Midbrain Control of the Swim Behaviour of the *Xenopus laevis* Tadpole

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I DECLARATION

I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than that of Master of Science (Research) being studied at the University of Kent. I also declare this work is the result of my own investigations except that I obtained raw extracellular electrophysiology recordings from a senior PhD student, but all the analyses were conducted by me.

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IV ABBREVIATIONS

HST	Head Skin Touch
LD	Light Dimming
PH	Press Head
CNS	Central Nervous System
L-DOPA	l-dihydroxyphenylalanine
CPG	Central Pattern Generators
MLR	Mesencephalic Locomotor Region
Cnf	Cuneiform Nucleus
PPN	Pedunculopontine Nucleus
GPi	Globus Pallidus Interna
SNr	Substantia Nigra Pars Reticulata
GABA	Gamma-Aminobutyric Acid
ADT	Anterior Dorsal Tegmentum
PAG	Periaqueductal Grey
vGlut2	Vesicular Glutamate Transporter
LPGi	Lateral Paragigantocellular Nucleus
EPSPs	Excitatory Postsynaptic Potentials
AMPA	Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid

NMDA	N-methyl D-aspartate
pgc	Pineal Ganglion Cells
D/MD	Diencephalic/Mesencephalic Descending Neurons
nMLF	The Nucleus of The Medial Longitudinal Fasciculus
TBF	Tail-Beat Frequency
OMR	Optomotor Response
MRN	Mesencephalic Reticular Nucleus
tSt	Trigeminal Sensory Touch Receptors
tINs	Trigeminal Sensory Pathway Neurons
mhrs	Mid-Hindbrain Reticulospinal Neurons
AP	Action Potential
dlc	Dorsolateral Commissural Neurons
dla	Dorsolateral Ascending Neurons
hdIN	Hindbrain Reticulospinal Neurons
CiD	Circumferential Descending Neurons
EINs	Excitatory Interneurons
dINr	Excitatory Descending Repetitive Interneuron
eCINs	Excitatory Commissural Interneurons
aINs	Ascending Interneurons

CoLos	Commissural local Ins
EXRC	European Xenopus Resource Centre
AWERB	Animal Welfare and Ethical Review Body
MS-222	Tricaine methanesulfonate
MHB	Midbrain-Hindbrain Border
fps	frames per second

V ABSTRACT

The survival of all animals is crucially dependent on motor responses that are timely and well-coordinated. In vertebrates, the central pattern generators (CPG) in the spinal cord, which generate the rhythmic pattern of locomotion, are influenced by descending commands from brainstem structures, particularly the midbrain. However, it remains unclear how the midbrain neurons interact with the CPG networks to initiate and control locomotion. Using behavioural and electrophysiological techniques, this study assessed the effects the disconnection of the midbrain from the rest of the brainstem has on the following parameters: (1) the side of the initiation of swimming, (2) the latency to swim initiation and (3) frequency of swimming. My behavioural data showed that the lesions did not change the ‘preference’ for first contralateral response seen among the control tadpoles. Differently, the latency to swim initiation in the lesioned tadpoles was significantly shorter than that of the control group. Extracellular ventral root recordings, indicative of fictive swimming, indicated that the control tadpoles strongly 'favoured' initiation on one side over the other based on the strength of the electrical stimulus applied to the trunk skin. These strong side preferences were eliminated following midbrain-hindbrain border (MHB) lesions, suggesting a lack of sensory discrimination caused by the midbrain lesion. In addition, within the same set of experiments I observed that the lesioned tadpoles responded significantly quicker than the controls to distinct strengths of electrical stimulus (at threshold and suprathreshold for swim initiation). Finally, assessment of the fictive swim frequency at the beginning of each swim episode revealed that MHB-lesioned tadpoles swim at significantly higher frequency in response to threshold electrical stimulus only. The conclusion, therefore, is that the midbrain is highly instrumental in survival by ensuring

the timely initiation of locomotion. The tadpole is no different to any other animal and even at this early developmental stage, the tadpole needs to make the right motor decisions.

1. INTRODUCTION

1.1 The Brain and Behaviour

The brain is an essential component of the nervous system. This system controls the general function of the body by transmitting electrical and chemical signals to and from the rest of the body. The brain and the spinal cord, which are occupants of the dorsal body cavity form one of the two major divisions of the nervous system, that is, the central nervous system (CNS). The CNS has three broad functions: the reception of sensory signals, the processing and integration of sensory signals and the generation of appropriate responses. These responses are influenced by factors, such as present situations and past experiences (Marieb and Keller, 2018). The collective name for all the generated responses is behaviour.

As indicated above, all our behaviours mirror the processes which occur in the nervous system. The brain particularly is responsible for all forms of behaviours. According to Schwartz et al. (2012b), “the brain is the seat of all behavior”. The present-day understanding of the relationship between the brain and behaviour is founded on the idea of Charles Darwin in the middle of the 19th century. Darwin went on to propagate the notion that human behaviour could be studied using animal models. This idea of studying evolution consequently led to the emergence of ethology. That is the study of animal behaviour under natural conditions and subsequently, experimental psychology - the study of both human and animal behaviour in controlled settings.

It is worth noting that prior to the emergence of Darwin's idea, the brain was not deemed the main source of all behaviours. René Descartes, among other early famous scientists, was a proponent of such theory. In the 17th century, Descartes believed that the soul, supported by the brain, was mainly responsible for generating human behaviour (Schwartz *et al.*, 2012b).

Behaviour can be defined as all overt activities of an organism undertaken in response to a change or a perceived change occurring internally (in the body) or externally (in its surroundings) (Kandel, 1976a). It is important to note that some behavioural responses are not explicitly overt or easily observable. For example, animals such as the possum, rabbit and polar bear sometimes favour the option of freezing or 'playing dead' as against escaping. Likewise, upon detecting a potential predator, the chameleon can subtly camouflage itself to blend with its immediate surroundings to evade an attack.

There are different types of behaviours according to the intended purpose of exhibiting them. Some of them are locomotor behaviour, reproductive or sexual behaviour, feeding behaviour, social behaviour and defence or avoidance behaviour. For example, as the name suggests, reproductive behaviours involve all activities, such as mating, which are performed with the primary aim of producing species of a particular kind. Also, feeding behaviour involves all activities primarily aimed at ensuring that we have access to food to ensure survival. All the above stated behavioural types can be broadly grouped into two classes according to the relationship between stimulus and response. The classes are the reflex and the

fixed-action behavioural responses. The characteristics, for example, the pattern and the amplitude of outputs generated in reflex behavioural responses are dependent on the characteristics (e.g., the intensity and pattern) of the corresponding stimuli or input. Conversely, the characteristics of the outputs in fixed-action behavioural responses are not determined by the characteristics of the corresponding input. Moreover, one major difference between the reflex and fixed-action behavioural response is that the former always needs an evoking input to generate a response. The latter, however, does not necessarily need an evoking stimulus to trigger a response because some responses can occur independently of any input (Kandel, 1976b).

Locomotor behaviour is virtually ubiquitous amongst all the other types of behaviours because of the association of the action movement to those types. For example, one of the characteristics of a feeding behaviour is the movement from one place to another to find food. Likewise, when we face adverse stimuli such as wildfires or dangerous animals, we tend to exhibit avoidance behaviour by moving away from the dangerous area to a safer place.

1.2 Locomotion

Locomotion is a common motor function that allows animals to move from one place to another, and most importantly, this ability to move is crucial for survival. Therefore, it is somewhat unsurprising that the greater majority of all activities of the nervous system of all animals results in locomotion (Arber and Costa, 2018). In my opinion, the view of Arber and Costa (2018) was also shared long ago by Charles Scott Sherrington, one of the most known influential neurophysiologists. According to Sherrington, “to move things is all that mankind can do, and for this the sole executant

is a muscle, whether it be whispering a syllable or felling a forest" (Grillner and El Manira, 2020). Also, even most episodes of non-motor behaviours, for example, the expression of emotions eventually end with motor behaviour. For instance, we often end an episode of joy by hugging someone. Likewise, after being annoyed for some time, we usually move away from the people who caused the annoyance.

There are many forms of locomotion, such as running, hopping, flying, crawling and walking (Schwartz *et al.*, 2012a; Kiehn and Dougherty, 2013). In the case of humans, we have a wide-ranging motor repertoire that allows us to perform many motor activities. This repertoire ranges from changing or maintaining body posture to directing the eyes to a particular area of a book (Grillner and El Manira, 2020). All forms of locomotion are characterised by the rhythmic and oscillating movements of the whole body or the appendages of an organism (Schwartz *et al.*, 2012a; Kiehn and Dougherty, 2013). These characteristics, particularly the rhythmicity of locomotion, often give the impression that locomotion is repetitive and stereotypical, as is the case in some neurodegenerative motor disorders (Schwartz *et al.*, 2012a). For example, Huntington's disease is associated with involuntary jerking or movements, also known as chorea. Parkinson's disease, another example, is characterised by a persistent resting tremor.

The various forms of locomotion can be broadly grouped into three depending on the behavioural demand of a situation. They are the exploratory, defensive and appetitive groups of locomotion (Jordan, 1998; Le Ray *et al.*, 2011). The general goal of every locomotor movement is established by the brain before the initiation of the motor function. The brain then recruits appropriate dedicated networks of neurons

responsible for ensuring that the perceived goal of the locomotion is subsequently achieved (Grillner and El Manira, 2020). Once a locomotor movement is initiated, the dedicated neural network makes regular adjustments to suit the current events of the environment especially in cases where locomotion is occurring in a foreign or an unknown territory (Schwartz *et al.*, 2012a). For example, our direction of walking has to be slightly or significantly modified when we notice an obstacle ahead of us. Again, the pace of our movement is normally increased when we feel threatened by happenings in our immediate surroundings.

Although common as locomotion is as a behaviour, the regular adjustments made from the point of initiation to the point of termination highlights the complexity of this seemingly simple motor function. This is because an episode of locomotion is not just all about the positional change of an organism, but also the involvement of other factors which are important in the achievement of the general goal of an episode of locomotion. One of such 'players' is the senses, which act through the sensory feedback mechanism to provide information to the brain for processing and issue appropriate commands for correction. The sensory information includes the visuomotor inputs, audiomotor inputs, cutaneous sensory inputs, olfactory sensory inputs and gustatory sensory inputs (Kiehn and Dougherty, 2013; Grillner and El Manira, 2020). Following the integration of the sensory impulses from the various sources, the visuomotor corrective motor command, for instance, is issued from the motor cortex to subsequently activate or engage the appropriate muscles via the posterior parietal cortex (Kiehn and Dougherty, 2013). It is worth noting that despite the modification and maturation of locomotion, this basic motor function remains an innate behaviour of vertebrates that are established in their nervous system before the

delivery of their offspring. However, this motor behaviour is not usually carried out until the maturation of, for example, the postural activity (Kiehn and Dougherty, 2013).

1.3 Control of Locomotion in Vertebrates: Brief Historical Account

Étienne -Jules Marey and Eadweard James Muybridge are credited for providing the first detailed information on locomotion through the use of photographic techniques in the late nineteenth century. Hence, allowing the observers at that time, to a lesser extent, appreciate different aspects of locomotion in humans and animals (Kiehn and Dougherty, 2013). However, in 1911 the work of Graham Brown an English neurophysiologist paved the way for the elucidation of the mechanisms underlying the neural control of locomotor movements in mammals. In Brown's experiments, he observed the extension and flexion movements of the hind limbs of cats whose spinal cords had been transected at the lower level of the thoracic vertebrae segment. The cats also had hind limb muscles that had been deafferented (the complete isolation or removal of all the dorsal roots that innervate the hind limbs) (Schwartz *et al.*, 2012a; Kiehn and Dougherty, 2013). Brown's conclusion on the aforementioned observations was that, the neuronal networks in the spinal cord can independently generate rhythmic locomotor movements without the involvement of sensory impulses (Goulding, 2009; Schwartz *et al.*, 2012a; Kiehn and Dougherty, 2013; Borisyuk *et al.*, 2017; Grillner and El Manira, 2020). This conclusion inspired the English neurophysiologist to develop the "half-centre" theory. This theory describes how two different groups of neuronal networks of the spinal cord reciprocally inhibit each other to ensure that locomotor movements are rhythmical and patterned. A classic example that explains this concept is the locomotor activities of flexors and extensors. In this example, the flexor neuronal network issues motor commands exciting corresponding

flexors and concurrently inhibiting the extensor neuronal network from activating corresponding extensors. On the contrary, when the extensors are activated, there is also the simultaneous inhibition of the flexors neuronal network (Schwartz *et al.*, 2012a; Guertin, 2013; Grillner and El Manira, 2020). Brown's theories cast serious doubts on the integrity of the well-established theory on locomotion preceding his. That is, locomotion was the product of repeated reflex reactions (Kiehn and Dougherty, 2013).

Fast forward, in the 1960s, nearly half a century following Brown's discoveries, and at a time when Brown's findings appeared to have been forgotten, a group of Swedish neurophysiologists in Gothenburg highlighted again the significance of Brown's work (Kiehn and Dougherty, 2013). This time, the Swedish group showed that rhythmic motor activity can be elicited in spinalized cats using a pharmacological approach. The pharmacological agent used in this experiment was L-DOPA (l-dihydroxyphenylalanine), an adrenergic drug and a precursor of noradrenaline (Schwartz *et al.*, 2012a; Kiehn and Dougherty, 2013). Following administration, L-DOPA increases the level of noradrenaline in the spinal cord, resulting in the spontaneous production of locomotor movements. It takes about 30 minutes for these effects to occur after administration (Schwartz *et al.*, 2012a). The ability of the spinal cord to generate locomotor movements without the presence of sensory input is due to the unique activities of its neural networks. These circuits are called the central pattern generators (CPG) (Goulding, 2009; Le Ray *et al.*, 2011; Kiehn and Dougherty, 2013; Severi *et al.*, 2014; Thiele, Donovan and Baier, 2014; Caggiano *et al.*, 2018; Haspel *et al.*, 2021). Now, in addition to the earlier studies that involved cats as model systems, there is a huge body of evidence from experiments involving several other

vertebrates, including humans, that corroborates these earlier findings on the activities of the CPG network.

