Beyond vertebrates: the amphioxus as a relevant model system to explore the formation, organization, and regeneration of neuromuscular synapses

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The neuromuscular junction (NMJ) is the peripheral synapse controlling muscle contraction and coordinated movement in a wide variety of animals. In humans, the mature NMJ is the primary target of morphological disassembly and functional decline in several physiological and pathological conditions, such as aging and motor diseases, respectively. Different paradigms of nerve damage in murine models have revealed that the peripheral nervous system bears regenerative abilities leading to functional NMJ recovery; however, this process is often inefficient in humans, likely due to their larger size and differences in the establishment of a still elusive regenerative niche at the NMJ (Zelada et al., 2021). One way to approach this obstacle is to understand the evolution of synaptic contacts, as they can provide novel insights into the fundamental cellular and molecular requirements that were ancestrally involved in the development and regeneration of neuromuscular synapses. Indeed, relevant information on the molecular mechanisms involved in peripheral neural organization and regeneration has been obtained from flies and worms (Richardson and Shen, 2019). The cephalochordates, also known as amphioxus, are a group of non-vertebrate chordate marine animals sharing many genomic and developmental features with the vertebrates. Interestingly, they also share several, although simplified, anatomical structures with vertebrates, including nervous and muscle tissues. Here, we specifically describe the distinctive neuromuscular contacts in amphioxus and briefly discuss how these features could provide valuable information to promote efficient vertebrate NMJ repair.

Anatomical structures involved in neuromuscular synapses in amphioxus: As benthic marine animals, muscle contraction waves allow amphioxus to swim to escape from predators and to bury themselves in the sand. The motor behavior in these animals relies on very peculiar neuromuscular contacts established between the neural tube and either the notochord (that has a muscular nature in this species) or the trunk muscles (myotomes) (Flood, 1966).

The central nervous system of the amphioxus consists of a short anterior region of the neural tube (a small cerebral vesicle) and a long caudal region (the spinal cord). The amphioxus neural tube is organized in a complex and segmented array of different neuronal cell types restricted to specific regions along the anteroposterior axis. Specifically, motor neurons organize into dorsal (DC) and ventral compartments (VC) within the neural tube. The DC is smaller in size and contains the axons of small motor neurons, whereas the VC has a large size and harbors bigger motor neurons (Flood, 1968).

A prominent notochord, that persists until adulthood, distributes along the entire length of the amphioxus body and extends more anteriorly than the neural tube. The embryonic notochord is formed by cells exhibiting large cytoplasmic vacuoles and plays a fundamental role as a source of key developmental cues. During metamorphosis, cytosolic vacuoles regress and become progressively replaced by myofibrils, providing the adult notochord with contractile properties (Suzuki and Satoh, 2000). As a consequence, the internal region of the adult notochord is organized in a series of transverse plates, called notochordal laminae, which are specialized muscle units with the ability to alter the mechanical properties of the entire notochord upon contraction (Flood, 1970). The notochordal cells of amphioxus contain transverse filaments with a striated pattern that resembles skeletal muscle sarcomeres (Flood, 1974). Consistently, gene expression studies have revealed that more than half of the muscle genes expressed in amphioxus notochordal cells are homologous to the genes coding for contractile proteins of vertebrate skeletal muscles (Suzuki and Satoh, 2000).

The notochord is surrounded by a thick sheath of collagenous connective tissue, similar to that of the neural tube (Flood, 1966, 1970). Phase-contrast microscopy has revealed the presence of “notochordal horns”, extensions of the notochordal laminae surrounded by connective tissue of the notochordal sheath. Notochordal horns are uniformly distributed in two anteroposterior rows all along the dorsal region of the notochord and make focal contacts with the ventral region of the neural tube through a paired series of pits of the notochord sheath (Flood, 1970). Transmission electron microscopy analyses have shown that the cytoplasmic processes of the notochordal horns are innervated by presynaptic nerve endings of the ventral neural tube (Flood, 1970) (**Figure 1**).

