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Inhibition and motor control in the developing zebrafish spinal cord

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Vertebrate locomotion relies on oscillatory activity along the spinal cord. Inhibition is involved in controlling the alternation of activity between each side and contributes in modulating propagation and termination of locomotor activity. Spinal inhibitory neurons are thought to regulate these mechanisms but the exact contribution of specific cell types remains difficult to tackle during active locomotion. In the past two decades, use of the transparent zebrafish larva has enabled morphological, functional, and genetic characterization of specific inhibitory spinal neurons. A wide range of new optical tools has been developed to monitor and to manipulate the activity of genetically targeted spinal populations. Combining these techniques with conventional electrophysiology will provide a better understanding of the contribution of inhibitory spinal interneurons in regulating essential features of locomotor patterns.

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Introduction

During sustained forward locomotion, activity of spinal motoneurons occurs as oscillations often propagating from the rostral to the caudal end of the cord [1,2]. The spatiotemporal pattern of discharge of motoneurons is tightly controlled by spinal interneurons that form central pattern generators (CPGs). Excitatory premotor interneurons contribute to the rhythmicity of the motor output by setting the frequency at which motoneurons fire action potentials [3–6]. Commissural glycinergic interneurons mediate reciprocal inhibition that enables activity to alternate between each side [3–6]. Precise timing of rostral to caudal propagation of the activity in the spinal cord is critical to ensuring proper locomotor patterns. Similarly, the termination of locomotor episodes and the reset of the activity upon sudden sensory stimulation rely on fine-tuning of the temporal recruitment of spinal circuits. Intracellular recordings of spinal interneurons combined with immunohistochemistry and pharmacological manipulation have suggested that inhibition contributes to these processes [6].

In the past twenty years, the function of spinal neurons involved in motor control has been established by combining electrophysiology, cell reconstruction, and pharmacology, in particular during fictive locomotion *in vitro*. This approach led to a general understanding of how oscillations are generated based on the recruitment of specific types of interneurons within the CPG. However, the relative functional diversity of inhibitory interneuron types in the spinal cord is still poorly understood. It is difficult to associate a cell type, defined functional anatomy, and expression of genetic markers, with its activation during specific motor patterns.

The investigation of the developmental origin of spinal neurons has revealed a map of progenitor domains defined by the combinatorial expression of transcription factors. These domains give rise to the many neuronal subtypes distributed along the dorsoventral axis in the vertebrate spinal cord [7,8]. The use of genetic model organisms facilitates the identification and targeting of specific cell types. Transparency of the zebrafish larva offers the opportunity to genetically target specific neurons and image their morphology while performing electrophysiological recordings in an intact animal. Larval zebrafish swim in discrete bouts and show relatively simple locomotor patterns with a finite repertoire of maneuvers including spontaneous slow forward swims, routine turns, J-turns during prey capture, sensory induced escape responses, and struggles [9,10]. The recent development of genetically encoded sensors and actuators facilitates the monitoring and the manipulation of the activity of specific populations of spinal neurons using light, while an animal is performing one of these maneuvers [11]. This review focuses on recent findings regarding the control of locomotor activity by inhibitory spinal and supraspinal neurons in the zebrafish larva. We provide an updated description of identified inhibitory spinal neurons and their putative roles in active locomotion with a particular focus on the escape response. Finally, we review the contribution of inhibitory cells to the development of spinal circuits.

Identity and contribution of spinal glycinergic interneurons to locomotion

The major neurotransmitter mediating fast inhibitory neurotransmission in the spinal cord is glycine [12], and downregulation or mutations in glycine receptors subunits are associated with major locomotor deficits in zebrafish. Thus, glycine release by spinal interneurons is essential to maintain functional spinal locomotor circuits [13–15]. In rodents, Renshaw cells and Ia interneurons originate from the V1 class of interneurons that differentiate from the medio-ventral p1 domain, and control high motor output frequencies in isolated embryonic mouse spinal cord preparations [16]. In zebrafish, V1 cells form the main class of Circumferential Ascending glycinergic interneurons (CiAs) and express Engrailed-1b (Eng1b). These interneurons fire rhythmically during fictive slow swimming and paired recordings revealed that they directly synapse onto motoneurons [17]. Additionally, connections with glutamatergic Commissural Primary Ascending interneurons (CoPAs) suggest that they can gait sensory information by blocking sensory excitation provided by touch sensitive Rohon–Beard cells via CoPAs [17]. There is evidence that the population of Eng1b positive neurons is composed of both glycinergic and GABAergic cells in both zebrafish and tadpoles but whether these two populations are functionally distinct is unclear [6,17].