Finally, the history of the neuronal control of locomotion cannot be complete without mentioning the monumental work (Shik, Severin and Orlovskiĭ, 1966) of a group of three Russian researchers. Their names are Mark Shik, Fidor Severin, and Grigori Orlovsky (Shik, Severin and Orlovskiĭ, 1966; Schwartz *et al.*, 2012a). In this study, Shik and his colleagues discovered that locomotor movements could be elicited in decerebrated (disconnection of the cerebral cortex from the spinal cord at the midbrain area) cat by electrically stimulating a circumscribed region in the midbrain (Jordan, 1998; Sirota, Di Prisco and Dubuc, 2000; Musienko *et al.*, 2012; Schwartz *et al.*, 2012a; Kiehn and Dougherty, 2013; Wang and McLean, 2014; Caggiano *et al.*, 2018; Ferreira-Pinto *et al.*, 2018; Chang *et al.*, 2020; Grillner and El Manira, 2020). The trains of electrical stimuli applied were delivered at constant low-threshold frequencies varying between 20-30 hertz (Chang *et al.*, 2020; Grillner and El Manira, 2020). Also, the decerebrated cats initiated locomotor movements in the form of stepping when they were placed on a treadmill belt (Schwartz *et al.*, 2012a).

This circumscribed region of the midbrain was, therefore, called the mesencephalic locomotor region (MLR). According to Sirota, Di Prisco and Dubuc (2000), the electrical stimulation was specifically performed in the caudal mesencephalon, activating the cuneiform nucleus (CnF). The CnF is one of the two groups of nuclei found in the mammalian MLR; the other group is the pedunculopontine nucleus (PPN). Another observation made during this study was that the strength of the electrical stimulation was directly proportional to the pace of the locomotor movements being generated (Le Ray *et al.*, 2011; Schwartz *et al.*, 2012a; Grillner and El Manira, 2020).

Since the discovery of MLR in cats in the 1960s, several studies over the years have demonstrated that all vertebrates studied to date possess MLR (Dimitri and Réjean, 2017; Caggiano *et al.*, 2018; Chang *et al.*, 2020).

1.4 Neuronal Control Components of the Locomotor System in Vertebrates

Overall, locomotion is controlled by spinal and supraspinal neuronal components that work together to achieve the goal of every locomotor episode. The organisation of these neuronal control components, as observed in different vertebrate species (ranging from salamanders to humans), are incredibly similar (Le Ray *et al.*, 2011; Grillner and El Manira, 2020). Examples of these components are:

1. Neuronal components in the forebrain responsible for selecting motor behaviour
2. Neuronal components in the brainstem responsible for initiating locomotion
3. Neuronal components in the spinal cord responsible for generating locomotion
4. Sensory signals generated in the muscles, joints and skin that regulate the activities of the locomotor CPGs of an ongoing locomotor activity
5. Neuronal components in the muscles and joints that send sensory impulses to the cerebellum and other supraspinal bodies, to regulate the activities of locomotor CPG networks of ongoing locomotor activity (for example, maintaining or adjusting body posture)
6. Neuronal components in the motor cortex responsible for adjusting ongoing locomotor movements based on visuomotor sensory inputs. For example, in situations where an organism faces an obstacle

7. Neuromodulatory components from the locomotor CPG networks of ongoing locomotor activity and other areas of the nervous system that are responsible for modulating the activities of locomotor CPGs. The changes made, for example, on locomotion frequency and burst amplitude, can be slow and long-lasting (Kiehn and Dougherty, 2013; Grillner and El Manira, 2020).

From here, the focus will be on the supraspinal control of locomotion, which according to the above list of examples involves the neuronal components responsible for the selection and initiation of locomotion. However, most of the attention will be given to the initiation of locomotion component. Figure 1 summarises the key spinal and supraspinal components of the locomotor control system.

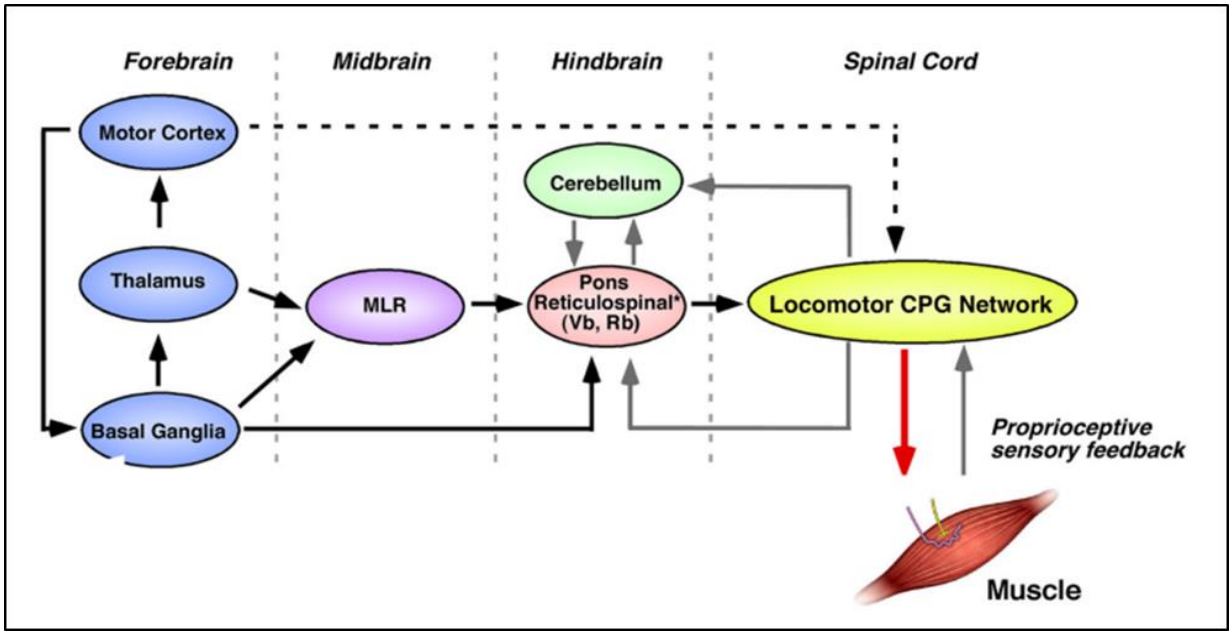


Figure 1. A schematic diagram summarising the general organisation of the neuronal control system of locomotion in vertebrates.

Following the receipt of sensory signals through the thalamus and other forebrain bodies, the basal ganglia recruit appropriate motor patterns based on the internal or external needs of a species. This first step in the locomotion control system can also be facilitated by neuronal

circuitry in the medial and lateral hypothalamus. The neurons of the output nuclei of the basal ganglia (globus pallidus interna and substantia nigra pars reticulata) eventually project to the mesencephalic locomotor region (MLR). Locomotion is initiated when the neurons in the MLR activates reticulospinal cells in the lower brainstem, which in turn excites appropriate locomotor central pattern generators (CPG) to execute locomotor movements. Descending vestibular (Vb) and rubrospinal (Rb) fibres control and maintain the equilibrium of the locomotor CPG network of ongoing locomotor activity. The cerebellum controls the locomotor CPGs of ongoing locomotor activities by mediating sensory signals generated by ongoing movements and internal feedback to fine tune the locomotor movement. The cerebellum also receives inputs from the proprioceptive sensory feedback for needed corrections of ongoing motor actions. Locomotor CPGs can be directly excited by projections from the motor cortex bypassing the MLR and the reticulospinal cells. This activity is mediated by the long axons possessing pyramidal cells. The black lines represent direct command pathways, whereas the grey lines represent feedback pathways. (Adapted from Goulding, 2009)

1.5 Supraspinal Control of Locomotion

As Figure 1 indicates, the spinal locomotor CPGs generate locomotor movements by exciting different muscles to operate in a well-coordinated fashion to suit a specific behavioural demand. The activities of the locomotor CPG networks are activated and controlled by some supraspinal bodies located in the three broader areas of the brain (forebrain, midbrain and hindbrain). These supraspinal bodies include the basal ganglia, motor cortex, MLR, diencephalic locomotor region (DLR) and the brainstem reticulospinal neurons (Le Ray *et al.*, 2011; Caggiano *et al.*, 2018; Ferreira-Pinto *et al.*, 2018; Grillner and El Manira, 2020).

1.5.1 Selection of motor behaviour

The basal ganglia represent a group of conserved interconnected nuclei located below the cortex. They control the activities of the other locomotor control elements in the

brainstem by dominating the process involved in the selection of appropriate motor behaviour following the receipt of sensory inputs. The striatum, one of the main components of the basal ganglia, is responsible for receiving the sensory information, which comes from different forebrain structures such as the cortex and thalamus. The striatal neurons then project their axons and form synapses with the neurons of the globus pallidus interna (GPi) and the substantia nigra pars reticulata (SNr). These two particular structures form the output nuclei of the basal ganglia (Kiehn and Dougherty, 2013). The neurons of the GPi and SNr, in turn, project their axons to the neurons in the MLR. The locomotor command areas in the mesencephalon and the diencephalon can only become activated to initiate locomotor movements when tonic disinhibition has taken place. This is because the neurons of the striatum, globus pallidus interna and the substantia nigra pars reticulata are inhibitory (Le Ray *et al.*, 2011; Kiehn and Dougherty, 2013; Grillner and El Manira, 2020). These neurons produce the main inhibitory neurotransmitter in the central nervous system, gamma-aminobutyric acid (GABA).

Moreover, under resting conditions, the neurons in the output nuclei of the basal ganglia have high tonic activity, hence, keeping both the MLR and DLR tonically inhibited (Kiehn and Dougherty, 2013; Roseberry *et al.*, 2016; Dimitri and Réjean, 2017; Grillner and El Manira, 2020; Messa and Koutsikou, 2021). It is suggested that the general aim of the tonic inhibition function is to save energy by preventing, most especially needless non-goal directed and energetically expensive locomotor activities from happening (Benjamin, Staras and Kemenes, 2010). When the exploratory system of locomotion is selected as the most appropriate type of locomotion in response to a behavioural demand, the inhibitory input from the striatum (Grillner and El Manira,

2020) reduces the tonic levels of the neurons in the output nuclei of the basal ganglia. This development, in turn, leads to the disinhibition of the neurons in the MLR and DLR. At this stage, these command centres become activated, paving the way for the initiation of locomotion (Jordan, 1998; Benjamin, Staras and Kemenes, 2010; Kiehn and Dougherty, 2013; Roseberry *et al.*, 2016; Dimitri and Réjean, 2017; Grillner and El Manira, 2020). Disinhibition of the MLR also occurs when dopamine reduces the excitation levels of striatal dopamine receptor D2-expressing neurons of the indirect dopaminergic pathway (Dimitri and Réjean, 2017). The findings from about half a dozen studies prove the existence of the disinhibition mechanism in the MLR of rodents (Benjamin, Staras and Kemenes, 2010; Roseberry *et al.*, 2016), lampreys (Ménard *et al.*, 2007; Ménard and Grillner, 2008), and cats (Benjamin, Staras and Kemenes, 2010).

It has been proposed that two other brain structures may also participate in the selection of appropriate motor patterns. These bodies are the lateral and medial hypothalamus. Starting with the lateral hypothalamus, this hypothalamic nucleus is understood to excite reticulospinal cells directly, without the involvement of neuronal components in the MLR. However, it is also suggested that neurons located in the anterior dorsal tegmentum (ADT) of the midbrain are integral in the locomotor movements initiated as a result of the stimulation of the lateral hypothalamus (Jordan, 1998). The locomotor activities initiated through the lateral hypothalamus are thought to meet behavioural demand where there is the need for an organism to be in contact with a stimulus of an appetitive and incentive value (Jordan, 1998; Kiehn and Dougherty, 2013; Ferreira-Pinto *et al.*, 2018). The lateral hypothalamus, therefore, is

considered part of the primary appetitive system of locomotion (Jordan, 1998; Ferreira-Pinto *et al.*, 2018).

On the other hand, the medial hypothalamus is thought to activate locomotor reticulospinal neurons directly or indirectly (through the MLR) for locomotor movements to be subsequently executed. The direct or otherwise connection to the reticulospinal neurons is thought to be relayed through the periaqueductal grey (PAG) matter in the midbrain (Jordan, 1998; Le Ray *et al.*, 2011; Ferreira-Pinto *et al.*, 2018). However, the existence of direct connections bypassing the PAG cannot be excluded (Jordan, 1998). Unlike the lateral hypothalamus, the medial hypothalamus functions as part of the primary defensive system of locomotion. The main function of this system is to ensure that dangerous or threatening stimuli are avoided (Jordan, 1998; Kiehn and Dougherty, 2013; Ferreira-Pinto *et al.*, 2018).

1.5.2 Initiation of Locomotion: MLR

The locomotion initiation system directly involves two neuronal components located in the brainstem of vertebrates. They are the MLR and the reticulospinal neurons. I will continue this section by first focusing on the MLR. The mesencephalic locomotor region, which is widely regarded as the most important supraspinal neuronal control element in the locomotion control system (Caggiano *et al.*, 2018), is a physiologically defined area in the midbrain. Anatomically, the MLR is located at the mesopontine border (Chang *et al.*, 2020; Grillner and El Manira, 2020), occupying the anterior region of the brainstem tegmentum (Chang *et al.*, 2020). Functioning as an integrating centre, that is, receiving sets of motor commands from superior brain regions such as the basal ganglia and hypothalamus, the MLR also operates as a control centre. This

unique attribute of the MLR is founded on an observation made by Mark Shik and his colleagues (Shik, Severin and Orlovskiĭ, 1966) in their original experiment that led to the discovery of this brainstem region (Shik, Severin and Orlovskiĭ, 1966; Jordan, 1998; Cabelguen, Bourcier-Lucas and Dubuc, 2003; Le Ray *et al.*, 2011; Schwartz *et al.*, 2012a; Dimitri and Réjean, 2017; Caggiano *et al.*, 2018; Chang *et al.*, 2020; Grillner and El Manira, 2020). In the words of Le Ray and colleagues (2011), "This initial stunning observation provided the basis for qualifying this particular brainstem region as 'dedicated to control a locomotor output'". The observation was that the rhythm of the locomotor movements of the decerebrated cats was directly proportional to the intensity of the electrical stimulation. That is, at low intensities, the cats stood up and began walking; as the stimulation intensities gradually increased, the pace of walking also increased, eventually leading to trotting and galloping, respectively (Le Ray *et al.*, 2011; Gariépy *et al.*, 2012; Schwartz *et al.*, 2012a; Kiehn and Dougherty, 2013; Grillner and El Manira, 2020). This phenomenon is explained by the fact that as the strength of the electrical stimulus increases, extra MLR neurons are recruited, hence, the relatively more intense locomotor output (Grillner and El Manira, 2020).