Most of the amphioxus body is occupied by skeletal muscles that are organized in 48 to 80 myotomes (depending on the species), each composed of mononucleated muscle laminae assembled by contractile proteins that, similar to notochordal cells, share a striated organization and functional associations with vertebrate sarcomeres (Suzuki and Satoh, 2000) (**Figure 1**).

Remarkably, trichrome staining and ultrastructural analyses have revealed that the ends of myotomal muscle laminae extend long processes that traverse the collagen sheath surrounding the neural tube, establishing neuromuscular contacts within the neural tube region (Flood, 1966) (**Figure 1**). Interestingly, the muscle processes that contact the VC region extend from deep sarcoplasm-poor muscle laminae having few mitochondria, whereas the muscle processes that contact the DC region of the neural tube originate from superficial muscle laminae exhibiting abundant mitochondria and glycogen within its extended sarcoplasm (**Figure 1**). Based on these features, these muscle cells have been classified as fast and slow muscle laminae, respectively (Flood, 1968).

Noteworthily, some of the particular features of the amphioxus neuromuscular contacts of myotomes are similar to those of vertebrates. For instance, in the adult zebrafish, the slow muscle fibers are located in the lateral regions while fast muscle fibers distribute in the deep portions of the myotomes, as in amphioxus (Ampatzis et al., 2013). However, zebrafish white muscle fibers, responsible for fast escape swimming, are innervated by dorsal large motor neurons, whereas red muscle fibers that allow prolonged swimming are innervated by ventral small motor neurons (Ampatzis et al., 2013), which is opposite to the motor innervation pattern observed in amphioxus. Although not identical, these studies at the cellular and histological levels reveal that the gross organization of the amphioxus locomotor system and its control is conserved with vertebrates.

Synaptic nature of the interaction of the neural tube with the notochord and myotomes: While mammalian NMJs are cholinergic in nature, flies and worms establish glutamatergic NMJs. Are the neuromuscular contacts of amphioxus more similar to other non-vertebrate species or to human beings? In this respect, it has been found that nerve endings of the amphioxus ventral neural tube contacting either the notochord or myotomes contain synaptic vesicles, mitochondria, and glycogen granules (Flood, 1970). Transmission electron microscopy studies have shown that these vesicles have a caliber of 70–117 nm and that the synaptic space is approximately 60 nm wide, similar to the vertebrate neuromuscular synaptic cleft (Flood, 1966). *In situ* hybridization studies have revealed the expression of the vesicular acetylcholine transporter and choline acetyltransferase along the anterior neural tube in amphioxus embryos and larvae (Candiani et al., 2012). In addition, following the classical Karnovsky & Roots technique, high cholinesterase enzyme activity has been detected at both the dorsal end of each notochordal horn contacting the neural tube, as well as in the VC and DC regions, where myotomal muscle processes contact the neural tube (Flood, 1974).

