Swimming with the current

Fictive locomotion reveals subtle phenotypes in the zebrafish locomotor network

HARMEN KORNELIS KONING
Abstract

Neural networks are the functional building blocks of the central nervous system. To better understand how these networks develop and operate, we turned to the zebrafish locomotor network, with a focus on subtypes of interneurons expressing dmrt3a and wt1a. These neurons first gained interest when a mutation in the Dmrt3 gene was found to be responsible for Icelandic horses’ ability to perform additional gaits, indicating a flexibility within their locomotor central pattern generator.

In zebrafish, the Dmrt3 population is known to be commissural, inhibitory and involved in escape behaviors and left-right alternation during locomotion. We characterized the locomotor behavior at embryonic, larval and juvenile stages in dmrt3a mutants. A strong phenotype was observed in larval escape behavior, showing reduced top speed while the animals spent more time accelerating. While the phenotype subdued as the animals developed, juveniles still maintained a lower maximum locomotor speed.

To get a more detailed understanding of the observed phenotypes, an experimental setup was established combining dual ventral root recordings with calcium imaging and various sensory stimuli to induce diverse locomotor outputs in fictively behaving larva. Implementing this method, we investigated the function of Dmrt3 and Wt1 expressing interneurons in escape behaviors and found that knock-down of Dmrt3a disturbed the fast phase of tail evoked escapes, while knock-down of Wt1a lead to aberrant looming evoked escapes, indicating sub-functionalization.

Finally, calcium imaging was employed to reveal the activity of Dmrt3 neurons at a population level. The fraction of active cells steadily increased during development and small clusters of correlated Dmrt3 interneuron ensembles were observed within a segment. This work provides insights into how parallel motor networks are orchestrated to generate a flexible behavioral output, revealing fundamental principles extending to the workings of our own brain.

Keywords: dmrt3a, wt1a, spinal cord, Mauthner cell, neural development, locomotor network, zebrafish

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“Everything is hard before it is easy”

Johann Wolfgang von Goethe
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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<td>AG</td>
<td>axonogenesis</td>
</tr>
<tr>
<td>CaP</td>
<td>Caudal primary (neuron)</td>
</tr>
<tr>
<td>CiD</td>
<td>Circumferential descending (neuron)</td>
</tr>
<tr>
<td>CoBL</td>
<td>Commissural bifurcating longitudinal (neuron)</td>
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<tr>
<td>CoLo</td>
<td>Contralateral local (neuron)</td>
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<tr>
<td>CPG</td>
<td>central pattern generator</td>
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<tr>
<td>Dmrt3</td>
<td>Doublesex and mab-3 related transcription factor 3</td>
</tr>
<tr>
<td>dpf</td>
<td>days post fertilization</td>
</tr>
<tr>
<td>eIN</td>
<td>excitatory interneuron</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>hox</td>
<td>homeobox</td>
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<tr>
<td>hpf</td>
<td>hours post fertilization</td>
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<tr>
<td>iIN</td>
<td>inhibitory interneuron</td>
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<tr>
<td>MCoD</td>
<td>multipolar commissural descending</td>
</tr>
<tr>
<td>MiP</td>
<td>middle primary (neuron)</td>
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<tr>
<td>nMLF</td>
<td>nucleus of the medial longitudinal fasciculus</td>
</tr>
<tr>
<td>GCaMP</td>
<td>Ca$^{2+}$ probe based on GFP and Calmodulin proteins</td>
</tr>
<tr>
<td>GECI</td>
<td>Genetically Engineered Calcium Indicator</td>
</tr>
<tr>
<td>GEVI</td>
<td>Genetically Engineered Voltage Indicator</td>
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<tr>
<td>MN</td>
<td>motor neuron</td>
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<tr>
<td>MYA</td>
<td>million years ago</td>
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<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
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<tr>
<td>RoP</td>
<td>rostral primary (neuron)</td>
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<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
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<tr>
<td>Tg</td>
<td>transgenic</td>
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<tr>
<td>UAS</td>
<td>upstream activating sequence</td>
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<tr>
<td>Wt1</td>
<td>Wilm’s tumor 1</td>
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Introduction

From nerve net to neocortex

The human brain is arguably the most complex structure in the natural world, comprising approximately 170 billion neurons, with each neuron establishing synaptic connections with thousands of other neurons within its immediate vicinity and across distant brain regions. This intricate assembly of neurons is organized into progressively larger elements, with diverse subtypes of neurons often arranged in anatomical layers, as observed in the cortex and cerebellum. Neurons within these layers form localized networks, large numbers of such networks contribute to the formation of brain regions. Brain regions, in turn, connect with other regions through neural tracts that facilitate communication within the brain and further central nervous system. The functioning of the entire brain is modulated by a range of hormones and signaling molecules. This orchestra of electrical and chemical signaling gives rise to a spectrum of behaviors, ranging from basic homeostatic functions and locomotion to complex processes like pattern recognition and associative learning. Ultimately, it culminates in an organ capable of introspection, self-awareness and even contemplating its own origins and operations.

Only knowing the blueprint of the brain does not directly answer the fundamental question neuroscience endeavors to answer: How does the brain function? How do physical cells produce intangible behavior? Whether investigating memory formation, sensory integration, pattern recognition, motor learning or locomotion, researchers invariably return to the core query of how networks of neurons process information and orchestrate simple and complex output. The questions posed and partially addressed in this thesis align precisely with the inquiry of how neural networks develop and generate behavior.

To address these questions effectively, we must first establish the fundamental and essential components that constitute a neural network. To do this, we rewind the tape of evolution to examine the earliest nervous systems produced by nature. These primitive nervous systems are found in cnidaria, which include the box jellies and true jellyfish, and consist of loosely organized neurons that form a nerve net spanning the organism's body. This nerve net consists of three primary types of neurons:
I. Sensory cells, responsible for detecting visual, chemical, and tactile environmental signals.

II. Intermediate cells, which process the patterned sensory signals.

III. Motor neurons, which execute the desired motor programs based on sensory input and intermediate processing.

This sequence of receiving sensory input, information integration by intermediate cells, followed by a generated output, serves as the foundational blueprint for all neural networks. Evolution, by means of natural selection and survival of the fittest [nervous system], flourished the development from pristine nerve net to the mighty neocortex\textsuperscript{15}.

### On the importance of models

The gap between early nerve nets and the human brain spans 500 million years of evolution and multiple orders of magnitude in cell number, cell types and general complexity (Fig. 1). Yet, the core principles of input, process, output remains the same. Finding a balance between complexity and simplicity in a topic of study is of great importance to generate knowledge that is both relevant and obtainable within reasonable effort.

![Figure 1: The locomotor network through evolution](image)

*Figure 1: The locomotor network through evolution.* Locomotion lies at the foundation of nervous system evolution and locomotor types throughout the evolutionary tree are variations on the same rhythmic concept. Thus, the basic building blocks of the locomotor network have been preserved through evolution. As locomotor behaviors grow in complexity, layers of network complexity are added and the number of involved cell types, indicated by colors, grows. Based on \textsuperscript{13,14,16}.

While the *cnidarian* nerve net might be too simple, the sheer size and complexity, not to mention ethical considerations, makes it a futile task to study neural networks in the human brain. Therefore, we need to peel back the layers of complexity to reduce the noise and convolution surrounding our neural network of interest and seek a simplification, what we need is a model.
Rat, fly, primate, fish, flatworm, mouse, all are well-established animal models used in neuroscience, each having their specific benefits and pitfalls. As the great statistician George Box once wrote, ‘All models are wrong, but some are useful’, hence we are not looking for the perfect model, but one that fits our purpose the best. To do so, we will set a few requirements.

1. **Input - processing - output**
   To be able to study the neural processing from input to output, it is advantageous to study a network with a simple input and a straightforward and quantifiable output. Therefore, if a perturbation occurs within the network, the change is measurable in the output.

2. **Balance of rigor, flexibility and predictability**
   A network that has too much rigor might collapse once it gets tempered with or altered. If A always leads to C through B, then a change in B makes it unlikely for C to occur.
   
   A network with too much flexibility generates too many different outputs and becomes unpredictable. Thus, leaving very little inference what change in B exactly leads to the altered outcome of C.
   
