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Developmental stage-dependent switching in the neuromodulation of vertebrate locomotor central pattern generator networks

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Abstract

Neuromodulation plays important and stage-dependent roles in regulating locomotor central pattern (CPG) outputs during vertebrate motor system development. Dopamine, serotonin and nitric oxide are three neuromodulators that potently influence CPG outputs in the development of *Xenopus* frog tadpole locomotion. However, their roles switch from predominantly inhibitory early in development to mainly excitatory at later stages. In this review, we compare the stage-dependent switching in neuromodulation in *Xenopus* with other vertebrate systems, notably the mouse and the zebrafish, and highlight features that appear to be phylogenetically conserved.

Introduction

During animal development, the requirements of locomotor control systems markedly change to facilitate the emergence of new or modified neuromuscular structures and behaviours. These changes are accompanied by profound alterations in the neuromodulatory systems acting on the underlying central pattern generator (CPG) networks, which generate the basic rhythmic motor activity, in order to sculpt appropriate network outputs throughout development. The effects of a neuromodulator at early stages of development can be precisely the opposite of its actions at later developmental stages. Thus, the roles of a neuromodulator are not static but, rather, can switch during development in response to changing environmental and organismal demands to adapt motor behaviours. Precisely timed changes in specific sources and targets of neuromodulators are involved in the regulation of these switches.

In principle, adaptive changes in the neuromodulatory control of motor behaviours can occur on multiple levels. At the systems level, entirely new networks can emerge to control additional body parts and behaviours, as is the case during metamorphosis in anuran amphibians, for example (Combes, 2019; Sillar et al., 2008), and these networks are subject to the coordinated actions of multiple neuromodulators. At the cellular level, new neurons can be formed and added to the complement of possible targets of neuromodulation, or already existing neurons may modify their

transmitter phenotype, including expression of modulatory co-transmitters (Nusbaum et al., 2001; Svensson et al., 2018; Vaaga et al., 2014). At the molecular level, single neurons can alter the array and balance of modulatory receptors, ion channels and even the ion pumps and transporters they express.

Acquiring a better understanding of the complex interactions that allow neuromodulation to adapt locomotor behaviours during development, both within and between levels, remains a major challenge and an important goal in the field of neuroscience. Comparing and contrasting neuromodulation of locomotor CPGs between different vertebrates is a fruitful avenue for study because it highlights those features that are specific to a particular animal or developmental stage, as well as features that have been conserved during evolution. In this review, we will outline recent advances in this area, focussing mainly on dopamine, serotonin and nitric oxide signalling during locomotor development in the *Xenopus* frog tadpole and compare this hybrid system (employment of axial- then limb-based locomotion) with the zebrafish (an axial swimmer) and the mouse (a quadruped).

Development of locomotor behaviour

Motor behaviours undergo two fundamental types of developmental change: progressive and transformative. The former, for example swimming in fish, involves newly hatched larval animals maturing into an adult with respect to animal growth and size whilst maintaining the same basic body format and swimming mechanism. In the latter, the body structure and behavioural repertoire of larval animals undergo radical transformations. Classic examples of this include the switch from caterpillar crawling to butterfly flight or from tadpole swimming to frog jumping. Another example occurs in the crustacean feeding system, which changes from filter feeding in the larval form to gastric mill chewing in the adult. In this particular case, much is known about the switch in CPG function in the stomatogastric ganglion (STG) (Casasnovas & Meyrand, 1995), which sits atop the stomach and controls the rhythmic food processing movements of the foregut. Interestingly in the context of what occurs in vertebrate motor systems, the CPG neurons are present from embryonic stages but are progressively influenced by neuromodulatory pathways that later project to the STG from the commissural ganglia during maturation of the feeding system.

Many mechanisms engaged in motor system development are highly conserved amongst vertebrates, therefore making comparative studies insightful. One animal model in which particularly dramatic changes occur during development - and which serve as the centrepiece for this review - is the South African clawed frog, *Xenopus laevis* (Figure 1). This anuran amphibian begins the first few days of life outside the egg as a small, ~5-7 mm long, hatchling tadpole (around stage 37/38 to 42 (Figure 1A; (Nieuwkoop & Faber, 1956)) and initially receives sustenance by absorbing its yolk sac. Tadpoles can swim when stimulated to escape from potential predators but spend most of their time lying dormant and undetectable. To simply remain stationary is the default behavioural status of these (and many other) small defenceless creatures. Within a few days, however, tadpoles become free swimming and begin filter feeding after the depletion of their yolk sac. At these stages (> stage 44/45), tadpoles swim freely and virtually continuously for the rest of their lives. This is either hovering in a head down orientation (with only the caudal tail tip oscillating; e.g. Figure 1B), whilst the tadpole gulps water and filter feeds, or in a propulsive burst swimming mode when more rostral trunk muscles are recruited.

During the ensuing weeks, the tadpoles will also undergo the remarkable transformation process known as metamorphosis, first acquiring hindlimbs (Figure 1C) and then forelimbs (Figure 1D), whilst retaining a functional tail for axial swimming throughout the metamorphic period, before transitioning into a juvenile anuran adult (Figure 1E). The emerging limbs are under the control of *de novo* motor networks that drive new motor neurons in the thoracic and lumbar enlargements of the spinal cord and that co-exist with the axial networks first established during late embryogenesis (for a recent review see (Combes, 2019)). The limb and axial rhythms are initially expressed at the same cycle frequency, with the early limb motor rhythm generating synchronous bursts in the extensor and flexor motor neurons of each hindlimb, and in alternation between the two sides (Combes et al., 2004). As the limbs develop, however, the limb rhythm becomes independent from the axial rhythm and adopts its own cadence. Therefore, by the time of the metamorphic climax, in contrast to the alternating left-right oscillations generating tail-based forwards swimming, the propulsive leg kick rhythm is bilaterally symmetrical, consisting of repeating extension (power stroke) and flexion (return stroke) cycles. Remarkably, the two rhythm generating systems can unify during powerful tail-based swimming or the limb and axial rhythms can be expressed separately, indicating the existence of two separate CPGs with flexible coupling between them. In addition to the principle transition to limb-based synchronous kicking following

post-metamorphosis, there is also the possibility that axial muscles remaining in the trunk of frogs may continue to contribute to locomotor rhythm generation, as is the case in other limbed vertebrates such as urodeles (Katz, 2016; Sillar et al., 2008) .

Throughout development, neuromodulators continuously regulate the locomotor CPGs to alter the animal's swimming behaviour in response to extrinsic and intrinsic cues, for example increasing motor burst intensity and locomotor rhythm frequency when being pursued by a predator. In addition, as mentioned above, the roles of some modulators can also dramatically switch during development to facilitate the maturation of locomotor CPGs and their behavioural outputs.

Key neuromodulators of developing locomotor networks

Although an extensive list of neuromodulators has been shown to alter locomotor output (Miles & Sillar, 2011), dopamine (DA), serotonin (5-HT) and nitric oxide (NO) have emerged as three key modulators of locomotor CPG activity and each exhibits a stage-dependent switching in their roles during locomotor network development. We will begin by reviewing the actions of each of these modulators in turn, first at early stages of swimming development in *Xenopus*, around the time of hatching, and compare their changing roles at later stages after the onset of free-swimming larval life, and then compare and contrast findings in *Xenopus* with other vertebrate locomotor systems.

