multiple circuits controlling the versatility of locomotion

Molecular and connectivity principles in zebrafish

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To my mother

ABSTRACT

Locomotion is produced by neuronal circuits in the spinal cord. These motor circuits rely on the activation of supraspinal centres and transform descending excitatory drive into coordinated rhythmic activity. Far from generating a mere stereotyped behaviour, the spinal locomotor circuit is endowed with mechanisms that allow for flexible motor output with appropriate changes in speed and strength to match specific external and internal demands. These mechanisms are not cemented after the initial assembly of the circuits at early developmental stages but face the necessity to adapt during maturation to the new behavioural needs of the animal. In this thesis, we take advantage of the genetic and experimental accessibility of the adult zebrafish as a model system to study the detailed organization of the spinal motor circuits that underlies the versatility of locomotion.

In adult zebrafish, the spinal locomotor circuit does not consist of a single network, but is modular, and comprises three types of rhythm-generating excitatory V2a interneurons (INs) that connect selectively to motoneurons (MNs) innervating the slow, intermediate or fast muscle fibers. The three V2a IN-MN circuit modules are recruited sequentially to drive swimming at different speeds. In the first study presented in this thesis, we reveal how the connectivity pattern of V2a INs, together with their intrinsic pacemaker properties, enables the initiation of rhythmic locomotion and allows for smooth transitions in speed. In the second study, we explore how the connectivity and function of the earliest-born V2a INs and primary MNs (pMNs) change during maturation from larval to adult stages. As the swimming behaviour shifts to lower frequencies in adult zebrafish, neurons that are involved in generating locomotion at fast speeds in larvae become embedded in different circuits and are redeployed to new functions. Finally in the third study included in this thesis, we reveal the molecular logic underpinning the diversity of MNs and V2a INs and their organization into three speed circuit modules. Overall, this work expands our knowledge of the molecular and functional organization of the spinal motor circuits, uncovering general principles that could be conserved in other vertebrate species.

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CONTENTS

1 INTRODUCTION 5
1.1 Locomotion and central pattern generators 5
1.2 CPG in vertebrates 6
1.2.1 Main CPG components 6
1.2.2 Transcriptional code and CPG classes in mammals 8
1.2.3 Diversity within neuronal populations and the quest for molecular markers 10
1.3 The locomotor CPG in adult zebrafish 11
1.3.1 Neuronal CPG classes in adult zebrafish 11
1.3.2 Modular organization of the spinal motor circuit 13
1.4 The escape circuit 14
1.5 From larva to adult: maturation changes 16
2 RESEARCH AIMS 19
3 MATERIALS AND METHODS 21
3.1 Animals 21
3.2 Zebrafish *ex vivo* preparation 21
3.3 Retrograde labeling of MNs 22
3.4 Electrophysiology 22
3.5 Immunohistochemistry and morphological analysis 22
3.6 Two-photon laser ablations 23
3.7 *In-vivo* behavioural analysis 23
3.8 Tissue dissociation and FAC sorting 23
3.9 Single-cell RNA sequencing and transcriptome analysis 24
3.10 *In situ* RNA hybridization 24
4 RESULTS AND DISCUSSION 25
4.1 Connectivity rules and pacemaker properties controlling the start and change in locomotion speed 25
4.1.1 Connectivity map of the rhythm-generating V2a IN population 25
4.1.2 Contribution of pacemaker properties to locomotor rhythm generation and acceleration 27
4.2 Maturation reshapes early-established motor circuit 29
4.2.1 Changes in connectivity, mode, plasticity, and strength of synaptic transmission 29
4.2.2 Circuit components are redeployed to a different function 30
4.2.3 Electrical coupling between V2a INs and pMNs and within the pMN population play complementary functional roles 32
4.3 Functional spinal circuit modules are molecularly defined 33
4.3.1 Molecular signature of MNs and V2a INs in adult zebrafish 33
4.3.2 The modular architecture of MNs and V2a INs is reflected in their molecular identity 36

5 CONCLUSION AND FUTURE PERSPECTIVES 39
6 ACKNOWLEDGEMENTS...41
7 REFERENCES...43

LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CIN	Commissural interneuron
CoLo	Commissural local interneuron
CPG	Central pattern generator
dIN	Descending interneuron
DLR	Diencephalic locomotor region
eIN	Excitatory interneuron
FACS	Fluorescence-activated cell sorting
IN	Interneuron
LPGi	Lateral paragigantocellular nucleus
MLR	Mesencephalic locomotor region
MN	Motoneuron
NMDA	N-Methyl-D-aspartic acid
pMN	Primary motoneuron
RNAseq	RNA sequencing

1 INTRODUCTION

1.1 LOCOMOTION AND CENTRAL PATTERN GENERATORS

Locomotion is one of the most basic yet crucial motor behaviours for both vertebrate and invertebrate species. It gives animals the ability to explore their environment, find food or mating partners, and avoid predators. It forms the basic motor repertoire of a species together with several other behaviours such as breathing, swallowing, balance control, and eye movements. To serve each of these motor behaviours, animals are endowed with specific neural circuits, called central pattern generators (CPGs), which are sufficient to generate and sustain rhythmic movements. CPGs are innate, but also very adaptable; they allow for a voluntary start and stop of movements as well as for their modulation according to the overall goal of the animal and combined with sensory feedback (Roberts et al., 1998; Marder and Bucher, 2001; Grillner, 2003; Marder and Rehm, 2005; Arber, 2012; Kiehn, 2016; Grillner and El Manira, 2020).

In vertebrates, the locomotor CPG is located in the spinal cord and generates the rhythmic and coordinated motor pattern underlying the sequential activation of axial and limb muscles (Grillner, 2003; Kiehn, 2006). Finally, CPGs act as processing centres, directly integrating information from sensory feedback and brain inputs that result in smooth changes of speed, direction, obstacle avoidance and corrective postural movements (Duysens and Pearson, 1976; Forssberg et al., 1977; Grillner and Wallén, 1982; Pearson, 1993; Grillner, 2006; Rossignol et al., 2006; Deliagina et al., 2008; Ferreira-Pinto et al., 2018).

Spinalization experiments in mammals, including adult cats, but also in amphibians, birds reptiles and fish, have demonstrated that the neural activity for locomotor movements can be generated by the spinal cord alone (Singer, 1884; Tarchanoff, 1884; Gray and Lissmann, 1940; Holst, 1954; Forssberg and Grillner, 1973; Grillner et al., 1976; Forssberg et al., 1977; Grillner and Rossignol, 1978; Forssberg et al., 1980a; Forssberg et al., 1980b; McDearmid and Drapeau, 2006). However, spinal CPGs need an appropriate external excitatory drive to initiate and control locomotion, which, in intact animals, comes from locomotor command areas in the mesencephalon (the mesencephalic locomotor region, MLR) and in the diencephalon (the diencephalic locomotor region, DLR) via reticulospinal brainstem nuclei (Shik et al., 1966; El Manira et al., 1997; Dubuc et al., 2008; Esposito et al., 2014; Capelli et al., 2017; Josset et al., 2018; Lemieux and Bretzner, 2019). In particular, the glutamatergic population of the lateral paragigantocellular nucleus (LPGi) directly contacts the spinal CPG (Capelli et al., 2017). These command areas provide a tonic excitatory drive to the spinal system, where an increase in stimulation will produce locomotion with increased speed which, in tetrapods, is associated with a switch of gate (Shik et al., 1966; Lemieux et al., 2016; Capelli et al., 2017; Caggiano et al., 2018; Koronfel et al., 2021). Additionally, hindbrain neurons have been shown to play a role in turning, postural control and termination of locomotor activity (Bouvier et al., 2015; Juvin et al., 2016; Capelli et al., 2017). Upstream of the MLR and DLR, the basal ganglia serve the function of action selection and decision

making, integrating inputs coming from cortex, thalamus and the dopaminergic substantia nigra pars compacta (Jordan, 1998; Ryczko and Dubuc, 2013; Grillner and Robertson, 2016). Finally, direct corticospinal projections play a role in locomotion for visuomotor coordination, which becomes important for instance on complex terrain (Liddell and Phillips, 1944; Lawrence and Kuypers, 1968; Georgopoulos and Grillner, 1989; Arber and Costa, 2018).

The spinal locomotor CPG can also generate rhythmicity and sustain activity without sensory feedback. This has been shown in deafferented and curarized animals, that can still produce coordinated and rhythmic movements (Harcombe and Wyman, 1970; Grillner and Zangger, 1975; Grillner et al., 1976; Kahn and Roberts, 1978; Grillner and Zangger, 1979; Delcomyn, 1980; Grillner and Zangger, 1984; Kyriakatos et al., 2011). Therefore, the locomotor CPG alone is sufficient to generate the coordinated pattern of rhythmic muscle activation which results in locomotion. However, sensory feedback is extremely important for adaptation to the external environment during ongoing activity and can effectively change the duration and intensity of muscle activation (Duysens and Pearson, 1976; Grillner and Rossignol, 1978; Grillner et al., 1981; Rossignol et al., 2006; Windhorst, 2007; Fidelin et al., 2015; Böhm et al., 2016; Koch et al., 2017; Knafo and Wyart, 2018; Mayer and Akay, 2018; Santuz et al., 2019; Picton et al., 2021).

1.2 CPG IN VERTEBRATES

1.2.1 Main CPG components

Most of what is known today about the general structure of vertebrate locomotor CPGs comes from studies in the lamprey (Grillner, 2003) and in *Xenopus* tadpoles (Roberts et al., 2010), in which locomotion consists of forward propulsion generated by a rostrocaudal propagation of left-right axial muscle contractions. In these animals, as well as in zebrafish and mice, locomotion is generated by a series of repeated CPGs in each hemi-segment of the spinal cord (Cangiano and Grillner, 2005; Arber, 2012; Kiehn, 2016; Berg et al., 2018). The locomotor CPG circuit emerging from the study of aquatic animals provides a conceptual framework for all the vertebrate lineage. The nervous system across vertebrates shares many similarities, and it is clear that organisms that need to solve similar problems use common strategies and building blocks, to which new circuit levels are added as the complexity of behaviour increases. Studying the locomotor CPG in aquatic animals such as lamprey, *Xenopus* and zebrafish has already provided information that can be extrapolated to mammals, which are less accessible experimentally. However, substantial work has been done in limbed animals, in which locomotion additionally involves the coordination between limbs and between the muscles of each limb.

Overall, the CPG consists of an oscillatory centre that receives the descending excitatory drive and is capable of producing coordinated rhythmicity. The main neuronal components of a locomotor CPG are ipsilateral, rhythm-generating INs and commissural, coordinating INs

generating left-right-alternation. For intra-limb coordination, circuits regulating flexor-extensor alternation are also needed. Finally, the coordinated rhythmic motor output is channelled to MNs that directly activate the muscles (Kiehn, 2016; D'Elia and Dasen, 2018; Dasen, 2018; Grillner and El Manira, 2020). MNs have long been seen as passive recipients of the CPG output, merely relaying the command to the muscles. However, recent work in zebrafish and mouse has shown that they have a more prominent role in the locomotor CPG and can retrogradely influence the rhythm and should therefore be considered an integral part of the CPG circuit (Song et al., 2016; Falgairolle et al., 2017).

Strong evidence from both limbed and non-limbed animals supports the hypothesis that the locomotor rhythm is generated by excitatory INs which use glutamate to provide direct rhythmic excitation to MNs and other INs on the same side of the spinal cord (including contralaterally projecting INs) via NMDA and AMPA receptors. They are the first recipients of descending brain input, and they transform these commands into rhythmic activity, often aided by their intrinsic neuronal properties (Grillner, 2003; Jankowska, 2008; Roberts et al., 2008; Goulding, 2009; Kiehn, 2011). Indeed, it has been shown that a single hemi-segment of spinal cord is able to produce rhythmic activity, independent of reciprocal inhibition (Kudo and Yamada, 1987; Soffe, 1989; Kjaerulff and Kiehn, 1996; Cangiano and Grillner, 2003, 2005; Grillner, 2006). The excitatory INs were first identified in *Xenopus* tadpoles (descending INs, dINs) and lamprey (excitatory INs, eINs) and they share many similarities with V2a INs in zebrafish and mouse (Jessell, 2000; Kimura et al., 2006; Eklöf-Ljunggren et al., 2012).

In both mammals and aquatic vertebrates, left-right alternation is thought to be controlled by excitatory and inhibitory commissural INs (CINs), which send axonal projections to the contralateral side of the spinal cord. Inhibitory CINs provide reciprocal inhibition through direct inhibition of MNs and INs on the other side of the spinal cord when activated by the ipsilateral excitatory INs. The inhibitory CINs were first identified using dual recordings in lamprey and *Xenopus*, and in both species it has been demonstrated that they play a crucial role in mediating left-right alternation by providing reciprocal inhibition (Grillner and Wallén, 1980; Buchanan and Cohen, 1982; Cohen and Harris-Warrick, 1984; Soffe et al., 1984).

A second way to achieve reciprocal inhibition was described in mammals and involves the activation of excitatory CINs that connect to ipsilateral inhibitory INs on the other side of the spinal cord (Butt and Kiehn, 2003; Jankowska et al., 2005; Quinlan and Kiehn, 2007; Jankowska, 2008; Talpalar et al., 2013). There is evidence in mouse that the coordination of axial muscles relies predominantly on inhibitory CINs, and not on excitatory CINs (Goetz et al., 2015). Excitatory commissural interneurons have also been shown to connect directly to fin motoneurons in the lamprey (Mentel et al., 2008). In zebrafish, both excitatory and inhibitory CINs have been described, similar to those found in mice (McLean and Dougherty, 2015; Björnfors and El Manira, 2016; Picton et al., 2022).