One important issue consists in determining the level of specialization of spinal interneurons. This question has been addressed by recording the activity of several subtypes of glycinergic spinal neurons during three electrically induced locomotor patterns, swimming, escape and struggle in the paralyzed larva [18]. Commissural Bifurcating Longitudinal (CoBL) and Commissural Secondary Ascending (CoSA) interneurons are active during all three fictive behaviors, suggesting that they are multifunctional. Conversely, subtypes such as Commissural Longitudinal Ascending (CoLA) interneurons were shown to fire only during fictive struggles, indicating that their activation may be behavior-specific. The observation of multifunctional glycinergic spinal neurons suggests that distributed spinal networks generate diverse locomotor patterns. Similarly, in invertebrates, sensory inputs, long projections neurons, and neuromodulators shape the activity of individual interneurons and motor output in a task dependant manner [19]. Modelling the spinal circuits as a network with multistable neural dynamics and modular organization could explain why certain spinal neurons are only active during a given fictive locomotor behavior and silent during others.

Genetic dissection of the circuit mediating the escape response

The circuits involved in escapes induced by touch, sounds, or water flow have been extensively studied in larval fish [20]. Following an acoustic or vestibular stimulation on one side, the activation of the Mauthner cell in the hindbrain leads to the fast recruitment of a large population of motoneurons on the contralateral side [21]. The Mauthner cell had been shown in goldfish to activate commissural interneurons through gap junctions on one side. These neurons consequently silence motoneurons located on the contralateral side [22]. Recently, an enhancer trap approach identified these neurons in zebrafish larva as spinal glycinergic Commissural Local (CoLo) interneurons [23]. Laser ablation of GFP-expressing CoLos altered the C-bend tail contraction following sensory stimulation indicating that their activation is critical for the execution of the behavior.

Local synaptic partners of Mauthner cells have been mapped in the goldfish spinal cord [20]. In zebrafish, glycinergic connections to the Mauthner cell are found as early as 27 hours post fertilization (hpf) [24]. A recent study described the structural organization of the hindbrain, based on the expression of transcription factors and transporters of neurotransmitters [25•]. The architecture of this network relies on three layers of glycinergic cells that are interspaced with stripes of glutamatergic cells. The medial glycinergic stripe contains Eng1b positive neurons that project ispsilaterally. The middle stripe contains only contralaterally projecting glycinergic neurons, and the lateral stripe contains cells with both ipsilateral and contralateral projections. Taking advantage of this organization, the authors investigated neuronal interactions between these inhibitory neurons and Mauthner cells by performing paired recording of cells labelled by dye injections at various position on the Mauthner cell [26••]. Feedforward inhibitory neurons that project on ipsilateral and contralateral Mauthner cells originate from the lateral glycinergic stripe. Their activation triggered short latency IPSPs in both Mauthner cells that were blocked by strychnine application, suggesting that these cells control the temporal spiking of the Mauthner cell. Feedback inhibition is necessary to control the duration of the Mauthner cell spiking and is mediated by glycinergic interneurons. Some of these neurons express Engrailed-1b, originate from the medial stripe, and project in the ascending direction. They were activated by the ipsilateral Mauthner and in turn sent a short latency inhibitory current back onto the same cell. Altogether, this work provides one of the most detailed descriptions to date of the inhibitory reticulospinal circuit controlling the Mauthner-dependant escape response in zebrafish.

Nature and function of GABAergic spinal interneurons

GABA is prevalent during early developmental stages compared to mature circuits, when multiple interneurons combine GABA and glycine release [27,28]. Pharmacological experiments in lamprey showed that GABA has a modulatory effect on spinal circuits involved in locomotion [29]. The possible roles of GABA appear

more subtle than glycine, but could play an important role in modulating the excitability of the cord, given the slow time course of GABA compared to glycine [30].