Dopamine

There are two main DA receptor sub-classes that possess different affinities for DA and mediate opposing physiological effects: 1) D1-like receptors (D_1 and D_5 subtypes) have a low DA affinity and are excitatory; and 2) D2-like receptors (D_2, D_3 and D_4 subtypes) that have a high DA affinity and are inhibitory (Beaulieu & Gainetdinov, 2011). At early larval stages in *Xenopus* (around 37/38-42), the primary effect of bath applied DA is a profound inhibition of most fictive swimming parameters, including reduced swim episode durations, ventral root burst amplitudes and intrinsic swim cycle frequencies (Picton & Sillar, 2016). These effects occur regardless of the bath applied DA concentration (0.5-100 μM). This strongly suggests that DA mediates its effects in young *Xenopus* tadpoles exclusively *via* D2-like receptors. Indeed, the D2-like receptor agonist quinpirole mimics the effects of bath applied DA on swimming, whilst the D1-like receptor agonist, SKF38393, has no effect (Picton & Sillar, 2016). The effects of quinpirole are blocked by

the selective antagonist, L745870, indicating that the inhibition is primarily mediated by the D_4 receptor subtype. The DA uptake inhibitor, bupropion, also mimics the effects of exogenous DA and the D2-like receptor agonists, which suggests that D_4 receptors have an endogenous role in inhibiting swimming activity. The inhibition of swimming by DA acting *via* D2-like receptors increases GIRK-type K^+ conductances, which results in widespread hyperpolarization of spinal CPG neurons. This hyperpolarization affects motor neurons, to decrease burst durations, and commissural interneurons to delay their firing and reduce post-inhibitory rebound activation of excitatory interneurons (Picton & Sillar, 2016).

We concluded from these data that at younger stages, when the tadpole is small and vulnerable to predation, DA helps maintain the tadpole in a quiescent state, except when it is stimulated to swim. Presumably, this facilitates a survival tactic in the tadpole as most predators respond predominately to the movements of a potential prey. The data also suggest that D1-like receptors are absent or not highly expressed in the swim CPG neurons at these early development stages, as exposure to high DA concentrations that are normally required to activate this receptor subtype have no obvious additional effects on swim parameters.

In their study of the effects of DA at the later free-swimming larval stages of *Xenopus* development, Clemens et al. (2012) found that low concentrations of DA (2 μM) reduced the occurrence of spontaneous fictive swim bouts in isolated CNS preparations and, as in the younger stages, increased the cycle periods and consequently decreased swim frequency. Thus, it appears D2-like receptors at later stages continue to exert an inhibitory influence on the locomotor CPG output. In contrast, higher concentrations of DA (50 μM) now exerted the opposite effect and switched inhibition to excitation, with increased swim bout occurrence and shorter cycle periods (Clemens et al., 2012). This suggests that D1-like receptors are functionally incorporated into the swim network during the transition from a sedentary to a free-swimming lifestyle. However, it remains unknown whether D2-like receptors de-sensitise when exposed to high DA concentrations at the older stages or whether the excitatory D1-like receptor influence merely overrides and dominates the inhibitory effects of D2-like receptors.

The transition in DA modulation during locomotor development in *Xenopus* tadpoles also appears to follow a similar trajectory in other vertebrate model systems. In zebrafish, for example, the tyrosine hydroxylase (*th)* positive orthopedia (*otp*)-expressing neurons of the midbrain that constitute the dopaminergic diencephalospinal tract (DDT) are solely responsible for DA release

within the spinal cord. The descending projections of the DDT appear in the spinal cord between 24-48 hours post-fertilization (hpf), but only begin to exert a modulatory effect on locomotor behaviour after 3 days post-fertilisation (dpf) (Lambert et al., 2012; McLean & Fetcho, 2004; Reimer et al., 2013). Locomotion in zebrafish larvae typically involves an intermittent beat-and-glide pattern that comprises distinct bouts of swimming followed by inter-bout intervals where the animal is gliding (Buss & Drapeau, 2001; Van Leeuwen et al., 2015). At 3 to 5 dpf there is a developmental switch in this locomotor pattern, from spontaneous swimming activity consisting of fewer longer bouts to more frequent swimming activity consisting of shorter bouts (Drapeau et al., 2002; Lambert et al., 2012; Thirumalai & Cline, 2008). This switch coincides with the emergence of foraging behaviour, which is required for survival as it enables zebrafish to find food as it matures and after it depletes its yolk sac. The spinal cord at 3 dpf can initiate high frequency spontaneous swimming but this is typically suppressed by DA acting at D_4 receptors, as in *Xenopus* tadpoles, an effect that can be unmasked by antagonising the receptors with L745870 (Thirumalai & Cline, 2008). However, it was found that increasing endogenous DA with bupropion at 5 dpf no longer leads to the attenuation of spontaneous swimming activity. This clearly suggests that the role of DA modulation in the initiation of spontaneous swimming activity differs between these two developmental stages.

Interestingly, DA also participates in sculpting locomotor network development by promoting neurogenesis within the spinal cord. Reimer et al. (2013), for example, revealed that DA after only 24 hpf facilitates motor neuron genesis at the cost of V2 interneurons by activating the hedgehog signalling pathway *via* D_{4a} receptors. It was also demonstrated that the reduced expression of $Hb9^{+}$ spinal motor neurons after morpholino knockdown of *th1* could be rescued in zebrafish larvae by utilising the D2-like receptor agonist, Pergolide, from 24-33 hpf to reinstate dopaminergic signalling within the spinal cord (Reimer et al., 2013).

In line with observations in *Xenopus,* DA modulation *via* D1-like receptors also emerges in zebrafish during the switch to a more mature and active locomotor swimming rhythm. A recent study by Jha and Thirumalai (2019) demonstrated in freely swimming larvae, between 6-7 dpf, that activation of D1-like receptors with SKF-38393 increases the speed of swim episodes and consequently the distance travelled per episode. The D1-like receptor antagonist, SCH-23390, when bath applied alone decreased these swim parameters, confirming that endogenous activation of D1-like receptors modulates swimming behaviour as development progresses. The excitatory

modulatory actions of D1-like receptors on locomotion are accomplished by recruiting and increasing the firing of primary motor neurons as well as reducing the spike threshold and first spike latency of secondary motor neurons (Jha & Thirumalai, 2019). It is currently not known whether D_{4a} receptors de-sensitise or whether their inhibitory actions are masked by excitatory D1-like receptor activation at early stages of development, between 3 dpf to 5 dpf, where the switch in the locomotor pattern commences.

Interestingly, Jay et al. (2015) conducted patch clamp recordings from the neurons of the DDT in 4 dpf larvae, a study which provides a rare insight into the activity patterns of modulatory neurons in the vertebrate brainstem. Their data show that the DAergic neurons are tonically active in the quiescent animal but switch into burst firing mode during swimming. This offers a possible way to titer up DA concentrations in order to control and modulate behavioural states; low DA levels from tonic firing activating inhibitory D2-like receptors, but higher levels from burst firing activating excitatory D1-like receptors.