1.2.2 Transcriptional code and CPG classes in mammals

Model organisms with high genetic accessibility, such as mice and zebrafish, allow targeting of specific neuronal populations by the expression of fluorescent proteins driven by molecular markers. In particular, the transcription factors involved in the differentiation of the developing spinal cord are broadly conserved in all vertebrates and clearly define the neuronal populations of the spinal cord (Figure 1). This approach has allowed the generation of many selective transgenic lines to interrogate CPG neuron types (Briscoe et al., 2000; Jessell, 2000; Goulding, 2009; Kiehn, 2011; Arber, 2012; Gosgnach et al., 2017; Berg et al., 2018; Grillner and El Manira, 2020). The different classes of neurons in the spinal cord develop following two gradients of morphogens from the roof and the floor plates, which generate 11 progenitor domains: 5 in the ventral part (V0 to V3 plus one giving rise to MNs) and 6 in the dorsal part of the spinal cord (dI1 to dI6). Studies in mammals and aquatic vertebrates show that the 5 ventral progenitor domains give rise to the MNs and excitatory and inhibitory INs in the locomotor CPG, whilst the dorsal domains give rise to neurons involved in sensory feedback as well as one class of CPG INs (dI6) (Ericson et al., 1997; Briscoe et al., 2000; Jessell, 2000; Liem et al., 2000; Lee and Pfaff, 2001; Goulding et al., 2002; Higashijima et al., 2004; Kimura et al., 2006; Stepien and Arber, 2008; Goulding, 2009; Alaynick et al., 2011; Kiehn, 2011; Andersson et al., 2012; Dyck et al., 2012; Satou et al., 2012; Vallstedt and Kullander, 2013; Gosgnach et al., 2017).

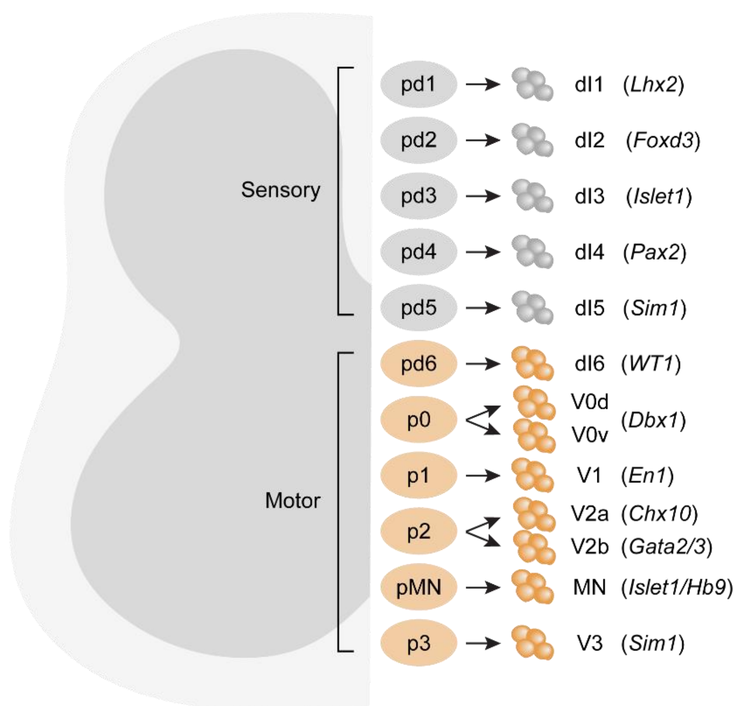

Figure 1. Schematic of the developmental origin of spinal cord populations. Gradients of morphogens specify 11 progenitor domains along the dorsoventral axis of the spinal cord (pd1-6, p0-3, pMN). Each progenitor domain gives rise to neuronal populations that persist in the adult animal (dI1-6, v0-3, MN) and that are defined by the expression of specific transcription factors (in brackets). The neuronal populations involved in motor circuits are highlighted in orange.

1.2.2.1 *Motoneurons*

MNs derive from one of the ventral progenitor domains, and the transcription factor *Islet1* is required to establish their identity (Pfaff et al., 1996; Ericson et al., 1997; Briscoe and Ericson, 1999; Jessell, 2000). In mammals they differentiate into separate motor columns along the rostrocaudal spinal axis, depending on the target muscles that they innervate. Within each motor column, MNs segregate into several motor pools, each innervating specific muscles (Jessell, 2000; D'Elia and Dasen, 2018). These anatomically defined MN types are also molecularly distinct, with specific transcription factor expression. However, there are skeletal muscle fibers with different contractile properties, namely the slow-twitch, fatigue-resistant fibers; fast-twitch, fast-fatigable fibers; and fibers with intermediate properties. In mammals, all fiber types are often present in the same muscle. These different fibers are innervated by distinct MNs with characteristic electrophysiological properties, forming slow, intermediate and fast motor units (ROMANES, 1964; Burke et al., 1981; Vanderhorst and Holstege, 1997; Dasen and Jessell, 2009; Nijssen et al., 2017). The slow-twitch MNs have the highest membrane resistance and excitability, while the fast-twitch have the biggest dendritic trees and largest axonal diameters (Henneman et al., 1965; Cullheim et al., 1987; Gardiner, 1993; Nijssen et al., 2017). Additionally, these MN types show different vulnerabilities to motoneuronal diseases such as amyotrophic lateral sclerosis (Nijssen et al., 2017).

1.2.2.2 *Rhythm-generating interneurons*

The V2a population is part of the V2 class of neurons and is defined by the transcription factor *Chx10*. In zebrafish, activation of the V2a IN population has been shown to be necessary and sufficient to drive locomotion (Eklöf-Ljunggren et al., 2012; Ljunggren et al., 2014). However, in mice, the search for an excitatory population underlying rhythm-generation has not yet yielded conclusive results. Developmental ablation of V2a INs in mice impacted left-right alternation and impaired the generation of locomotor activity by electrical, but not pharmacological stimulation (Crone et al., 2008; Crone et al., 2009). As it is known that optogenetic activation of glutamatergic neurons initiates locomotor-like activity, several other excitatory ipsilateral populations have also been investigated (Hägglund et al., 2010; Hägglund et al., 2013). Among these, perturbation of *Shox2*-expressing neurons, which partially overlap with the V2a IN population, affects the locomotor rhythm at high frequencies (Dougherty et al., 2013; Ha and Dougherty, 2018). Finally, the *Hb9*-expressing neurons appeared to be good candidates, as they display pacemaker properties and are rhythmically active during locomotion. However, their firing is delayed compared to that of MNs (Kwan et al., 2009; Masino et al., 2012; Caldeira et al., 2017), and locomotion remains unaltered when glutamatergic transmission from these neurons is eliminated (Koronfel et al., 2021).

1.2.2.3 Left-right coordinating interneurons

One of the major classes of commissural INs is the V0 population, which is conserved from zebrafish to mice, and is characterized by the expression of the transcription factor *Dbx1*. In mice, genetic deletion of this population synchronizes the motor activity on the left and right side of the spinal cord during locomotion, demonstrating their role in left-right coordination (Lanuza et al., 2004; Talpalar et al., 2013). The V0 class comprises the excitatory V0v and the inhibitory V0d INs (Moran-Rivard et al., 2001; Pierani et al., 2001; Lanuza et al., 2004). They have been shown to be involved in coordination at slow and high speeds, respectively (Talpalar et al., 2013). In addition, the V0 population has been shown to be necessary for correct left-right coordination during backward locomotion (Zelenin et al., 2021).

1.2.2.4 Burst-terminating interneurons

The V1 INs are defined by the expression of the transcription factor *En-1*. They are a heterogenous class of ipsilateral inhibitory INs that form monosynaptic connections to MNs (Saueressig et al., 1999; Wenner et al., 2000; Moran-Rivard et al., 2001; Pierani et al., 2001; Alvarez et al., 2005; Gosgnach et al., 2006; Zhang et al., 2014; Bikoff et al., 2016). Silencing of V1 INs via ablation or hyperpolarization increased the synaptic drive to MNs and increased the burst duration, resulting in a slower locomotor rhythm in the neonatal mouse spinal cord (Gosgnach et al., 2006; Falgairolle and O'Donovan, 2019). These results suggest that V1 INs affect the speed of locomotion through a burst termination mechanism.

1.2.3 Diversity within neuronal populations and the quest for molecular markers

The genetic approach based on transcription factors has helped shed light on the respective contribution of each CPG population to locomotion, while considering these populations as homogeneous sets of neurons with the same properties and function in the network. However, a growing body of research in vertebrates has begun to address the functional heterogeneity within each of the major spinal cord classes, which is reflected in differences in intrinsic properties, connectivity patterns and ultimately in function (Ampatzis et al., 2013; Dougherty et al., 2013; Bikoff et al., 2016; Björnfors and El Manira, 2016; Song et al., 2016; Chopek et al., 2018; Ha and Dougherty, 2018; Hayashi et al., 2018; Song et al., 2018; Sweeney et al., 2018; Menelaou and McLean, 2019; Pedroni and Ampatzis, 2019; Song et al., 2020; Picton et al., 2022). Therefore, opto- or chemo- genetic studies in which whole spinal neuron classes are manipulated (Lanuza et al., 2004; Gosgnach et al., 2006; Crone et al., 2008; Zhang et al., 2008; Talpalar et al., 2013; Zhang et al., 2014) could be insufficient to uncover the detailed organization of the spinal networks. For example, considerable diversity has been demonstrated within the V2a population in mice, with the identification of two types of V2a INs (type I and II) that have distinct rostrocaudal distribution, connectivity and function. While type I V2a INs have descending axonal projections and are enriched at lumbar levels,

type II V2a INs project to both spinal and supraspinal targets and are enriched at cervical levels (Hayashi et al., 2018). A better understanding of the diversity within each CPG class is of pivotal importance to clarify the function of these major spinal populations. Furthermore, this approach may reveal new subpopulations contained within the major classes that could have nuanced contributions towards behaviour.

In recent years, dedicated efforts have begun to investigate the molecular characterization of this heterogeneity within different spinal populations in mammals to enable selective manipulation and functional insights (Bikoff et al., 2016; Gabitto et al., 2016; Hayashi et al., 2018; Häring et al., 2018; Sathyamurthy et al., 2018; Delile et al., 2019; Osseward and Pfaff, 2019; Alkaslasi et al., 2021; Blum et al., 2021; Osseward et al., 2021; Russ et al., 2021). Despite the growing evidence of high molecular diversity, the functional identity of the proposed subpopulations has remained elusive. A particular interest has been devoted to MNs (Nijssen et al., 2017; Alkaslasi et al., 2021; Blum et al., 2021) due to their pivotal role in the generation of movements and because their dysfunction underlies human neuromuscular disorders. However, in mammals, the transcriptionally defined MN subpopulations have been shown to correspond to distinct motor pools, therefore segregating based on their muscle innervation pattern, rather than their electrophysiological and functional diversity (Alkaslasi et al., 2021; Blum et al., 2021).

1.3 THE LOCOMOTOR CPG IN ADULT ZEBRAFISH

Zebrafish is an advantageous model system to analyze in detail the connectivity and function of the neural circuits for locomotion. At both larval and adult stages, it is accessible for electrophysiological recordings, optogenetic manipulations, behavioural analysis following acute ablations in the intact animal (larva and juvenile) or using an *ex vivo* preparation (adult) (Fetcho et al., 2008; Gabriel et al., 2011; Grillner and El Manira, 2015; Koyama et al., 2016; Berg et al., 2018; Knafo and Wyart, 2018). This experimental accessibility combined with genetic amenability allows for the generation of transgenic lines that afford selective manipulation of different neuronal populations (Higashijima et al., 2004; Kimura et al., 2006; Arrenberg et al., 2009; Satou et al., 2012; Satou et al., 2013; Kimura et al., 2014; Sternberg et al., 2016).

1.3.1 Neuronal CPG classes in adult zebrafish

1.3.1.1 Motoneurons

The first MNs to develop in zebrafish are the pMNs, followed by a larger number of secondary MNs. In adult zebrafish (7-12 weeks of age), pMNs are never recruited during locomotion and are part of a specialized circuit that is active during escape behaviour (Gabriel et al., 2011; Ampatzis et al., 2013; Song et al., 2015; Guan et al., 2021). Secondary

MNs are organized into three types, each projecting to one specific type of muscle fiber (Ampatzis et al., 2013). Like mammals, zebrafish have slow, intermediate or fast fibers, however, unlike mammalian fibers, they are spatially segregated in the axial musculature (van Raamsdonk et al., 1982; Devoto et al., 1996). The three MN pools are topographically organized from ventro-lateral to dorso-medial and sequentially recruited during swimming. At slower speeds of locomotion (below 4 Hz) the ventro-lateral slow MN pool is recruited and remains active at higher speeds, followed by the intermediate pool (which starts to be recruited from 4 to 8 Hz) and finally by the dorso-medial fast MN pool (recruited at higher frequencies than 8 Hz) (Gabriel et al., 2011; Ampatzis et al., 2013). The distinct MN pools also differ in firing and intrinsic electrophysiological properties that correlate with the muscle fiber they innervate. Fast MNs have low membrane resistance and narrower action potentials, with a large afterhyperpolarization, and accordingly fire at high frequency with strong adaptation. In contrast, slow MNs fire bursts of action potential at lower thresholds, with no afterhyperpolarization, and wide action potentials. The intermediate MNs display tonic firing and less pronounced spike frequency adaptation (Ampatzis et al., 2013).