   Thus, a balance should be struck between rigor and flexibility.

3. **Accessibility**
   So, we want a network that processes input and delivers a quantifiable output. But, if we are to modulate and measure the network, we also need the nervous system to be accessible. A network so accessible that the input can be maintained as a constant, the processing can be observed or modulated, and finally, the output can be precisely measured.

**Fishing for networks, reeling in zebrafish**

At the basis, the nervous system is thought to have evolved to facilitate movements. Locomotion is the primary way for an animal to interact with, and navigate through its environment. As locomotion used a considerable amount of energy, all elements of it are optimized through evolution and development. And finally, because the goal of locomotion is to move, no matter the animal or mode of movement, locomotion is a highly quantifiable behavior.

By focusing on the neural networks of locomotion, the shift from brain to spinal cord is inevitable. This is beneficial for the sake of simplification, as spinal cord anatomy is highly somatotopic and input and output are topologically separated in ventral and dorsal areas respectively. Brain and spinal cord, being part of a single central nervous system, are highly homologous in cell type, topology and network architecture. Therefore, lessons learned in spinal cord will translate to studies of the brain.
Because the invertebrate modes of transportation, and body plan, are by enlarge too dissimilar from the mammalian, they are not good as a proxy. Soft bodies and exoskeletons require such different muscle recruitment patterns that the underlying networks will be unsuitable for extrapolation. Our pool of model animals is thus narrowed to vertebrates.

Within the vertebrate subphylum, rodents are the most prevalent animal model by number of publications and animals used\(^\text{17}\), resulting in that the rodent nervous system is likely the most well described vertebrate nervous system of all. When it comes to mammals, the locomotor output becomes complicated. Their limbed anatomy results in a spinal cord that is heterogeneous along its length, with increased complexity at the height of front and hind limbs. Multiple joints and pairs of antagonizing flexor/extensor muscles add layers and layers of complexity. Additionally, rodents display complex locomotor behaviors and have to maintain its body posture. Finally, muscles, skin and bones, obscure the nervous system and its development is contained within a womb.

By using the larval zebrafish as a model, one can circumvent many of these issues. Their small size and translucent nature during their ex-utero development make their central nervous system readily accessible for both optical and electrical measurement and modulation\(^\text{18,19,20}\). Zebrafish display a number of stereotyped locomotor behaviors, which can be consistently triggered by providing sensory stimuli. Their behavior is flexible enough to show variation but the repertoire is small enough so it can be easily categorized and quantified.

Probing the network

On top of the complexity of the normally developing nervous system, we are also challenged by the never-ending plasticity. If we remove one component of the network, the rest of the network will adopt to find a way to perform its task to the best of its abilities. So, we need a way of identifying subtle and perhaps transient phenotypes during development of the circuitry. With the monumental complexity of the brain, probing the relative simplicity of the locomotor network is advantageous, while still providing crucial information regarding the orchestration of a behavioral output.

By using the zebrafish locomotor system as a model, we are left with a network that is reduced to the governance of simple frequency and amplitude modulated left-right alternation. We can start probing the function of network elements by eliminating components and observing how this change alters the output.
The locomotor network

Locomotion

The origin of locomotion goes hand in hand with the origin of the nervous system. The earliest nervous systems developed to read basic environmental signals, such as luminescence levels and chemical gradients in the environment.

The processing of these cues by interneurons translates into an output in the form of a motor program that propels the animal towards, or away from, an external stimuli. Even though complexity has increased over the course of evolution, both in terms of neural networks and behavioral repertoires, the basis of it all pivots around a single concept: the repetitive sequences of stereotyped rhythmic body movements.

Patterns in history

The groundwork to understand the source of locomotor rhythmicity was performed at the turn of the twentieth century by the Scottish physiologist Thomas Graham Brown. Brown posed the idea that the rhythmic signaling underlying locomotion was locally produced in the spinal cord. He proved this through a series of experiments in decerebrated cats and guinea pigs, showing that the spinal cord alone could still produce coherent stepping (Fig. 2A). This idea challenged the then leading hypothesis by his mentor, Charles Scott Sherrington, who rebutted that decerebrate locomotion was produced by activation of reflex loops under the stimulation of proprioceptive sensory input. Brown then strengthened his point by showing that his results persisted in deafferented animals, a preparation in which the spinal cord is deprived of both descending input and sensory input. These experiments were proof that the network generating the variable rhythmic output needed for locomotion was located completely within the spinal cord.

Ahead of his time, and without the methodological means to proof his ideas, Brown proposed the idea of the spinal cord central pattern generator (CPG), a pair of half-centre oscillators, one on each side of the spinal cord, which through mutual inhibitory connections produce the rhythmic alternating bursting needed for flexor-extensor or left-right alternation (Fig. 2B). In this model, two neurons that fire stochastically in isolation are coupled through bidirectional inhibitory connections. This reciprocal inhibition drives
the two half-centres to start anti-phase rhythmicity through spike frequency adaptation or other intrinsic membrane properties 24.

Figure 2: Conceptualization of the central patter generator A) Brown’s recordings of the antagonistic tablis anterior (top) and gastrocnemius (bottom) in decerebrate and deafferented cat are the first proof of the spinal origin of locomotor rhythm. (Brown, 1911). B) Brown’s first conceptualization of the half-center model with reciprocal inhibition of flexor (F) and extensor (E), lower and higher case lettering indicates strong and weak inhibition. (Brown, 1916) C) Proof for Brown’s half-centre CPG model by experimental cellular recordings on decorticate cat knee joint. Based on (Lundberg, 1981).

The topic of CPG’s was left mostly untouched until the 1960s, when the Swede Anders Lundberg proved the basis for spinal half-center oscillators through intercellular recordings in motor and interneurons 25(Fig. 2C). CPGs have since been studied and described in various rhythmic behaviors and animal models ranging from mammals 26 to invertebrates 27,28. In this context, an honorary mention to the research performed in lamprey (Petromyzon marinus), as the majority of spinal components and descending inputs of the locomotor CPG were first discovered and described in the lamprey model 29,30. As Gillner put it: ‘the lamprey can be considered a vertebrate prototype, a Ford Model T, if primates are equated with the most recent Ferrari. Although the latter is more advanced, the same general control principles apply.’ 29. And so, due to its simplicity compared to mammals, it has been feasible to delineate not just the distinct networks within the lamprey, but also to elucidate the inherent functioning of these networks in terms of interplay among neurons, neurotransmitters and ion channels 31–33. Furthermore, a comprehensive computational model was established, grounded in experimentally derived data spanning from the molecular and genetic level to that of networks and motor behavior 34,35. Both direct and indirect evidence suggests that the insights gained from primordial vertebrate models like the lamprey, are also applicable to later vertebrates.
The locomotor central pattern generator

Because biology deals in spectra, not categories, some fluidity and overlap distort the hard outlined definition of what is contained in the locomotor network or in the locomotor CPG. As the latter is contained within the former, we adhere to a somewhat lose border in where the locomotor CPG ends and the greater locomotor network starts.

In the term locomotor network, we include the descending input, spinal cord cells involved, the motor neurons that ultimately project to the peripheral musculature and the proprioceptive and other sensory neurons that feedback and refine execution of locomotion.

The CPG however, will be considered as the local network contained withing the spinal segments that processes descending input into the rhythmic ouput comprising the motor plan. From in-depth work in lamprey and frog we can conclude that the aquatic locomotor CPG consists of four major components (Fig. 3)

1. excitatory drive, provided by ipsilateral excitatory interneurons;
2. left-right alternation, governed by inhibitory commissural interneurons;
3. burst termination, governed by ipsilateral inhibitory interneurons;
4. program execution by primary and secondary motor neurons;

![Diagram of the generalized segmental CPG for swimming in lamprey and zebrafish.](image)

Figure 3: The generalized segmental CPG for swimming in lamprey and zebrafish. Four primary components of a half-centre drive the swimming aquatic vertebrate CPG: commissural interneurons (cIN) regulate left-right alternation, burst termination is provided by ipsilateral inhibitory interneurons (iIN), motor neurons (MN) drive the musculature and the whole network is under excitatory drive from ipsilateral excitatory interneurons (eIN). Two half-centres lay the basis for CPG which is repeated in every segment along the body. Based on 38,39.
Now that we have delineated the primary elements of the locomotor CPG, we can dive into their development and function.