Since 1966, this remarkable observation has been made in other animal models involved in studies investigating locomotion in vertebrates. Examples of such vertebrate species are carp (*Cyprinus carpio*) (Kashin, Feldman and Orlovsky, 1974), goldfish (Budick and O'Malley, 2000), salamander (Cabelguen, Bourcier-Lucas and Dubuc, 2003), rabbit (Musienko *et al.*, 2008), mouse (Roseberry *et al.*, 2016), Atlantic stingray (*Dasyatis sabina*) (Livingston and Leonard, 1990) and lamprey (McClellan and Grillner, 1984; Sirota, Di Prisco and Dubuc, 2000). For example, in semi-intact preparations of larval and adult sea lampreys (*Petromyzon marinus*), Sirota, Di Prisco and Dubuc (2000) found that the stimulation intensity was inversely proportional to the

delay of swim initiation. Put differently, as the strength of the electrical stimulus increased, the delay between the onset of stimulation and the start of swimming decreased. Conversely, there was a positive correlation between the stimulation intensity and the delay of termination of swimming. In other words, as the stimulation intensity increased, the delay between the termination of swimming activities and cessation of stimulation increased. In addition to the above findings, the investigators of this study also found that the amplitude of body movements and the frequency of muscle contractions were dependent on the strength of the electrical stimulus applied to the MLR (Sirota, Di Prisco and Dubuc, 2000).

The MLR has also been viewed as a command hub for initiating forward walking or forward locomotion, the principal form of locomotion for quadrupeds and bipeds, such as humans. This view originates from the study of Musienko et al. (2012), a study that compared the locomotor activities of decerebrate cats elicited by the stimulation of the MLR and the spinal cord. The cat preparations walked on a treadmill belt that moved in different directions. The investigators observed that the stepping movements evoked following the spinal cord stimulation were well-coordinated at diverse treadmill directions. The direction of the stepping movements was also opposite to the treadmill direction. Differently, following MLR stimulation, the cat preparations generated well-coordinated stepping movements only when the treadmill belt moved backwards. Remarkably, at any other treadmill directions or angles, no stepping movement was generated. In rare cases where stepping movements were produced, the movements were significantly distorted. Analysis of these observed distortions suggested that following the MLR activation, the CPG networks generated motor patterns for forward

stepping movements, which were unrelated to the treadmill direction (Musienko *et al.*, 2012).

1.5.2.1 Neuronal Composition of MLR

The activities of the vertebrate MLR is controlled by superior brain elements such as the basal ganglia. Following the receipt and integration of the motor commands from the superior structures, the MLR eventually controls the generated locomotor movements by indirectly (through the reticulospinal cells) activating the dedicated locomotor CPG networks in the spinal cord. Historically, there have been several arguments regarding the neuronal composition of the MLR, as some even believed that the MLR was a single element (Jordan, 1998; Caggiano *et al.*, 2018). Some quarters even proposed that the MLR concept should be deemed outmoded. This arguably extreme suggestion sprang up after findings of studies aimed at finding the exact location of MLR neurons were not convincing enough. Additionally, the findings from lesion studies aimed at demonstrating the role of MLR neurons in initiating locomotion were inconclusive (Jordan, 1998).

Now, there appears to be a consensus on this bone of contention because currently, it is widely accepted that the MLR contains the CnF and PPN groups of neurons (Jordan, 1998; Sirota, Di Prisco and Dubuc, 2000; Le Ray *et al.*, 2011; Roseberry *et al.*, 2016; Dimitri and Réjean, 2017; Caggiano *et al.*, 2018; Chang *et al.*, 2020; Grillner and El Manira, 2020). Anatomically, the population of the CnF and PPN are located mostly in the dorsal and ventral MLR, respectively (Caggiano *et al.*, 2018; Chang *et al.*, 2020). Furthermore, the MLR at the mesopontine region, is anatomically close to both neurons (Grillner and El Manira, 2020), but the CnF population is considered the

closest. This is because of the distance between the MLR and the inferior colliculus, which is estimated to be 6mm (Schwartz *et al.*, 2012a). Both the CnF and PPN neuronal populations are excitatory in nature; the CnF neurons produce glutamate whereas PPN neurons produce both acetylcholine and glutamate (Le Ray *et al.*, 2011; Kiehn and Dougherty, 2013; Roseberry *et al.*, 2016; Caggiano *et al.*, 2018; Ferreira-Pinto *et al.*, 2018; Chang *et al.*, 2020). Although excitatory, the MLR also contains GABAergic neurons (Roseberry *et al.*, 2016; Caggiano *et al.*, 2018; Ferreira-Pinto *et al.*, 2018; Chang *et al.*, 2020). This neurochemical population provides the localised inhibitory effects in the locomotor region prior to the activation of the disinhibition mechanism (Kiehn and Dougherty, 2013; Dimitri and Réjean, 2017; Grillner and El Manira, 2020).

For many years, the issue of the most effective MLR neuron for initiating and controlling locomotion has long been debated and divided opinions (Jordan, 1998; Le Ray *et al.*, 2011; Ferreira-Pinto *et al.*, 2018; Chang *et al.*, 2020; Grillner and El Manira, 2020). Some people argued in favour of the PPN population despite the inconsistencies associated with its electrical mapping findings over the years (Jordan, 1998; Chang *et al.*, 2020). As expected, other people also strongly believed that the CnF is the most effective MLR area for controlling and initiating locomotion (Le Ray *et al.*, 2011), given its close proximity to the MLR (Schwartz *et al.*, 2012a; Caggiano *et al.*, 2018). Unlike the PPN neurons, the electrical mapping results reported on the CnF neurons in many related preclinical studies, including the monumental work of Shik and his colleagues (Shik, Severin and Orlovskiĭ, 1966) are consistent (Chang *et al.*, 2020). Lastly, on the varied opinions on this subject matter, some people were also of

the belief that the two neuronal populations work synergistically to control locomotion (Jordan, 1998; Grillner and El Manira, 2020).

Three pivotal optogenetic studies in mice (Roseberry *et al.*, 2016; Caggiano *et al.*, 2018; Josset *et al.*, 2018) have settled this debate, as findings from these studies further elucidated the organisation and distinct functions of the MLR neuronal population (Ferreira-Pinto *et al.*, 2018; Chang *et al.*, 2020; Grillner and El Manira, 2020). In addition to the application of optogenetic techniques in all these three key studies, the use of other techniques (for example, electrophysiological recordings, viral tracing and kinematic analysis) other than electrical stimulation, lesioning or pharmacological techniques was significant in the quest to improve our understanding of the cellular organisation and function of the MLR. The reason for this assertion is that the different groups of MLR neurons are located close to each other, and some are even intermingled (Le Ray *et al.*, 2011; Caggiano *et al.*, 2018; Ferreira-Pinto *et al.*, 2018; Chang *et al.*, 2020). Therefore, making it extremely challenging to clearly distinguish the unique function of each neuronal component using the latter experimental approaches (Caggiano *et al.*, 2018; Ferreira-Pinto *et al.*, 2018; Grillner and El Manira, 2020).

The findings from all the three optogenetic studies in mice (Roseberry *et al.*, 2016; Caggiano *et al.*, 2018; Josset *et al.*, 2018) clearly favour the glutamatergic CnF neurons as the most effective MLR site for initiating and controlling locomotion, thus, dismissing the long-held view that the cholinergic PPN neurons are fundamental in initiating and controlling locomotion (Roseberry *et al.*, 2016; Caggiano *et al.*, 2018; Ferreira-Pinto *et al.*, 2018; Josset *et al.*, 2018; Chang *et al.*, 2020; Grillner and El Manira, 2020). For example, the study of Caggiano et al. (2018) showed that

optogenetic stimulation of PPN neurons expressing the vesicular glutamate transporter, vGlut2, (PPN-vGlut2) evoke locomotor movements with alternating gaits, which are characterised by low speed and long latency. Conversely, the activation of CnF-vGlut2 neurons generate locomotor movements at shorter latencies. Also, the speed of locomotion is dependent on the intensity of the stimulation (Caggiano *et al.*, 2018; Ferreira-Pinto *et al.*, 2018). Additionally, Roseberry *et al.* (2016) demonstrated that the cholinergic PPN neurons can modulate locomotor activities following electrical stimulation, but are not capable of initiating locomotion at shorter latencies (Roseberry *et al.*, 2016).

Together, the findings from these three studies (Roseberry *et al.*, 2016; Caggiano *et al.*, 2018; Josset *et al.*, 2018) support the classifications assigned to the two excitatory MLR neurons, with respect to the famous classification of locomotion based on behavioural demand. The CnF neuronal group is considered part of the “primary defensive system” (Jordan, 1998; Le Ray *et al.*, 2011) of locomotion, whereas the PPN neuronal population is regarded as part of the “exploratory system” (Jordan, 1998; Le Ray *et al.*, 2011) of locomotion (Jordan, 1998; Le Ray *et al.*, 2011; Caggiano *et al.*, 2018; Ferreira-Pinto *et al.*, 2018; Josset *et al.*, 2018; Chang *et al.*, 2020; Grillner and El Manira, 2020). In other words, the glutamatergic CnF neurons are mostly involved in escape locomotor activities. The cholinergic PPN neurons, on the other hand, takes part in locomotor activities aimed at bringing organisms closer to a stimulus of interest. Concluding this section, it is worth noting that the PPN neuronal population has two subdivisions (Grillner and El Manira, 2020). One of them is characterised by ascending projections that send inputs into forebrain elements, such as the basal ganglia and the thalamus, whereas the other division involves descending projections that receive

information from forebrain structures, including the basal ganglia (Ferreira-Pinto *et al.*, 2018; Grillner and El Manira, 2020). Moreover, the descending glutamatergic PPN projections receive motor inputs from multiple brain structures involved in behaviour selection and voluntary movements. Differently, the CnF neurons receive specialised motor inputs from superior bodies (for example, the PAG and inferior colliculus), which are known to be involved in escape locomotor responses (Caggiano *et al.*, 2018; Ferreira-Pinto *et al.*, 2018). In summary, the glutamatergic PPN and CnF neurons are respectively considered functionally heterogeneous and homogenous. The above reasons explain why the role of the PPN neurons, especially the glutamatergic component, in the control and initiation of locomotion is not as clear as the role of CnF neurons (Chang *et al.*, 2020).

1.5.3 Reticulospinal Cells

The reticulospinal cells represent the last part of the supraspinal locomotor system. The neurons of MLR indirectly activate the dedicated locomotor CPG networks by recruiting a specialised population of reticulospinal cells to initiate and control locomotion. The concept of reticulospinal cells functioning as a gateway to the locomotor CPGs is evolutionarily conserved (Ferreira-Pinto *et al.*, 2018). Reticulospinal cells are part of the reticular formation, and they are thought to be located in the lower brainstem, (specifically in the ventromedial medulla) (Cabelguen, Bourcier-Lucas and Dubuc, 2003). The exact functional identity of the reticulospinal cells is not fully understood (Kiehn and Dougherty, 2013; Grillner and El Manira, 2020). Two systems appear to be involved in the initiation of locomotion: the glutamatergic and serotoninergic locomotor pathways.

The glutamatergic pathway is thought to be present in all vertebrates, whereas the serotoninergic pathway can be found mainly in mammals (Kiehn and Dougherty, 2013). The existence of the glutamatergic pathway was proven when locomotion was inhibited as a result of interference with the activities of the glutamatergic receptors in the spinal cord (Kiehn and Dougherty, 2013). Additionally, the work of Capelli, Pivetta and Arber (2017) has demonstrated in mice that the glutamatergic neurons present in the lateral paragigantocellular nucleus (LPGi), a small caudal brainstem area, can evoke well-coordinated locomotor movements at varying speeds. Also, LPGi can positively adjust the locomotor speed after receiving inputs from glutamatergic MLR neurons (Capelli, Pivetta and Arber, 2017; Grillner and El Manira, 2020).

On the issue of the existence of serotoninergic locomotor pathways, experiments in rats have shown that electrical stimulation of the serotoninergic neurons-rich region of the caudal brainstem, parapyramidal area, elicits locomotor activity, which is blocked when there is an interference with the excitation of serotoninergic receptors in the spinal cord (Kiehn and Dougherty, 2013). Findings from several studies have also shown that monosynaptic excitatory postsynaptic potentials (EPSPs) are generated in the reticulospinal cells after being excited by MLR neurons (Le Ray *et al.*, 2011; Grillner and El Manira, 2020). The motor information conveyed to the reticulospinal cells is sent via both the cholinergic and glutamatergic transmissions. The receptors involved are the nicotinic (cholinergic), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl D-aspartate (NMDA) ionotropic receptors (glutamatergic). The AMPA and NMDA receptors work synergistically in the locomotor network (Grillner and El Manira, 2020).

In addition to the direct neuronal projections to the reticulospinal cells, MLR neurons excite a specific population of muscarinoceptive cells in the brainstem, which in turn, provide the reticulospinal cells with additional excitation (Le Ray *et al.*, 2011; Roseberry *et al.*, 2016; Dimitri and Réjean, 2017). The additional excitation is intended to amplify the locomotor output that will be subsequently generated by the dedicated locomotor CPG networks (Le Ray *et al.*, 2011).

1.6 Midbrain Control of Locomotion in Non-Mammalian Vertebrates

Several lines of evidence show that the midbrain controls the initiation and different locomotor kinematic parameters in lower or non-mammalian vertebrates. Looking at the work of Jamieson and Roberts (1999) aimed at characterising the neuronal pathway underlying the swimming behaviour of the hatchling *Xenopus laevis* tadpole in response to light dimming, it was revealed that the pineal ganglion cells (pgc) in the pineal eye of the tadpole do not directly excite the appropriate hindbrain population in the pathway. Rather, the investigators found that pgc project to hindbrain neurons through a group of third-order neurons in the midbrain, the diencephalic/mesencephalic descending (D/MD) neurons. Through pharmacological experiments, the light dimming pathway was known to be excitatory with glutamate being one of the main neurotransmitters involved.

One of the parameters measured in a number of lesion experiments conducted was the latency between light dimming and swimming onset. The results from these works suggested that the midbrain generally controls swim initiation and the latency to swim initiation in the tadpole. When all the axons leaving the pineal eyes were severed on both sides, all the 22 tadpoles involved in the experiment failed to initiate swimming in response to light dimming. Moreover, when all the axonal projections exiting the pineal

on only one side were severed, the investigators reported a significant increase in mean latencies to swim start following the lesion (after lesion: 98.7±3.48 ms vs before lesion: 88.9±2.2 ms) (Jamieson and Roberts, 1999).

In a different but related study (Jamieson and Roberts, 2000), the results, when taken together, suggest that the midbrain through the D/MD neurons controls swimming frequency and vertical swimming in the hatchling *Xenopus laevis* tadpole. In physiological experiments, immobilised tadpoles reacted to dimming, which is known to generate vertical turns, with increased fictive swimming movements. Now, it can be said that the midbrain is essential to the survival of tadpoles, given the knowledge that attachment to floating shadow-casting objects is one of the ways tadpoles avoid predation (Jamieson and Roberts, 2000).