On the basis of morphological and genetic studies, few GABAergic spinal subtypes have been identified in zebrafish. Dbx1-expressing neurons, originating from progenitor domain P0, represent a mixed population of glutamatergic, glycinergic, and GABAergic neurons [31•]. Among them, ventral GABAergic *Dbx1+ Pax2+* cells are commissural and project locally, with both ascending and descending axons. An elegant study in newly born mice using an intersectional genetic ablation system described a dual mode operation of the Dbx1 population. Dbx1 positive cells control the left-right alternation of activity in a frequency-dependant manner [32••]. Dorsal GABAergic *Dbx1+/Pax7+* cells control left-right alternation at low locomotor frequencies. Conversely, ventral glutamatergic *Dbx1+/Evx1+* were involved in alternation at high frequencies. Whether these cells contribute to left-right alternation in zebrafish remains to be demonstrated.

GABAergic Ventral Lateral Descending (VeLD) interneurons originating from V2 progenitors exhibit pacemaker-like patterns of activity and are recruited during early spontaneous activity in zebrafish embryos [33,34]; however, their function remains unknown. Similarly, Dorsal Longitudinal Ascending (DoLA) neurons are GABAergic neurons for which the function is largely unknown. They are located on the dorsal side of the cord and extend long processes ventrally [35]. On the basis of their unique morphology and location, they could relay sensory information from dorsal neurons to more ventral interneurons and motor neurons. GABAergic cerebrospinal fluid-contacting neurons called Kolmer–Agduhr neurons (KAs) in zebrafish lie around the central canal in the ventral spinal cord and originate from both p3 and pMN progenitor domains [36,37]. An observation based on the use of light-gated channels for remote activation in intact zebrafish larva revealed that these cells could modulate the excitability of spinal networks involved in locomotion [38]. Their physiological recruitment in normal conditions *in vivo* and how they are relevant to active locomotion remains to be further investigated.

GABA/glycine neurotransmission at early stages of development and neurogenesis

During early development, concentration gradients of morphogens sculpt the spatial organization of spinal progenitor domains, allowing combinatorial expression of specific genes along the dorsoventral axis of the cord [39]. This process contributes to the generation of diverse population and subpopulation of spinal neurons [40]. Spinal progenitors are exposed to neurotransmitters released from newly differentiated neurons. Recent studies in the zebrafish embryo indicate that endogenous release of neurotransmitters could influence cell fate acquisition in spinal cord. The disruption of the alpha-2 subunit of glycine receptors has been associated with a decrease of the pool of interneurons and an increase of the pool of cycling neuronal progenitors [41]. Accordingly, the blockade of glycinergic receptors with strychnine recapitulates the effect of a glycine receptor knockdown suggesting that glycinergic neurotransmission regulates neurogenesis in the spinal cord [42•,43]. Alteration of glycine signalling did not alter the differentiation of motoneurons or sensory neurons, indicating that the effect on neurogenesis may be restricted to specification of interneurons only. However, the source of glycine affecting interneurons specification during these early developmental stages in the spinal cord has not yet been characterized.

Although early GABAergic neurotransmission is crucial for the onset of spontaneous activity in mammals [44,45], evidence suggest that early spontaneous activity patterns in zebrafish embryonic spinal cord may rely more heavily on gap junctions than on synaptic transmission in the spinal cord [34,46]. Even though GABA has been shown to be expressed in neurogenic niches around the central canal in turtle spinal cord [47], it is not clear yet whether GABA signalling plays a role in the specification of spinal interneurons during development in other vertebrates.

Challenges and perspectives

Identifying specific neuronal lineages and cell types in the spinal cord is a critical step to unravel their function, patterns of connectivity, and dynamics of recruitment. Most of our knowledge of the function of spinal neurons is based on electrophysiological and pharmacological studies coupled with reconstruction of cellular morphology and immunohistochemistry [35,48,49]. Since the development of the Tol2-mediated transgenesis in zebrafish [50,51], the library of cell-specific reporter lines has been rapidly expanding (Table 1). These tools have the ability to reveal new categories of interneurons, such as locally projecting ones [23], that would be difficult to label with retrograde tracing approaches. Stable transgenic lines provide new ways to investigate the function and the developmental origin of specific cell types [23,52••]. While an investigation of circuits with electrophysiology is essential, it is usually performed on one or two neurons recorded simultaneously. Novel optical approaches offer means to probe the dynamic recruitment of populations of identified spinal neurons during locomotor pattern generation.