1.3.1.2 V2a interneurons

As previously mentioned, the main excitatory drive to MNs in zebrafish comes from the ipsilateral glutamatergic V2a population, marked by the expression of the transcription factor Chx10 (Kimura et al., 2006). It has been demonstrated through optogenetic activation and two-photon ablation that in zebrafish this neuronal population is necessary and sufficient to drive the locomotor behaviour (Eklöf-Ljunggren et al., 2012; Ljunggren et al., 2014). In larvae, it also has been shown that dorsal V2a INs provide excitatory drive to pMNs and they have been suggested to have a role in swimming and postural control during swimming (Kimura et al., 2006; Bagnall and McLean, 2014; Menelaou and McLean, 2019). These results strengthen the hypothesis that the zebrafish V2a IN population corresponds to the excitatory population found in lamprey and *Xenopus* which generates the locomotor rhythm.

However, until recently, it was assumed that the locomotor CPG and the excitatory IN population consist of a single network and that increase in speed results from an increase in overall excitation in the network. Instead, it has been shown that in adult zebrafish the V2a INs are a heterogeneous population composed of three types mirroring the MN pools, which are sequentially activated at slow, intermediate, and fast swimming speeds (Ampatzis et al., 2014; Song et al., 2016; Song et al., 2018). V2a IN types also show differences in their electrophysiological features, although each of these properties alone does not unambiguously discriminate the module types. The V2a INs recruited at slower speed show higher input resistances, lower firing thresholds, and non-adapting firing patterns, and the ones recruited at faster speeds show lower input resistances, higher firing thresholds, and adapting firing (Ampatzis et al., 2014; Song et al., 2018).

1.3.2 Modular organization of the spinal motor circuit

The detailed connectivity between V2a INs and MNs has been studied, and the results show a precisely structured CPG that comprises three speed circuit modules (Figure 2). Each V2a IN type connects preferentially to the MN counterpart belonging to the same speed module, with a mixed chemical and electrical synapse. At the start of a locomotor episode, the slow module is engaged, namely, the slow V2a INs become active and drive the slow MN pool. With increasing swimming speed, presumably following an increase in descending drive from the brain, the intermediate and then the fast modules are sequentially recruited (Ampatzis et al., 2014; Song et al., 2016).

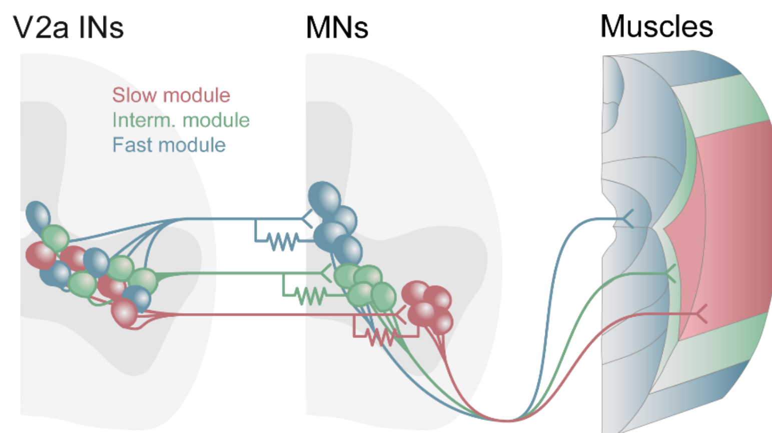

Figure 2. The modular locomotor CPG in zebrafish. The locomotor circuit in adult zebrafish is subdivided into three modules, each comprising one type of rhythm-generating excitatory V2a interneuron (V2a IN) controlling fast (blue), intermediate (green), and slow (red) motoneurons (MNs) and the selective muscle fibers that these innervate. The connections between V2a INs and MNs are mixed with electrical and chemical components. The gap junctions (indicated by the resistors in the drawing) enable MNs to provide feedback to the V2a INs, amplifying the chemical synaptic transmission when depolarized.

Additionally, MNs can retrogradely influence, through gap junctions, the firing and synaptic transmission of the presynaptic V2a INs of the same module and of adjacent modules. The gap junctions allow back-propagation of changes in the MNs' membrane potential to V2a INs so that post-synaptic depolarization enhances transmitter release, and conversely, hyperpolarization causes depression of synaptic transmission. These gap junctions are axo-dendritic, and the change in membrane potential from the axon can also back-propagate to the soma of the V2a INs and affect their firing. The combined change in synaptic transmission and firing of V2a INs through gap junctions allow MNs to affect ongoing swimming (Song et al., 2016).

The connections between V2a INs and MNs in different speed modules also show differences in the location and properties of their synaptic transmission. In each of the three modules, there are two types of V2a INs displaying distinctive morphological and electrophysiological

characteristics. The first type displays a pacemaker firing pattern and elicits NMDA-mediated short-term potentiation in the target MNs. The second type is non-pacemaker and elicits weaker synaptic responses in MNs that display linear summation (Song et al., 2018). Morphologically, the pacemaker type shows purely descending axonal projections and targets the dendrites of MNs, while the non-pacemaker type projects bidirectionally and targets MN somata. Most of the pacemaker V2a INs target slow MNs via strong synapses displaying short-term potentiation, while most of the fast MNs receive inputs from non-pacemaker V2a INs that do not display short-term potentiation. These results show that the slow MN pool can be activated by a small number of pacemaker V2a INs involving NMDA-mediated short-term potentiation, while the fast module relies on a different strategy requiring convergence of precisely timed input from several V2a INs (Song et al., 2018). From a behavioural point of view, this flexibility in the CPG is important, as the slow module needs to be more responsive and easier to engage, while the fast module, involved in behaviours such as escaping a predator, must be activated only in specific situations.

The modular organization extends to other IN populations, such as the V0d and V0v populations of CINs. In each of these populations, three classes have been described that are recruited respectively at slow, intermediate or fast speeds of locomotion (Björnfors and El Manira, 2016; Picton et al., 2022). Interestingly, the majority of the V0v population belongs to the fast module, suggesting that in adult zebrafish this population is prevalently involved in coordinating swimming at high speeds (Björnfors and El Manira, 2016). Conversely, the V0d population comprises mostly slow INs in adult zebrafish, and participates in slow swimming speeds (Picton et al., 2022).

1.4 THE ESCAPE CIRCUIT

In addition to swimming, teleost fish including zebrafish, display another evolutionarily important behaviour: the escape response. Escape behaviour is a fast, stereotyped maneuver that allows fish to move away from a threat. This highly reliable circuit has been studied in detail in adult goldfish and larval zebrafish (Domenici and Hale, 2019). The escape response is known as “C-start” manoeuvre because it consists of the activation of all axial muscles on the contralateral side to the stimulus, producing a C-shaped bend (escape stage 1) that is followed by counter-bending in the opposite direction (escape stage 2) (Weihs, 1973; Eaton et al., 1977; Domenici and Hale, 2019). This sequence is also often followed by a short phase of fast swimming (Escape stage 3) (Weihs, 1973; Domenici and Hale, 2019; Xu et al., 2021).

Escape is initiated in response to certain sensory stimuli that could indicate the presence of a predator, such as a sound, a visual input or a movement. This sensory information coming from the vestibular and auditory branches of the VIIIth cranial nerve, the optic tectum or the lateral line, is relayed to populations of reticulospinal neurons which function as sensory integrators. Among these, the most relevant and well-studied are the Mauthner cells, a bilateral pair of commissural brainstem neurons characterized by large soma and axon size,

which guarantees high conduction velocities (Mauthner, 1859; Eaton and Farley, 1975; Zottoli, 1977; Faber et al., 1991; Guan et al., 2021; Xu et al., 2021). Unilateral activation of the Mauthner cell is ensured by feedforward inhibition by glycinergic neurons as well as by feedback inhibition from the cranial relay neurons in the brainstem to generate the appropriate directionality of the C-bend (Koyama et al., 2011; Koyama et al., 2016). Mauthner cells project to the contralateral side of the spinal cord and provide direct excitatory inputs to pMNs, fast secondary MNs, spinal descending INs, and inhibitory contralateral INs involved in the execution of the escape movement (Eaton and Farley, 1975; Fetcho and Faber, 1988; Faber et al., 1991; Fetcho, 1992; Hale et al., 2016; Guan et al., 2021). A recent study in adult zebrafish identified an excitatory cholinergic V2a IN type that expresses the transcription factor Chx10 and has a central role in amplifying and delivering the escape command from the Mauthner cell (Guan et al., 2021). This specialized V2a IN receives direct mixed chemical and electrical axo-axonic synapses from the Mauthner cell and delivers this excitation locally to other escape V2a INs and to pMNs via mixed synapses. Spinal inhibitory INs are contacted by the Mauthner axon and by the escape V2a INs, such as the commissural local INs (CoLos), active only during escape and not in swimming or struggling (Liao and Fetcho, 2008; Satou et al., 2009). CoLos are recruited by the Mauthner cell through electrical synapses, and by escape V2a INs through mixed synapses, and provide glycine-mediated inhibition to the whole contralateral escape circuit, including primary and fast secondary MNs as well as CoLos and some V2a INs (Liao and Fetcho, 2008; Satou et al., 2009; Guan et al., 2021). This powerful mechanism not only inhibits the output on the contralateral side of the spinal cord but also releases the inhibition to the ipsilateral side. Activation of both Mauthner cells seldom occurs, and in these cases, the CoLos serve to ensure a unilateral circuit activation (Satou et al., 2009).

The escape circuit has a unique organization of synaptic connections with axo-axonic contacts and electrical or mixed synapses. The axo-axonic distribution of synapses within this circuit likely enables a fast and reliable propagation of stimulus via direct recruitment of the elements of the circuit. Electrical synapses appear to play a particularly important role, contributing to the speed and coordination needed for this highly stereotyped behaviour. Gap junctions are intercellular channels, each composed of two hemichannels, that form a cytoplasmic continuity between neurons, ensuring a fast and direct transmission of the stimulus. Interestingly, it has been shown that gap junctions located on the Mauthner cell (contacting the VIIIth cranial nerve afferents and the CoLos) are composed of two distinct connexin proteins, one forming the pre-synaptic hemichannel (cx35.5) and one the post-synaptic hemichannel (cx34.1). Mutants in the genes encoding for the two connexin proteins show defects in the escape behaviour, such as slower speed or coordination defects (Miller et al., 2017).

1.5 FROM LARVA TO ADULT: MATURATION CHANGES

In vertebrates, motor circuits undergo reconfiguration during an animal's life, and we have a broad understanding of how the motor output and modulation of these circuits change (Saint-Amant and Drapeau, 2000; Combes et al., 2004; Marder and Rehm, 2005; Husch et al., 2015; Landmesser, 2018; Hachoumi and Sillar, 2020). However, how the underlying connectivity is reshaped during maturation has remained an ongoing challenge.

The zebrafish is one of the few vertebrate model systems that permits the study of neural circuits and their function with high levels of detail both at larval and adult stages (Buss and Drapeau, 2001; Fetcho et al., 2008; Arrenberg et al., 2009; Ahrens et al., 2012; Dunn et al., 2016; Berg et al., 2018; Chow et al., 2020). Therefore, this model offers a unique opportunity to study the refinement and transformation of early neural circuits to adapt to maturation changes.

Several systemic changes occur in zebrafish during maturation to adulthood. Larval zebrafish swim with the so-called "burst and glide" pattern, comprising short bursts of fast left-right oscillations followed by a passive gliding phase. Adult zebrafish instead can sustain longer episodes of swimming with smooth speed transitions. The body oscillations occur at a much higher frequency in the larvae (20-60 Hz) than in the adult (up to 20 Hz) (Saint-Amant and Drapeau, 1998; Budick and O'Malley, 2000; Müller et al., 2000; Buss and Drapeau, 2001; Gabriel et al., 2011; Kyriakatos et al., 2011; Ampatzis et al., 2013). These changes in swimming pattern and frequency mirror the changes in muscle composition. Larval muscle is composed mainly of fast fibers, with a thin superficial layer of red fibers, which will fully differentiate at maturation into the adult slow fibers at the time when intermediate fibers emerge (van Raamsdonk et al., 1982; Devoto et al., 1996).

In line with the differences in swimming pattern, frequency and muscle composition, there are also changes in the organization of locomotor circuits. Locomotor movements in larvae are mainly driven by fast circuits involving early-born neurons such as pMNs and dorsally located V2a INs, while the smallest and most ventral neurons are not yet integrated into the circuit (Myers, 1985; Myers et al., 1986; Westerfield et al., 1986; Liu and Westerfield, 1988; Kimura et al., 2006; McLean et al., 2007; McLean et al., 2008; McLean and Fetcho, 2009; Menelaou and McLean, 2012; Svara et al., 2018; Pujala and Koyama, 2019). Ventral V2a INs and MNs are active at slow frequencies (20 Hz) and these V2a INs become de-recruited at higher frequencies (Kimura et al., 2006; McLean et al., 2007; McLean et al., 2008; McLean and Fetcho, 2009). In contrast, the slow circuit module remains active throughout the swimming episode at adult stages (Ausborn et al., 2012; Eklöf-Ljunggren et al., 2012; Ampatzis et al., 2013, 2014). Recent work in larval zebrafish has suggested that two types (I and II) of dorsal early born V2a INs selectively control the timing (type I) and amplitude (type II) of pMN activity during swimming and were hence suggested to form a hybrid hierarchical circuit (Bagnall and McLean, 2014; Menelaou and McLean, 2019). In adult zebrafish, pMNs have become selectively integrated into the escape circuit and do not participate in the production of swimming (Gabriel et al., 2011; Ampatzis et al., 2013).

Therefore, the zebrafish locomotor circuit seems to undergo significant changes during maturation from larval to adult stages. However, the precise nature of these changes has remained unclear.