In support, using electrical stimulation, laser ablation and electrophysiological recording techniques, the nucleus of the medial longitudinal fasciculus (nMLF) in the midbrain was shown to play an instrumental role in initiating and regulating some swimming bout parameters in the larval zebrafish. The parameters were speed, duration, distance and tail-beat frequency (TBF) (Severi *et al.*, 2014). For example, in one of the laser ablation experiments involving RoM-ablated larvae, Mauthner neuron-ablated larvae, and nMLF-ablated larvae, the failure of the nMLF-ablated larvae to swim at a high speed in response to optical stimuli was more consistent and significant than the behaviour of the other ablated groups. This particular observation was also made in all other experiments that measured different parameters. “In this study we attribute to the nMLF a major role in the control of the speed of locomotion.”, Severi and colleagues (2014) stated in their concluding remarks.

Again, Thiele, Donovan and Baier (2014) showed through their work on larval zebrafish that the nMLF neurons play a significant role in controlling the posture of tail orientation and direction of swimming as part of the optomotor response (OMR) (Thiele, Donovan and Baier, 2014). Concluding on the evidence associating the midbrain to the initiation and control of swimming in lower vertebrates, the findings of the study (Wang and McLean, 2014), when taken together, implicate the nMLF neurons of controlling the swimming behaviour (for example, TBF and duration of swim episodes) of larval zebrafish. The nMLF neurons achieve this by interacting and imposing motor commands on preferred spinal motoneurons or spinal motor pool (Wang and McLean, 2014).

Despite the technological advances (for example, brain scanners which can identify and relate specific brain areas to specific functions (Roberts, Li and Soffe, 2010)), and decades of research in the area of neurobiology, including the almost half a dozen studies cited above, our understanding of the interactions between the midbrain neurons and the CPG networks in the locomotor initiation pathway remains limited. This problem is mainly due to the complex nature of the adult vertebrate brain especially, higher vertebrate systems particularly, humans (Buhl, Roberts and Soffe, 2012; Roberts *et al.*, 2019). The mammalian brain has millions of descending neurons that synapse with the CPG networks in the spinal cord (Kandel, 1976b; Budick and O'Malley, 2000). In addition to this extremely high number, the mammalian neurons are typically small in size, making it quite challenging to easily identify them (Kandel, 1976b).

An effective way of reducing the magnitude of this problem is to investigate and understand the neuronal mechanism responsible for locomotor behaviour in

invertebrates and lower vertebrates. Unlike the higher vertebrates, higher invertebrate systems such as crayfish, leeches, locusts, roaches, and snails have central nervous systems that are made up of a relatively fewer number of neurons (between 10^5 to 10^6). The neurons are also larger than that of mammals (Kandel, 1976a). Likewise, non-mammalian vertebrate species are less problematic in terms of understanding their neuronal architecture underlying motor behaviour. This is because of their relatively fewer number of neurons and other advantages over mammals, such as amenability to different techniques and experimental accessibility. Most importantly, the neuronal architecture of sensorimotor networks across vertebrate systems is incredibly similar (Goulding, 2009; Berg *et al.*, 2018). This highly useful feature in the vertebrate world has allowed neurobiologists to study numerous model species. Through those studies, our understanding and appreciation of the relationship between the midbrain neurons and the CPG network of the spinal cord are improving (Berg *et al.*, 2018; Grillner and El Manira, 2020) – an exciting development in the quest to have a comprehensive knowledge of the pathophysiology of neurodegenerative motor disorders most notably, Parkinson's disease.

The lamprey, *Xenopus laevis* tadpoles and zebrafish are a group of model organisms that have been well studied with regard to their motor behaviours (Grillner and El Manira, 2020). For example, the lamprey, which belongs to the oldest vertebrate group (cyclostome) currently living (Grillner and El Manira, 2020), has a nervous system that is remarkably similar to a mammalian nervous system in terms of organisation (Le Ray *et al.*, 2011; Ferreira-Pinto *et al.*, 2018; Grillner and El Manira, 2020; Grillner, 2021). Lamprey, as a model system, among other benefits, is amenable to *in vitro* techniques, and through these techniques, much knowledge is now known about the various

brainstem neurons involved in the initiation and control of locomotion (Sirota, Di Prisco and Dubuc, 2000; Le Ray *et al.*, 2011). For example, the reticulospinal cells of lamprey are both anatomically and physiologically characterised.

Consequently, we now understand that reticulospinal cells make up about 90% of all projections that synapse with CPG networks in the spinal cord. Moreover, the lamprey's reticulospinal cells are housed in one mesencephalic reticular nucleus (MRN) and three rhombencephalic reticular nuclei (RRN). Nearly 90% of the entire reticulospinal cells population in the RRN resides in the middle and posterior RRN (PRRN). Lastly, the reticulospinal cells in the PRRN are considered homologous to the gigantocellular reticular nucleus (Gi) found in mammalian systems (Le Ray *et al.*, 2011).

In spite of the immense benefits and advantages of studying the preceding model species over complex vertebrate systems, it is more advantageous and less problematic to study the locomotor initiation system of models at an early developmental stage (Roberts, Li and Soffe, 2010; Roberts *et al.*, 2019). In light of this, the model chosen for this study was the hatchling *Xenopus laevis* tadpole at developmental stage 37/38.

1.7 *Xenopus laevis* Tadpole

The *Xenopus laevis*, a species of the African clawed frog, serves as an attractive model system in the neurobiology field because their genome is characterised. Between the *Xenopus laevis* species and the teleost group (for example, zebrafish), the genome of *Xenopus laevis* is the most similar to the genome of humans. The genome of the teleost group is known to be variable and at the same highly specialised

(Nakatani *et al.*, 2007; Rash *et al.*, 2012; Pratt and Khakhalin, 2013; Thiele, Donovan and Baier, 2014). The smaller cousin of *Xenopus laevis*, *Xenopus tropicalis*, also has a genome closer to humans' (Hellsten *et al.*, 2010). The Xenopus laevis species also has a very high fecundity rate.

The hatchling *Xenopus laevis* tadpole as an independent entity is also a useful and one of the ideal model choices for studying the basic neural architecture of the circuitry underlying motor behaviour in vertebrates. Studying the functional organisation of the nervous system of the hatchling *Xenopus laevis* tadpole (Buhl, Roberts and Soffe, 2012; Roberts *et al.*, 2014; Buhl, Soffe and Roberts, 2015; Koutsikou *et al.*, 2018) has allowed neurobiologists to unearth some remarkable novel discoveries about the fundamental functional and organisational principles of the vertebrate's nervous system (Roberts, Li and Soffe, 2010; Roberts *et al.*, 2019). Additionally, the somatosensory system of the *Xenopus laevis* tadpole is well characterised, and this achievement is due to the simplicity of the system's organisation. As a result, we know about the three sensory pathways of modalities of the tadpole. These are the "touch", "pressure", and "noxious" modalities (Roberts, 1998). Furthermore, we know about the neuronal function and structure of the spinal cord, as well as the characterisation of the reticulospinal neurons of the tadpole (Buhl, Soffe and Roberts, 2015; Koutsikou *et al.*, 2018).

There is also a good understanding of some key events in the development process of the *Xenopus laevis* tadpole's nervous system. Examples of such events are axon guidance, neuronal differentiation, neurogenesis and synaptogenesis (Katz, Potel and Wassersug, 1981; Villinger, 2007; Villinger and Waldman, 2012; Pratt and Khakhalin, 2013). Concluding on the benefits of the *Xenopus laevis* tadpole model system, it is

also highly amenable to techniques, such as electroporation (Pratt and Khakhalin, 2013) and calcium imaging (Tao *et al.*, 2001; Junek *et al.*, 2010; Xu *et al.*, 2011; Pratt and Khakhalin, 2013; Saccomanno *et al.*, 2021). The electroporation technique, for example, allows investigators to genetically manipulate the *Xenopus laevis* tadpole (Haas *et al.*, 2001; Bestman *et al.*, 2006; de Miera, Parr and Denver, 2018).

1.8 Summary of the Development of the Tadpole's Nervous System

The CNS of the *Xenopus laevis* tadpole develops 24 hours prior to the closure of the neural tube. Following this period, the CNS continues to form rapidly and can be divided into its main parts. They are the three broader brain regions (forebrain, hindbrain and midbrain) and the spinal cord. In the spinal cord, about 200-300 touch-sensitive Rohon-Beard cells (Roberts, 1998) form a continuous column along the dorsal midline, extending rostrally into the brainstem (Clarke *et al.*, 1984; Roberts, 1998; Roberts, Li and Soffe, 2010; Borisyuk *et al.*, 2017). Located between this column are five types (ten subtypes) of disorganised interneurons columns, whose identity can be established through the use of antibodies against neurotransmitters and their respective transcription factors (Roberts, Li and Soffe, 2010). The entire body of the tadpole is innervated by touch sensitive neurons, with their cell bodies located in the trigeminal ganglion cells (head skin touch) and the spinal cord (trunk skin touch) (Roberts, 1998; Roberts, Li and Soffe, 2010). Rohon-Beard cells in particular innervate the trunk skin and are characterised by longitudinal central axons that project to the upper and lower parts of the dorsal sensory tracts (Roberts, 1998).

1.9 Behaviour of *Xenopus laevis* Tadpole

In its resting state, the tadpole spends a lot of time on the floor of ponds or laboratory tanks. Curiously, a newly hatched tadpole spends nearly all 24 hours on its first day completely idle (Roberts, Li and Soffe, 2010). Also, the tadpole regularly attaches itself to objects with the help of the mucus secreted by the cement gland. The period of attachment to objects such as water surfaces is shorter compared to solid objects. This is because the tadpole's mucus forms a more secure connection with solid objects (Roberts, Li and Soffe, 2010). Once detached, the tadpole moves to the floor of a dish or container it is in, and after about 60-90 seconds (sec) (Borisyuk *et al.*, 2017), starts to swim spontaneously (Roberts, Li and Soffe, 2010; Borisyuk *et al.*, 2017).

Swimming, the most noticeable motor behaviour of the newly hatched *Xenopus laevis* tadpole (Roberts, Li and Soffe, 2010) can be initiated by reducing light intensity and the application of brief mechanical stimuli on the skin (e.g., trunk and head skin touch) (Clarke and Roberts, 1984; Roberts, 1998; Roberts, Li and Soffe, 2010; Buhl, Roberts and Soffe, 2012; Roberts *et al.*, 2014, 2019; Buhl, Soffe and Roberts, 2015; Koutsikou *et al.*, 2018). Before developing the ability to swim, the hatchling tadpole reacts to touch stimuli with a simple flex of the body in a direction contrary to the side of stimulation (contralateral direction).

This characteristic begins to manifest soon after the closure of the neural tube during the development of its CNS. As the tadpole gets older, its flexion in response to mechanical stimuli becomes stronger, and these flexions are characterised by alternations on both sides of the tadpole's body (Roberts, 1998). At this point, the

tadpole can be said to have developed its swimming abilities. The swimming frequency of the tadpole is normally between 10-25 Hertz (Li, Roberts and Soffe, 2009; Roberts, Li and Soffe, 2010; Borisyuk *et al.*, 2017).

2.0 Sensory Pathways

At this point, I consider four sensory pathways of the tadpole, namely, the trunk skin touch (TST), head skin touch (HST), light dimming (LD) and press head (PH) pathways. The main focus will be on the TST pathway, but I will provide brief accounts of the other pathways (Figure 2).

2.0.1 Head Skin Touch (HST) Pathway

The skin of the tadpole's head is innervated by trigeminal sensory touch (tSt) receptors located in the trigeminal ganglia. Following the detection of head skin touch, tSt receptors directly excite trigeminal sensory pathway neurons (tINs) (Buhl, Soffe and Roberts, 2015; Borisyuk *et al.*, 2017; Koutsikou *et al.*, 2018; Roberts *et al.*, 2019), and it is assumed that tINs directly activate a group of extension neurons (exNs), and after processing the excitations, the exNs then activate the hdIN population (Borisyuk *et al.*, 2017; Koutsikou *et al.*, 2018; Roberts *et al.*, 2019). Motoneurons are subsequently excited to initiate swimming following the firing of the electrically coupled hdIN population (Roberts *et al.*, 2014, 2019; Buhl, Soffe and Roberts, 2015; Koutsikou *et al.*, 2018).

2.0.2 Light Dimming (LD) Pathway

The pgc and D/MD neurons on both sides of the tadpole's body are innervated by the photoreceptors of the pineal eye (Jamieson and Roberts, 1999; Borisyuk *et al.*, 2017).

The assumption is that when the pineal eye is excited by light dimming, the excitation is delivered to the hdIN population (Roberts, Li and Soffe, 2010; Borisyuk *et al.*, 2017; Roberts *et al.*, 2019) without initially passing through the exNs (Borisyuk *et al.*, 2017). The tadpole subsequently responds to the light dimming by initiating swimming after the firing of the hdIN population, which in turn activates the motoneurons. The speed of swimming increases throughout the light dimming period in a situation where the tadpole was already swimming before the light dimming (Jamieson and Roberts, 2000; Borisyuk *et al.*, 2017).

2.0.3 Press Head (PH) pathway

The tadpole stops swimming when, for example, it bumps into objects or is pressed on the head skin. Trigeminal sensory neurons cement gland receptors (tSp), which detect the stimuli innervate a group of GABAergic mid-hindbrain reticulospinal neurons (mhrs) on both sides of the body (Roberts, Li and Soffe, 2010; Borisyuk *et al.*, 2017). The hdIN population receives inhibition and together with other CPG neurons terminates swimming activities of the tadpole (Li *et al.*, 2003).

2.0.4 Trunk Skin Touch (TST) Pathway

The TST pathway, also known as the trunk touch system (Roberts, 1998) involves Rohon-Beard cells which innervate the trunk skin of the tadpole. This feature of the hatchling *Xenopus laevis* tadpole is very similar to the one observed in the newt *Triturus* (Roberts, 1998). When a stimulus is applied to the trunk skin of the tadpole, a group of Rohon-Beard cells are excited, and in turn, will activate dorsolateral commissural (dlc) and dorsolateral ascending (dla) interneurons (Clarke and Roberts, 1984; Roberts, Li and Soffe, 2010; Roberts *et al.*, 2014; Buhl, Soffe and Roberts, 2015;

Borisyuk *et al.*, 2017; Koutsikou *et al.*, 2018; Messa and Koutsikou, 2021). These two types of sensory neurons are responsible for the strictly controlled amplification of the sensory signal originating at the trunk skin. Therefore, dla and dlc distribute the sensory signals from the Rohon-Beard cells to targeted areas of both sides of the tadpole's brainstem (Roberts *et al.*, 2019). In the next stage of the TST pathway, it is suggested that a group of exNs receive inputs from both dla and dlc (Borisyuk *et al.*, 2017; Koutsikou *et al.*, 2018; Roberts *et al.*, 2019; Messa and Koutsikou, 2021). The exNs process these signals by significantly extending the duration and also determining the action potentials that will be fired eventually by the hindbrain reticulospinal neurons (hdIN) (Koutsikou *et al.*, 2018; Roberts *et al.*, 2019; Messa and Koutsikou, 2021).