In contrast, the role of DA modulation during mammalian locomotor development is less well understood. This is partly due to the incredible complexity of the spinal networks and their locomotor outputs, and partly because the locomotor networks *in vitro* are usually activated by a cocktail of drugs that can include DA itself. In mammals, dopaminergic input to the spinal cord primarily originates from the 150-300 dopaminergic neurons of the A11 nuclei located in the posterior hypothalamus (Skagerberg et al., 1982). Descending dopaminergic innervation of the spinal cord develops slowly compared to other monoaminergic systems, with projections initially detected from (postnatal day) P20 in rodents (Commissiong, 1983). In the mouse, now the most widely utilised mammalian model for studies of locomotion, DA can act on all five DA receptor subtypes (D_1-D_5) that are heterogeneously distributed in the ventral horn of the spinal cord where the motor networks reside, and the motor neurons themselves express all subtypes (Zhu et al., 2007). It has recently been shown that D_2 receptor activation inhibits both spontaneous network activity and reduces burst amplitudes and cycle periods of the fictive locomotor rhythm *in vitro* (Sharples et al., 2019). Interestingly, D_2 receptor activation hyperpolarises ventral spinal interneurons but has no obvious effect on motor neurons, suggesting that D_2 receptor inhibition at early stages of mouse development primarily takes place at the CPG level (Sharples et al., 2019).

DA has also been shown to inhibit locomotor activity in the disinhibited *in vitro* spinal cord preparation of neonatal mice by acting on D2-like receptors, which in turn attenuate the recurrent

excitatory pathway projecting to the spinal CPG (Humphreys & Whelan, 2012; Maitra et al., 1993). Thus, D2-like receptor control of the spinal locomotor CPG is state-dependent (Sharples & Whelan, 2017). In addition, DA modulates sensorimotor integration; decreasing the monosynaptic stretch reflex in wild type mice but increasing it in D3-receptor knockout mice (Clemens & Hochman, 2004). These knockout mice display a phenotype reminiscent of restless leg syndrome in humans (Clemens et al., 2006). This suggest that D2-like receptors normally function as an important sensory gain control mechanism in spinal reflex pathways that in turn affect CPG network activity.

Exogenous DA promotes locomotor activity in neonatal mice through D1-like receptor activation, which stabilise the rhythmic motor pattern and increase motor burst amplitude. For example, Madriaga et al. (2004) illustrated this in P2-P3 mice where application of the D1-like receptor antagonist, (*R*)-(+)-SCH 23390, disrupted the stability of the alternating rhythmic pattern in a dose-dependent manner as reflected in the lower peak-to-trough correlation coefficient (PTCC) score. DA increases locomotor activity by increasing the excitability of motor neurons *via* the depression of two K^+ currents, including I_A to reduce first spike latency and Ca^{2+}-dependent K^+ currents that in turn reduce the medium afterhyperpolarisation (Han et al., 2007). DA increases postsynaptic AMPA/NMDA conductances in motor neurons *via* D1-like receptors, but not D2-like receptors (Han & Whelan, 2009). DA is also necessary (along with 5-HT and NMDA), but not sufficient in itself, to produce the facilitation of oscillatory activity in the proposed rhythm generating $Hb9^+$ interneurons of the spinal cord (Hinckley et al., 2005; Kwan et al., 2009; Madriaga et al., 2004; Wilson, 2005). The mechanisms underlying DA modulation of $Hb9^+$ interneurons have not yet been explored. In addition, DA increases excitability by regulating sensory synaptic inputs to motor neurons and between motor neurons and Renshaw cells (inhibitory interneurons) in adult spinalised rats (Maitra et al., 1993; Seth et al., 1993). Although DA acting *via* D_1 receptors has an excitatory influence on locomotor output in neonates, blocking DA uptake with GB3R12909 fails to replicate the high concentration DA effects, but replicates the low concentration DA effects (Sharples et al., 2019). The implication of this result is that whilst the locomotor network already has the modulatory machinery in place at the time of birth, there is insufficient endogenous DA to produce a physiological effect *via* D_1 receptors.

There is evidence that the excitatory modulatory role of D1-like receptors and the inhibitory role of D2-like receptors in the spinal cord persists into adulthood in rodents (Lapointe et al., 2009;

Schindler & Carmona, 2002). However, the roles of each subtype may be state- dependent, with D2's promoting quiescence and D1's supporting locomotion. In keeping with this idea, activating the A11 dopaminergic neurons optogenetically, presumably increasing DA activation of D1-like receptors, enhances locomotor behaviour (Koblinger et al., 2018). In mice, the motor pattern evolves from ataxia at birth (P0), crawling with forelimbs around P4, developing postural reflexes and weight bearing at P7-9, and displaying an adult-like gait by P14 (Whelan, 2003). It is likely this sequence is accompanied by changes in the modulatory role of DA. Indeed, preliminary evidence from real-time PCR and laser-capture microdissection experiments has revealed that motor neurons predominantly express D_2 receptors in juvenile P14 mice (Zhu et al., 2007). Since motor neurons do not respond to D_2 receptor activation in neonates, this suggests a modulatory switch that results in D_2 receptors having a more prominent role in controlling motor neuron activity as the architecture of locomotor networks matures and become more complex at later stages of development.

The idea that G-protein coupled receptors such as dopaminergic receptors exist only as functionally independent monomers has been recently revised due to growing evidence that dopaminergic receptors can form multi-receptor complexes such as homodimers (e.g. D1R-D1R, D2R-D2R) and heterodimers (e.g. D1R-D2R, D1R-D3R), or even higher-order oligomers (Ferré et al., 2014; Guo et al., 2008; Wouters et al., 2019). With such structural arrangements, it is possible for DA to engage simultaneously different second messenger pathways. For example, Guitart et al. (2014) showed in transfected cells that two interacting D1R and D3R homodimers (the D1R-D3R heteromer) were coupled to G_s and G_i proteins, respectively, and that crosstalk between these receptors upon co-activation leads to a negative interaction at the level of adenylyl cyclase signalling. With respect to locomotion, the putative D3 receptor agonist, PD 128907, potentiates locomotor activity in mice induced by the D1 receptor agonist, SKF 38393 (Marcellino et al., 2008). It has also been shown that the D1R-D3R heteromer are expressed in striatal neurons. Thus, it is feasible that the actions broadly attributed to D1-like and D2-like receptors in spinal locomotor networks may results from the direct interactions between different DA receptor subtypes existing in a multi-receptor complex. Whilst this has yet to be resolved in any detail, this mechanistic feature would diversify, amplify and fine-tune dopaminergic control of locomotor activity.

Serotonin

The serotonergic system profoundly modulates most aspects of spinal cord function using a variety of 5-HT sources (predominantly in the midline raphe nuclei of the midbrain, pons and medulla) and acting on a wide array of different 5-HT receptor subtypes across development (Hochman et al., 2001).