Developmental origin of spinal neurons

Like many “earlier” vertebrates, the zebrafish anatomy is still of a highly segmental nature. Mid- and hindbrain are unique and highly differentiated, but the structure of the nervous system, musculature and general physiology in the trunk is more or less a repetition of the same in every segment. This repetition is also represented in the locomotor network for the axial musculature, where the locomotor CPG is repeated in every segment. Each segment contains the same number and constellation of CPG cells, their projections form repetitive and similar connections to its associate CPGs located in adjacent segments.

![Figure 4: Spatial patterns for temporal patterns.](image)

During early spinal cord development dorso-ventral gradients of morphogens such as Wnt and Ssh are released from the roof and floor plate. Combinatorial differential concentrations of morphogens cue development of 11 cardinal classes of spinal neurons. The dorsal pd1-5 are progenitors to the somatosensory department of the spinal cord. Ventral p0-p4 supplemented with pd6, give rise to the cardinal classes that make up the locomotor CPG. Based on 40,41.
The segmental body plan originates during embryonic development. To put it briefly, the spinal cord develops and organizes spatially based on expression gradients of a handful of crucial morphogens. In the rostro-caudal axis directionality is established by RA, FGF and WNT, orchestrating the development of midbrain, hindbrain and spinal cord. The dorso-ventral axis of spinal cord is patterned by opposing gradients of Shh and Wnt/Bmp, which leads to combinatorial expression of transcription factors, which ultimately give rise to a total of eleven progenitor domains and subsequent cardinal neuron classes. The six dorsal classes, dI1-6, are primarily involved in sensory processing, with an exception of the borderline dI6 class, which is also known to be involved in locomotion. We will return to the dI6 class at a later stage.

The five remaining ventral classes (V0, V1, V2, MN and V3) give rise to the neurons governing and orchestrating locomotion. Naturally, these cardinal classes form only the fundamental differentiating factor between spinal neurons as each class contains a whole spectrum of subclasses based on positioning, projection patterns and transcriptomics amongst others. Most importantly, the genetic identification of the 11 cardinal classes of spinal cord neurons allows for translational comparison of neural subtypes between species. Most data suggests that projection patterns, neurotransmitter phenotypes and function are to a large extend conserved within cardinal classes between vertebrate species.

Components of the zebrafish locomotor CPG

The CPG’s workhorse: primary and secondary motor neurons

Motor neurons are the end point and workforce of the CPG, providing the excitation needed for the musculature to contract. Zebrafish motor neurons are born in two distinct waves during development and are thus clustered as early born primary motor neurons and late born secondary motor neurons.

In larval zebrafish, a hemi-segment harbors four primary motor neurons, each innervating a dorso-ventral quadrant of non-overlapping musculature of their respective segment (Fig. 5). Primary motor neurons are active during fast swimming and also recruited during struggling behavior and fast escape swims. A rationalization for the firstborn motor neurons being fast, can be found in ethology, young larvae are at their most vulnerable and thus a high selection pressure is present for having a fast, reflex-based motor program in place as soon as the larvae hatch from the chorion.

Secondary motor neurons are smaller in size, located more ventrally in the spinal cord and innervate both distinct and overlapping parts of the axial musculature. Making up the majority of the roughly 60 motor neurons per segment, secondary motor neurons present close to 20 unique projection patterns.
Primary and secondary motor neurons are mostly recruited in a size and position dependent manner. During slow swimming, few, small secondary motor neurons are recruited. As swim speed increases, cumulative recruitment increases the engaged motor neuron pool until finally, the fast secondary motor neurons are recruited in a ventral to dorsal manner\textsuperscript{54,60,63}.

**Cracking the whip: excitatory drive by V2a’s**

Spinal cord V2a interneurons are excitatory ipsilaterally projecting interneurons of a glutamatergic nature, also known as circumferential descending (CiD) neurons in zebrafish\textsuperscript{64–66}. V2a’s are known to form both electrical and chemical synapses on the motor neuron pool in a manner adhering to the speed-dependent modularity\textsuperscript{65,67}. Additionally, fast V2a’s are born before their slow counterparts, which is in line with birth dating of slow and fast motor neurons\textsuperscript{64}.

In addition to birth date and speed-dependency, V2a’s can also be divided on a functional and connectomic basis. In this framework, type I V2a’s connect to other V2a’s and additional inhibitory V0d pools in order to control the timing within the CPG\textsuperscript{68}. A stronger connection is formed between type II V2a’s and motor neurons. This type II subnetwork controls the tail amplitude during locomotor episodes\textsuperscript{68}.

The speed-dependent and function-dependent subdivision of the V2a interneuron pool are not mutually exclusive perse and elude on the complex interaction and specialization within the locomotor network. This also emphasized the intricacy and interdependent relation of even a relatively “simple” network as the locomotor network, where multiple facets like frequency, amplitude, vigor and even posture all coincide in adjacent networks and an overlapping nature.

A clear consensus is present on the role of V2a interneurons as being the powerhouse of the locomotor CPG. Multiple lines of evidence suggest that the V2a pool is the main driver of excitation to the locomotor CPG. Optogenetic stimulation of V2a interneurons generates bouts of coherent locomotion in fictively behaving juveniles\textsuperscript{69} and laser ablation of V2a’s lead to a higher threshold for swims to occur\textsuperscript{70}. Dual patch clamp experiments also revealed that V2a’s tend to spike before initiation of motor neuron bursting\textsuperscript{65}. All results indicate that V2a’s provide excitatory drive to the CPG and specifically the motor neuron pool.

The V2a population fulfils several additional roles during locomotion\textsuperscript{71,72}. An abundancy of Chx10 positive V2a neurons are present in the zebrafish tectum and hindbrain, with several long descending reticulospinal neurons being chx10 positive. Optogenetic stimulation at a population level of hindbrain V2a neurons elicits locomotor-like output while inhibition of the same cells terminates ongoing locomotor activity\textsuperscript{73}.

Finally, a subset of cholinergic V2a neurons, dubbed esV2a’s, has been found to play a role in the escape network in adult zebrafish. These esV2a
neurons receive input from the Mauthner cell through chemical synapses and amplify its trigger to initiate an escape response to fast motor neurons and contralateral local (CoLo) neurons. The other part of the V2 class, the V2b interneurons, can be subdivided in fast glycinergic V2b-gly neurons and slow gaba- and glycinergic V2b-mixed neurons. The two populations are known to make reciprocal connections locally, which leads to stabilizing the locomotor speed.

To spur on the movement: V3 modulation

V3 interneurons, a fairly homogeneous population of ventromedial glutamatergic spinal neurons, is known to provide drive to motor neurons in the locomotor CPG. Ablation of V3 neurons reduces the fraction of active motor neurons during swim bouts without modulating the output frequency. Furthermore, the V3 population is involved in modulation of swim strength and duration. An exciting study pioneering in vivo voltage imaging, recorded spinal V3 interneuron activity and ventral root output during fictive swimming events. They observed an increase in swim strength in locomotor episodes that coincided with high V3 activity as compared to episodes with low V3 activity. A similar positive correlation was observed for the relationship between V3 activity and bout duration. Interestingly, no relation was found between V3 activity and swim frequency. These findings, together with the observation that V3’s display non-rhythmic spiking, lead the researchers to infer that the V3 population modulates the locomotor CPG but is not an integral component of it.

Giddyup! Don’t shake your head! V0

Commissural interneurons are neurons with an axon that cross the midline of the nervous system. The ventral V0 (V0v) interneurons in the zebrafish spinal cord are commissural and project caudally to four segments rostrally where they connect to the motor neuron pool. This population has been shown to contribute to stabilizing the head and in generation of axial the S-shape during locomotion.

V0v are known to be involved in slow swimming while remaining inactive during fast swims. The dorsal V0d population, on the other hand, is active during faster movements.