The level of sensory processing activities of the exN population reduces once swimming starts, but the level remains steady until swimming terminates (Borisyuk *et al.*, 2017). Following the receipt of excitation in the form of trains of variable summating EPSPs, the hdIN population (about 60 in total on both sides of the body) (Koutsikou *et al.*, 2018), depending on their membrane potential, initiate swimming by firing. This action, in turn, excites the motoneurons (Buhl, Soffe and Roberts, 2015; Koutsikou *et al.*, 2018; Roberts *et al.*, 2019; Messa and Koutsikou, 2021). In addition to the motoneurons, the hdIN population also activates reciprocal inhibitory neurons (Roberts *et al.*, 2019), and once swimming starts, the hdIN population fires only once per swimming cycle.

The reciprocal inhibition mechanism coordinates the firing of the hdIN population, ensuring that the population on both sides of the spinal motor pathway do not fire at the same time in one swimming. In other words, reciprocal inhibition tries to prevent

the synchronous contraction of opposing muscles. Despite this control mechanism, it is surprising to note that the hdIN population on both sides of the spinal motor pathway occasionally fires concurrently during a swimming cycle (Li *et al.*, 2014; Roberts *et al.*, 2014, 2019; Buhl, Soffe and Roberts, 2015; Koutsikou *et al.*, 2018). In addition to the above function, the reciprocal inhibition crucially sustains swimming by evoking rebound firing in the hdIN population (Li, Roberts and Soffe, 2009). It is proposed that this feature of the hdIN population is the main mechanism for maintaining swimming in the tadpoles (Li *et al.*, 2014). The hdIN population in the hatchling *Xenopus laevis* tadpole is homologous to the circumferential descending (CiD) neurons in the zebrafish embryo, excitatory interneurons (EINs) in the lamprey and V2a neurons (Gata3+) in the mouse (Goulding, 2009).

2.1 Struggling – A Surviving Tool of the Tadpole

Struggling is among the behavioural repertoire of the hatchling *Xenopus laevis* tadpole, and it is only employed when the tadpole is grasped. When grasped by predators or held firmly, the tadpole struggles by making a slower but highly vigorous series of alternating movements in an attempt to escape (Roberts, 1998; Roberts, Li and Soffe, 2010; Borisyuk *et al.*, 2017). Some of the predators of the tadpole are the adult *Xenopus laevis* frog, water scorpions, larvae of dragonflies, damselflies and beetles (Roberts, Li and Soffe, 2010; Buhl, Soffe and Roberts, 2015). Interestingly, the muscle contraction frequencies observed in struggling are slower than the flexions seen in swimming. Moreover, the waves of alternating flexions observed in struggling begin from the tadpole's tail and then proceed to its head, as is also the case in the larval zebrafish (Wang and McLean, 2014). This characteristic of the tadpole is

opposite to that of its swimming movements (Roberts, 1998; Roberts, Li and Soffe, 2010; Borisyuk *et al.*, 2017).

During struggling, additional CPG neuronal populations are recruited, including excitatory descending repetitive interneuron (dINr), excitatory commissural interneurons (eCINs), (Figure 2) (Li *et al.*, 2007). Each burst of action potentials fired during struggling last for about a maximum of 200 ms (Roberts, 1998; Borisyuk *et al.*, 2017). The tadpole stops struggling once the related stimulation stops. Following the cessation of stimulus delivery, the tadpole begins to swim even in situations where the tadpole was not swimming before the struggling period (Borisyuk *et al.*, 2017).

The escape response in fish, such as the zebrafish is characterised by extremely swift bending movements that look like the shape of the letter C. This response is mediated by Mauthner neurons (McDiarmid and Altig, 1999; Budick and O'Malley, 2000; Berg *et al.*, 2018; Koutsikou *et al.*, 2018; Roberts *et al.*, 2019; Haspel *et al.*, 2021), a group of large hindbrain neurons. During escape responses, the Mauthner neurons electrically synapse with Commissural local INs (CoLos) and also inhibit contralateral motoneurons (Berg *et al.*, 2018).

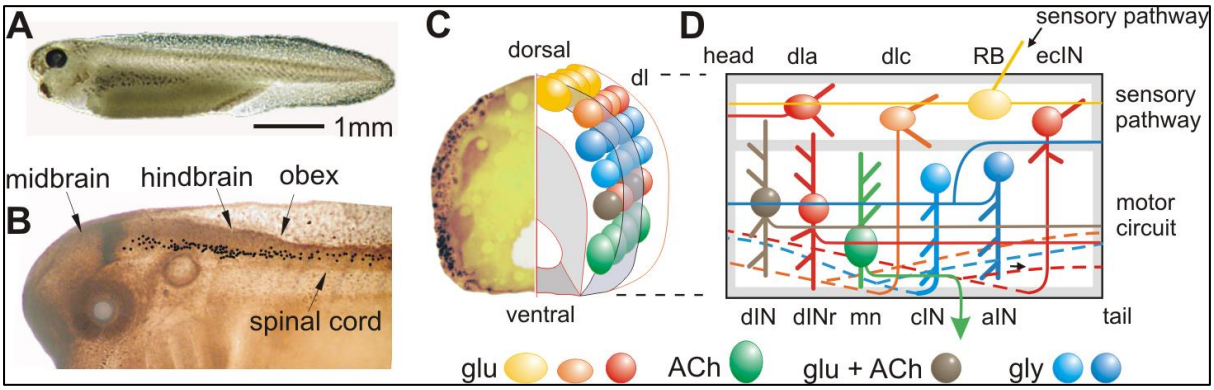


Figure 2. A schematic diagram summarising the sensorimotor neuronal pathway of the hatchling Xenopus laevis tadpole at developmental stage 37/38. *(A) Photo of a resting hatchling tadpole at stage 37/38. (B) Photo showing the side view of the head of a tadpole and also indicating with arrows some elements of its CNS. (C) Diagram illustrating the location as well as the orientation of the different neuronal types within the dorso-lateral space. (D) Schematic showing details about the different neuronal populations responsible for locomotion (for example, swimming and struggling) in the tadpole.*

2.2 Aims and Objectives

Given the lack of clarity on the interactions between the midbrain neurons and CPG network in the locomotion initiation system, this study was aimed at gaining an understanding of the role of the midbrain in initiating locomotion in the hatchling *Xenopus laevis* tadpole. The objective was to use behavioural and electrophysiological experiments to assess the effects of midbrain lesions on the tadpole's direction of swim initiation and its latency to swim onset. I hypothesise that the midbrain lesion will hinder the latency to onset and the direction of swimming, which will make the tadpole more vulnerable to predation.

3.0 MATERIALS AND METHODS

3.1 Ethical Approval

Xenopus laevis embryos were supplied by the European *Xenopus* Resource Centre (EXRC, Portsmouth). All experimental procedures were carried out on *Xenopus laevis* tadpoles at developmental stage 37/38, and they were conducted under the approval of University of Kent Animal Welfare and Ethical Review Body (AWERB). The temperature at which experiments was conducted was about 19 °C.

3.2 Animal Care

Xenopus laevis embryos (Figure 3C) were contained in Petri dishes and delivered in refrigerated boxes. Following delivery, the eggs that appeared to be non-viable were disposed of (Figure 3C). The healthy ones were kept in Tupperware storage containers containing tap water treated with a commercially available water conditioner (please refer to Appendix Aii for information on the preparation of tadpole water). The treatment was to remove heavy metals (dangerous to the eggs) from the tap water. The eggs were stored in an incubator, ensuring that the storage containers were not completely closed. The incubator was kept at a fixed temperature (16.6°C) to slow down the development of the embryos. The eggs were left undisturbed until they developed to the tadpole developmental stage of 37/38 (Figure 3B). At 16°C, the eggs normally reached this stage within 3 days. It was ensured that the developing tadpoles were kept in clean conditions by discarding the non-viable eggs and replacing the water daily.

Figure 3. Different Xenopus laevis Development Stages.

(A) Dorsal view of stage 1 (egg). Post fertilisation (pf) period of 0 minutes. Temperature of 23 °C. Drawing by Xenbase (http://www.xenbase.org/).

(B) Lateral view of stage 37/38 developing tadpole. Post fertilisation period: 53 hours. Temperature: 23°C. Photo by Xenbase (http://www.xenbase.org/, RRID: SCR_003280; Taken from Messa, Li and Koutsikou, 2018)

(C) Xenopus laevis embryos in a container of conditioned water following delivery from EXRC. Arrows show non-viable embryos.

3.3 Preparation for Behavioural Experiments

Behavioural experiments were carried out on three experimental groups of tadpoles at developmental stage 37/38 to measure the following swimming kinematic parameters: direction of the first bend and latency to swim initiation. All but one tadpole group were anaesthetised by being kept briefly in saline solution that contained 0.1% MS-222 (tricaine methanesulfonate, Sigma Aldrich, UK). The saline solution was

always made fresh in 1L batches (for more information on the 1L volume, please see Appendix Ai). The anaesthetised tadpoles were pinned on a rotatable Sylgard block inundated with saline (Figure 4), and different lesions were performed. The lesions were performed under a dissecting microscope with the following items: etched tungsten microneedles mounted to the tip of a glass pipette and fine forceps. In the control group of tadpoles, both trigeminal nerves were severed, and in the lesioned group, there was a midbrain-hindbrain border lesion in addition to the severing of both trigeminal nerves (Figure 5).

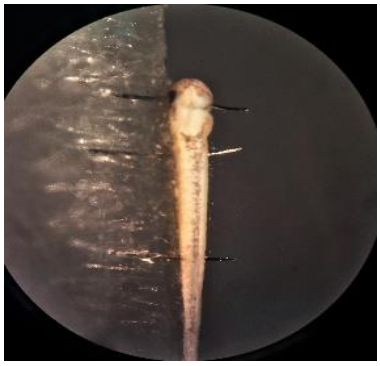


Figure 4. *Photo of an anaesthetised tadpole pinned on a Sylgard block prepared for lesioning. The tadpole was purposely pinned at the edge of the block to easily access the CNS. (Adapted from Messa, Duah and Koutsikou, 2019)*

3.3.1 Establishing the 'Control' group

The tadpole group with both trigeminal nerves severed (Figure 5B) was incorporated into this study to ensure that the skin impulse does not reach the CNS. The skin impulse generated by a brief touch on the trunk skin can lead to initiation of swimming. However, severance of the trigeminal nerves ensures that the swimming behaviour of the tadpoles was solely due to the excitation of the Rohon-Beard cells (Trunk skin pathway described on page 44) via brief touch applied to the trunk skin (James and

Soffe, 2011; Messa, 2021; Messa and Koutsikou, 2021). Severance of the trigeminal nerves does not cause significant changes to the side and latency of swim initiation (Figure 6A and B, respectively).

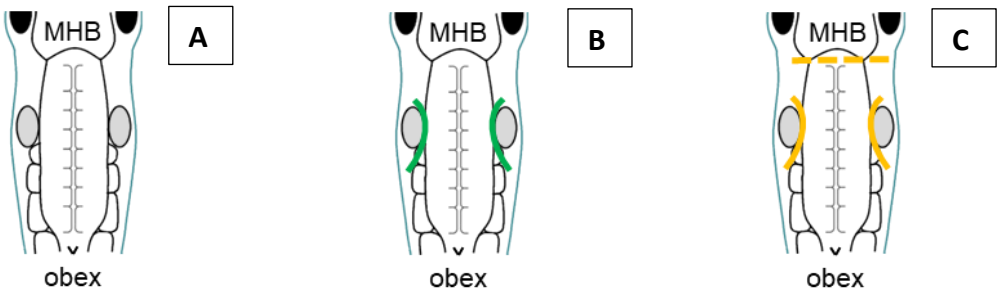


Figure 5. Schematic representation of all the tadpole groups involved in behavioural experiments.

(A) Schematic of the central nervous system of an intact tadpole.

(B) Schematic of the central nervous system of a tadpole with only the trigeminal nerves severed (control). The green marks indicate the cuts at the level of the otic capsule, an area of entrance to the brain for trigeminal nerves.