Beside the morphological changes in body shape and muscle composition, one example of circuit alterations from larvae to adult is represented by the V0d IN population. These INs switch activity profile as the animal develops from larval to adult stages. During larval stages, V0d INs contribute to the swimming behaviour only at the highest speeds, while following maturation most of these INs are recruited at slow swimming speeds (Satou et al., 2020; Picton et al., 2022). This functional switch is accompanied by an overall diversification in morphology, intrinsic properties, and function of the V0d population, that in the adult comprises neurons recruited at slow, intermediate, and fast speeds (Picton et al., 2022).

2 RESEARCH AIMS

The overall aim of this thesis is to understand the circuit organization underlying the flexibility of locomotor behaviour. This includes the functional and molecular mechanisms that produce a flexible change in speed during locomotion as well as the adaptive changes dictated by the maturation of the organism. The specific aims addressed in this work are:

1. To uncover the organization of the spinal networks underlying the transformation of descending tonic drive into rhythmic activity and speed changes during swimming.
2. To determine at the level of single neurons and synapses how early-born motor circuits change their connectivity and function during maturation.
3. To investigate the molecular logic underlying the functional diversity of MNs and V2a INs and their modular circuit organization.

3 MATERIALS AND METHODS

This is a short overview of the methods used in the thesis. For details refer to the methods section of each paper.

3.1 ANIMALS

In most experiments, 7-12 week old zebrafish (*Danio rerio*) of both sexes were used, except for acute ablations in papers I and II that were performed in 6-7 week old animals, and chronic ablations in paper II that were performed at 4-5 days post fertilization. Different transgenic animals were used to label specific neuronal populations or for optogenetic manipulations.

In all the papers presented, Tg(*Chx10*:GFP) or Tg(*Chx10*:RFP) were used to specifically target the V2a IN population in which the expression of green or red fluorescent proteins (GFP or RFP) was driven by the promoter of the transcription factor *Chx10*. Additionally, in paper II the Tol-056 enhancer trap line (Tol-056) was used, in which the Mauthner cells and cholinergic escape V2a INs express GFP. To elicit swimming with optogenetic stimulation of channelrhodopsin 2 (ChR2) selectively expressed in glutamatergic neurons, we used Tg(*Vglut2a*-loxP-dsRed-loxP-Gal4) crossed with Tg(UAS:ChR2-YFP), and Tg(*Elavl3*:cre), referred to as Tg(*Vglut2*-ChR2-YFP). In paper III, the transgenic line Tg(*Islet1a*:GFP), in which GFP expression is driven by the promotor of *Islet1a*, was used to sequence the transcriptome of MNs. Finally, the lines Tg(*Esrrga*-hs:loxP-RFP-loxP-GFP), Tg(*Shox2*:GFP) and Tg(*Vachta*:GFP), each crossed with a reporter line for V2a INs were used to target respectively Esrrga$^+$, Shox2$^+$ or vAChTa$^+$ V2a INs.

3.2 ZEBRAFISH *EX VIVO* PREPARATION

Electrophysiological recordings were performed using a spinal cord-brainstem *ex vivo* preparation of adult zebrafish (7-12 weeks old) of either sex according to previously published protocols (Gabriel et al., 2008; Gabriel et al., 2011; Kyriakatos et al., 2011). Animals were deeply anesthetized with MS-222 and then dissected in a slush of frozen extracellular solution. The internal organs, axial musculature, skull, and vertebral arches were removed. A portion of the axial caudal muscles was left intact to record ventral root motor activity by placing an extracellular recording electrode at an intramyotomal cleft. The preparation was then placed in the recording chamber and perfused with oxygenated extracellular solution at room temperature (20-22°C) for the duration of the experiment.

3.3 RETROGRADE LABELING OF MNS

Animals were anaesthetized in MS-222 and the dextran dye tracers were injected using a small pin in a selective type of muscle fibers (slow and intermediate or fast) or in a selective quadrant of the axial musculature. Animals were left to recover for at least 2 hours prior to electrophysiology experiments, immunohistology or in situ hybridization assays.

3.4 ELECTROPHYSIOLOGY

Neurons were identified by their expression of fluorescent proteins (GFP and/or RFP) driven by the promoters of different genes or by their labeling with fluorescent dyes achieved through muscle injections. All recordings were performed using the whole-cell patch-clamp techniques. Intracellular signals were recorded in current clamp with no bias current or in voltage clamp while holding the neuron at its resting membrane potential. Sequential paired recordings were performed by keeping the recording of one of the neurons while extracting the other pipette and changing it to a clean one for the next recording. Synaptic connectivity between pairs of neurons was tested by triggering a single action potential in the presynaptic neuron for 100–200 consecutive sweeps. To measure synaptic plasticity, trains of ten action potentials were elicited in the presynaptic neuron. Electrical coupling between pair of neurons was tested by injection of hyperpolarizing current in each neuron of the pair for 20–30 consecutive sweeps. In some experiments, the NMDA and/or AMPA receptors antagonists and cadmium were bath applied to block the glutamatergic and all synaptic transmission, respectively. Riluzole was bath applied to block persistent sodium currents. For post-hoc morphology reconstruction, 0.25% neurobiotin was added to the intracellular solution used for whole-cell recordings. Fictive swimming was elicited by electrical stimulation of descending axons with an extracellular glass pipette that was placed at the junction between the brainstem and the spinal cord, or by optogenetic stimulation of glutamatergic neurons in the hindbrain. Escape was elicited with electrical stimulation of the Mauthner cell with an extracellular glass pipette.

3.5 IMMUNOHISTOCHEMISTRY AND MORPHOLOGICAL ANALYSIS

Spinal cords were dissected and fixed in 4% paraformaldehyde (PFA). Non-specific protein binding sites were blocked and spinal cords were incubated with anti-GFP, anti-mCherry or with antibodies for connexin proteins (paper II). The tissue was then incubated with the appropriate Alexa Fluor-conjugated secondary antibody. To label neurobiotin-filled neurons, Alexa Fluor-conjugated streptavidin was added.

A laser scanning confocal microscope was used to acquire whole-mount imaging of the spinal cords with a 20x or a 40x water objective. The soma size was measured as the maximum surface area from confocal images. The position of axonal projections was measured from confocal images two spinal segments away from the soma. The full

morphologies (soma, axons and dendrites) of neurobiotin-filled neurons or retrogradely labelled MNs were traced and reconstructed manually in Adobe Illustrator.

3.6 TWO-PHOTON LASER ABLATIONS

Animals were anesthetized and embedded in 1.5% low-melt agarose in a Petri dish. For juvenile animals, the gills and mouth were exposed and the Petri dish was filled with fish water containing MS-222. The neurons were then photoablated bilaterally using a two-photon laser. Control animals were embedded alongside the ablated zebrafish but were not subjected to ablation. Successful ablations were confirmed by the permanent loss of GFP fluorescence. Animals were allowed to recover from anesthesia and acclimate for 1 h before behavioural analysis at room temperature.

3.7 *IN-VIVO* BEHAVIOURAL ANALYSIS

For behavioural analysis, animals were placed in a circular dish containing fish water positioned on a plexiglass platform, illuminated from below by an LED lightbox and imaged from above with a high-speed camera. Control and ablated fish were tested in randomized order. Fish were placed in a circular glass dish 8 or 12 cm in diameter respectively for testing escape or swimming behaviour and were allowed to acclimate for 20 min. Fast swimming was induced by a tactile stimulus applied to the tail using a fine tungsten pin and was recorded at 300 fps. Escape was evoked by a brief sound stimulus delivered by an audio speaker and recorded at 470 fps.

3.8 TISSUE DISSOCIATION AND FAC SORTING

Adult animals (7 weeks old) of either sex were deeply anesthetized in a slush of frozen extracellular solution and the spinal cords were quickly dissected. Two samples with 6-10 isolated spinal cords were prepared from each of the transgenic lines Tg(*Islet1a*:GFP) and Tg(*Chx10*:GFP) line. The samples were incubated with papain on a heated shaker at 37 °C and then mechanically triturated with fire-polished glass pipettes. The cell suspension was filtered through a cell-strainer and the nuclear DNA stain DRAQ5 was added. Using fluorescence-activated cell sorting (FACS), cells positive for GFP and DRAQ5 in each sample were sorted into a 384-wells plates.

3.9 SINGLE-CELL RNA SEQUENCING AND TRANSCRIPTOME ANALYSIS

The RNA in the single isolated cells was sequenced following the previously published protocol Smart-seq2 (Picelli et al., 2013; Picelli et al., 2014) by the Eukaryotic Single Cell Genomics Facility at SciLifeLab, Stockholm.

The reads from each sequenced cell were mapped to the zebrafish reference genome "Danio_rerio, Ensembl, GRCz11". Most of the following analysis was performed in R using the Seurat package (Stuart et al., 2019). Analysis of each dataset was conducted separately. Genes that were expressed in less than 3 cells, and cells that had more than 30% reads from mitochondrial genes or from spike-ins, or a number of genes higher than 9000 or lower than 2000 were excluded. The gene expression measurements within each cell were scaled by a constant factor then log-normalized and regressed. The 2000 most variable features or a list of all transcription factors present in the dataset were used for the t-distributed stochastic neighbour embedding (tSNE) two-dimensional visualization and for graph-based clustering using the FindClusters Seurat function. Differentially expressed genes in each cluster compared to all clusters were identified using the MAST test implemented in the Seurat function FindMarkers. Gene ontology analysis of all differentially expressed genes per cluster was performed using Panther 17.0 overrepresentation test. Regulon analysis was performed on all differentially expressed genes in the transcription factors-derived clusters, using the Cytoscape plugin iRegulon (Janky et al., 2014).

3.10 *IN SITU* RNA HYBRIDIZATION

For RNA hybridization analyses, custom probes were designed. The RNAscope Multiplex Fluorescent v2 Assay was performed according to the manufacturer's instructions on whole spinal cords. If required, the RNAscope protocol was followed by immunohistochemistry. Images were acquired using a laser scanning confocal microscope with a 40x water objective. RNAscope puncta were counted manually on z-stacks of confocal images and neurons were classified as positive for the RNA probe if they had at least 3 puncta at the level of the cell body.

4 RESULTS AND DISCUSSION

In this thesis, three projects were carried out to study different aspects of the organization of the locomotor network underlying the flexibility of motor output.

4.1 CONNECTIVITY RULES AND PACEMAKER PROPERTIES CONTROLLING THE START AND CHANGE IN LOCOMOTION SPEED

4.1.1 Connectivity map of the rhythm-generating V2a IN population

In adult zebrafish, V2a INs are necessary and sufficient to drive locomotion. They are subdivided into three types, which through their selective connection with MNs form three modules that become activated at different locomotor speeds (Eklöf-Ljunggren et al., 2012; Ampatzis et al., 2014; Eklöf-Ljunggren et al., 2014).

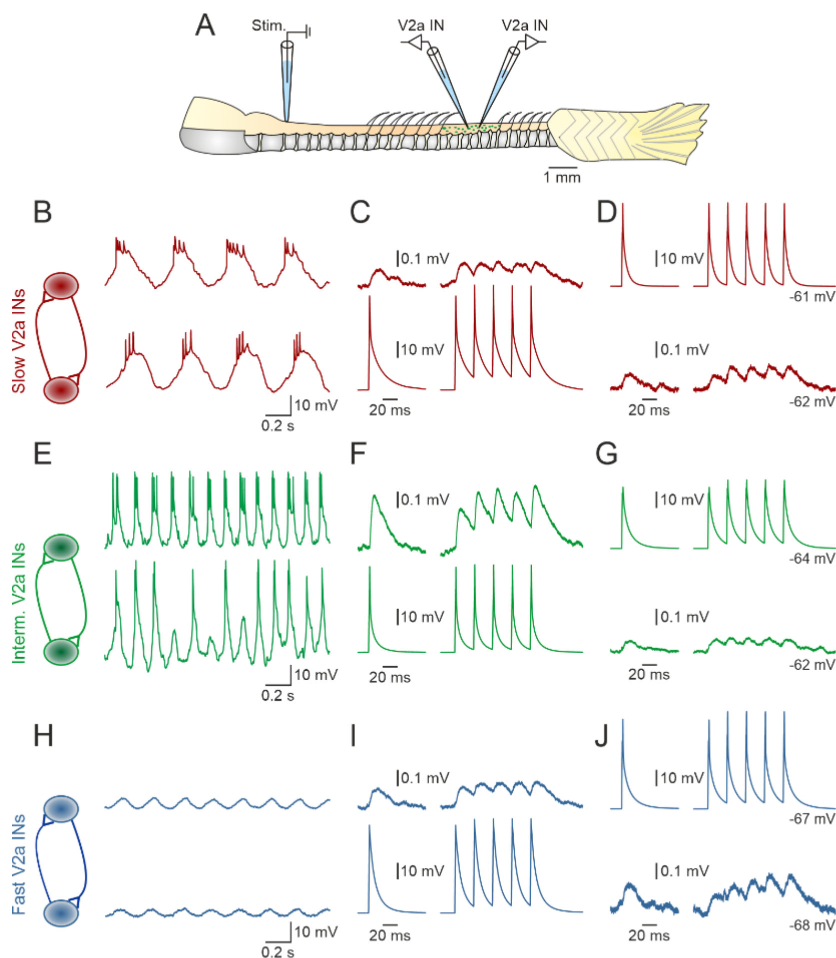

Figure 3. V2a INs form excitatory circuits within each speed module. (A), Experimental setup for paired-recordings and evoking fictive swimming **(B-D),** Dual patch-clamp recordings of a pair of V2a INs recruited during slow swimming frequencies, shows reciprocal synaptic connections. **(E-G)**, V2a INs recruited at intermediate swimming frequencies are reciprocally connected. **(H-J)**, Fast V2a INs that are not recruited at slow or intermediate swimming speeds show reciprocal connections.