Pulling the reins: left-right alternation governed by commissural interneurons from dI6

The dorsal and ventral regions of the vertebrate spinal cord are generally highly compartmentalized, the ventral classes modulate motor output while the dorsal spinal cord orchestrates sensory processing. The zebrafish dI6 class is an exception to this case as it plays pivotal roles within the locomotor CPG.
Zebrafish dI6 interneurons are glycinergic, commissural and known to inhibit contralateral motor neurons \(^{83}\), providing in-phase inhibition during locomotion\(^{81}\).

A subtype of dI6 interneurons are the CoLo’s, displaying a morphology of a distinct spherical soma, thin initial axon which thickens and projects contralaterally after contacting the Mauthner cell axon \(^{82}\). One CoLo neuron is present per hemi-segment and this subpopulation is known to be involved in escape swims \(^{82}\).

Genetic ablation of dI6 neurons leads to a loss of left-right coordination and increased output frequency during fictive swimming \(^{81}\).

Both the CoLo’s role in escapes and general role of dI6 interneurons will be elaborated on later in this work.

Finally, dI6 interneurons have been shown to have a function in pectoral fin alternation in larval zebrafish \(^{84}\), which in itself is also a speed-dependent locomotor output \(^{85}\).

### Speed module modulation by V1

V1 is a population of inhibitory ipsilateral interneurons \(^{86}\). V1’s shorten the cycle length, thus increasing swim frequency by providing in-phase inhibition \(^{87}\). V1 have slow and fast subtypes \(^{87}\). V1’s select the active sets of neurons during specific locomotor speeds, during slow swims, the slow V1’s manage locomotor speed by tuning the cycle length \(^{87,88}\). Fast V1’s inhibit the slow V2a, and thus slow motor neurons during fast swimming episodes \(^{87}\). Additionally, V1’s have been shown to inhibit the locomotor network locally, but additionally inhibit the ventral spinal cord at more distal locations \(^{89}\), indicating dual functionality within the same cell.

### The locomotor network in action

The zebrafish locomotor network is built up from speed-dependent modules. The adult zebrafish has slow, intermediate and fast muscles \(^{90}\), which receive input from slow, intermediate and fast motor neurons \(^{91}\). Each speed module receives excitatory drive from respective subgroups of V2a interneurons \(^{65,92}\), which are in turn thought to be regulated by their own speed dependent subgroups of V0v and V0d MCoD interneurons \(^{67,93}\).

This speed dependent modular division is also present early in development, albeit less pronounced. The larval locomotor network only comprises a slow and fast module, with slow swims having a tailbeat frequency below 30 Hz and fast swims higher \(^{94}\). This speed module duality might originate from the fact that larvae only have two types of skeletal muscle; aerobic red fibers, which are rich in myoglobin and are used for sustained slower movements, and larger white fibers, which are recruited during stronger movements of shorter duration \(^{90,95,96}\). Slow muscles span a superficial medial band along the
trunk of the animal, while fast muscles make up the bulk of the musculature. Slow swims are powered by the incremental recruitment of small, ventrally located secondary motor neurons. This pool of motor neurons receives their input from a class of CiD in the V2a population. These, in turn receive input from multipolar commissural descending (MCoD) interneurons from the V0v population.

![Spinal cord network diagram](image)

**Figure 5: The spinal cord network underlying fast and slow swimming in zebrafish larva.**
A) During slow swimming, secondary motor neurons receive excitatory drive from slow CiD interneurons from the V2a pool and contralateral MCoD cells from the ventral V0 subpopulation. As swim speed increase through command from supraspinal descending input, more secondary motor neurons are recruited before the ~30Hz threshold is reach and the fast module gets involved. Dorsal CiD and MCoD provide drive for primary motor neurons while ventral CiD and MCoD are actively de-recruited. Cells involved in slow swim are indicated by dashed stroke. B) Primary and Secondary motor neurons innervate slow and fast muscle groups specifically. Based on 58,94.

When a movement intensifies from slow to fast, the fast module comes into play. Fast movements recruit the primary motor neurons, which have larger somae and are located more dorsally in the spinal cord than secondary motor neurons (Fig. 5). The CiD and MCoD interneurons of the slow circuitry are actively de-engaged, simultaneously dis-CoD/CiD (V2a) interneurons increase activity. These receive their input from commissural longitudinal bifurcating (CoBL) interneurons from the V0d pool. Although direct synaptic
connectivity has not been proven yet, the V0 interneuron pool is likely to receive their input from a group of reticulospinal neurons that have their soma located in the nucleus of the medial longitudinal fasciculus (nMLF) in the hindbrain \(^{101,102}\). The nMLF has been shown to be involved in key locomotor processes such as locomotor initiation and cessation in the lamprey \(^{31,103}\). These roles have been confirmed in zebrafish larvae; nMLF activity is correlated with swim duration and frequency and lateral optogenetic stimulation of nMLF cells results in ipsilateral deflection of the tail \(^{101,102}\).

### Zebrafish locomotor behavior repertoire

Throughout its lifetime, zebrafish adapt several different locomotor behaviors \(^{104,105}\). These motor programs change and adapt to the environment and selection pressures at their contemporary life stage. Zebrafish spent their first day inside a protective chorion where motor programs are “just practice”. After hatching from the chorion, the free moving larvae is more vulnerable to predation, stressing the importance of fast reflexes and escape programs. Interestingly, at the larval stage, the animal is so small that water viscosity and inertial forces have a more significant impact on the body-medium interaction compared to parameters in the adult, where drag and boundary layers are closer to negligible \(^{106,107}\).

During the larval stage, locomotor behaviors are rather linear and segregated. During burst and glide swimming, for example, each episode consists of a high frequency burst of the tail, followed by a glide. One whole sequence is fulfilled before executing another. This is in great contrast to locomotor behaviors in adult zebrafish, at which state, low frequency and more continuous and variable locomotor bouts can be observed. Except for when they sleep, adult animals are continuously in motion and locomotor episodes effortlessly morph over from one to the next.

It goes to show that even a network as fundamental as the locomotor network still maintains a level of plasticity and undergoes development and adaptation throughout an animal’s life span. This is also an exhortation for researchers in developmental biology that a developing organism is always in flux, and not to only see the benefits of the model but also keep its limitations and variability in mind.

### Coiling

While still in the chorion, and only 3 hours after the onset of axonogenesis (AG), embryonic zebrafish initiate a low-frequency (1 Hz) coiling behavior driven by periodic depolarization of motor neurons \(^{105,108}\) (Fig. 6). A cluster of ipsilateral caudal (IC) neurons is known to provide the excitatory drive needed
for motor neuron depolarization during coiling\textsuperscript{109}. IC cells later provide bursting activity to motor neurons, allowing for high frequency output\textsuperscript{109}, eluding at a similarity in function between IC and V2a cells\textsuperscript{94}. However, the IC clusters generate rhythmic drive by providing a pacemaker function originating from endogenous bursting properties of IC neurons. As the switch from coiling to swimming commence, the oscillatory drive changes from a pacemaker to local half-center oscillators\textsuperscript{110}.

Coiling starts as slow, single-sided contractions at 17 hours post fertilization (hpf), gradually progressing into alternating left-right double or triple coils\textsuperscript{111,112}. The frequency of this behavior increases until reaching its peak at 21 hpf, after which it gradually diminishes until it completely disappears by 31 hpf\textsuperscript{112}.

The coiling behavior can be seen as serving the purpose of training and strengthening the synaptic connections within the motor neuron pool, and its connection to the musculature\textsuperscript{112}. At the onset of coiling at 17 hpf, motor neurons fire more or less individually. Over the course of the following four hours, as coiling frequency rises, coherence of activity within the motor neuron pool increases. By 21 hpf, when coiling frequency peaks, all motor neuron activity is organized within two neural ensembles, one for each half of the spinal cord\textsuperscript{113}.

Interestingly, coiling is a behavior solely driven by and activity produced locally in the spinal cord as the only descending input to the spinal cord are the Mauthner cell and its homologues, MiDcm2 and MiDcm3, involved in escapes, which are present from 7 hpf\textsuperscript{105}. Coiling behavior also persists in spinalized larvae\textsuperscript{112}. It is striking that left-right ensembles of motor neurons are established before the wave of long descending projections of the reticulospinal system, which reach the spinal cord at 22-24 hpf\textsuperscript{114–116}.