(C) Schematic of the central nervous system of a tadpole with a midbrain-hindbrain border lesion and severed trigeminal nerves. The short yellow dashes show lesions at the level of the midbrain-hindbrain border. The slightly curved yellow marks also indicate the cuts at the level of the otic capsule, an area of entrance to the brain for trigeminal nerves. (Adapted from Messa, Duah and Koutsikou, 2019)

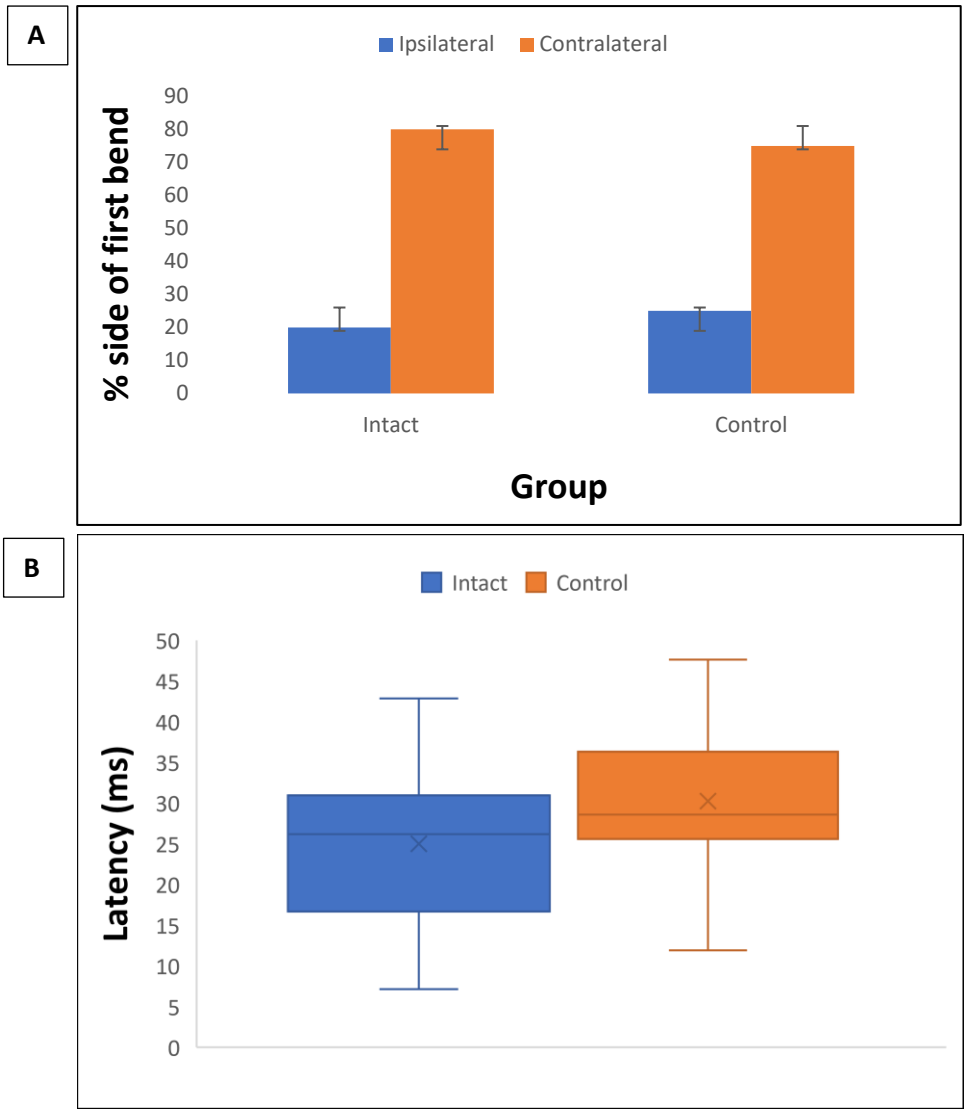


Figure 6. Comparing the behavioural data of the intact and control lesion groups

(A) Percentage of the direction of the first body bend following single trunk skin stimulus. N = 18 tadpoles (9 tadpoles in each group); n = 35 trials (intact), n = 40 trials (control). Error bars indicate standard deviation.

(B) Delays to swim initiation following trunk skin stimulation. N = 18 tadpoles (n = 9 for each group). n = 35 trials (intact), n = 40 trials (control). p=0.06 (unpaired t test). Milliseconds (ms).

3.3.2 Experimental Group: Midbrain-Hindbrain Lesioned Group (MHB)

For the experimental group, a lesion was made across the entire midbrain-hindbrain border (MHB), as well as severing both trigeminal nerves (Figure 5C). This group was called the “lesioned” group. Following the MHB lesion, the tadpoles were kept in saline until they recovered from anaesthesia and could resume swimming.

3.4 Behavioural Experiments

The tadpoles were put in a Sylgard-lined petri dish containing saline. The dish was placed on a bright-lit box illuminating the tadpole from underneath (Figure 7B). Using a fine rabbit hair (approximately 5 mm long) mounted to the tip of a glass pipette, the tadpoles were drawn into the small groove created in the Sylgard, ensuring that they were in an upright position (Figure 7A). Positioning the tadpoles upright allowed them to move freely in any direction, ensuring that initiation of swimming was not biased towards a particular direction. Moreover, this position prevented the activation of the cement gland through contact with the Sylgard platform. Slow-motion videos of the tadpoles were recorded at 420 frames per second (fps) using a Casio EX-F1 digital camera fitted on a metal support stand (Figure 7B). Strokes to the trunk skin were delivered using a fine hair.

The recording was stopped once the swim movements appeared to indicate a consistent trajectory. There was a gap of 2 minutes between the application of consecutive touch stimuli to allow the animal to return to a resting state. In total, 121 videos of 29 tadpoles were recorded, and here is the breakdown with respect to the tadpole groups: the intact group had 35 videos of 9 tadpoles, the control group had 40 videos of 9 tadpoles and the lesioned group had 46 videos of 11 tadpoles.

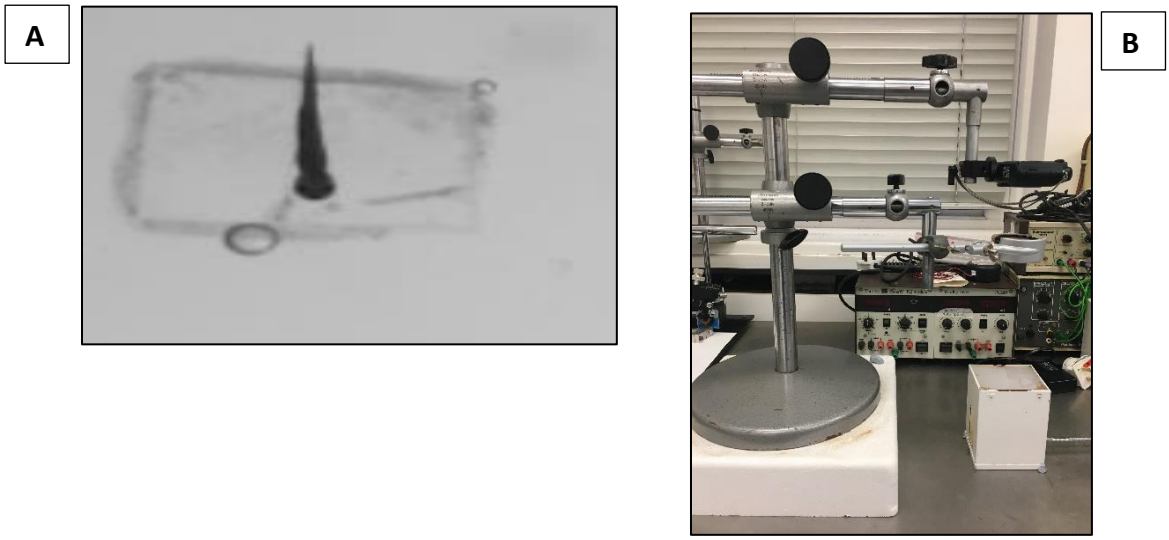


Figure 7. Behavioural experiments measured two swimming parameters.

(A) A photo of the whole view of a Sylgard-lined dish containing a tadpole in a behavioural experiment. The tadpole is positioned upright in a groove at the centre of the dish, with its head and tail free.

(B) A photo showing the equipment used in behavioural experiments, for example, a lightbox, metal support and Casio EX-F1 digital camera.

3.4.1 Data Analyses

Video analysis was conducted using ImageJ software by determining the latency (in number of frames) to swim start (Figure 8B) following a single trunk skin stimulus (Figure 8A). ImageJ was also used to measure the direction of the first swimming movement. Videos recorded in the AVI format were directly opened in ImageJ, but the videos recorded in the MOV format were first converted to single frame JPEG/PNG images using Adobe Photoshop software. The generated stacks of images were then opened in ImageJ in a sequential order. The ImageJ data were graphically represented in Microsoft Excel. The distribution of all sets of behavioural data was tested using, the D'Agostino-Pearson normality test (GraphPad Prism 9.0). The

outcome of normality test is presented in Table 2 (Appendix B). Statistical analyses were selected based on the outcome of the normality test and performed using IBM SPSS Statistics (version 27). The level of statistical significance of all the tests was set at p=0.05.

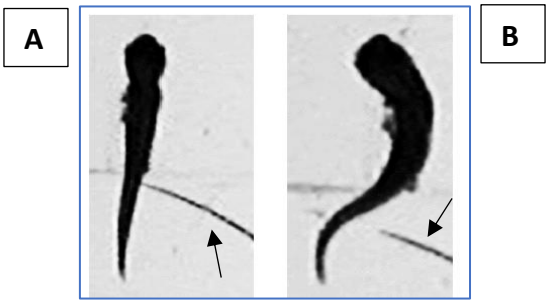


Figure 8. Single trunk skin stimulus was delivered in the behavioural experiments.
(A) A photo of a tadpole in an upright position being prodded. The arrow shows a strand of fine hair.
(B) A photo showing the first movement of a tadpole in response to a stimulus. The tadpole responded contralaterally to the trunk skin stimulus. (Adapted from Messa, Duah and Koutsikou, 2019)

3.5 Electrophysiology Experiments

Extracellular ventral root recordings, which indicate fictive swimming, were employed on two groups of immobilised tadpoles (control and lesioned groups) to assess the implication of midbrain lesion on their fictive swimming behaviour. Tadpoles were anaesthetised in MS-222 as above, followed by paralysis using 0.01mM α-bungarotoxin (Sigma-Aldrich, Dorset, UK) in saline for an hour. Paralysed tadpoles were pinned through the notochord onto a rotatable Sylgard block under a dissecting microscope. The electrical stimuli (constant current stimulator made in-house by Dr Steve Soffe) were applied on the tadpole's right trunk skin at the level of the anus (Figure 9A-B). The electrical stimuli were in two forms; one had the minimal voltage

needed to evoke fictive swimming, whereas the other had a voltage that was 1.5 V stronger than the threshold voltage. Ventral root activities on both sides of the trunk skin were recorded with two electrodes; each was placed close to a side (Figure 9A). Data were collected using Signal version 7 (Cambridge Electronics Design Ltd., Cambridge, UK) (Figure 10). Some of the data collected included frame number, side of the first spike/burst and the delay between stimulation and onset of fictive swimming (Figure 9A-B).

3.5.1 Data Analyses

The distribution of all sets of the electrophysiological data was tested using the D'Agostino-Pearson normality test (GraphPad Prism 9.0). The outcome of the normality tests is presented in Table 2 (Appendix B). Statistical analysis was selected based on the outcome of the normality test and conducted using IBM SPSS Statistics (version 27). The level of statistical significance of all the tests was set at $P = 0.05$.

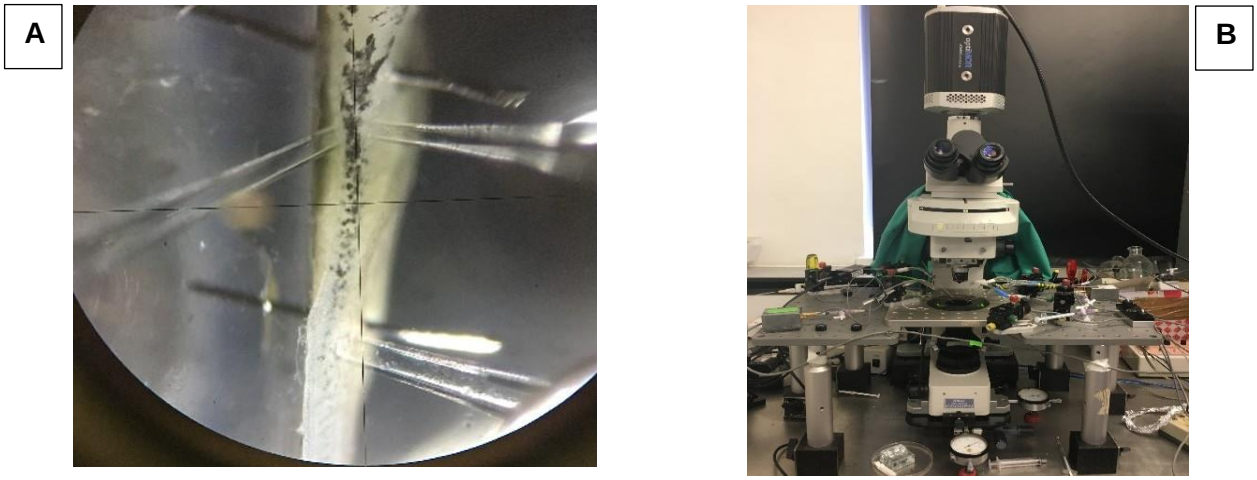


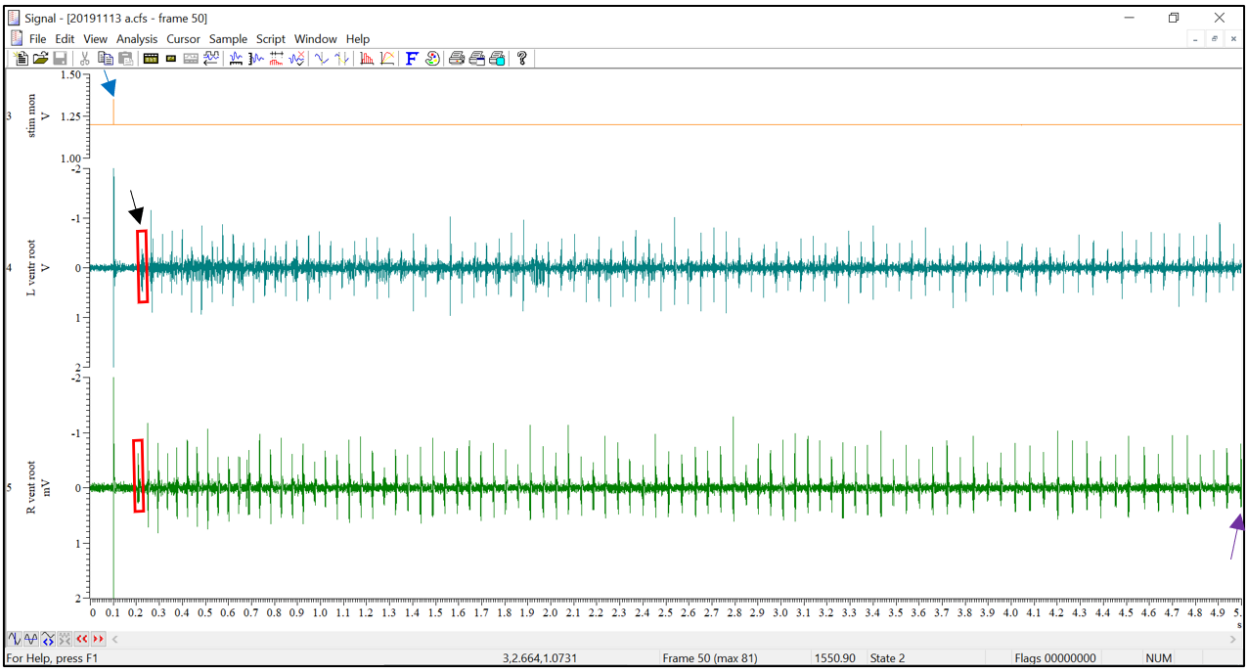
Figure 9. Extracellular ventral root recordings set up.