How the connectivity of these rhythm-generating V2a INs is organized to mediate changes in swimming speed has remained unclear. To answer this question, we have mapped the pattern of connectivity between V2a INs belonging to the same or different speed modules using dual patch-clamp recordings.

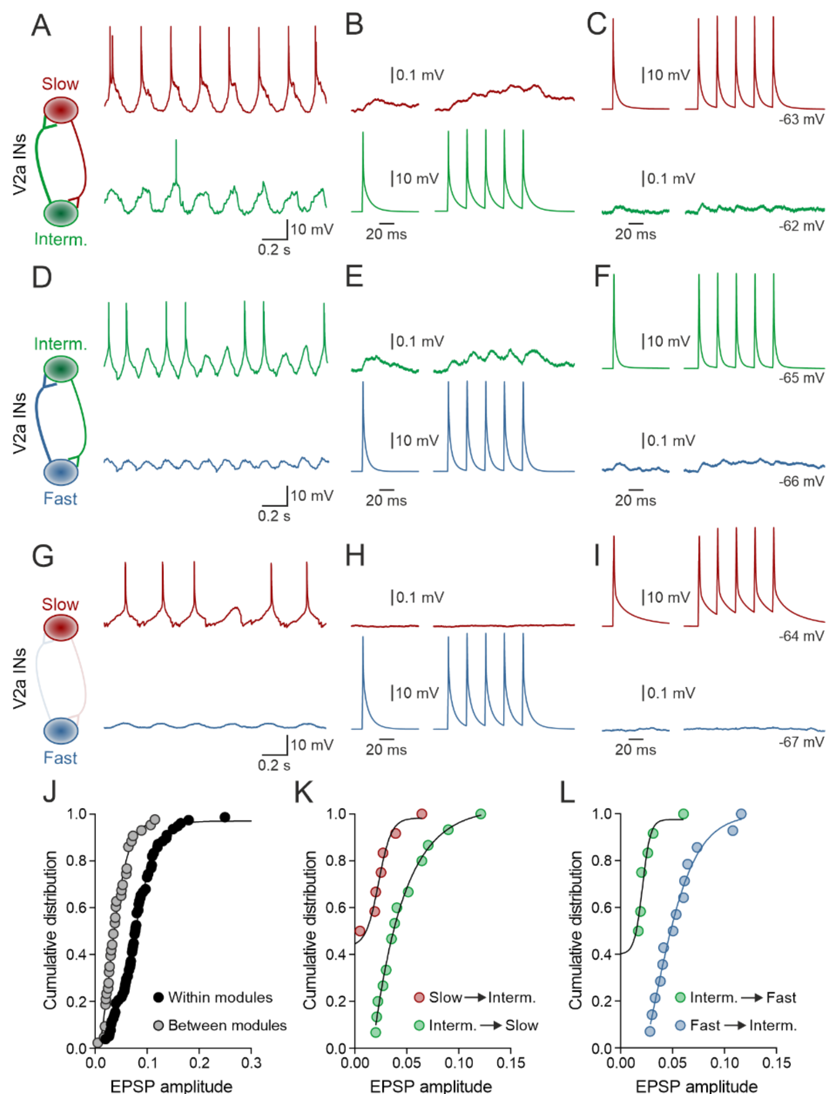

Figure 4. Asymmetrical connectivity between V2a INs of different speed modules. (A-C), Dual patch-clamp recordings in pairs of slow/intermediate V2a INs shows a stronger drive from intermediate to slow V2a INs. **(D-F)**, Interconnectivity between a pair of intermediate/fast V2a INs shows a stronger drive from fast to intermediate V2a IN. **(G-I)**, Pairs of slow/fast V2a INs did not have synaptic connections. **(J)**, Cumulative distribution of EPSP amplitude of V2a IN pairs within and between modules. **(K, L)**, Cumulative distributions of the EPSP amplitude of slow/intermediate and intermediate/fast V2a IN pairs shows asymmetrical connectivity strengths between V2a INs of different modules.

Our results reveal reciprocal excitatory connections between V2a INs within each module (Figure 3). Most importantly, V2a INs of each module are connected to those of the adjacent speed module, but with the faster module exerting a stronger drive onto the slower one (Figure 4). These results suggest that the recurrent excitation within each speed module serves to amplify the excitation and stabilize the speed. To increase the speed of locomotion a

faster module is then recruited and the asymmetric connections between adjacent modules ensure that the faster module becomes the leading one, driving the slower module to match the new frequency, thus guaranteeing smooth speed transitions.

4.1.2 Contribution of pacemaker properties to locomotor rhythm generation and acceleration

Next, we show that a subset of V2a INs in each module display pacemaker properties. These pacemaker V2a INs have a specific morphology and distribution across the three-speed modules (Figure 5). The proportion of pacemaker neurons is highest in the slow module, followed by the intermediate, and lowest in the fast module. Within each module, the pacemaker V2a INs had lower firing thresholds than the non-pacemaker type, and their pacemaker frequencies matched their recruitment frequency during swimming. These properties allow the pacemaker V2a INs to be recruited first and transform the tonic descending drive into rhythmicity.

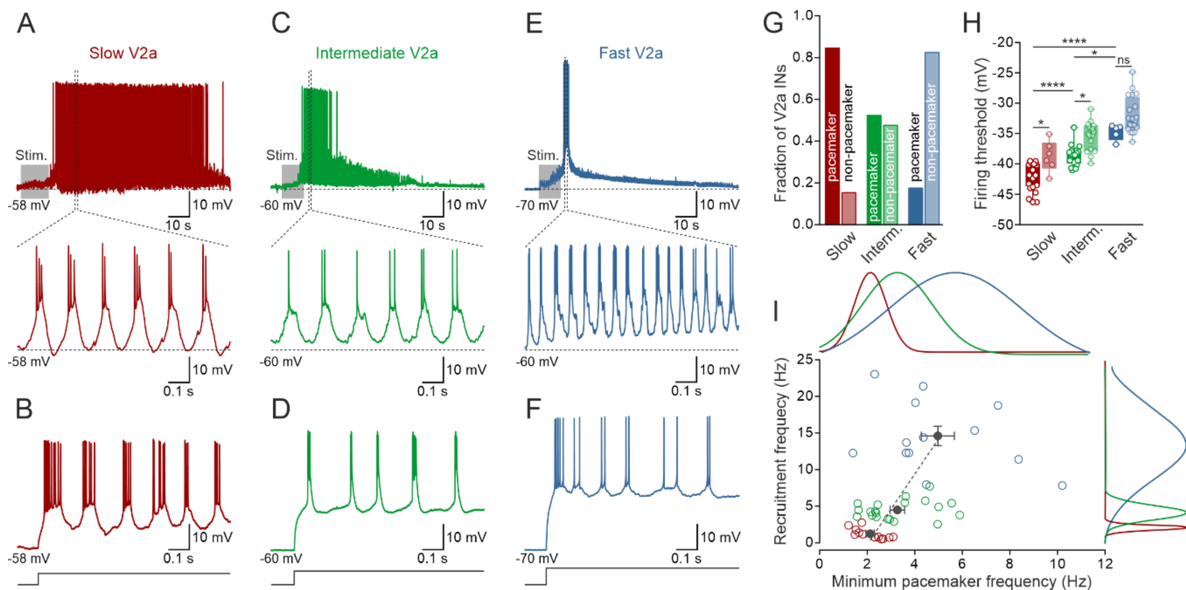

Figure 5. Intrinsic pacemaker properties of V2a INs. (A-F), A subset of V2a INs in each of the three speed modules are endowed with intrinsic pacemaker properties. **(G)**, The fraction of pacemaker V2a INs is highest in the slow module and lowest in the fast module. **(H)**, In each module, the firing threshold of pacemaker V2a INs is significantly lower than that of the non-pacemaker V2a INs **(I),** Correlation of minimum burst frequency and minimum recruitment frequency of V2a INs belonging to the three speed modules.

We then probed the role of the pacemaker properties *ex vivo* using riluzole, a blocker of persistent Na^+ current, which abolished pacemaker firing in V2a INs. Riluzole application delayed the start of the swimming episode induced by electrical stimulation of descending axons from the brainstem. This was associated with a reduction in swimming speed and duration. Finally, we used *in vivo* two-photon laser ablation of pacemaker V2a INs and *in vivo* application of riluzole. Both *in vivo* approaches showed a markedly impaired ability to

generate spontaneous swimming, together with a reduction of swimming speed and distance travelled (Figure 6). These results are consistent with the role of pacemaker V2a INs in swimming initiation and speed build-up.

Overall, our results show that the spinal network relies on specific connectivity patterns between V2a INs, combined with their intrinsic pacemaker properties, to transform the tonic drive from the brain into coordinated swimming and to control its speed.

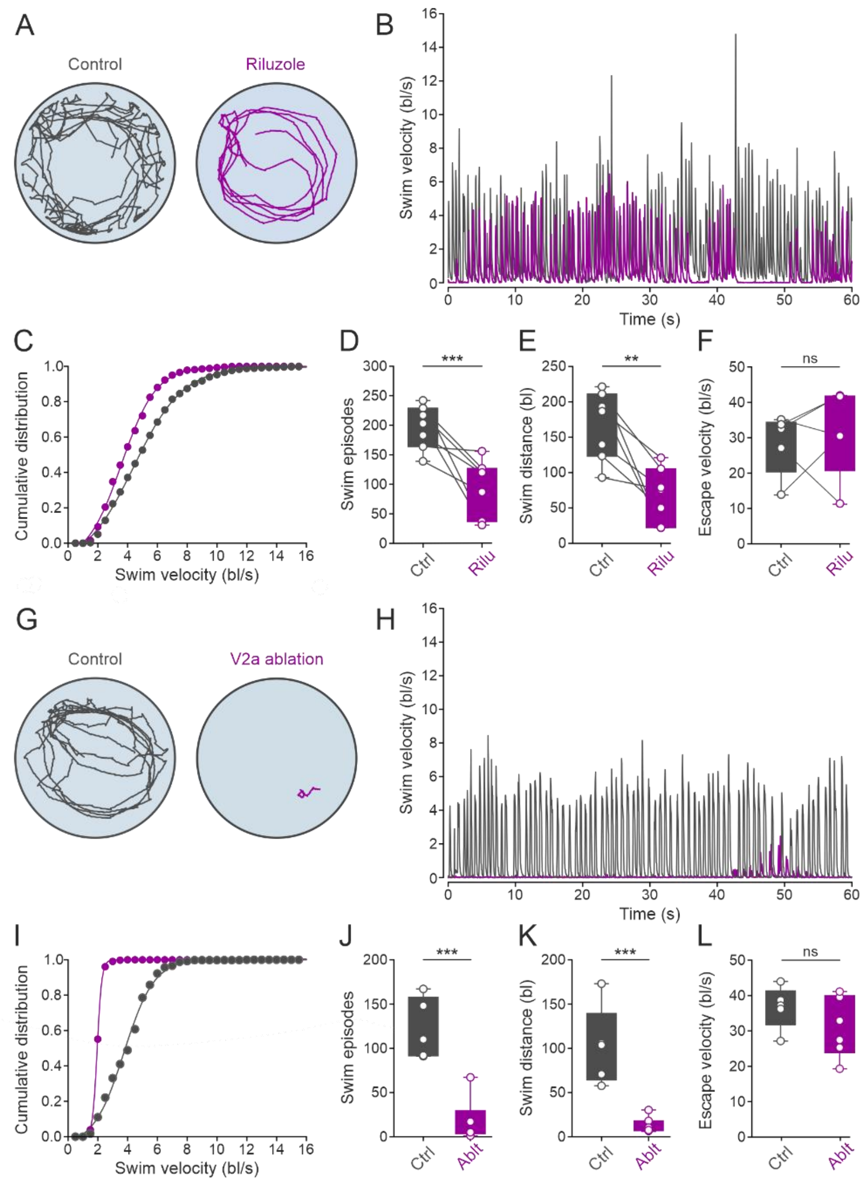

Figure 6. The effect of riluzole and ablation of pacemaker V2a INs during *in vivo* swimming. **(A)**, Centroid tracking of juvenile zebrafish in control (black) and during application of riluzole (purple). **(B)**, Swim velocity in control and with riluzole application. **(C)**, Swim velocity decreases when riluzole is applied. **(D-F)**, Riluzole reduces the number of spontaneous swimming episodes and the distance travelled but escape velocity is not affected. **(G)**, Tracking of juvenile zebrafish in control (black) or following ablation of pacemaker V2a INs (purple). **(H)**, Swim velocity in control and animals where pacemaker V2a IN were ablated. **(I)**, Swim velocity decreases in animal with ablated pacemaker V2a INs. **(J-L)**, Ablation reduces the number of spontaneous swimming episodes and the distance traveled without affecting escape velocity.