In *Xenopus* tadpoles, 5-HT contributes to the maturation and flexibility of spinal locomotor networks. The tadpole swim CPG initially is sensitive to bath applied 5-HT from stage 37/38 where the amine produces a modest increase in the duration of motor bursts during a fictive swim episode - though only in the rostral-most regions - and a reduction in swim episode duration, but has very little effect on swim frequency (Sillar et al., 1992). The influence of 5-HT then expands over the next day or so of development (stages 40 to 42) to more caudal segments and appears to mirror the normal acquisition of a "bursty" axial swim pattern during development (Sillar et al., 1991). The sensitivity of the locomotor CPG to 5-HT broadly coincides with, but just precedes, the arrival of descending projections from the raphe nucleus of the tadpole brainstem (Sillar et al., 1995; van Mier et al., 1986), suggesting that the normal development of larval swimming might be causally linked to the ingrowth of serotonergic fibres. This notion was supported by experiments demonstrating that the neurotoxic deletion of these fibres with 5,7 DHT resulted in stage 42 larvae continuing to exhibit an embryo-like swimming rhythm (Sillar et al., 1995). Altogether, these data indicate that the descending fibres of the tadpole raphe nucleus play a developmental as well as a modulatory role. This is similar to findings obtained for DA in zebrafish, as detailed above, and clearly highlights the fact that both these monoaminergic systems are involved in constructing the very networks they subsequently modulate.

In hatchling tadpoles, 5-HT_{1A} receptors are involved in mediating the inhibitory actions of 5-HT on the tail swimming rhythm. More specifically, activation of these receptors with R(+)-8-OH-DPAT mimics the actions of 5-HT that can be reversed with the 5-HT_{1A} receptor antagonist NAN-190 (Sillar et al., 1995). The cellular and synaptic mechanisms underlying 5-HT actions in the spinal CPG are far from fully understood. 5-HT leads to hyperpolarization of spinal neurons, probably *via* 5-HT_{1A} receptor activation, but also causes presynaptic inhibition of transmitter release from commissural glycinergic interneurons (McDearmid et al., 1997; Sillar et al., 2002). Although the mediating receptor subtype involved is still not known, it is possible that a 5-HT_{1D}

receptor underlies this effect as has been shown in the adult lamprey spinal cord where this subtype reduces glutamate release from presynaptic reticulospinal neurons (Schwartz et al., 2005).

The modulatory role of 5-HT switches during pro-metamorphic stages and at metamorphic climax (Figure 1C, D), where the amine begins to mediate a dual effect. Whilst 5-HT maintains its suppressive influence on the axial CPG network, it simultaneously exerts an excitatory effect on the now fully functional limb CPG network (Rauscent et al., 2009). Thus, the opposing modulatory actions of 5-HT regulate the functional coupling between the axial- and limb-based rhythms. After metamorphosis, there is a final switch in the locomotor pattern whereby the axial rhythm ceases and its network disappears due to resorption of the tail resulting in the presence of only the limb network in the spinal cord. Here, the appendicular rhythm accelerates due to a decrease in both cycle periods and motor burst durations, assisting the generation of limb kicks that propel the froglet forward (Rauscent et al., 2009). The receptor mechanisms underlying the evolving role of 5-HT modulation from metamorphic climax to post-metamorphosis are not fully understood, although it is likely that 5-HT continues to inhibit the axial rhythm (when the network is present) *via* 5-HT_{1A} receptors.

The zebrafish spinal cord receives 5-HT innervation from descending raphe spinal projections and intraspinal neurons (McLean & Fetcho, 2004; Montgomery et al., 2018). In young zebrafish larvae (3-4 dpf), 5-HT does not influence the frequency of spontaneous swimming episodes or any intra-swim episode parameters, but instead has an excitatory influence by reducing the quiescent period between consecutive swimming bouts (Brustein et al., 2003; Brustein & Drapeau, 2005). The quiescent period is regulated by an inward cation-chloride cotransporter, most likely NKCC1, which maintains high intracellular Cl^- concentrations (Brustein & Drapeau, 2005). This means that activation of Cl^- gated ion channels by conventional inhibitory transmitters (GABA and glycine) leads to Cl^- efflux and depolarizations that can induce firing and spontaneous network activity. This is a consistent feature of immature vertebrate motor systems, including zebrafish, chick and rodents (Bos et al., 2013; Brustein & Drapeau, 2005; Vinay et al., 2010), and one that is important in network development. In zebrafish, blocking the inward Cl^- cotransporter with bumetanide increases the silent intervals and the same effect occurs with the 5-$HT_{1/2}$ receptor antagonist, methysergide (Brustein & Drapeau, 2005). This suggests that endogenous 5-HT reduces swim bout intervals *via* an action on Cl^- cotransporters.

The lack of serotonergic modulation on locomotor frequency in zebrafish larvae is only transient and by 5 dpf its effects begin to manifest due to changes in intraspinal neurons. Between 3 and 4 dpf, intraspinal neurons undergo extensive morphological alterations that involves their neurites increasing in length and projecting over greater distances (Montgomery et al., 2018). In addition, the axons of intraspinal neurons terminate with enlarged growth cones (McLean & Fetcho, 2004) and at 5 dpf contain synaptic vesicles (Montgomery et al., 2018). The growth cones of the intraspinal neurons transform into synaptic terminals that mediate 5-HT transmission. These changes in the intraspinal neurons stabilise at 5 dpf (Montgomery et al., 2018). What are the consequences of these developmental changes to serotonergic intraspinal neurons for the locomotor pattern? At 5-7 dpf, increasing endogenous 5-HT using the re-uptake blocker citalopram in spinally-transected preparations reduces motor bursting and the episodic organisation of the bursts during NMDA-induced fictive swimming (Montgomery et al., 2018). Furthermore, these effects can be replicated when serotonergic intraspinal neurons are activated optogenetically, consistent with a direct action of these neurons on the swim CPG circuitry.

Unlike *Xenopus,* the inhibitory modulatory role of 5-HT persists as zebrafish larvae mature into adulthood. Intracellular recordings of spinal neurons in juvenile (> 30 dpf) and adult (>90 dpf) zebrafish reveal that the decrease in fictive locomotor frequency mediated by 5-HT is due to an increase in mid-cycle inhibition and a delay to the onset of excitatory synaptic inputs onto motor neurons (Gabriel et al., 2009). These results in zebrafish clearly indicate that 5-HT maintains its inhibitory modulatory role in the spinal cord and does not switch as it enters adulthood. Perhaps this is linked to the switch that occurs in DA modulation from inhibitory to excitatory between 3 to 5 dpf; thus, the serotonergic signalling systems may act homeostatically to counteract this excitatory dopaminergic effect on the locomotor pattern.

In more complex vertebrates such as the mouse and chick, for example, a switch in the role of 5-HT modulation during development occurs, analogous to that observed during *Xenopus* development. During embryonic development in the chick, 5-HT acting on 5-HT_1 and 5-HT_2 receptors switches from hyperpolarizing spinal motor neurons at (embryonic day) E12 to the opposite response by E18, reducing and increasing motor neuron discharge frequency, respectively (Hayashi et al., 1997).

From birth in the mouse, descending serotonergic fibres from the raphe nuclei of the brainstem are present in the spinal cord (Ballion et al., 2002; Deneris & Gaspar, 2018; Schmidt & Jordan, 2000),

although, it takes approximately three weeks post-birth for the full development of descending 5-HT innervation in rodents to occur (Deneris & Gaspar, 2018; Tanaka et al., 1992). During the perinatal period, the effects of 5-HT on rat lumbar motor neurons change from small amplitude (1-2 mV), long-lasting depolarizations associated with a decrease in input resistance at E16-17, to much larger depolarizations (> 7 mV) after birth (Ziskind-Conhaim et al., 1993). Around this period, there is a switch in receptor expression, with a significant decrease in $5HT_{1A}$ receptors occurring in concert with an upregulation of 5-HT_2 receptors (Talley & Bayliss, 2000). Interestingly, 5-HT acting on 5-HT_2 receptors supports the induction of plateau properties in motor neurons that are implicated in the postural control required to support the body weight (Li et al., 2007).