(A) Photograph of an immobilised tadpole prepared for extracellular ventral root recordings. Three electrodes were positioned on different areas of the tadpole. The electrodes positioned

on the upper left and upper right sides of the trunk skin recorded ventral root activities coming from the respective sides. The electrode positioned on the lower right side applied electrical stimulus. All stimulations were carried out on the right side.

(B) Photograph of extracellular ventral root recordings set up. Equipment involved include a Nikon Eclipse E600FN microscope, stimulating and recording electrodes connected to amplifiers and digitisers, and an air/anti-vibration table, which protects the setup from external forces such as vibration.

A



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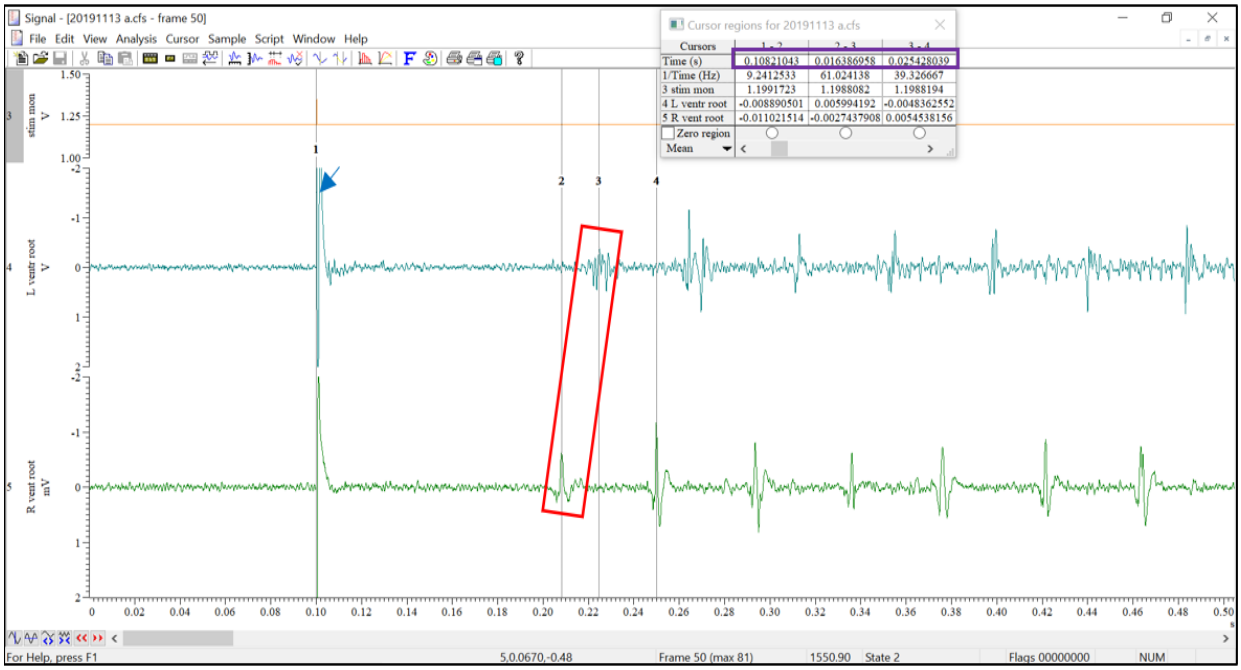

Figure 10. Screenshots of raw extracellular electrophysiology recordings with description on data analysis, using Signal version 7 (Cambridge Electronics Design Ltd., Cambridge, UK).

(A) A screenshot of a recording frame (frame number: 50; filename: 20191113 a; tadpole group: control) with raw data captured within a 5-second window. The top orange panel (channel 3) measures the strength and timing of the applied electrical stimulus, which was delivered by a homemade constant current stimulator made by Dr Steve Soffe. The electrical stimulation was performed on the right side of the tadpoles. The blue arrowhead points to the stimulus artefact, and in this recording frame, the stimulus was applied at 0.1 seconds. The strength of the stimulus is measured in volts (V). Extracellular ventral root activity from the left and right sides of the body are captured in the next two traces. The blue panel (channel 4) and the green panel (channel 5) measure in mV extracellular ventral root activities from the left and right sides, respectively. The black arrow indicates the initiation of fictive undulatory swimming on both sides of the body, with the left side starting first. The continuous ventral root activity indicates fictive undulatory swimming. The purple arrow indicates the end of the recorded ventral root (VR) activity within the 5-second window. It is worth noting that the purple arrowhead does not necessarily signify the end of the swimming episode because the tadpole may have swum beyond the 5-second window. The two red boxes represent one cycle of

fictive undulatory swimming. The number of individual swim cycles was measured using a pair of red boxes as presented above to count the total number of swim cycles within the 5-second window. I used this approach in all the 99 recording frames. The total number of swim cycles within this frame was 93.

(B) A screenshot of a recording frame (frame number: 50; file name: 20191113 a; tadpole group: control) with raw data captured within a 0.50-second window. The blue arrowhead points to the electrical stimulus located on the orange panel. The latency to swim initiation was measured by looking at the difference between cursor 1 (stimulus artefact on blue and green traces) and cursor 2 (first ventral root activity on green trace). Cursor 3 and cursor 4 represent the second and third ventral root activities, respectively. As shown in the table, the data highlighted in purple indicate the time (in seconds) between electrical stimulation and initiation of fictive swimming. The red box indicates the first recorded swim cycle initiated on the right side (green trace).

4.0 RESULTS

Both behavioural and electrophysiological experiments were employed to assess the effects of midbrain lesions on different aspects of the tadpole's swimming. These aspects include the latency to swim onset (behaviour), the direction of first body flexion (behaviour) and latency to the first ventral root activity, indicative of fictive swimming initiation (electrophysiology).

4.1 Behavioural Experiments

4.1.1 Effect of Midbrain Lesions on the Direction of Flexion of Swimming

In response to trunk skin stimulation, the tadpole first flexes its trunk before initiating swimming (Roberts, 1998; Buhl, Soffe and Roberts, 2015; Roberts *et al.*, 2019). The direction of first bend was measured behaviourally in tadpoles after gently stimulating their trunk skin with a fine hair. Slow-motion videos recorded at 420 fps were analysed in ImageJ. The analysis demonstrated that the two groups of tadpoles, that is, the control (N = 9 tadpoles) and lesioned groups (N = 11 tadpoles), 'preferred' responding contralaterally to the trunk skin stimuli (contralateral incidence for control group = 75% and lesioned group = 72%; Figure 11). Interestingly, the percentage incidence of ipsilateral response increased by 3% following the midbrain-hindbrain border lesion (ipsilateral incidence for control group = 25% and lesioned group = 28%; Figure 11). In summary, these data show that the disconnection of the midbrain from the rest of the brainstem does not alter the preference for contralateral swim initiation in the hatchling tadpole.

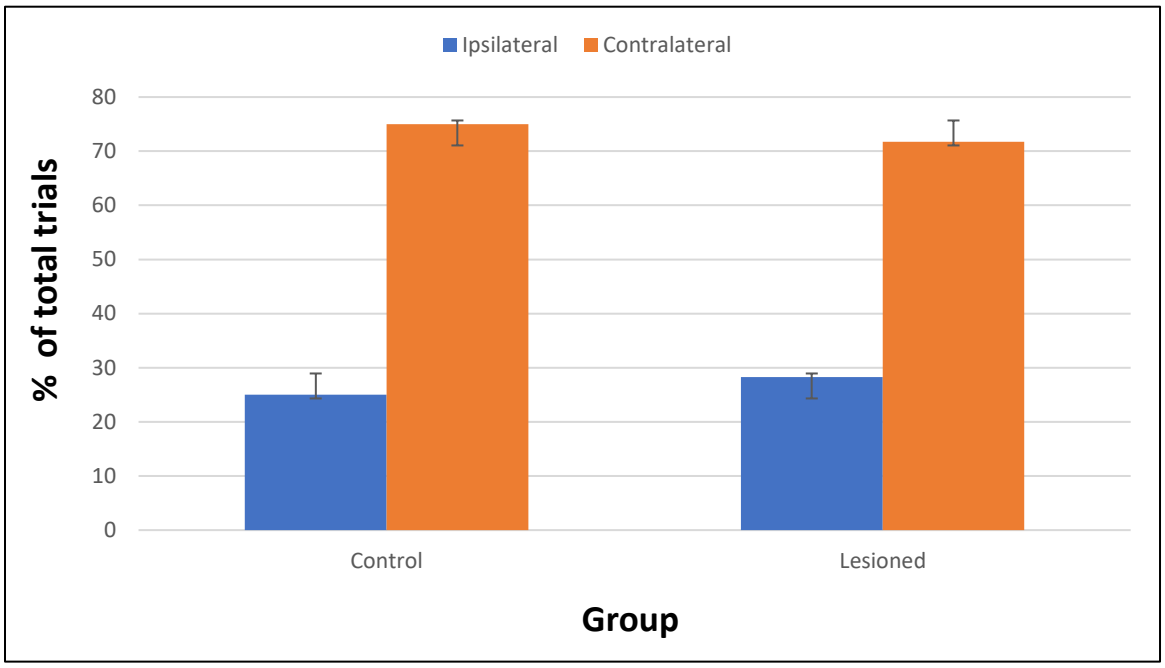

Figure 11. Side preference in swimming initiation following mechanical stimulus application in behavioural experiments.

Percentage values for direction of first body bend following trunk skin stimulation in control and lesioned animals. N = 20 tadpoles (9 control tadpoles and 11 lesioned tadpoles); n = 40 trials (control), n = 46 trials (lesioned). Black lines indicate standard deviation.

4.1.2 Effect of Midbrain Lesions on Latency to Swim Onset

The delay often observed between the time of trunk skin stimulation and the first bend allows the tadpole to make the right motor decisions. By doing this, the tadpole becomes less predictable, therefore, less susceptible to predatory attacks (Roberts *et al.*, 2019). The latency to swim onset measurements for the tadpole groups were obtained using ImageJ. An unpaired *t* test was conducted to identify the degree of influence of midbrain lesion on the latency to swim response. There was a significant increase in the latency to swim response between the control (N = 9 tadpoles; trials: n = 40) and lesioned (N = 11 tadpoles; trials: n = 46) tadpoles (mean: 29.35 vs 22.05;

mean difference: 7.30; S.E.M: 1.29 vs 1.11; t (84) = 4.307 p<0.0001; unpaired t test; Figure 12). Taken together, these findings suggest that the midbrain significantly controls the response time of tadpoles following the delivery of trunk skin stimulus.

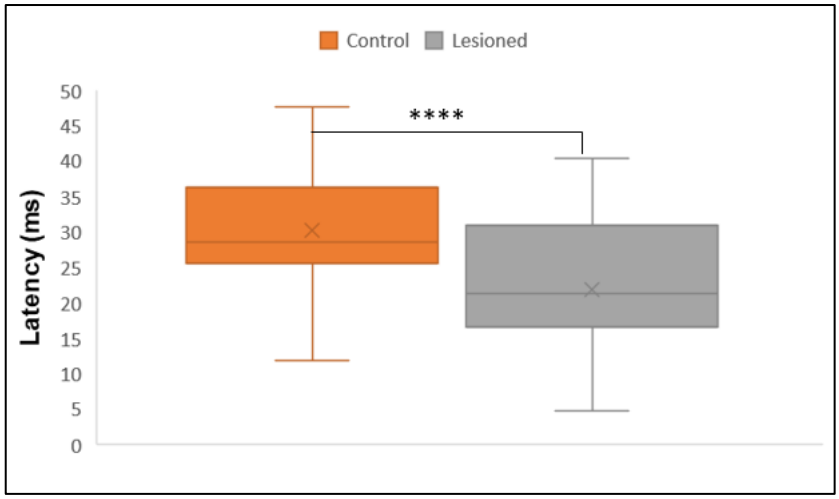

Figure 12. Latency to swimming initiation following trunk skin stimulation in behavioural experiments.

Latencies to first head bend following hair touch stimulation in control and lesioned animals. N = 20 tadpoles (9 in the control group and 11 in the lesioned group). n = 40 trials (control), n = 46 trials (lesioned). p<0.0001 (unpaired t test). Milliseconds (ms).

4.2 Extracellular Electrophysiology Recordings

Extracellular ventral root activity was recorded from immobilised tadpoles (control and lesioned) in response to threshold and suprathreshold electrical stimuli applied on their trunk skin at the level of the anus. The following parameters were measured.

4.2.1 Latency to Fictive Swim Initiation

The delays between most skin (including trunk skin) stimulation and swim starts are long and varied in *Xenopus* tadpoles (Buhl, Soffe and Roberts, 2015; Roberts *et al.*, 2019). Among the control group of tadpoles, the latencies to swim initiation following

threshold electrical stimulation were long and variable (95.84 ± 41.45 ms; Figure 13A). However, following suprathreshold electrical stimulation, the latencies of the control group were short and less variable (37.29 ± 21.90 ms, Figure 13B). Between the control (N = 5 animals; trials (n) = 18), and lesioned groups (N = 7 animals; trials (n) = 19), the response time of the lesioned group was significantly shorter than that of the control group after receiving threshold electrical stimuli (mean: 28.54 vs 95.84; mean difference: -67.30; S.E.M: 3.25 vs 9.77; $t(35) = 6.682$, $p<0.0001$; unpaired *t* test).

Likewise, following the delivery of suprathreshold stimulation, the response to swim initiation within the lesioned group (N = 7 animals; trials (n) = 19) was significantly faster (median: 17.91, IQR: 13.33) than the response of the control group (N = 5 animals; trials (n) = 18) (median: 17.91 vs 30.34; median difference: -12.43; IQR: 13.33 vs 15.55 U=162, $p=0.002$; Mann–Whitney U test). Collectively, these results suggest that at early developmental stages in tadpoles, the midbrain is capable of influencing the response time of swimming based on the strength of the stimulus applied.