4.2 MATURATION RESHAPES EARLY-ESTABLISHED MOTOR CIRCUIT

4.2.1 Changes in connectivity, mode, plasticity, and strength of synaptic transmission

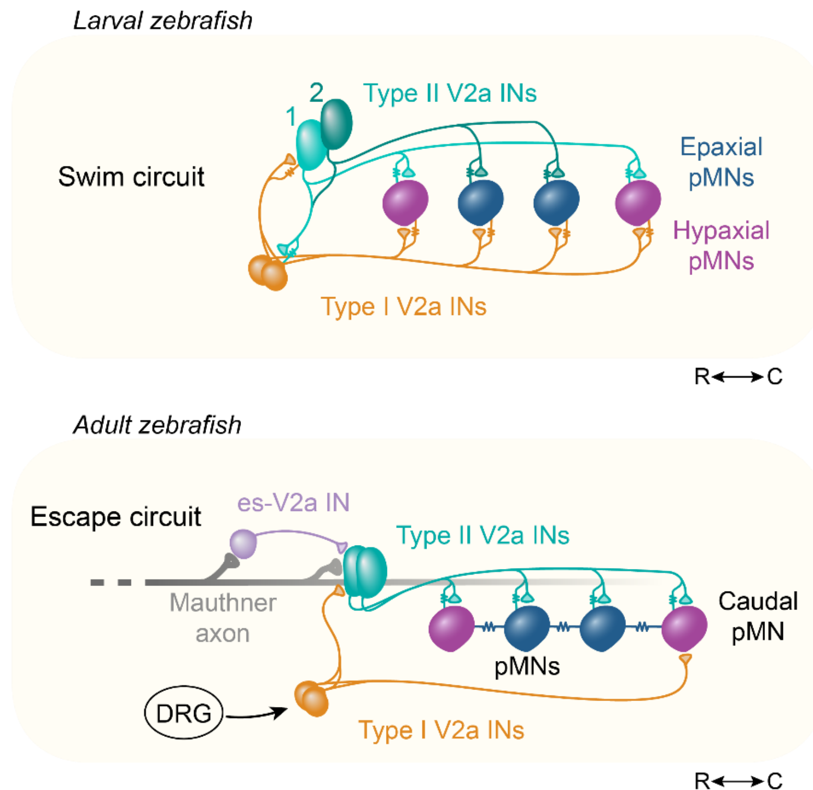

Figure 7. The first established fast motor circuit in zebrafish includes the two types of early-born V2a INs (type I and type II) and the four primary MNs (pMNs). Recent work in larval zebrafish has shown a distinct pattern of synaptic connections within this circuit. In larvae, type I V2a INs connect indiscriminately with chemical or mixed synapses to all pMNs in a spinal hemisegment while type II V2a INs connect either to pMNs projecting to epaxial (blue) or hypaxial (purple) muscles. The two V2a IN types form a reciprocal excitatory circuit acting as a swim rhythm generator that drives swimming at fast speeds. In this study, we reveal connectivity and functional changes at maturation, with loss of existing connections and establishment of new ones, changes in synaptic modalities, plasticity and function.

Locomotor circuits in the spinal cord are assembled and are already functional at early developmental stages (Hamburger 1963; Saint-Amant and Drapeau, 1998; Branchereau et al., 2000; Clarac et al., 2004; Tong and McDearmid, 2012; Falgairolle et al., 2017). However, the locomotor output, and the organization and functional connectivity of locomotor circuits change during maturation to adulthood (van Raamsdonk et al., 1982; Saint-Amant and Drapeau, 1998; Budick and O'Malley, 2000; Müller et al., 2000; Buss and Drapeau, 2001, 2002; Masino and Fetcho, 2005; Kyriakatos et al., 2011; Hale, 2014; Picton et al., 2021; Picton et al., 2022). To study the precise nature of these changes at the level of single neurons and synapses, we analyzed an early-established motor circuit comprising two types of V2a INs (type I and type II) and pMNs (Figure 7) (Menelaou and McLean, 2019).

All the components of this circuit are still present in adult zebrafish, but their organization undergoes several key changes during maturation. The connectivity pattern of the two types of V2a INs to pMNs was studied with sequential paired recordings in adult zebrafish and

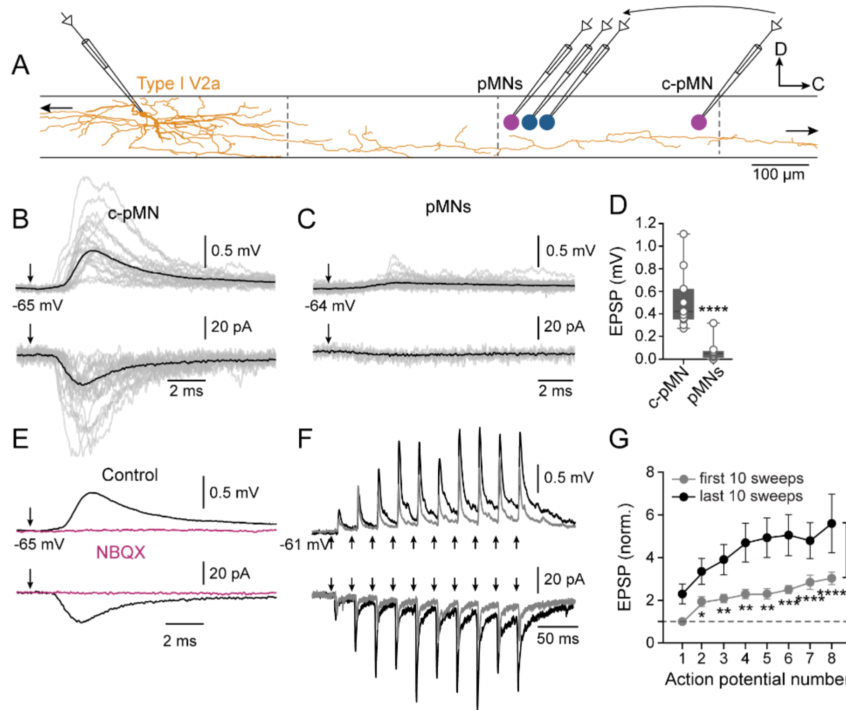

Figure 8. Selectivity and short-term plasticity of type I V2a IN-pMN synapses. (A), Experimental setup for sequential paired recordings showing a type I V2a IN reconstruction (orange) and the four pMNs in a hemisegment. Dashed lines indicate spinal segment borders. **(B-D)**, Type I V2a IN stimulation elicited EPSP/Cs in the caudal most pMN (c-pMN) in a hemisegment but not in the three other pMNs. Arrows indicate V2a IN action potentials. **(E)**, The response in c-pMNs was purely chemical and was abolished by the AMPA receptor blocker NBQX. **(F-G)**, 40 Hz stimulation of a type I V2a IN elicited short-term potentiation in c-pMNs.

compared to that reported in larvae. While type I V2a INs become more selective and connect only to the most caudal pMN of each spinal segment, type II V2a INs become less selective and instead broadly connected to all pMNs along several segments of the spinal cord (Figure 8, 9). The connectivity between type I and II V2a INs also shows age-related changes as the two types no longer form a recurrent excitatory circuit to drive locomotion at high speeds. Furthermore, our results show changes in the modalities of type I V2a IN synapses, which become strictly chemical and display use- and time-dependent potentiation.

4.2.2 Circuit components are redeployed to a different function

Unlike in zebrafish larvae, our results from *ex vivo* and *in vivo* experiments show that both type I and II V2a INs no longer drive swimming activity in adult zebrafish. Instead, we show that type I receives sensory information from dorsal root ganglia, and the selective circuit with the caudal pMNs could be involved in sensory integration for steering movements during escape. On the other hand, type II V2a INs receive drive from escape V2a INs and the Mauthner cells and are recruited during escape behaviour. Type II V2a INs *in vivo* are necessary to control the speed and force of the counter-bends following the C-bend during the escape behaviour (Figure 10). The broad connectivity of type II V2a INs with pMNs combined with the employment of gap junctions, allows a rapid and simultaneous relay of the excitation to all pMNs along the whole spinal cord. A more efficient and powerful movement could be required in adult zebrafish in which the length and body mass has increased. Therefore, our work shows that there is a retention, but functional redeployment, of components of early established motor circuits to adapt to the new behavioural demands that emerge during maturation to adulthood.

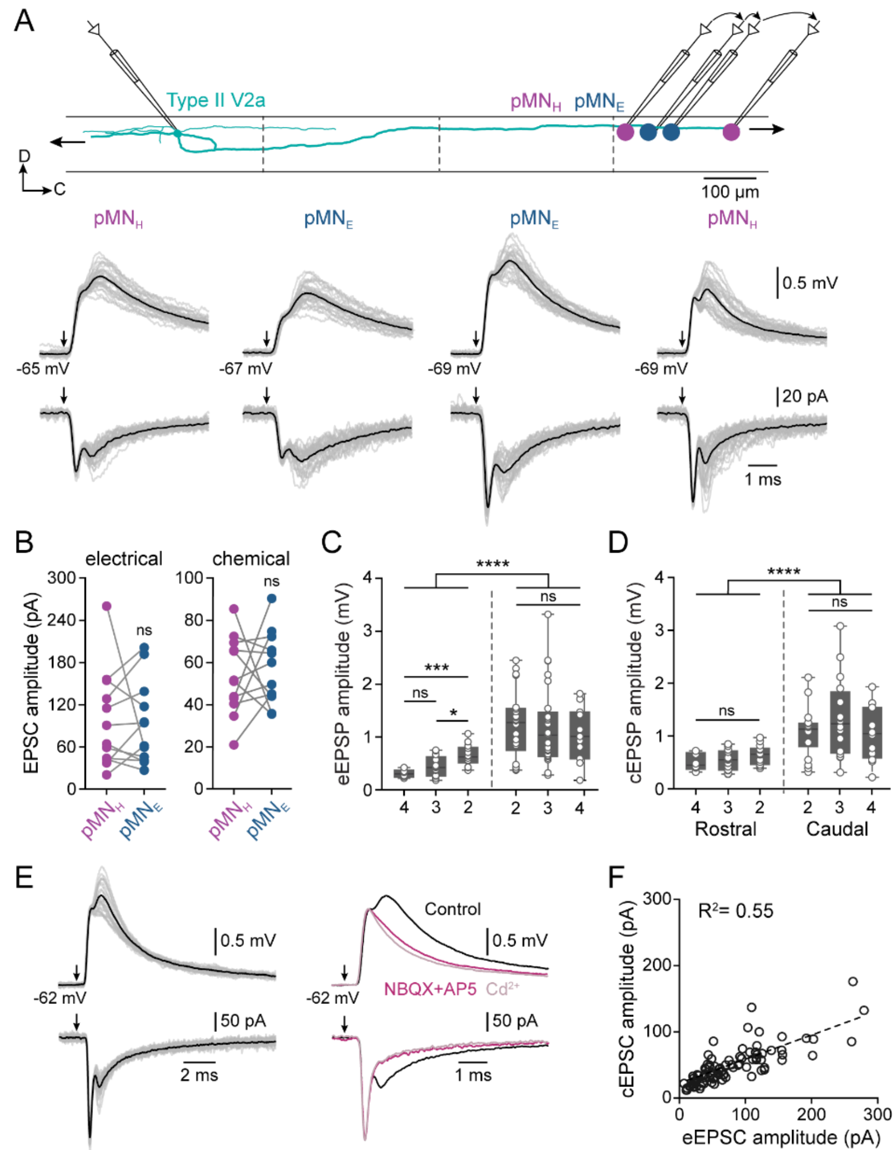

Figure 9. Broad synaptic connections between type II V2a INs and pMNs. (A), Top: Experimental setup of sequential paired recordings showing a reconstruction of a type II V2a IN (green) and pMNs. Bottom: Type II V2a INs send highly divergent inputs to all pMNs in a spinal segment regardless of their innervation of epaxial (pMN_E) or hypaxial (pMN_H) muscles. **(B)**, There was no difference in the chemical or electrical EPSC amplitude of pMN_H and pMN_E. **(C, D)**, Amplitude of eEPSP (C) or cEPSP (D) in pMNs recorded in caudal or rostral segments relative to the stimulated type II V2a IN. **(E)**, Type II V2a IN stimulation elicited a mixed chemical and electrical EPSP/C in a pMN. The chemical component was abolished by NBQX and AP5 leaving only an electrical component that remained also in presence of cadmium (Cd^{2+}). **(F)**, Correlation between cEPSC and eEPSC amplitude

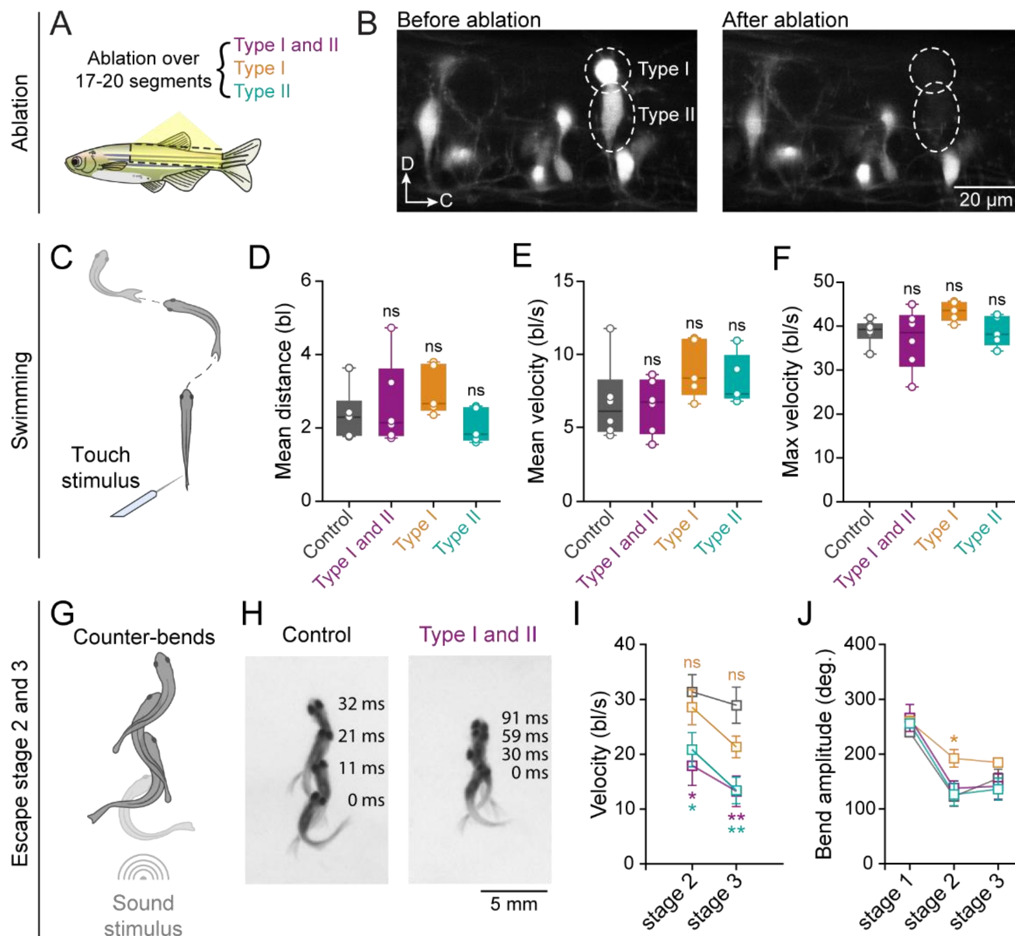

Figure 10. Type II V2a INs control the propulsive phase during escape. **(A)**, Ablation of V2a INs over the body region indicated in yellow and dashed lines. Three experimental groups were tested in which both type I and II were ablated (purple), or only type I (orange) or type II V2a INs (green, control in grey). **(B),** Image of the spinal cord before and after ablation of type I and II V2a INs encircled by the dotted lines. **(C-F)**, Ablations had no effect on swimming elicited by touch stimulus. **(G)**, Escape response was elicited with a touch stimulus and consisted in a C-bend (stage 1, not shown) followed by two or three counter-bends (stage 2 and 3). **(H)**, Ablation of type I and II V2a INs impaired the escape response by decreasing the distance travelled. **(I, J)**, Ablation of type II V2a INs alone decreased the velocity of escape stage 2 and 3 but had no effect on bend amplitude.