In neonatal mice (P0-P3), in contrast, citalopram bath applied to isolated brainstem-spinal cord preparations has an inhibitory effect on the locomotor rhythm (Dunbar et al., 2010). There is an increase in cycle periods as well as a decrease in burst amplitudes, flexor burst durations and a deterioration in flexor-extensor coupling. The inhibitory actions of endogenous 5-HT are mediated in part *via* 5-HT_1 receptors as it was demonstrated that the combination of the 5-HT_{1A} antagonist, WAY-100635, and 5-HT_{1B} antagonist, SB-216641, attenuated some of the inhibitory effects of citalopram on locomotion (Dunbar et al., 2010).

5-HT_{2A} and 5-HT_7 receptors were also identified as having an excitatory role in the modulation of locomotion during this period of development. The 5-HT_{2A} antagonist, ketanserin, and the 5-HT_7 antagonist, SB-269970, when combined reduced the capacity of 5-$HT1_{A/B}$ antagonists to reverse citalopram's actions by potentiating the inhibition of the locomotor rhythm (Dunbar et al., 2010). Thus, locomotor networks in neonatal mice are bi-directionally modulated by endogenous 5-HT with excitation mediated by 5-$HT_{2A/7}$ receptors and inhibition mediated by 5-$HT_{1A/B}$ receptors, reminiscent of the situation at broadly equivalent stages in *Xenopus* development described above.

5-HT modulation of Cl^- homeostasis is not restricted to zebrafish and is a recurring feature in the locomotor networks of other models such as rodents. NKCC1 inward Cl^- transporters in immature neurons are gradually replaced during development by the K^+/ Cl^- cotransporter, KCC2, that has the opposite effect to NKCC1 and expels Cl^- ions to maintain low levels intracellularly (Vinay et al., 2010). This ensures that the reversal potential for inhibitory inputs is at or below the resting membrane potential. Recently it has been shown that the KCC2 cotransporter in developing neonatal rat spinal neurons is modulated by 5-HT acting *via* the $5HT_{2A}$ receptors and this

modulation shifts the chloride reversal potential (Bos et al., 2013). Activation of the 5-HT_{2A} receptor upregulates KCC2, rendering the reversal potential for inhibition more negative and strengthening IPSPs as a result. Moreover, 5-HT_{2A} receptor agonists increase KCC2 expression, restores endogenous inhibition and reduces spasticity after spinal cord injury in rats (Bos et al., 2013).

The consensus from studies in mature rodents is that 5-HT modulation switches to largely having an excitatory role capable of evoking locomotor pattern generation. For example, experiments conducted by Liu et al. (2009) in an adult *in vivo* mouse preparation revealed that applying SB-269970 directly to the spinal cord, to block 5-HT_7 receptors, hindered locomotor activity and resulted in prolonged extension of the hindlimbs. This mimicked the phenotype of 5-$HT_7^{-/-}$ adult mice where there was also a loss in voluntary locomotion (Liu et al., 2009). The other 5-HT receptor subtypes also heavily implicated in the generation of locomotor output as mice mature include 5-HT_{1A} and 5-$HT_{2A/C}$ receptors. The modulatory roles of these receptors have predominately been examined in the context of spinal cord injury (SCI) where their activation in the spinal cord promotes recovery of locomotor function (Antri et al., 2003; Liu et al., 2009; Ung et al., 2008). However, whether 5-HT_{1A} and 5-$HT_{2A/C}$ receptors inherently modulate locomotor activity in healthy adults or only do so in response to the changing functional environment following SCI is unclear.

Nitric oxide

The enigmatic gaseous signalling molecule nitric oxide (NO) is generated in a wide range of tissues from the degradation of L-arginine by nitric oxide synthase (NOS) enzymes and plays a diversity of important biological roles. In the CNS, NO is produced in neurons by the constitutively active neuronal isoform, nNOS. From its point of synthesis, NO diffuses readily within neurons and across lipid bilayers to alter the function of a variety of target proteins, notably activating guanylate cyclase leading to cGMP formation, but also modulating other proteins such as NMDA type glutamate receptors *via* S-nitrosylation, for example (Ahern et al., 2002).

In the developing CNS of *Xenopus* tadpoles, NOS^{+ve} neurons first appear in the brainstem very early in development, around mid-way through embryogenesis, a mere day or so after fertilization. By the time of hatching at stage 37/38, three bilaterally symmetrical clusters are established, and

these populations remain relatively stable until stage 42, in the prelude to free feeding larval life (McLean et al., 2000; McLean & Sillar, 2000). NO at these early stages exerts an overall inhibitory effect on swimming by reducing episode durations, swim frequencies, ventral root burst durations and intensities (McLean & Sillar, 2000). A primary mechanism underlying this inhibitory control over motor output is the presynaptic facilitation of inhibitory transmitter release (McLean & Sillar, 2002), including GABA from descending mid-hindbrain reticulospinal (mhr) neurons (to prematurely terminate episodes) and glycine from spinal commissural interneurons (to slow swim cycle frequencies). The mhr neurons are activated by trigeminal sensory neurons that innervate the rostral cement gland (Figure 1A), which terminates swimming if the tadpole encounters an object in its swim path. The effect of NO on glycinergic transmission is indirect, involving the nitrergic enhancement of noradrenergic signalling, which in turn strengthens glycinergic connections in the spinal cord (McDearmid et al., 1997; Sillar et al., 2002). This organisation elevates NO's status to that of a serial "*metamodulator*" (McLean & Sillar, 2004).

Metamodulation by NO raises the possibility that switches in NO's role during development will exert additional downstream effects on potentially connected aminergic pathways. Indeed, there is evidence in the CNS for such interactions. For example, Smith & Whitton (2000) demonstrated that microinjection of NO inhibitors such as 7-nitroindazole (7-NI) in the raphe nucleus (that also project nitrergic fibres) blocks NMDA-induced 5-HT release. In rodents, this has a biphasic effect on locomotion where high concentrations of 7-NI increases locomotion and low concentrations decreases it (Spiacci et al., 2008). Another study demonstrated that pre-treatment with 5-HT1a receptor agonist, 8-OH-DPAT, blocked the increase in locomotor activity induced by the microinjection of peroxynitrite donors in the raphe nucleus (Gualda et al., 2011). Whether stage-development switching of nitrergic and aminergic signalling in spinal locomotor networks alters the relationship between these neuromodulatory systems is an interesting and important avenue that has yet to be explored.

In relation to the preceding discussion of neuromodulators promoting a sessile existence that favours survival in the small vulnerable post-hatching stages of the tadpole's life, NO functions similarly, in part by orchestrating the inhibitory actions of subordinate transmitter systems. It is interesting to note that the synthetic enzyme, NOS, like other proteins has a Q10, which strategically positions NO at the interface between the environment and the behaviour of this poikilothermic organism. For example, the behavioural response of the tadpole to increasing water

temperatures (Sillar & Robertson, 2009), is controlled at least in part by the NO system (Robertson & Sillar, 2009).