Recording the activation of genetically targeted populations of neurons during specific behaviors can be performed using genetically encoded calcium indicators (GECIs) or bioluminescent reporters [53,54]. These approaches are based on the estimation of intracellular calcium variations following neuronal activation and can be used efficiently to reveal activation of specific cells

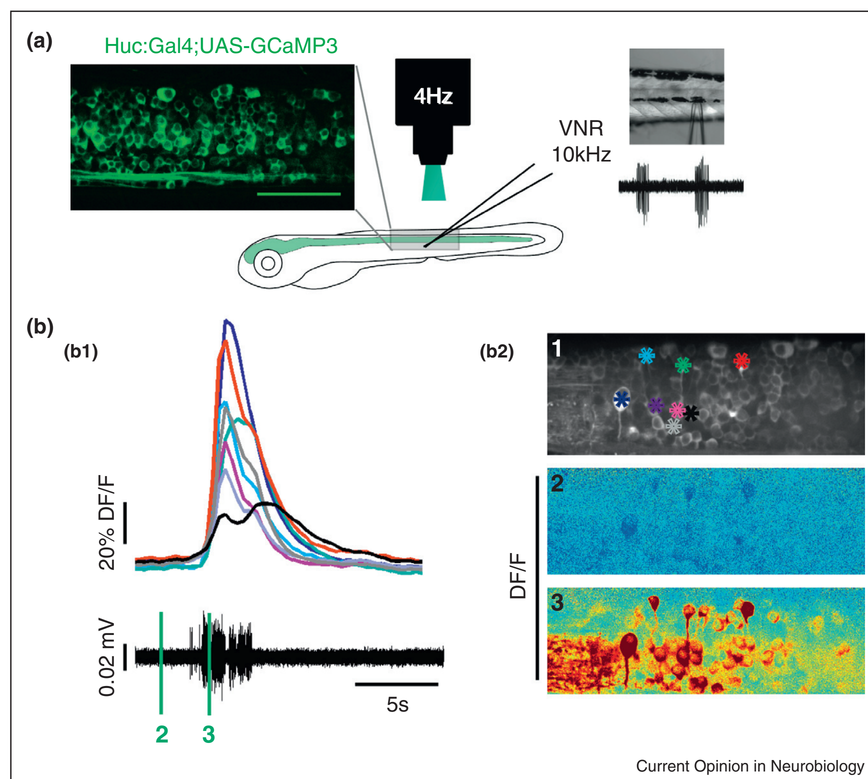
Table 1

Summary of recently generated zebrafish transgenic lines targeting inhibitory spinal neurons

Construct/Line	Genetic markers	Targeted neurons in spinal cord	References
glyt2:GFP glyt2:lRl-GFP (glyt2:RFP) glyt2:Gal4 glyt2:lRl-Gal4 (glyt2:RFP)	Glycine transporter 2 (glyt2)	Glycinergic neurons	McLean *et al.* [66] Satou *et al.* [31•] Satou *et al.* [52••]
Tol56:GFP	Enhancer trap	Glycinergic and GABAergic neurons	Satou *et al.* [23]
gad1b:GFP	Glutamate decarboxylase type 2-gad1b	GABAergic neurons	Satou *et al.* [31•]
gad1b:lRl-GFP (gad1b:RFP)	Glutamate decarboxylase type 2-gad1b	GABAergic neurons	Satou *et al.* [52••]
dbx1b:GFP	dbx1b transcription factor	Mixed GABAergic/glycinergic/glutamatergic	Kinkhabwala *et al.* [25•] and Koyama *et al.* [26••]
dbx1b:lRl-GFP	dbx1b transcription factor	Mixed GABAergic/glycinergic/glutamatergic	Kinkhabwala *et al.* [25•] and Koyama *et al.* [26••]

during a fictive locomotor event (Figure 1). However, the use of GECIs presents some limitations. First, sensor responses are not linear, which means that subtle changes in calcium concentration induced by few action potentials might not be sufficient to generate a signal. Consequently, sparse or single spikes might not be recorded. In this context it can be difficult to distinguish between a silent neuron and a neuron whose activity is not detected.