4.2.3 Electrical coupling between V2a INs and pMNs and within the pMN population play complementary functional roles

Gap junctions are present at different synapses in the above described circuit, and they serve complementary functions. Axo-somatic gap junctions at mixed synapses between type II V2a INs and pMNs allow pMNs to retrogradely influence synaptic transmission. A similar mechanism has been demonstrated between V2a INs and MNs of the swimming circuit (Song et al., 2016). In contrast, gap junctions between pMNs effectively synchronize their firing and enable them to act as an ensemble. With this circuit organization, simultaneous activation of pMNs by type II V2a INs synchronizes their output and further strengthens the excitatory drive with retrograde feedback, ensuring a reliable and coordinated escape response.

4.3 FUNCTIONAL SPINAL CIRCUIT MODULES ARE MOLECULARLY DEFINED

4.3.1 Molecular signature of MNs and V2a INs in adult zebrafish

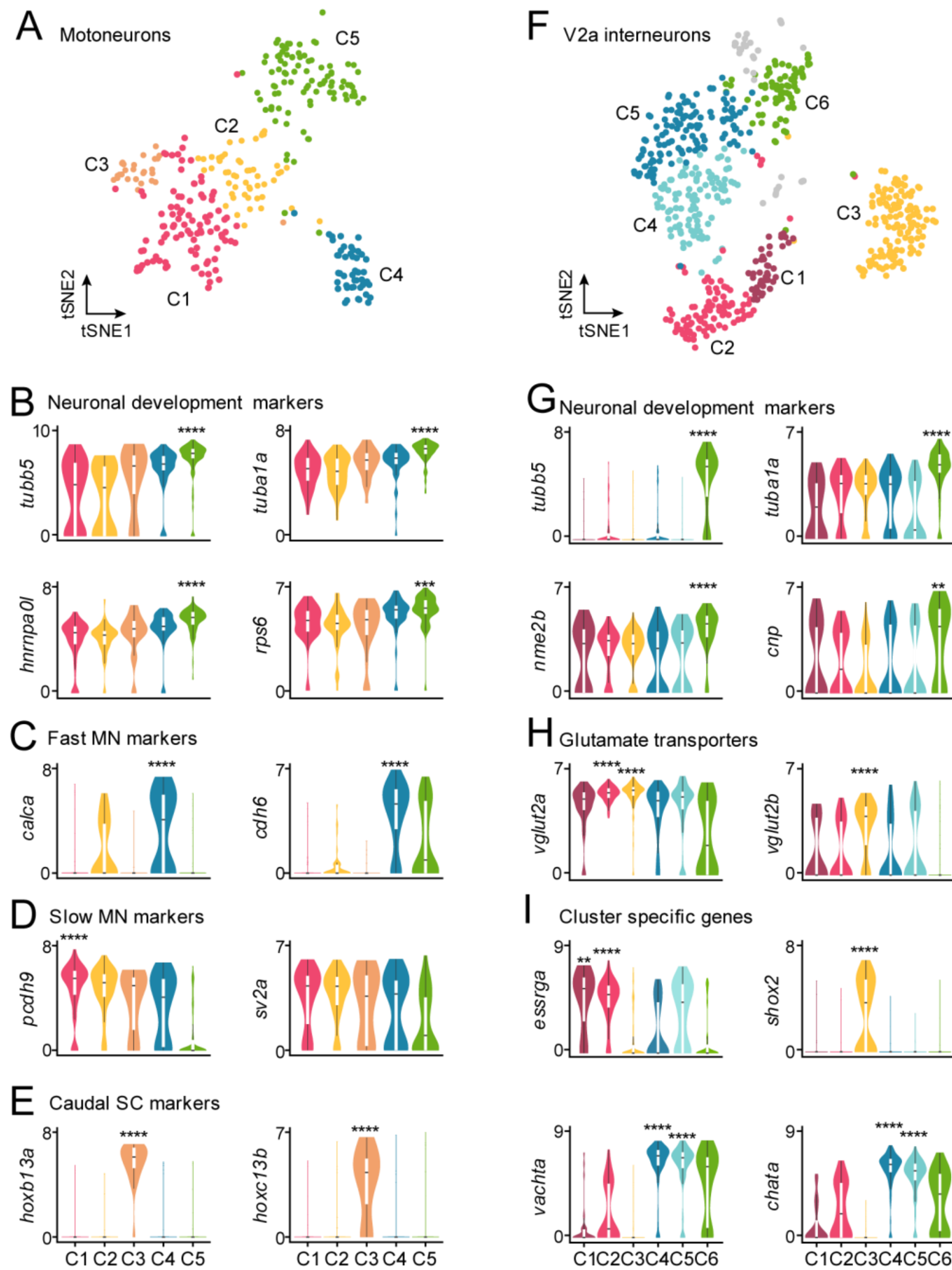

Figure 11. Molecular diversity within MN and V2a IN populations. (**A**), MN single-cell transcriptomes visualized with t-distributed stochastic neighbour embedding (tSNE), color-coded for five molecularly defined clusters. **(B)**, The levels of expression of markers for neuronal development suggest that cluster C5 represents immature MNs. **(C,D)**, Cluster C4 has enriched expression of markers for fast MNs while clusters C1 and C2 have high expression of a slow MN marker. **(E),** Cluster C3 represents caudal MNs. **(F)**, V2a IN single-cell transcriptomes visualized with t-distributed stochastic neighbour embedding (tSNE), color-coded for six molecularly defined clusters. **(G)**, Expression of neuronal development markers indicates that cluster C6 is composed of immature V2a INs. **(H)**, Clusters C1-5 express high level of glutamatergic markers. **(I)**, Expression levels of selected marker genes for clusters C1/2, C3 and C4/5.

In adult zebrafish, the flexibility of locomotion is ensured by the modular organization of the swimming network, composed of three V2a IN types selectively connected to slow, intermediate or fast MNs. Whether the heterogeneity within and across spinal neuronal populations is molecularly encoded has remained unclear. Using single-cell RNAseq, combined with RNAscope, neuronal tracing and whole-cell patch-clamp recordings during swimming, we reveal the molecular signatures for the known functional types of MNs and V2a INs.

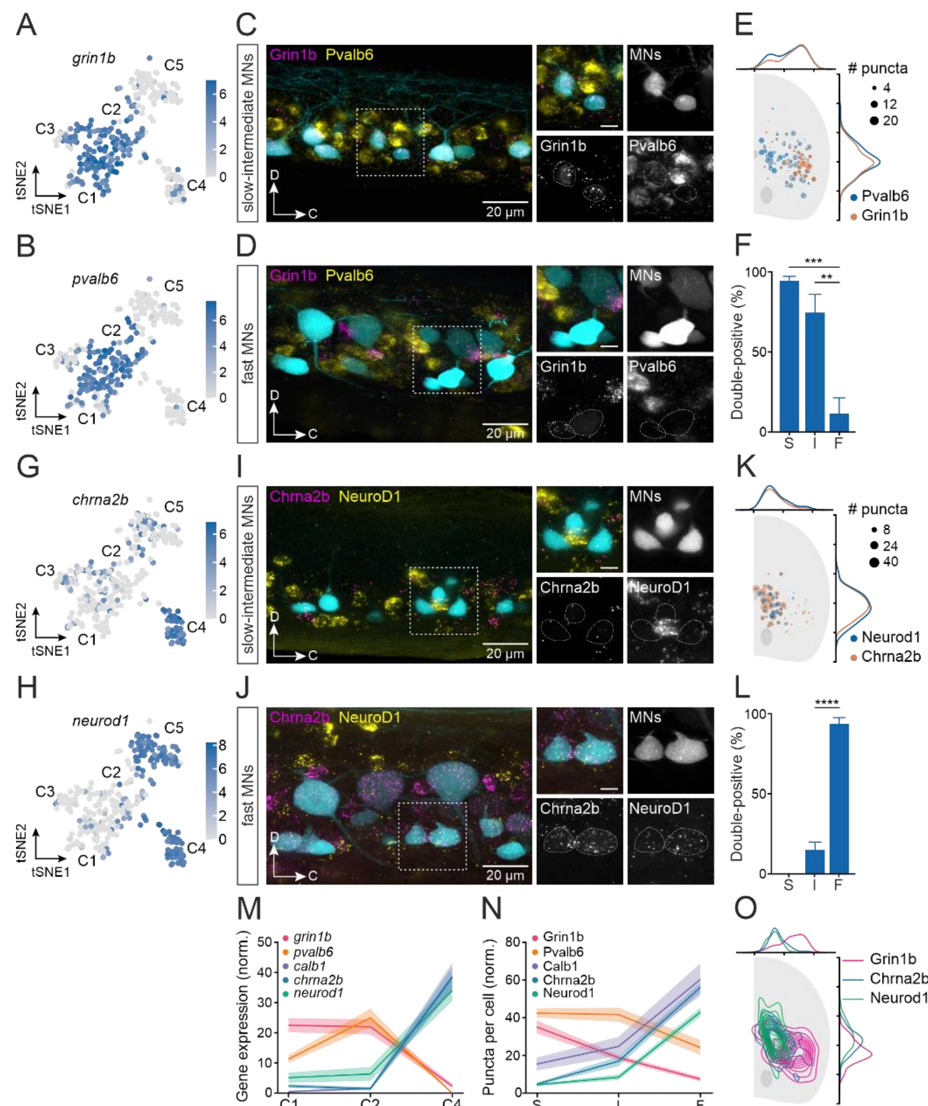

Figure 12. Validation of MN clusters. **(A, B)**, tSNE showing the normalized expression of C1-C2 marker genes *grin1b* and *pvalb6*. **(C, D)**, *In vivo* validation of C1-C2 markers by RNAscope and muscle injection of slow-intermediate or fast muscle fibers. RNA scope shows markers expression in the lateral (C) but not in the medial (D) spinal cord. **(E)**, Normalized soma position of MNs positive for *grin1b* and *pvalb6* RNAscope probes in the coronal section of the spinal cord. The size of the circles reflects the number of puncta per cell (# puncta). **(F)**, Quantification of MNs with colocalization of *grin1b* and *pvalb6* RNAscope probes. **(G, H)**, tSNE showing the normalized expression of C3 marker genes *chrna2b* and *neurod1*. **(I, J)**, *In vivo* validation of C4 markers by RNAscope and muscle injection of slow-intermediate or fast muscle fibers. RNA scope shows expression in the medial (J) but not in the lateral (I) spinal cord. **(K)**, Normalized soma position of MNs positive for *chrna2b* and *neurod1* RNAscope probes in the coronal section of the spinal cord. **(L)**, Quantification of MNs with colocalization of *chrna2b* and *neurod1* RNAscope probes. **(M)**, Normalized gene expression of the markers used to validate the clusters C1-C2 and C4. **(N)**, The number of RNAscope probes puncta per cell reflects the gene expression pattern of each marker gene. **(O)**, Spatial distribution of the positive MNs to RNAscope of marker genes.

In the MN population, five molecular clusters could be identified (Figure 11). One of these clusters (C5) had features of immature MNs, while a second cluster (C3) expressed *Hox13* genes and corresponded to MNs located in the caudal spinal cord. Two of these clusters (C1 and C2) were molecularly similar and showed upregulation of genes related to high metabolic activity and the expression of a slow MN marker (*pcdh9*). In contrast, cluster C4 was distinct, had downregulation of genes implied in metabolism, and expressed two mammalian markers of fast MNs (*calca* and *cdh6*). Using RNAscope for selected markers for clusters C1-C2 (*grin1b* and *pvalb6*) and cluster C4 (*chrna2b*, *neurod1*, and *calb1*) we show that these clusters correspond respectively to MNs innervating slow, intermediate and fast muscle fibers (Figure 12). Thus, we reveal that the functional MN types are molecularly diverse and we propose new molecular markers and features to identify slow/intermediate vs fast MNs that can be tested in mammals and other species.