Later in development the tadpole must somehow break free from this inhibitory control, and in this respect, the degeneration of the cement gland that connects to the GABAergic mhr neurons is an important change. However, what changes in the anatomy and physiology of the NO system occur during later larval development? From an anatomical perspective, a large increase in nNOS expression takes place (Ramanathan et al., 2006), with the appearance of NOS^{+ve} neurons and processes in additional populations throughout the brain and now also in the spinal cord (McLean & Sillar, 2001; Ramanathan et al., 2006). Functionally, the influence of NO switches from inhibitory to facilitatory (Currie et al., 2016). The effects, however, are subtle and the underlying mechanisms have yet to be fully addressed. For example, between stages 42 and 45 there is a gradually increase in the number of spontaneous swimming episodes and the probability of occurrence of these episodes increases when NO donors are applied. Interestingly, there are no significant effects of NO on intra-episode swim parameters such as burst durations and cycle frequencies. Presumably the facilitation of inhibitory synaptic transmission by NO is somehow developmentally disengaged or significantly reduced, and/or it is counterbalanced by broadly equal and opposite effects of NO on excitatory processes. The fact that the occurrence of swim episodes decreases with NOS inhibition, or after scavenging NO with PTIO, points to an endogenous NO tone regulating neurons involved in swim initiation. Moreover, these pharmacological manipulations are effective only when drugs are applied to the brainstem (Figure 3), which in turn suggests that NO excites the descending pathways that activate locomotor sequences.

Unravelling how NO's role in locomotor control switches during development will be a long and complex task, not least because of the multiple levels on which NO can modulate the motor system and the progressively changing sources and targets of NO. The identification of the NOS^{+ve} neurons is another important challenge. Assuming that NO is a co-transmitter, what is the phenotype of the nitrergic brainstem populations that promote inhibition at early stages? When new groups of neurons appear during pre- and pro-metamorphic stages, were they pre-existing and subsequently triggered to express NOS according to a prescribed developmental timetable or are they *de novo* components of new spinal networks, for example those controlling the limbs, that produce NO from the outset?

Understanding the targets and actions of NO at the cellular and synaptic levels of motor and pre-motor elements will also be important. Perhaps the most familiar actions of NO are in the mammalian hippocampus where it is proposed to play a role in LTP (Garthwaite & Boulton, 2003), strengthening excitatory synaptic transmission *via* a sGC-cGMP dependent pathway that increases vesicular release of glutamate. However, direct actions on ion channels and receptors are also recognised mechanisms underlying NO's actions making it likely that in the tadpole, for example, the global influence of NO emerges from the sum of its effects on multiple targets.

The overarching impact of NO as a metamodulator, like DA and 5-HT, switches from nett inhibition to excitation between early post-hatching stages and larval and metamorphic stages of *Xenopus* development (Currie et al., 2016). The inhibitory effect matches that reported for the locomotor CPG in the isolated mouse spinal cord (Foster et al., 2014) at roughly the equivalent (neonatal) stage of development. Interestingly, there is evidence to suggest that nNOS-specific inhibitors decrease locomotor activity in adult mice (Dzoljic et al., 1997; Engelhardt et al., 2006), suggesting that an inhibitory influence is retained during development, distinct from the switching role in *Xenopus* tadpoles. However, the loci or mechanisms of action of NO in adult mammals are unknown.

Curiously, the modulation of the zebrafish swim CPG by NO has not been extensively studied and much less is known compared to *Xenopus*. nNOS mRNA expression is detected from 19 hpf in the forebrain and there is a large increase in NOS^{+ve} neurons in the brainstem in the course of embryonic development and the prelude to free swimming (Holmqvist et al., 2004). Within the spinal cord, a class of cells close to the central canal labels by 55 hpf. Despite the expression in brainstem neurons, as in *Xenopus* tadpoles, there are no reports of NO directly modulating the swim CPG, although the respiratory rhythm of zebrafish is affected by manipulating the NO system (Porteus et al., 2015). In larvae, NO donors increase ventilation while in adults the same manipulation inhibits ventilation (Porteus et al., 2015).

Although the zebrafish locomotor CPG itself is apparently unaffected by NO, in contrast to *Xenopus* tadpoles, the formers swimming system as a whole is profoundly modulated. NO signalling plays a key role in the innervation of the myotomes by motor neurons by impairing neurite outgrowth, regulating muscle innervation and neuromuscular transmission (Bradley et al., 2010; Jay et al., 2014). These effects impact on the locomotor drive for swimming and the likely source of endogenous NO that causes these effects are the aforementioned cells near the central

canal that are in fact a class of previously unreported spinal interneuron, neighbours of serotonergic VeSe, glutamatergic VeMe and GABAergic KA neurons (Bradley et al., 2010).

Conclusions and future perspectives

One of the fundamental goals in the motor control field is to understand how the complex integration of neuromodulatory inputs contributes to the development and function of locomotor CPG networks. There is emerging evidence that the roles of key and highly conserved neuromodulators often alter throughout development in order to accommodate (and arguably to orchestrate) the changing behavioural requirements of an animal.

In this review, we have focussed, for brevity, on three neuromodulators - DA, 5-HT and NO - and highlighted their contributions in one hybrid (frog tadpole), one axial (zebrafish) and one limbed (mouse) model system across broadly equivalent developmental stages. The three neuromodulators initially mediate an inhibitory influence on locomotor networks at early stages of development in order to promote a predominantly sessile lifestyle. This is important as most developing vertebrates emerge from the egg or womb precociously and the immature, often defenceless, animal enters the world facing a high predation risk. Thus, the default mode is usually to remain still until more effective escape mechanisms have developed. Evidence generated in each model system suggests that there is a switch during later stages of development whereby the three neuromodulatory influences leads to a more excited state overall in locomotor networks. This higher activity state is important for survival as it enables animals to mediate escape responses and find food. In each model, DA appears to exert its excitatory and inhibitory actions on locomotor networks *via* D1-like and D2-like receptors, respectively. This suggests that the mechanisms underlying dopaminergic signalling during motor control development are highly conserved, phylogenetically. The weight of evidence concerning 5-HT modulation during development is that inhibitory effects are mediated *via* 5-HT_{1A} receptors, while 5-HT_{2A} and 5-HT_7 receptors facilitate locomotor network output. However, the underlying mechanisms and their developmental incorporation remain to be deciphered and very little is known in detail about the involvement of other 5-HT receptor subtypes in promoting and inhibiting locomotor activity. The same is true for NO where even less is known about its dynamics and mechanisms during motor control development, particularly in mammals.

There is still much to learn about the conventional targets (transmitter receptors and ion channels) that underlie modulatory control of locomotor networks at different stages of development. An additional avenue for future research is to explore more novel targets of neuromodulators in locomotor networks such as ion transporters and pumps, which may have important roles during development. As described in this review, cation-chloride cotransporters for example, such as NKCC1 expressed in immature spinal neurons and KCC2 expressed in mature spinal neurons are subject to modulation by 5-HT that regulates Cl^- homeostasis during development and subsequently the excitability status of the network. Whether other neuromodulators such as DA and NO modulate these cotransporters have yet to be examined. Another novel target that has recently garnered much attention is a subtype of the Na^+/K^+ exchange pump (expressing the $\alpha 3$ catalytic subunit) that functions in a "dynamic", activity-dependent manner. These pumps are exclusively recruited following intense locomotor activity to produce an ultra-slow afterhyperpolarisation (usAHP) lasting up to a minute. In young *Xenopus* larvae, this underlies a short-term motor memory mechanism in the spinal cord that decreases network excitability and minimises fatigue (Zhang & Sillar, 2012). The usAHP is known to be subject to modulation by DA, 5-HT and NO (Picton, 2017; Hachoumi, Zhang, Picton and Sillar *in preparation*). We need to understand how the modulation of these dynamic pumps are altered during development and what consequence this has on the maturation of short-term motor memory, an important activity-dependent mechanism in spinal locomotor networks.