Swimming

A second birth wave of reticulospinal neurons\textsuperscript{114}, secondary motor neurons\textsuperscript{52,61} and various types of interneurons\textsuperscript{56,116,117} occurs around 27 hpf, coinciding with the time point at which the first evoked swimming behavior is observed. The recurrent timing of expansion of neuron pools and motor behaviors eludes a connection between network- and motor program number\textsuperscript{118,119}. It is well worth noting that swimming behavior and their associated motor neurons and interneurons are strictly tied to birth order. High frequency and speed locomotion appears before lower frequency and speed swimming\textsuperscript{80,119}.

Touch evoked swimming is observed when larvae are removed from the chorion and receive a tactile stimulus (Fig. 6). Tailbeat frequencies for touch evokes swimming start at ~7 Hz at 27 hpf and increases up to 30 Hz by the age of 36 hpf\textsuperscript{112}.

Naturally, the first spontaneous swimming behavior\textsuperscript{120} is only observed after the embryo hatches from the chorion, which happens between 48 to 72
This *burst swimming* consists of episodes that often last for seconds, at which the larva swims with a tailbeat frequency around 50 Hz. [note: even within the niche field of aquatic vertebrate locomotor studies, ‘burst swimming’ has a polysemous nature. In some cases burst swimming refers to the behavior described in the previous section, in others it is used as a collective term, separating burst swims from turning swims. The latter definition will apply to the text from this point on.]

During day 3-5 of development, the long swim episodes shorten to roughly 200 ms per episode and average tailbeat frequency drops to 35 Hz. At this point in development the beat-and-glide swim is the most prevalent locomotor behavior. Beat-and-glide swims consist of a period of several hundreds of milliseconds of 60 Hz swimming followed by a period in which the animal straightens its body and glides out until all momentum gained during the beat phase is lost. Beat-and-glide swimming is accompanied by various turning behaviors.

**Figure 6: Zebrafish locomotor repertoire during development.** Zebrafish embryos/larvae experience a rapid succession of developmental milestones and locomotor behaviors. First motor action is coiling at 17 hpf which coincides with the first wave of primary motor neuron and interneuron births and axonogenesis (AG). At 21 hpf embryos respond to touch and by 30 hpf first swimming is observed after the second wave of motoneurons and interneuron birth. By 50 hpf, after embryos hatch from the chorion, they first display long burst swims which over the course of the next two days get finetuned into beat-and-glide swims. Based on.

By the end of the first week of development, the larval locomotor repertoire is greatly expanded and fine-tuned. By this age the larva has consumed all of the nutrients in the yolk and turns to hunting various small prey, a behavior that requires fast, flexible and precise locomotor programs. An impressive
body of work that applied machine learning based clustering on a dataset of over 2 million swim episodes captured in high speed provided the most in-depth analysis of larval zebrafish locomotor repertoire. Concluding that the zebrafish larva at 7 dpf displays 13 basic swim patterns that are used in various combinations and contexts \(^{124,130}\).

By the time that zebrafish reach the juvenile stage, swim frequencies bottom-out at 1-7Hz and locomotion becomes more or less continuous \(^{131–133}\) with one locomotor behavior seamlessly flowing over into the next \(^{134}\). The behavioral repertoire keeps expanding to facilitate new feeding and mating behaviors \(^{134}\).

Escapes

In relation to the many locomotor behaviors zebrafish display during development, the escape response is by far the most conserved and stereotyped one. Because of the high selection pressure on having a fast and reliable escape response, this network is highly conserved throughout evolution. Analogues to the giant Mauthner cell, the key component to the escape response, are present in both invertebrates \(^{135}\) and mammals \(^{136,137}\).

The most prevalent escape swim behavior in zebrafish is the C-startle. C-startles start with a bend along the body’s axis, forming a C-like shape bending away from the stimulus that induced the escape (Fig. 7A, B). The C-bend is followed by a high frequency swim burst \(^{138}\).

The main driving force in this escape response is the Mauthner cell, a giant reticulospinal neuron residing its soma in the hindbrain and sending an exceptionally thick axon along the length of the contralateral spinal cord (Fig. 7C). When a stimulus occurs that is strong enough to cross the Mauthner cell firing threshold \(^{139,140}\), the Mauthner cell gets triggered by auditory, visual, mechanosomatosensory input \(^{141–143}\), which initiates a single action potential \(^{140}\). Because the Mauthner cell directly innervates the primary motor neuron pool, its firing results in a C-bend away from the side on which the Mauthner cell was triggered \(^{140}\). Gap junctions between Mauthner cell and CoLo interneurons provides inhibition to the motor pool on the contralateral side to the contraction \(^{81,82}\).

Two subtypes of C-bends are seen in larval zebrafish, a fast and slow, or short and long latency C-startle. Fast C-startles show a weaker C-bend and are elicited by caudal stimuli like a poke to the tail (Fig. 7A). The slower C-startle is elicited by rostral stimuli and involve a stronger C-bend, as the animal aims to bend away from the stimulus before swimming off (Fig. 7B). The network for both fast and slow C-startles is overlapping and follows a predominantly similar pathway (Fig. 7C).

In the case of a fast C-startle, just the Mauthner cell is the driver of the movement \(^{115}\). In the case of a slow C-startle an additional two reticulospinal
neurons are involved. The MiD2cm and MiD3cm are Mauthner cell homologues which fire a train of action potentials and innervate a different, unknown, pool of spinal neurons (Fig. 7A).

Figure 7: C-startle in larval zebrafish. A) Tail directed stimuli evoke a short latency C-startle with weaker C-bend and more forwards oriented escape. This response only involves the Mauthner cell. B) Head directed stimuli result in a long latency C-startle with a longer reaction time and stronger C-bend. This response involves the entire Mauthner array. C) Basic elements involved in the larval C-startle. Sensory input triggers an action potential in the Mauthner cell which sends a large action potential along its commissural axon. Chemical synapse onto the primary motor neuron pool initiates a C-bend away from the triggering stimulus. Simultaneously, electric coupling between Mauthner cell and CoLo interneurons provides contralateral inhibition during the C-bend. V2a and secondary motor neurons are omitted from this schematic for simplicity’s sake.

Changes in the locomotor network

Cardinal class dI6: Dmrt3 as the gait-keeper

The locomotor network and CPG are highly conserved through evolution, likely because they have had to “solve” the same problems over and over. In a few interesting cases, CPG output can be found to be divergent in different breeds within the same species. Commonly, domestic horse breeds display four motor programs, or, natural gaits: walk, trot, canter and gallop. Icelandic horses deviate from the norm however, as they display two additional gaits, tölt and pace.
A genome wide association study among 20 horse breeds identified that a nonsense mutation in the *doublesex and mab-3 related transcription factors (Dmrt3)* gene is permissive for the ability to display additional gaits \(^{145}\). The ability to open up the CPG to produce additional gaits dubbed the *Dmrt3* mutation as ‘the gait-keeper gene’ in the equestrian world \(^{146}\).

In addition to linking the *Dmrt3* mutation to a CPG phenotype, this foundational paper also established *Dmrt3* expression, in combination with Wilms tumor 1 (*Wt1*) expression, as specific markers for the dl6 cardinal class \(^{145}\).

It is rather difficult to generate animal models with locomotor phenotypes through random mutations. This is largely due to the essential role that locomotion plays in an animal’s survival and developmental plasticity often circumvents said mutations. Hence, the “naturally” occurring and stable *Dmrt3* mutation is an outstanding opportunity to study a defined CPG mutant with clear phenotype and genetic background.

A *Dmrt3* knock-out mouse has been generated, which displayed a distinct lack of left-right and front-hindlimb coordination in neonatal animals \(^{145}\). The locomotor phenotype reduced in severity during development due to functional plasticity. Adult mice were unaffected at low locomotor speeds, only losing left-right coordination in the hindlimbs at high locomotor speeds \(^{145}\).

*Dmrt3* interneurons in mice are commissural with ascending and descending projections \(^{147}\) and make primarily glycinergic inhibitory connections to motor neurons \(^{145,147}\). Patch-clamp and calcium imaging experiments showed *Dmrt3* neuron activity during fictive locomotion, but no predictable firing pattern of *Dmrt3* interneurons in relation to ventral root output \(^{147,148}\).