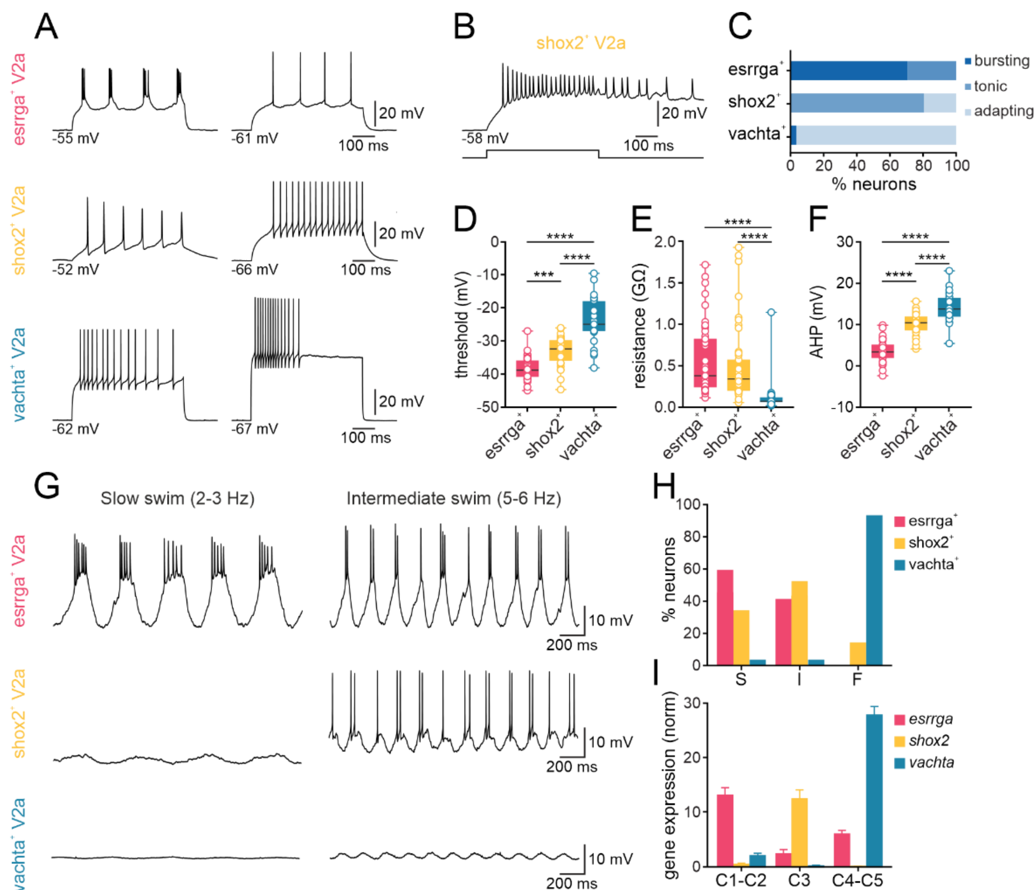

Figure 13. Electrophysiological validation of V2a INs. (A), Top: two examples of Esrrga$^+$ V2a INs displaying bursting (left) or tonic (right) firing in response to depolarizing current pulses. Middle: two examples of Shox2$^+$ V2a INs displaying tonic firing. Bottom: two examples of vAChTa$^+$ V2a INs displaying adapting firing. **(B)**, A proportion of Shox2$^+$ V2a INs shows plateau potentials in response to depolarizing current pulses. **(C)**, Differences in firing patterns in the three V2a IN types. **(D-F)**, Box and whiskers plot showing the difference in firing threshold (D), input resistance (E) and after-hyperpolarization (AHP, F) in the three V2a INs types. **(G)**, Top: recordings of an Esrrga$^+$ V2a IN showing that it was recruited at all swimming frequencies. Middle: example of a Shox2$^+$ V2a IN that became recruited at intermediate swimming frequencies. Bottom: example of a vAChTa$^+$ V2a IN that received subthreshold synaptic inputs at slow and intermediate swimming frequencies. **(H)**, Classification of V2a INs belonging to the three molecular types into slow (S), intermediate (I) or fast (F) types based on electrophysiological properties and recruitment frequencies. **(I)**, The normalized gene expression of the markers that were used to validate the V2a IN clusters matches with the electrophysiological classification in (H).

The V2a IN population was segregated into six molecularly distinct clusters (Figure 11). One of these clusters (C6) expressed features of immature V2a INs, similar to the immature MN cluster. Given the lack of spatial segregation of the functional V2a IN types, we validated the clusters using electrophysiological recordings in transgenic reporter lines for selected marker genes (*esrrga* for clusters C1/C2, *shox2* for cluster C3 and *vachta* for cluster C4/C5). Analysis of the morphology, intrinsic properties and activity during swimming reveal that these molecular clusters corresponded to the functional types driving swimming at slow, intermediate and fast speeds. In particular, Esrrga$^+$ V2a INs are the most excitable and are recruited at the slowest swimming speeds. Shox2$^+$ V2a INs are recruited mostly at intermediate swimming speeds. VAChTa$^+$ V2a INs are the least excitable and are never recruited at slow or intermediate swimming speeds (Figure 13). These results show a tight correspondence between the anatomical, electrophysiological and functional properties of V2a IN types and their distinct molecular profiles. Interestingly, some of the adult zebrafish markers of V2a IN types are similar to those revealed in mice (e.g., Shox2, Zfhx3, Neurod2, Nfib and Esrrga) (Hayashi et al., 2018; Sathyamurthy et al., 2018; Delile et al., 2019; Osseward et al., 2021; Russ et al., 2021), supporting the notion that these markers could be conserved among species. However, the functional relevance of the molecularly defined V2a IN types in mice has not yet been demonstrated.

4.3.2 The modular architecture of MNs and V2a INs is reflected in their molecular identity

Our analysis reveals similarities in the transcriptomic space of MN and V2a IN types belonging to the same speed circuit module (Figure 14). Furthermore, we found shared molecular signatures that define the slow/intermediate vs fast module and show that each of the three modules has a matching expression of transcription factors and differentially expressed genes. These shared genes include functionally relevant genes that could play a role in the specification of the common circuit identity. Thus, our study reveals a molecular logic underlying circuit organization into functional units, which could be a general principle conserved among vertebrate species.

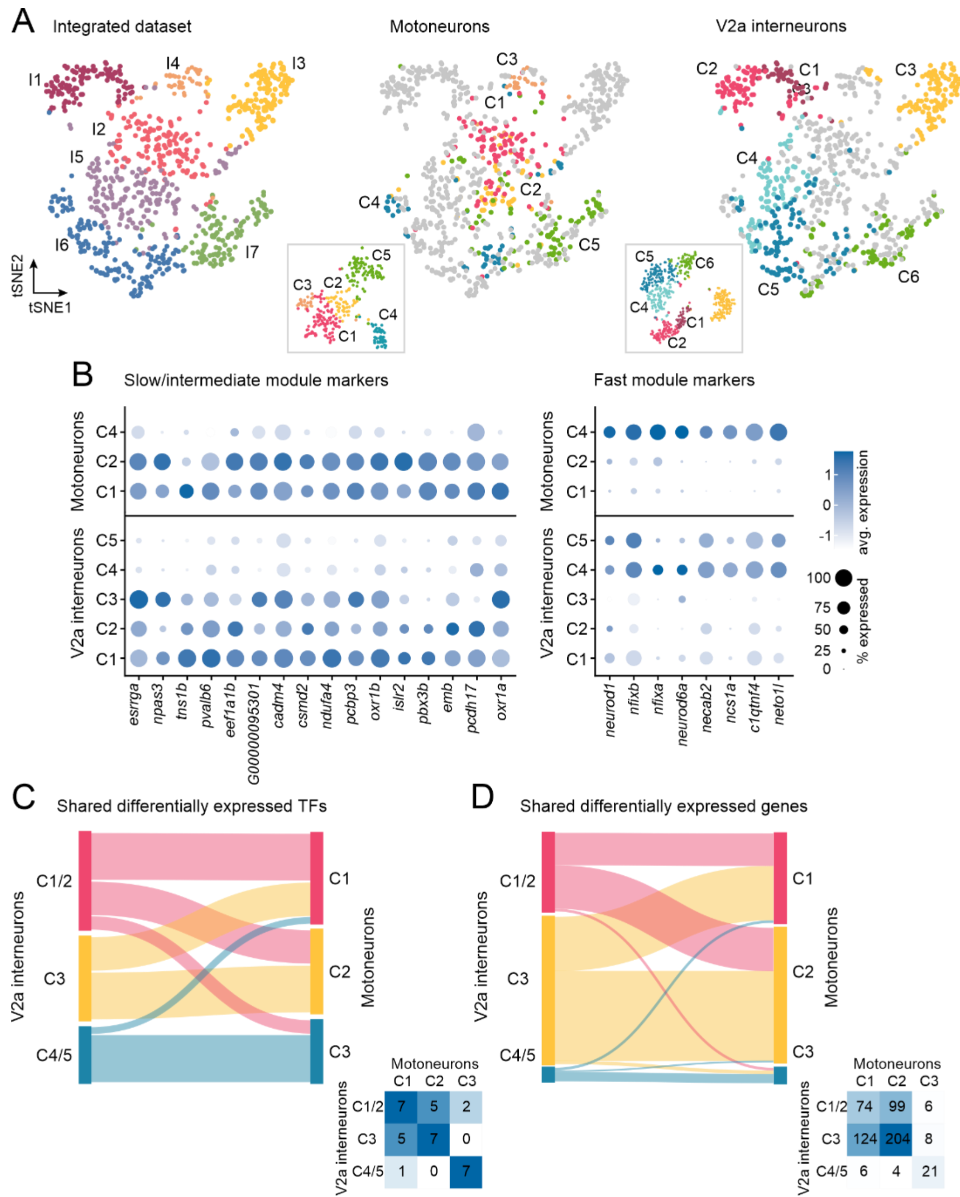

Figure 14. Shared features of functional speed modules across spinal populations. (A), Left: t-distributed stochastic neighbour embedding (tSNE) of the MN and V2a IN integrated dataset, color-coded for seven molecularly defined clusters. Middle: tSNE of the integrated dataset, color-coded for original MN clusters (shown in inset). Right: tSNE of the integrated dataset, color-coded for original V2a IN clusters (shown in inset). **(B)**, List of differentially expressed genes in the different circuit modules. Left: slow and intermediate modules; right: fast module. **(C, D)**, Sankey plot of differentially expressed TFs (C) and overall genes (D) shared by different modules of V2a IN and MN clusters.

5 CONCLUSION AND FUTURE PERSPECTIVES

This thesis explores the molecular and connectivity principles that underlie the versatility of the zebrafish locomotor circuit. We provide insights into the mechanisms underlying the initiation and speed changes during locomotion that involves a combination of the connectivity between the rhythm-generating V2a INs and their pacemaker properties. We demonstrate how changes in connectivity and synaptic modalities play a role in shaping motor circuits during development, and allows for functional adaptions to produce a mature behavioural output. Finally, we reveal a previously unappreciated molecular diversity within and across different spinal neuronal populations that reflects their functional diversity.

The detailed study of connectivity, intrinsic properties, and molecular diversity of neurons is of pivotal importance to understand the mechanisms that enable circuits to fulfil their diverse functions. As the work of this thesis demonstrates, any of these aspects taken singularly would not suffice to explain how a neural circuit can produce a versatile behaviour that can adapt to different needs of the animal. Zebrafish is one of the few model organisms that affords the combined study of connectivity and electrophysiological properties at the level of single neurons and synapses while relating them to function at an organism level at different developmental stages. While other model organisms such as *Xenopus* and lamprey share these advantages, zebrafish is also genetically accessible, which allows the selective manipulation and molecular interrogation of different neuronal populations. Furthermore, the genetic code that defines neuronal populations is phylogenetically conserved, permitting a more immediate translation of the results to other species. Therefore, the results obtained in this thesis can be extrapolated and used to guide future investigations in less accessible species where such in-depth levels of analysis are almost impossible to achieve. One example is the molecular architecture of speed circuit modules revealed in the third study, which provides the tools to investigate whether this modular organization is a general organizational strategy in the spinal cord of adult zebrafish as well as in other species. A first step in future investigations could include the molecular characterization of V0d and V0v INs in adult zebrafish, which have already been shown to respect the functional division in speed modules.

The locomotor circuit can also be used as a model to reveal circuit dynamics that can be found in other central nervous system networks. For example, our work highlighting the changes in motor circuits during maturation shows that early established circuits should not be considered predictive of the adult. Furthermore, a general circuit mechanism described in this thesis is the asymmetric recurrent excitatory circuit formed by V2a INs of the three speed modules combined with pacemaker activity at different preferred frequencies. This configuration allows the sequential recruitment of subcircuits and an increase in the frequency at which the circuit is tuned. Oscillatory networks are a recurrent building block of the central nervous system and it is reasonable to assume that similar strategies could be

employed for different functions. Finally, an open question in neuroscience that has been attracting attention in recent years regards the role of gap junctions in the central nervous system. In paper II of this thesis, we reveal a circuit structure with two different, but complementary, functions of gap junctions that allows the reliable broadcasting of a relevant excitatory signal. This finding may also apply to other neural circuits with similar demands.

In conclusion, the work presented in this thesis offers detailed insights into the organization and function of the locomotor network of adult zebrafish from the perspective of intrinsic properties, connectivity and molecular diversity. Moreover, studying this network at the level of single neurons and synapses permitted to uncover general rules of the assembly of neural circuits that can be translated to other systems and organisms.

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