A fascinating feature of neurons that warrants further exploration is their ability to switch neurotransmitter phenotype during development, which also likely contributes to changes in locomotor behaviours as animals mature. In pre-metamorphic *Xenopus*, there is evidence that developing limb motor neurons at these early developmental stages transiently utilise glutamatergic neurotransmission at neuromuscular junctions before later switching to cholinergic neurotransmission (Lambert et al., 2018). Interestingly, the plasticity of a neuron's neurotransmitter phenotype during development has also been documented in zebrafish where spinal motor neurons in adults acquire the capacity to co-transmit glutamate alongside acetylcholine (Bertuzzi et al., 2018). In light of this evidence it is possible that stage-dependent developmental switches in the modulatory roles of DA, 5-HT and NO also stem from spinal neurons undergoing neurotransmitter re-specification as spinal locomotor networks reorganise to facilitate the maturation of locomotor behaviours.

In conclusion, there are many gaps in our understanding of the mechanisms responsible for functional transitions in neuromodulation of CPGs during development. More knowledge is required about the receptors, ion channels, transporters and pumps that are modulated in each neuromodulatory system and how these systems interact with each other and are re-organised throughout development. The phylogenetic conservancy of the DA, 5-HT and NO systems and their significance for diseases and malfunctions in humans underscore the importance of future research in this area.

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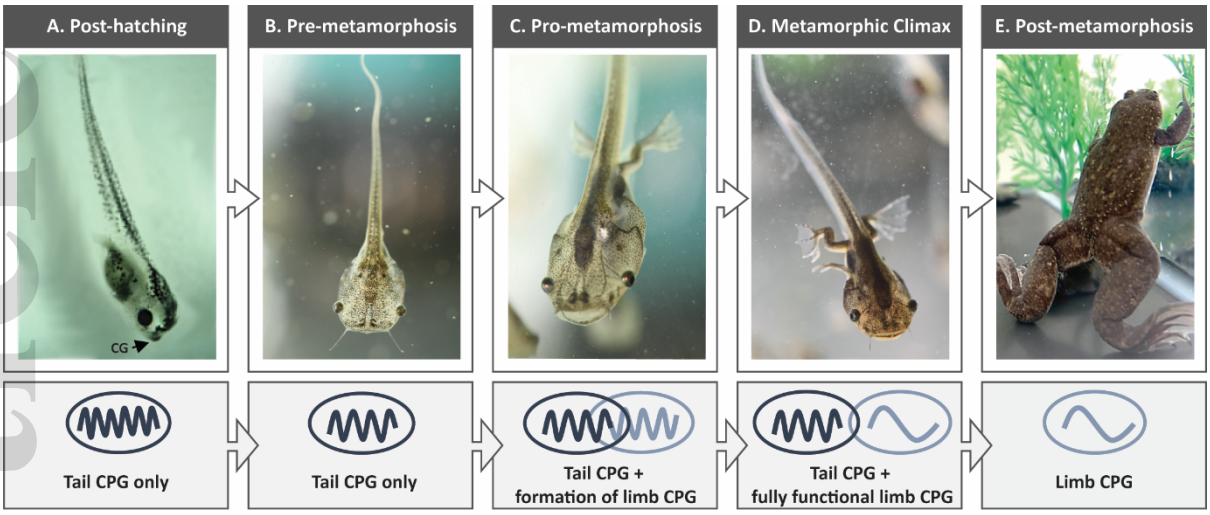

Figure 1. ***Xenopus laevis*** **metamorphosis.** (A) In small, post-hatchling tadpoles (~stage 42 displayed; ~7mm long), high frequency swimming is generated by rhythmic tail undulations. (B) As the pre-metamorphic tadpoles get older and larger (stage 51 displayed, ~ 3cm long), these tail undulations occur at lower frequencies. (C) During pro-metamorphosis (~stage 57 displayed; ~ 7cm long), a functional limb CPG network begins to emerge in the spinal cord, but at the same coordination and frequency as the tail rhythm. (D) At metamorphic climax (stage 60 displayed; ~ 8-10 cm long), the tail and now fully functional limb CPG network co-exist within the spinal cord but can function independently from each other and at different rhythm frequencies. (E) Following the resorption of the tail after metamorphosis, the limb CPG network now provides the main locomotor drive in young adult frogs. CG = cement gland; see text for details. Note, images not to same scale. (Photos generated by KTS and LH at the University of St Andrews).