To access the *Dmrt3* population in these *ex vivo* experiments, the complete dorsal horns needed to be removed, a procedure which is very likely to disturb and sever the network greatly \(^{147}\). Therefore, a deeper *in vivo* study of the *Dmrt3* population in zebrafish would greatly strengthen our knowledge regarding this population.

The role of *dmrt3* expressing neurons in zebrafish

Ablation of *dmrt3a* expressing interneurons (referred to as ‘dmrt3 interneurons’ from here on out) in zebrafish results in disturbed left-right alternation during fictive swimming \(^{81}\), confirming their role in the locomotor CPG. Further characterization of *dmrt3* interneurons in zebrafish revealed that the population is glycinergic, commissural and connects to contralateral motor neurons and CPG neurons \(^{81}\). The *dmrt3* interneuron population fires in phase with ipsilateral motor neurons during fictive locomotion, hinting at a function whereby they provide mid-cycle inhibition to the contralateral spinal cord \(^{81}\).

The *dmrt3* population can be subdivided in three subpopulations based on morphology. *Dmrt3* type A and B are grossly similar in morphology and of the CoBL type: commissural and bifurcating contralaterally, projecting both
rostrally and caudally. Morphologically, A and B type dmrt3a neurons are distinct from each other in primary dendrite length, with type A projecting further than 100 µm and type B shorter. Functionally, firing probability of type A peaks between 25 and 35 Hz fictive swims, this lies between 35 and 45 Hz for type B. During escape swimming, fast CoBL’s fire directly after CoLo’s do and remain active during the fast period of the escape. Slow CoBL’s however, never fire during the fast phase and only become active during the later, slow phase of the escape swim.

Type C displayed the striking CoLo morphology with short dendrite, thick commissural axon and spherical soma. CoLo neurons are known to only be active during C-startles, firing primarily during the initial strong bend of the C-startle. CoLo interneurons are known synapse onto motor neurons ventromedially, while CoBL’s do so in a medial and lateral topology.

Those who are one, the other, or both...

The cardinal class of dI6 interneurons can be subdivided in three groups based on transcription factor expression: those expressing Dmrt3, those expressing Wt1 and those expressing both. The Wt1 expressing subpopulation in mice has also been shown to have a role within the CPG. Ablation of Wt1 interneurons lead to disturbed left-right alternation early in development, a reduction in locomotor frequency and increase in stride length in adulthood. During ex vivo analysis, the population was found to predominantly consist of inhibitory commissural neurons with axons terminating close to local commissural interneurons.

In zebrafish, virtually nothing is known about the Wt1 positive subpopulation of the dI6 class. It has only been confirmed by single cell transcriptomics data that wt1a marks a sub-population of the dmrt3a lineage in the larval spinal cord.

Summary

This initial theoretical framework described the rationale and motivation to study the neural circuitry underlying locomotion in larval zebrafish. After placing the topic in a historical perspective, a detailed description was given regarding the developmental origin of the components making up the locomotor CPG. By describing how the cellular components come together in networks we can ultimately understand what is driving the various locomotor behaviors displayed through development.

On this theoretical framework, we based the practical experimental endeavor described in the second half of this thesis. Setting out to use before-mentioned models to investigate the role of Dmrt3 and Wt1 interneurons in the zebrafish locomotor network.
Aims

The overarching aim of the work represented in this thesis is to get a better understanding of the role of Dmrt3 and Wt1 interneurons within the locomotor network. How do these interneurons modulate the output of the network they reside in and what does that tell us about the network architecture?

This general aim was broken down into the following four aims and respective papers:

**Paper I**
To generate and verify a zebrafish *dmrt3a* mutant and morphant model emulating the mutation found in Icelandic horses and to subsequently describe the phenotype in locomotor behavior that arises in the absence of gene.

**Paper II**
To establish an experimental setup, and concomitant data analysis pipeline, with which to conduct experiments combining bilateral ventral root recordings with calcium imaging and various sensory stimulation protocols to measure subtle locomotor phenotypes in fictively behaving zebrafish larvae.

**Paper III**
To describe and explore the *wt1a* expressing lineage in zebrafish. Focus was on characterizing the morphology and topology of the newly discovered W1\(^{CoLo}\) interneuron and describe the functional difference in locomotor network output in absence of the Dmrt3\(^{CoLo}\) or Wt1\(^{CoLo}\) interneuron population.

**Paper VI**
To investigate if calcium imaging is a viable tool for making inferences about population level activity patterns of Dmrt3 interneurons.
Methods

Transgenic animals

The zebrafish model is well known for its relative short generation time, large number of offspring and translucent nature during development. These factors facilitate an unprecedented ease to produce transgenic animals resulting in a large battery of available transgenic tools in zebrafish. The GAL4/UAS system was used to combine various genetic markers and molecular tools\textsuperscript{154,155}. In this system, driver lines (TG(dmrt3-GAL4), TG(mnx1-GAL4)) are used in which expression of the transcription factor GAL4\textsuperscript{154} is regulated by the promoter of a gene of interest. In separate reporter lines, the sequence for molecular tools such as fluorescent reporters or calcium indicators are linked to an upstream activating sequence (UAS). By crossing a driver line and reporter line one creates offspring in which the molecular tool is produced solely in cells expressing the gene of interest. The GAL4/UAS system hence provides a flexible system allowing for the combination of various drivers and tools with the easy of a simple cross of two transgenic animals.

Knock-outs and knock-downs

In order to probe the function of the Dmrt3 and Wt1 interneuron populations we made use of morpholino antisense oligomers. Morpholino oligomers are injected in into the single cell stage of fertilized eggs and are present for three to five days in the developing embryo where they bind to their complimentary mRNA sequence. Morpholinos can be targeted either towards the ATG translation start, sterically preventing the translation machinery, or towards a splice site, preventing the spliceosome to bind, resulting in intron retention and mRNA degradation\textsuperscript{156}. Both strategies result in a lack of the protein encoded by the targeted mRNA.

Ventral root recordings and fictive locomotion

Paradoxically, even though this thesis is focused on the neural circuits underlying movement, a completely still and motionless animal is required for observation of neural circuit activity through methods like calcium imaging or
electrophysiological recordings. To infer the unaltered locomotor activity in motionless animals we made use of the fictive locomotion paradigm. Here, larvae are immobilized by an injection of alpha bungarotoxin, a neurotoxin that binds competitively and irreversibly to post synaptic nicotinergic acetylcholine receptors. This blocks signal transduction from motor neurons to the musculature, rendering the animal motionless while leaving the central nervous system unaffected. By placing recording electrodes at the animal’s musculature, a readout is obtained from the underlying motor neuron end plates. The dual recording allows us to recreate the animal’s fictive locomotor output during spontaneous behaviors or during activity elicited events using visual or sensory stimuli.

Data analysis
Ventral root recordings of fictively behaving animals harbor a wealth of information, but requires a high amount of processing, filtering and precise extraction of quantifiable components. A lack of available software packages to handle this process forced us to develop a custom data processing and analysis pipeline in-house.

A Matlab pipeline was developed that takes in folders with experimental data, reads and filters the raw data files. Bouts of fictive locomotor activity, ranging from zero to tens of episodes per recording, are then automatically detected and stored to a library. Further processing includes extraction of detailed swim bout, half-beat and spike parameters such as output frequency, phase-shifts, quantification of left-right alternation, burst length, etc. This library containing hundreds of episodes can then be indexed by treatment and type and plotted and compared on population, animal, bout and half-beat resolution.

Importantly, this extensive pipeline facilitates automated and unbiased processing and analysis of large complex data type. Only at one point, early in the processing, is human selection of bouts happening in a quality control setting. Unbiased processing was of high importance because we expected a phenotype in our mutant fictive locomotor data. Hence, human interference was avoided and very low exclusion criteria were set during data sorting.