Figure 1



The recruitment of large populations of neurons can be estimated during spontaneous fictive locomotion using genetically encoded calcium indicators GCaMP. **(a)** Schematic of the experimental setup. GCaMP3 is expressed under the control of the pan neuronal HuC (*elav3*) promoter in a 5-day-old zebrafish larva (scale bar is 50 μm). Motor output is recorded from the ventral nerve root (VNR) and GCaMP3 fluorescence signals are acquired using fast scanning microscopy. **(b)** Pattern of activation of a population of spinal neurons during fictive locomotion. (b1) Calcium transients from eight spinal neurons (colored in b1 and b2) during a fictive slow swimming. Top panel in (b2) shows the fluorescence (F) of GCaMP3 in spinal neurons located in the field of view. Middle panel in (b2) shows the variation of fluorescence (DF/F) before a burst. Bottom panel shows the DF/F during a fictive swim event. Calcium transients are concomitant with the fictive swim event detected by the VNR recording.

In addition, the temporal dynamics of GECIs are still very slow compared to electrophysiological recordings. Finally, calcium sensors allow detection of neuronal activation but cannot be used to record inhibition. Despite these limitations one should be aware of, GECIs are fast and sensitive enough to monitor the activity dynamics during a single locomotor event. They can be combined with genetic targeting to reveal how specific inhibitory populations defined by a combination of molecular markers are recruited during locomotor patterns.

New generations of GECIs raise the hope that single action potentials can be detected without averaging trials, and cell activation can be monitored at high frequencies in a paralyzed animal [55,56,57••]. These new generations of sensors enable recording many neurons at once and as a consequence visualize dynamics of activity and recruitment over time during different behaviors. In mice, preliminary results have revealed collective patterns of activation of En-1 positive interneurons in the ventral horn of neonate mouse lumbar spinal cord, half of the neurons recorded were active in phase with the motor output recorded from ventral nerve roots while the other half were active out of phase [58]. This split pattern of activity for Renshaw cells had been reported with electrophysiological recordings [59]. Similarly antiphase activity has also been shown for Ia interneurons and Renshaw cells in the cat [60]. Imaging techniques combined with genetic targeting should resolve the molecular origins of the functional heterogeneity for these two populations. The recent development of light-gated channels or pumps allows probing the modulation of active locomotion by defined cell types in many model organisms such as fish, frogs and rodents. These actuators could be used in combination with calcium sensors to understand how the activation or inhibition of defined spinal neurons affects the recruitment and activity of other spinal populations [61–63,64••,65].

Most of the data described here have been acquired during embryonic or larval stages, when the animal is able to perform different stereotyped maneuvers and the circuits are formed but not fully mature. Physiological changes occur during transitions to juvenile and adult stages at cellular, morphological, and behavioral levels, suggesting a refinement of spinal network organization during morphogenesis [66–68]. Studies on motoneurons and premotor excitatory interneurons in adult zebrafish have revealed that the patterns of recruitment rely as well on a combination of their intrinsic and synaptic properties, but the organization of the network and contribution of intrinsic versus synaptic properties may change with time. Inhibitory premotor interneurons have not been yet investigated in the juvenile and adult zebrafish. The ability to perform recordings from genetically identified premotor and motor networks in young and old fish will provide ample opportunity to investigate the cellular and synaptic mechanisms underlying the maturation of locomotor behaviors [69–71]. Regarding inhibitory cells, a recent study has investigated the profile of expression of the glycine transporter GLYT2 in the adult [72••] showing evidence for glycinergic reticulospinal projections. The role of the descending glycinergic inputs in the adult spinal cord needs to be further characterized but could play an important role in shaping spinal activity.

All together the combination of stable transgenic lines established in recent years with innovate optical methods for remotely monitoring, activating or silencing neurons *in vivo* will enable the functional investigation of the spatio-temporal recruitment of inhibitory interneurons and their contribution to the dynamics and plasticity of the spinal circuits.

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