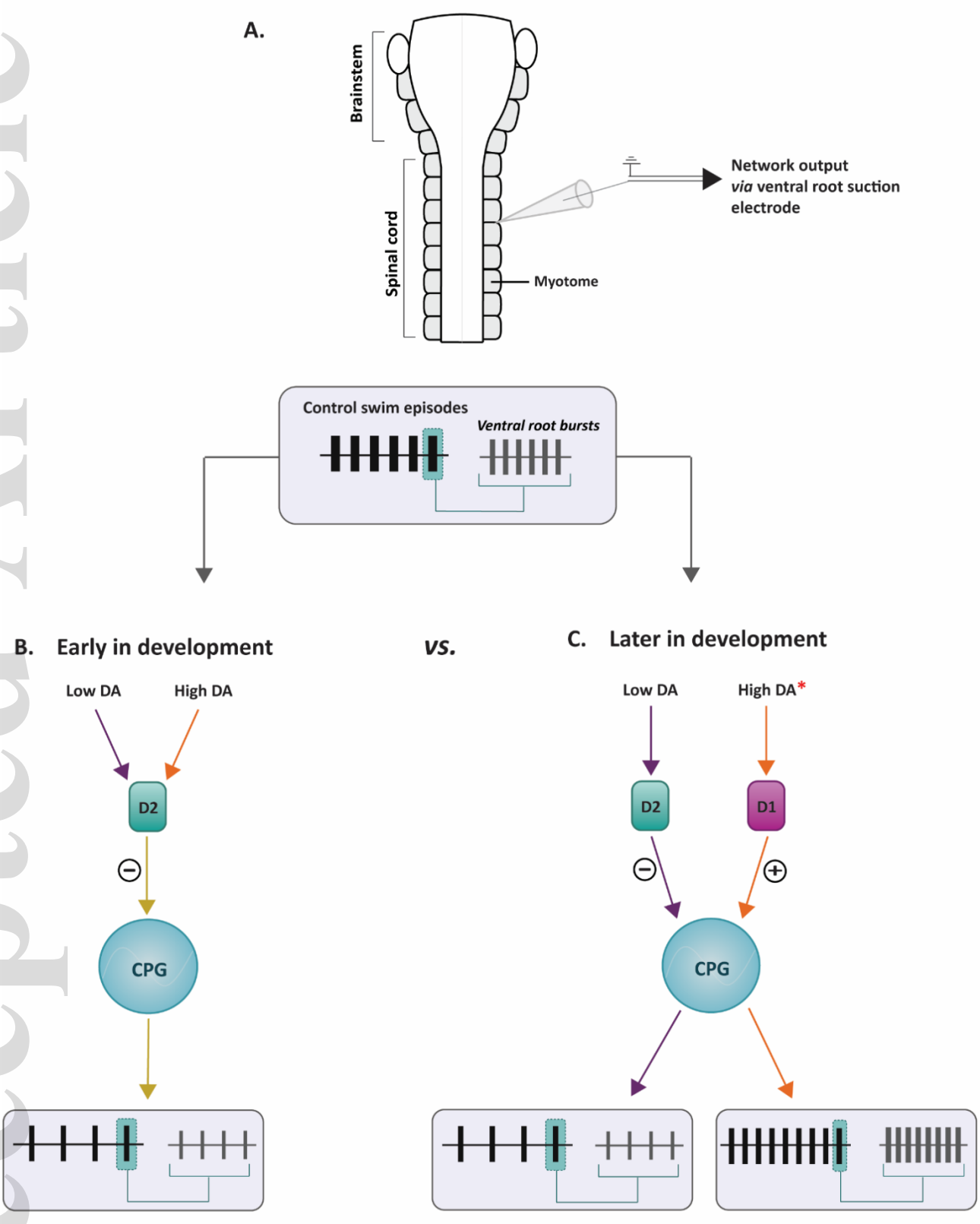

Figure 2. Overview of dopamine's role in the developing locomotor CPG network of *Xenopus laevis.* (A) Illustration of an isolated spinal cord preparation utilised to examine the effects of DA on fictive swimming *via* ventral root recordings. (B) During early tadpole development, both low and high levels of DA within the spinal cord activate D2-like receptors to exert an inhibitory effect on swimming. This is accomplished by a decrease in swim episode frequency and duration as well a decrease in the number of ventral root bursts and their duration (compare schematised recording traces with the control in part A). (C) However, at later stages of larval development there is a switch in the modulatory role of DA. Whilst low DA levels in the spinal cord still activate the inhibitory D2-like receptors, high DA levels now activate D1-like receptors that promote locomotor activity and in turn supersedes the inhibitory D2-like effect The increase in locomotor activity by D1-like receptor activation is associated an increase in swim episode frequency, a decrease in episode duration as well as an increase in ventral root bursts and a decrease in cycle (compare schematised recording traces with the control in part A and early development in part B). Figure adapted from findings by (Picton & Sillar, 2016) and (Clemens et al., 2012).

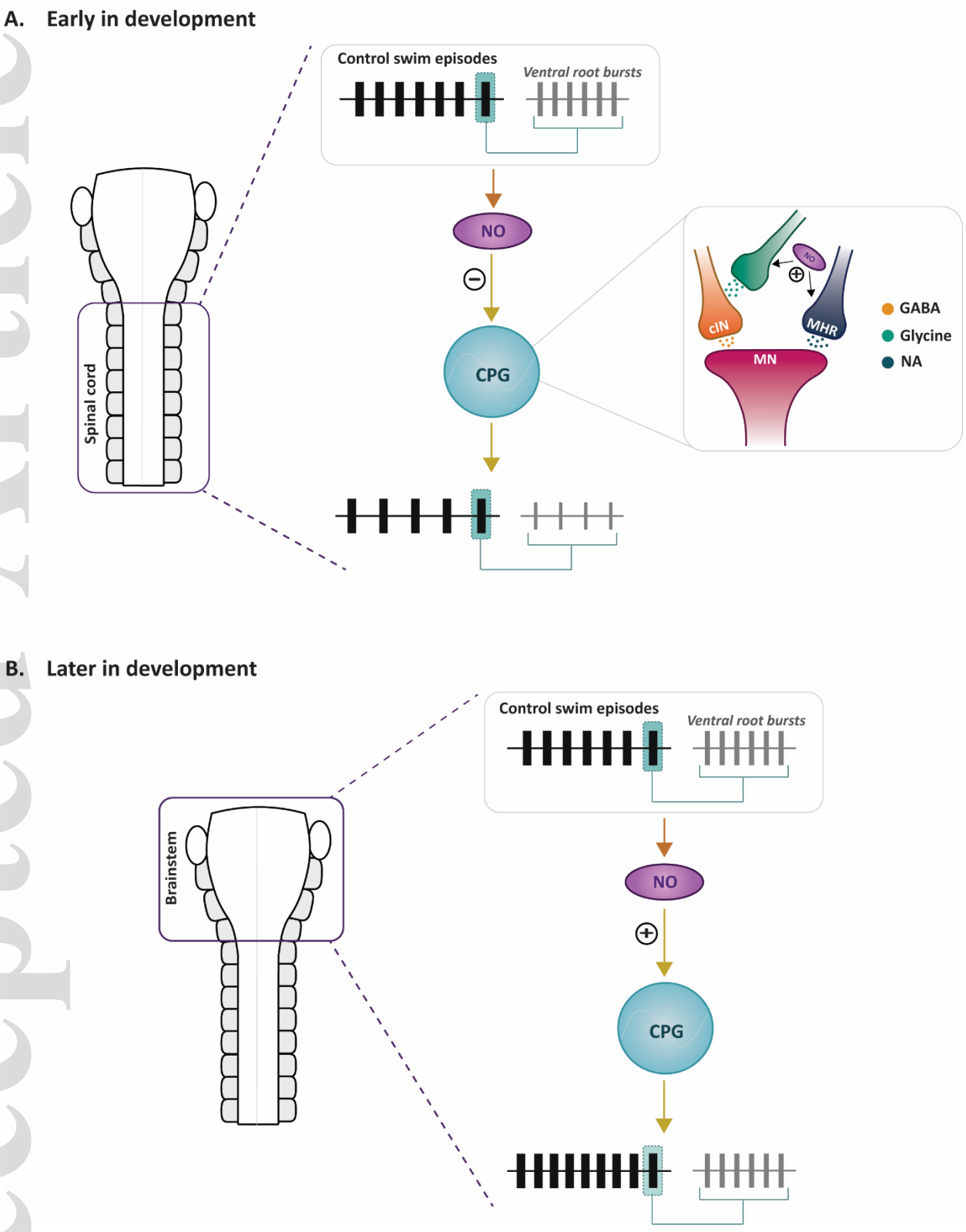

Figure 3. Overview of the role of NO in the developing locomotor networks of *Xenopus laevis*. (A) At early stages of development (~stage 37/38-42), NO predominately mediates an inhibitory effect on swimming output *via* the spinal cord. The expanded schematic of a locomotor swim episode illustrates that the inhibitory effect of NO on episode duration is accompanied by an attenuation of ventral root burst frequency and amplitude. This inhibition by NO is mediated by an increase in presynaptic facilitation of inhibitory neurotransmitter release onto motor neurons (MNs), including glycine directly from mid-hindbrain reticulospinal (mhr) neurons and GABA from commissural interneurons (cINs) indirectly *via* the release of noradrenaline (NA). (C) Later in tadpole development, the role of NO switches where it exerts an excitatory effect on the frequency of swim episodes *via* the brainstem that is not accompanied with any changes to the intra-swim parameters. Figure adapted from findings by (McDearmid et al., 1997), (McLean & Sillar, 2000, 2002) and (Currie et al., 2016).

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