Calcium imaging
To observe the activity patterns of neuronal populations we made use of the genetically encoded calcium indicator GCaMP5. GCaMP is a synthetic fusion protein consisting of a green fluorescent protein, the calcium binding protein calmodulin, and the peptide chain M13. In the absence of calcium, the protein is a state of low fluorescence, but when the calcium concentration in
the environment increases, the protein changes its conformation resulting in an increase of fluorescence. The depolarization of neurons during action potential goes paired with an inflow of calcium into the cytoplasm, hence, by imaging and quantifying the fluorescent change GCaMP a strong inference can be made about the activity of said neurons and the presence of neural ensembles.

Calcium imaging data requires rather extensive data processing and analysis. The standard pipeline involves motion correction to correct for motion artefacts, ROI detection to find the active region in the field of view, extraction of fluorescent timeseries, deconvolution and normalization, and finally computation of neural ensembles. Luckily, packaged algorithms are available for all steps along this process, but every step needs to be optimized and tailored to the specific quality and nature of the recording in question. Because calcium imaging data consists of timeseries of images, computational steps are heavy and the required computing power scales linearly with increases in recording length, frame rate or resolution.

In addition to the standard pipeline, multiple in-house written user interfaces were written to simplify the topological annotation of recordings. To be able to easily view and interpret the large datasets, another user interface was written to interactively view compiled activity traces, ROIs and ensembles.
Results and discussion

Paper I

In paper I we compared three different zebrafish models emulating the nonsense mutation that allows Icelandic racehorses to display higher flexibility in their CPG (Fig. 8A). A Dmrt3a knock-down morphant, in which the translation of dmrt3a was blocked by morpholino injection. A mutant with a late truncating single nucleotide polymorphism, closely mimicking the Icelandic horse mutation. A CRISPR/Cas9 generated mutant line with a 5 base-pair deletion in the first exon.

Free moving locomotor behavior of all mutants was measured at embryonic age 24 hpf, larval age 3-6 and 10 dpf, and 6-week-old juveniles. No effect was observed for any model during coiling behaviors. The late truncating mutant only showed a minor locomotor phenotype at 3 dpf, where embryos swam less and short distances (Fig. 8B).

Figure 8: Knock-down of Dmrt3 expression produces a locomotor phenotype in zebrafish. A) Three knock-down strategies were implemented; a late truncating and early truncating mutation and a translation blocking morphant. B) The strongest locomotor phenotype was observed during tap evoked escape swims in which larvae spent more time accelerating but did not manage to reach the same locomotor speeds as controls. C) Juveniles with the early truncating mutation reached lower maximum swimming speed.
The translation blocking morphant and early truncating mutant showed somewhat similar phenotypes in both spontaneous and tap-evoked escape swimming (Fig. 8B). In day 4 to 10, number of movements, displacement per movement, acceleration and maximum speed all reduced in spontaneous swimming. During escape swims, both models spent more time accelerating while reaching lower maximum velocity and displacement. High-speed kinematics of the early truncating mutant revealed a reduced ability in left-right alternation and larger amplitude between half-beats. Interestingly, both morphants and mutants showed a similar recovery where 22 dpf larvae appeared to behave normally. However, swim tunnel analysis of the early truncating mutant revealed lower maximum swimming speed in juveniles (Fig. 8C).

In conclusion, we confirmed and characterized the involvement of dmrt3 interneurons in coordinating zebrafish locomotion. Much like the findings in mice \(^{145}\), the observed locomotor phenotype weakened through development. Interestingly, for juvenile fish that were forced to swim at high speed, early truncated mutants failed earlier than wild type fish. A finding resembling the adult mice managing to move without problem at low speeds but displaying left-right coordination problems when forced to run at high speeds.

The high selection pressure on locomotion and plastic nature of the developing nervous system renders it possible that the lack of \textit{dmrt3a} expressing cells is compensated for by another cell type. \textit{In situ} hybridization data showed increased \textit{wt1a} expression in \textit{dmrt3a} mutants. A finding, again, similar to observations in mouse spinal cord tissue of \textit{Dmrt3} knock-outs \(^{145}\).

### Paper II

The phenotype described in paper I through behavioral analysis gives a clear indication of a function for the Dmrt3 population in the zebrafish CPG, but the method lacks a depth of output that enables inferences about possible changes in the network. Hence, in paper II, an experimental setup was established that combines synchronized bilateral ventral root recordings with calcium imaging and diverse sensory stimuli to induce various types of locomotor behaviors (Fig. 9). This paper was published with a strong focus on describing the methodology in detail.

A comparison was made between spontaneous swimming and locomotion induced by electrical stimulation to the tail, application of NMDA to the bath and presentation of a visual stimulus.

Chemical induction of locomotion through application of NMDA, a frequently used approach, lead to various artifacts in the locomotor output. Although electrical stimulation introduces a methodological artifact in ventral root recordings, this stimulus did induce escapes consistently. The optomotor response came out as the preferred method to induce locomotor episodes in a
wide range of fictive swim frequencies. Finally, we presented a proof of concept of the modularity of the experimental setup by combing it with calcium imaging and optogenetic inhibition. Overall, this paper laid the foundational methodology to evoke, measure and analyze fictive locomotor behaviors in larval zebrafish, used for testing locomotor phenotypes in paper III and IV.

**Figure 9: Methodology established in paper II.** An experimental setup was established that combines dual ventral root recordings with an array of stimuli to elicit locomotor behavior; electrical, chemical and visual stimulation. The modular setup can easily synchronize recording and stimulation with calcium imaging and optogenetic neuromodulation. An accessory data processing and analysis pipeline was established to extract and analyze global and local parameters from large sets of fictive swim bouts.

**Paper III**

In **paper III**, the locomotor phenotype found in paper I, using the methodology of paper II, was further pursued. In addition to the Dmrt3a translation blocking morphant, the Wt1 lineage was investigated using a similar approach. The \textit{wt1a} expressing \textit{dl6} subpopulation is an interneuron population that has largely been omitted in studies.

We generated a fluorescent reporter line for the Wt1 lineage, which lead to the discovery of a novel type of CoLo interneuron. $Wt1^{\text{CoLo}}$ neurons appear, just like their $Dmrt3^{\text{CoLo}}$ counterparts, as a singlet per hemisegment, but they exhibit a distinct morphology and different (Fig. 10A, B) electrophysiological properties. Elimination of either $Dmrt3^{\text{CoLo}}$ or $Wt1^{\text{CoLo}}$ revealed that $Dmrt3^{\text{CoLo}}$ neurons are involved in the fast phase of short latency escape responses while $Wt1^{\text{CoLo}}$ play a role in slower directional escapes (Fig. 10C, D, E). Additionally, startle behavior mice under chemokinetic inactivation of $Dmrt3$ neurons showed a distinct forward jumping phenotype in response to foot shock (Fig. 10F) Based on these findings and current literature, we hypothesize that these various CoLo interneurons in combination with subsets of the hindbrain Mauthner cell array expand the cellular repertoire to allow
for a more varied escape response enabling for flexible escape trajectories tuned to the requirements of varied environmental threats.

**Figure 10: Newly discovered Wt1\textsuperscript{Colo} plays role in slow directional escapes.** A) Image of well-described dmrt3\textsuperscript{Colo}. B) Image Wt1\textsuperscript{Colo} with distinct axon with elaborate arborizations before crossing the midline. C) Tail evoked swims, Dmrt3\textsuperscript{MO} lack the distinct fast phase during escape swims. D) During head evoked escapes, Wt1\textsuperscript{MO} swim slower than Dmrt3\textsuperscript{MO} which swim slower than control\textsuperscript{MO}. Wt1\textsuperscript{MO} escape bouts are shorter than control\textsuperscript{MO}. E) During loom evoked escapes, Wt1\textsuperscript{MO} show increased spike number and spike frequency. F) Dmrt3\textsuperscript{KD} mice display aberrant behavior in response to foot shock.