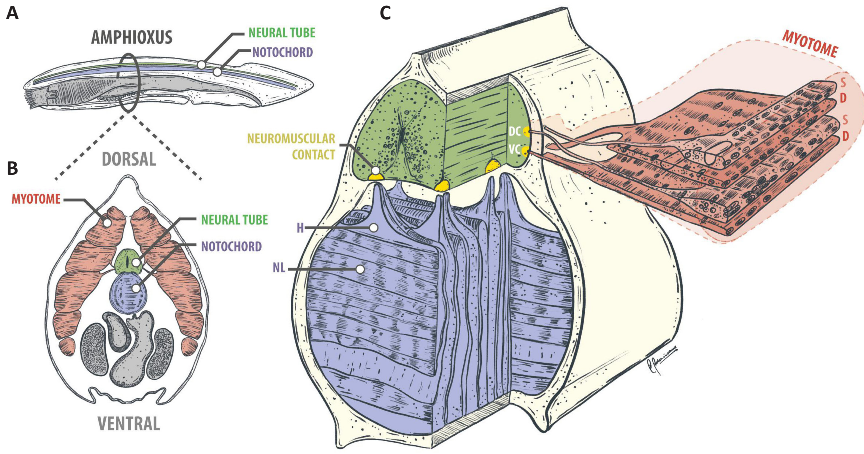

Figure 1 | **Neuromuscular contacts in the amphioxus.**
The main structures involved in neuromuscular synapses in adult amphioxus are schematized. (A) Longitudinal scheme of the adult amphioxus, highlighting the neural tube (green) and the notochord (purple). (B) Cross-sectional scheme of the transverse area depicted in A, showing the myotomes (red), the neural tube (green), and the notochord (purple). (C) Neuromuscular contacts (yellow) occur within the neural tube area with the myotomes and notochord: notochordal laminae (NL) extend notochordal horns (H) that contact ventral areas of the neural tube; in turn, the myotome, composed of superficial (S) and deep (D) laminae, protrudes cell projections that contact the dorsal (DC) and ventral compartments (VC) of the neural tube, respectively. The neural tube and the notochord are surrounded by a thick collagen sheath (light yellow). Artwork by Felipe Serrano @biolustrador.

Although these studies indicate that the amphioxus neural tube forms cholinergic synapses with both the notochord and myotomal muscles, a still-unsolved issue involves the identity of the receptors participating in amphioxus neuromuscular synapses. In vertebrate cholinergic synapses, ligand-activated AChRs exist as metabotropic muscarinic, present in neurons and peripheral tissues, or ionotropic nicotinic, expressed by skeletal muscles at the NMJ and somatic neurons. Neuronal nicotinic AChRs include nine α (α2–α10) and three β (β2–β4) subunits. Although sequence analyses have shown that amphioxus expresses homologous genes for the α1, α2, α4, α9, α10, and for β2, β4, and β5 subunits of nicotinic AChR, the specific receptors potentially involved in neuromuscular connectivity remain unknown (Pedersen et al., 2019). In vertebrates, muscle nicotinic AChRs switch from a fetal α2β1γδ composition to an adult α2β1εδ stoichiometry. The replacement of γ for ε subunits is crucial for NMJ organization and function (Cetin et al., 2020). On the one hand, fetal AChRs have a higher sensitivity to choline, which could facilitate neuromuscular transmission soon after muscle innervation. On the other hand, adult AChRs display faster recovery rates from desensitization and higher Ca^{2+} permeability, which are two relevant features to regulate NMJ efficacy. Studies with chimeric fetal/adult receptors have shown that fetal AChRs determine a proper distribution and innervation pattern of nascent NMJs (Cetin et al., 2020). Therefore, we envision that the identification and characterization of AChRs in amphioxus neuromuscular contacts will significantly extend the usefulness of this attractive model system to understand vertebrate NMJs.

The amphioxus, as the earliest divergent representative of the chordate phyla, is currently considered a chordate archetype from a genomic and morphological perspective. Their unique features position this non-vertebrate species as a key intermediate to understand the emergence of the vertebrates. A main relevant feature for the study of the amphioxus NMJ is related to their regenerative capacity, which involves the regeneration of the notochord, the neural tube, and myotomes (Somorjai et al., 2012). Indeed, transversal amputation experiments in different sections of the amphioxus revealed strong regeneration abilities at both ends of the anteroposterior axis, unless the damage is generated close to the pharynx or the hepatic diverticulum, which leads to death. Although juvenile amphioxus displays higher regeneration rates, adult animals also exhibit remarkable regenerative abilities (Somorjai et al., 2012). Therefore, considering its many experimental advantages, we propose that the amphioxus has a relevant potential to approach the complex molecular mechanisms that are conserved in different animal species, including future research on the activation of neuromuscular regeneration mechanisms.

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