**Paper VI**

**Paper VI** is focused on the activity of Dmrt3 interneurons on the population level. Much of the knowledge about the Dmrt3 population is obtained through single cell patch-clamp experiments. Although this provides and in-depth characterization of single cells, it is not feasible to get population level knowledge through this approach. Therefore, we turned to calcium imaging, a method in which activity of tens to hundreds of cells can be measured simultaneously. First, using conventional microscopy, we developed a developmental time line of Dmrt3 neuron activity between 1 and 5 dpf. We observed
that at the coiling stage (1dp) only a small portion of the Dmrt3 cells were active. As the larvae and their locomotor network developed from 2 to 5 dpf, the percentage of active cells increased to 100% at 5 dpf. We then assessed the population dynamics of Dmrt3 interneurons in relation to motor neurons by conducting high-speed light-sheet imaging experiments on double transgenic animals (tg(dmrt3-Gal4; UAS-GCaMP5g; mnx1-GCaMP5g)). We describe, for the first time, the presence of small, localized, clusters within the Dmrt3 population. These clusters displayed high levels of correlation in their activity pattern and consist of 2-6 cells, which align in the medio-lateral axis across the spinal cord. It is possible that these clusters represent the functional and morphological subtypes known to exist within the Dmrt3 lineage.
Conclusion

The work presented in this thesis consists of the generation and characterization of a \textit{dmrt3a} knock-out model in zebrafish, development of experimental and analytical tools to further investigate the locomotor phenotype, a description of the functional role of Dmrt3 and Wt1 CoLo interneurons in the escape network and analysis of Dmrt3 neuron activity at a populational level.

The work provides a description of the development and function of two specific interneuron populations within the zebrafish locomotor network. By expanding our knowledge of how neuronal networks are formed and how these hardwired neuronal circuits are coordinated to produce a flexible behavioral output, we can better understand how complex organisms evolved to survive in a dynamic and ever-changing environment.

Building on the well-established idea that the zebrafish locomotor network is a speed-dependent modular system, where different pools of neurons are recruited as the fish accelerates, the results herein suggest a similar concept regarding the larval escape network. Different escapes, elicited by different stimuli, seem to recruit partially overlapping networks to produce variations on the same locomotor behavior, the C-startle.

Currently, it is difficult to conclude whether we are looking at a single hardwired network that produces variable outputs, or various networks that share overlapping components. Further studies, into the connectomics and mutual activity exclusivity, are needed to resolve this unanswered question. This thesis provides a small piece of the puzzle to understand how millions of subnetworks are coordinated to generate the complex outputs of the human brain.
Future perspectives

Chronic ablation to acute control

The work presented in this thesis all revolves around chronic modulatory methods. Mutants and morphants lack the knocked-down protein from the start of development. An approach that is likely to lead to developmental compensation; closely related subpopulations that compensate or take over the function of the lost population, the connectome is altered.

As described in the literature and paper I and III, a chronic loss of Dmrt3 during development results in an increased presence of Wt1 cells and vice versa.

A much cleaner approach is by way of optogenetic neuromodulation. Light-gated ion channels are expressed in neuronal populations of interest and can be instructed to either hyperpolarize or depolarize with millisecond precision. This approach completely circumvents the backfalls of chronic ablation and the temporal resolution opens many options for precise inference of function. For example, a population can be modulated solely during the fast, slow or transitional phase of an escape response, giving high temporal insight into the functionality.

Four new transgenic lines carrying UAS-driven optogenetic modulators have already been created. However, some optimization is still needed before the experiments can be conducted.

A wt1a driver line

The dI6 class is made up of subpopulations that expresses either dmrt3, wt1 or both. Having a wt1a-Gal4, in addition to our dmrt3a-Gal4, would allow us to address questions regarding the entire dI6 lineage. In the past year, we have invested a considerable amount of time and effort in trying to generate a tg(wt1a-Gal4) zebrafish line. A founder for a wt1a-GF2 transgenic has been confirmed to be swimming in our facility days before the printing of this thesis. Having this transgenic line will add it considerable depth to our studies regarding the dI6 interneuron class.

We are establishing several driver and reporter lines in the QF2-QUAS system in parallel to our Gal4-UAS lines. This duality in our transgenic zebrafish colony will allow us to more easily mix and match double transgenic drivers.
with genetic tools. Simplifying characterization and functional probing of populations. One could, for example, combine two optogenetic neuromodulators driven under the \textit{dmrt3a}-gal4 and \textit{wt1a}-QF2, in a single experiment probing the function of all three dI6 subpopulations. Or, one could express an optogenetic neuromodulator under \textit{dmrt3a}-gal4 while driving a fluorescent marker under \textit{wt1a}-QF2 as a visual guide to patch the sister population.

**Box up the spectrum**

A recurrent theme in this thesis has been the fact that neural populations are almost always made up by multiple subpopulations, which in turn can be subdivided even further. An added complexity is that these subdivisions can be made based on birth timing, morphology, connectivity, function, transcriptomics, thus often requiring post hoc identification of a cell that what imaged or recorded from.

Having a specific marker to probe a population of interest is thus key in all these studies. This asks for a large top-down and bottom-up screen to identify markers for known and new populations of neurons that may be involved in shaping locomotor and behavioral output.

We are collaborating with a research group at an agricultural university to identify new candidate genes in horses that are able to pace, a fifth gait. In parallel we aim to use genetic screens in mice to, in a high-throughput manner, generate zebrafish reporter lines in the hope to mark a small subpopulation to dissect its functionality.

**Closed-loop experiment**

The conversion of the experimental setup described in paper II from a linear experimental setup to a closed-loop set-up has been a desire from early on in the project. In a closed-loop experiment, data is filtered and interpreted live, while the experiment is running. Experimental parameters like visual stimuli or feedback and optogenetic modulation can be altered in the moment in reaction to a criterium being met. For example, to examine the function of a neuronal population during fast swimming, one can inhibit or excite that population specifically during a slow, fast, or frequency changing state during a swim bout. Thus decoupling the role of that population during each phase of the bout.

Additionally, by running a feedback loop from ventral root output frequency to visual stimulation, the immobilized larvae will actually receive sensory input. This can increase the animal’s motivation to move and provides a range of options for more complex behavioral paradigms to be studied like social behavior and foraging. Finally, this closed-loop experimental setting
opens an avenue into studying the role of sensory feedback and sensory processing during locomotion. One can, for example exaggerate or understate the visual feedback an animal receives during/after a swim, leading to the animal adjusting its swim over multiple trials\textsuperscript{160}. In this way, by tuning variables like feedback speed, duration, acceleration, direction, one can probe specific elements of locomotor output.

Genetically engineered voltage indicators

As discussed in paper IV, there can be a huge benefit in the ability to observe the activity of large numbers of neurons from a population simultaneously. The common method to visually measure population activity currently is through genetically engineered calcium indicators (GECI’s), such as GCaMP. New and improved versions of GECI proteins are developed continuously and the signal to noise ratio and protein dynamics get better and better with each generation. However, to be able to record useful data in the spinal cord form neurons active in the CPG, GECI’s will never do the job because they report calcium concentrations, an inherently slow process.

Recent advances in genetically engineered proteins have led to more accessible genetically engineered voltage indicators (GEVI’s). GEVI’s combine the non-invasive measuring of large numbers of cells as seen in calcium imaging with the fast dynamics of electrophysiology. Being able to measure many cells at temporal resolutions capable of detecting single action potentials will revolutionize neurobiology.

Genetic vectors are now readily available and the contemporary GEVI’s seem to have solved previous issues around dye stability and signal to noise ratio. The methodological requirements for voltage imaging are a little steep though. It requires a microscope able to manage high sensitivity recording at 500-1000 frames per second, combined with illumination methods with decent photon economy like two photon or light-sheet.

Voltage imaging in the spinal locomotor network has the potential to provide a wealth of knowledge about function and interactions of populations, functional subpopulations. Use of GEVI’s will greatly benefit from being combined with a previously described closed-loop setup, pushing the network to a specific state and then precisely time bursts of high frequency imaging. Thus, minimizing heavy data collection, imaging only moments of interest while gathering more substantive data.

Machine learning

Three key aspects for a dataset suitable for machine learning based analysis are quality, quantity and variation\textsuperscript{162,164}. In paper II and III we produced large
datasets containing many trials of the same set of behaviors with slight variations and combinations. In current analysis bouts were categorized by the stimulus type or grouped based on threshold or binned ranges of set parameters. It would be interesting to analyze the libraries of fictive swim episodes with the help of machine learning algorithms to produce unbiased deep clustering of the libraries and see if it can pinpoint other aspects or perspectives on the phenotypes found.
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