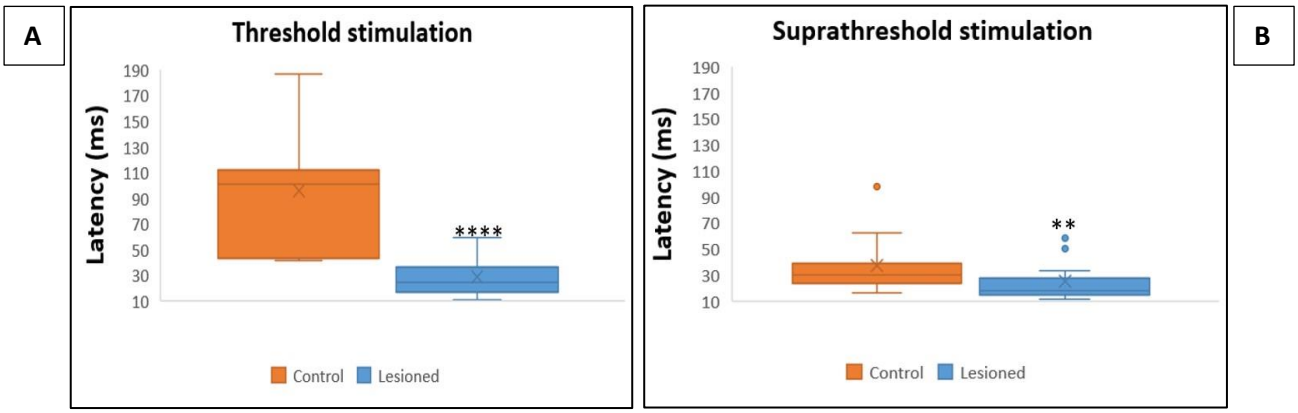

Figure 13. Latency in swimming initiation following threshold and suprathreshold electrical stimulus in extracellular ventral root recordings.

Latencies to the first VR burst following threshold (A) and suprathreshold (B) electrical stimulus delivered to the trunk skin of control and lesioned animals. N = 12 tadpoles (5 in the control

group and 7 in the lesioned group). n = 41 (control), n = 47 (lesioned). p<0.0001 (unpaired t test) (A). p=0.002 (Mann–Whitney test) (B). Milliseconds (ms).

4.2.2 Effect of midbrain-hindbrain border lesion on the side of fictive swim initiation

Following trunk skin stimulation, the tadpole has to decide whether to initiate swimming contralaterally or ipsilaterally (Buhl, Soffe and Roberts, 2015; Koutsikou *et al.*, 2018; Roberts *et al.*, 2019). The main observation made here was that the two different stimulation strengths evoked different outcomes in terms of the direction of the first movement. For example, when the control tadpoles were electrically stimulated at the threshold level, there was 64% chance of swim initiation on the ipsilateral (stimulated) side (right) (N = 5 tadpoles; n = 22 trials) (Figure 14A, Table 1). Conversely, when the stimulation intensity was increased to suprathreshold level (threshold voltage plus 1.5 V), the control tadpoles initiated contralateral responses more often (74%; N = 5 tadpoles; n = 27 trials; Figure 14B).

The suggestion that can be drawn from this observation is that the midbrain is highly influential in separating, and also evaluating different stimuli intensity to help the tadpole make the right motor decision. Deviant to the main observation above, and as Figure 14A-B and Table 1 shows, the percentage incidence of both the contralateral (48%) and ipsilateral (52%) response was similar among the lesioned group following threshold and suprathreshold stimulation. Moreover, the clear distinction in the ‘choice’ of direction observed in the control group has practically disappeared in the lesioned group following both stimulation intensities, as the probability of observing either a contralateral or ipsilateral response is nearly 50%. There was no statistically significant

association between the two stimulation intensities in the lesioned group; that is, both stimulation intensities exerted an equal level of influence on the side of fictive swim initiation ($\chi(1) = 0.029$, p=0.864, Pearson's chi-square test).

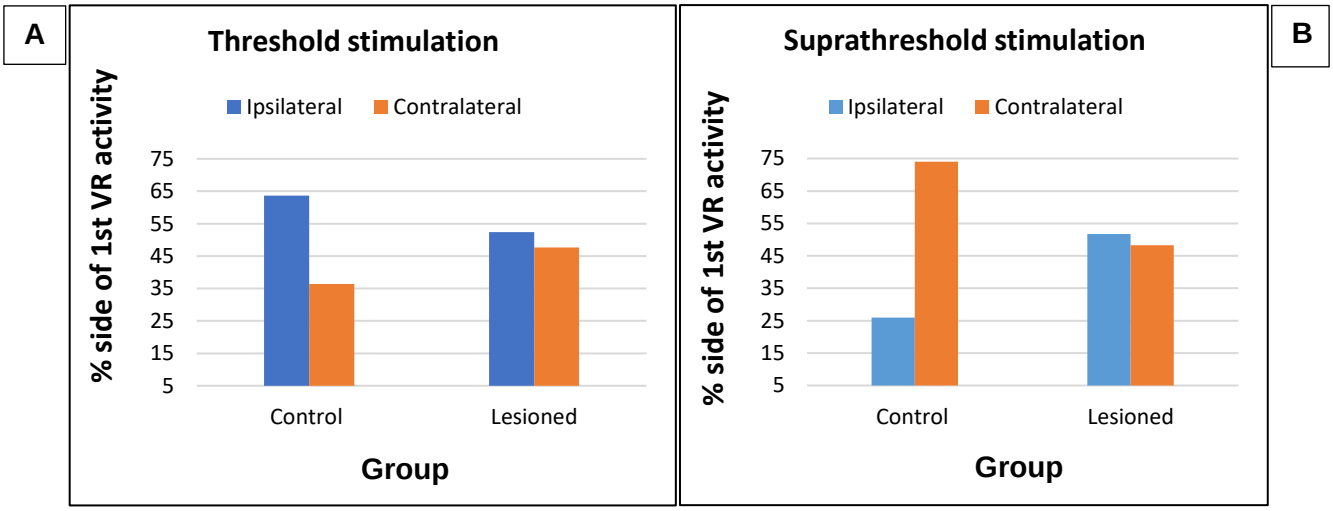

Figure 14. Side preference in swimming initiation following threshold and suprathreshold electrical stimulus in extracellular ventral root recordings.

Percentage values for the side of the first VR burst in response to threshold (A) and suprathreshold (B) electrical stimulus delivered to the trunk skin of control and lesioned animals. N = 12 tadpoles (5 in the control group and 7 in the lesioned group). n = 49 trials (control), n = 50 trials (lesioned). VR = ventral root.

	Threshold (%)		Suprathreshold (%)	
	Ipsilateral	**Contralateral**	**Ipsilateral**	**Contralateral**
Control	64	36	26	74
Lesioned	52	48	52	48

Table 1: Percentage occurrence of first ventral root (VR) activity on the contralateral versus ipsilateral side in response to threshold and suprathreshold electrical stimulation of the tadpole's trunk skin. N = 12 tadpoles (5 in the control group and 7 in the lesioned group). n = 49 trials (control), n = 50 trials (lesioned).

4.2.3 Frequency of fictive undulatory swimming

The undulatory locomotor movement of the tadpole (Grillner and El Manira, 2020) progresses from the head to the tail, and it is also characterised by the alternation of the left and right sides of its trunk/body (Buhl, Soffe and Roberts, 2015; Koutsikou *et al.*, 2018; Roberts *et al.*, 2019). The frequency of fictive swimming was measured using the formula below.

$$Freqency\ of\ swimming\ (Hz) = \frac{number\ of\ fictive\ swim\ cycles\ (events)}{t2 - t1}$$

The variables t1 and t2, respectively, represent the times of the first and last ventral root activity within the initial 5-second window of fictive swimming. Even at this early stage of development, the tadpole CNS can discriminate between different stimulus intensities and respond accordingly (Figure 14). When the control group of tadpoles (N = 5 animals; trials (n) = 21) were subjected to a threshold electrical stimulation of the trunk skin, the observed frequency of fictive swimming at the start of the episode (within the first 5-seconds) was significantly lower, compared to when the stimulation strength was increased to suprathreshold level (N = 5 tadpoles; trials (n) = 26) (threshold median: 18.66 Hz vs suprathreshold median: 21.26 Hz; median difference: -2.6 Hz; IQR: 1.38 vs 4.86; U=126.5, p=0.002; Mann–Whitney U test; Figure 15A).

The midbrain lesion at the MHB led to significant changes in frequency between the control (N = 5 tadpoles; trials (n) = 21) and lesioned groups (N = 7 tadpoles; trials (n) = 21) following the delivery of threshold electrical stimulus only (control mean: 18.66 vs lesioned mean: 20.92; mean difference: -2.26; control S.E.M: 0.30 vs lesioned S.E.M: 0.26; t(40) = -5.858, p<0.0001; unpaired t test; Figure 15B). In contrast, following delivery of suprathreshold electrical stimulus only, the frequency of fictive

swimming was not statistically different between the control (N = 5 tadpoles; trials (n) = 26) and lesioned groups (N = 7 tadpoles; trials (n) = 28) (control median: 21.26 Hz vs lesioned median: 20.67; control IQR: 4.86 vs lesioned IQR: 2.51; U = 299.5, p=0.264; Mann-Whitney U test; Figure 15C).

Interestingly, the difference in frequency of fictive swimming we observed in response to different strengths of stimulation in Figure 15A has been lost following the MHB lesions. Lesioned tadpoles respond in a similar way, and at a higher frequency to both types of stimulation (threshold mean: 20.92 vs suprathreshold mean 20.44; mean difference: 0.48; S.E.M: 0.26 vs 0.29; t(47)=1.171, p=0.247 unpaired t test; Figure 15D). Taken together, these results suggest that the midbrain influences how fast or slow the tadpole swims, as it is known to do through the nMLF in the larval zebrafish (Severi et al., 2014).

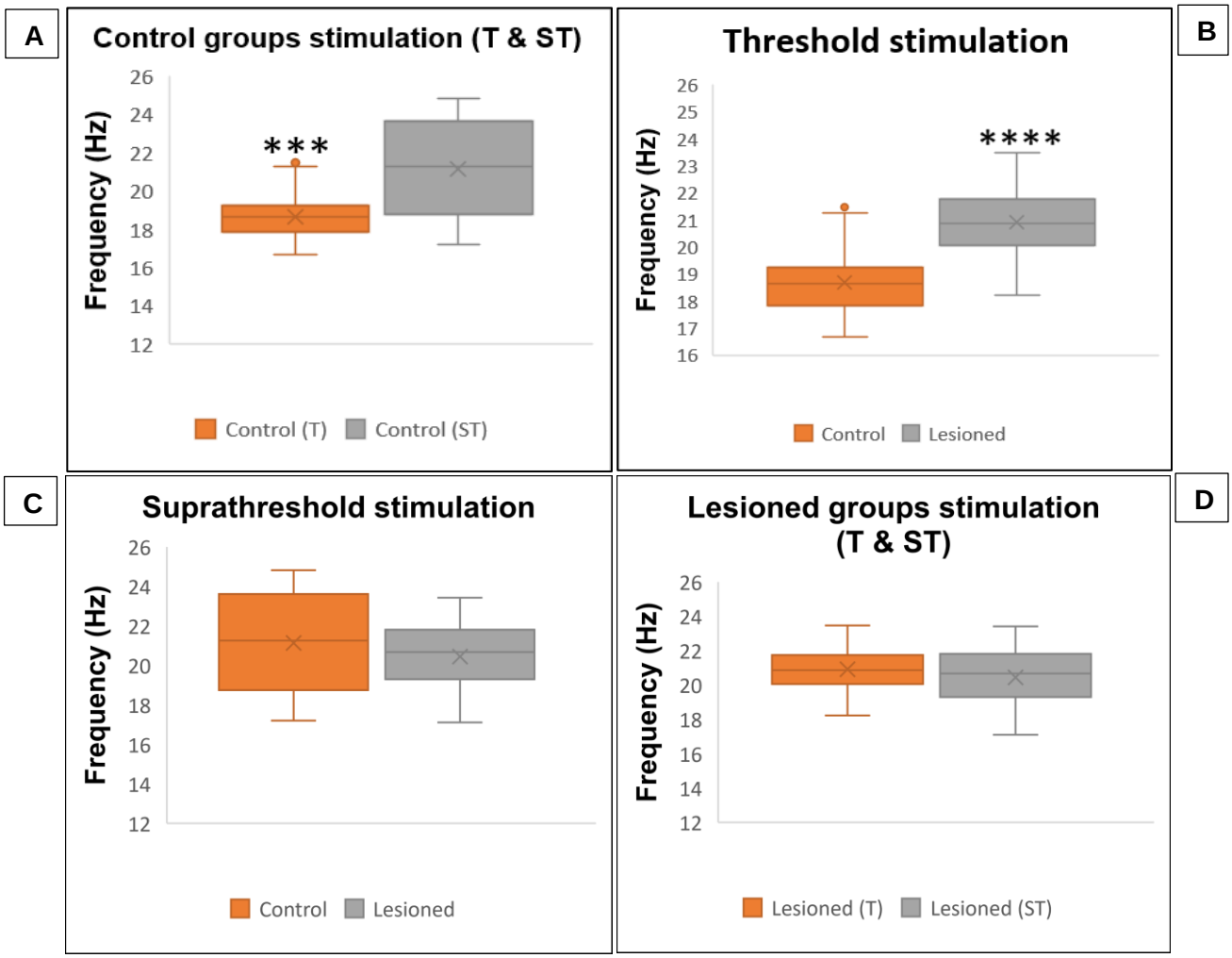

Figure 15. Frequency of fictive swimming following threshold and suprathreshold electrical stimuli in extracellular ventral root recordings.

(A) Comparing the effect of threshold and suprathreshold electrical stimuli on the frequency of fictive swimming among the control tadpoles (threshold: N = 5 tadpoles, trials (n) = 21; suprathreshold: N = 5 tadpoles, trials (n) = 26; p=0.002, Mann–Whitney U test).

(B) Comparing the effect of only threshold electrical stimulus on the frequency of fictive swimming among the control and lesioned tadpoles (control: N = 5 tadpoles, trials (n) = 21; lesioned: N = 7 tadpoles, trials (n) = 21; p<0.0001; unpaired t test).

(C) Comparing the effect of only suprathreshold electrical stimulus on the frequency of fictive swimming among the control and lesioned tadpoles (control: N = 5 tadpoles, trials (n) = 26; lesioned: N = 7 tadpoles, trials (n) = 28; p=0.264; Mann-Whitney U test).

(D) Comparing the effect of threshold and suprathreshold electrical stimuli on the frequency of fictive swimming among the lesioned tadpoles (threshold: N = 5 tadpoles; trials (n) = 26; suprathreshold: N = 7 tadpoles, trials (n) = 28; p=0.247 unpaired t test).

Hertz (Hz). Threshold (T). Suprathreshold (ST).

4.2.4 Effect of midbrain-hindbrain border lesion on the relationship between the side of first VR burst and latency to fictive swimming

The data have been grouped according to the side of the first VR burst and Figure 16 presents median latencies for those first bursts in response to threshold (Figure 16A) and suprathreshold stimulation (Figure 16 B). The first interesting observation made from this presentation is that despite the effect of the electrical stimulation intensity on the side of swim initiation in control tadpoles, with threshold stimulation leading to more ipsilateral starts while suprathreshold stimulation leads to more contralateral starts (Figure 14), the latency to ipsilateral starts is always significantly longer compared to the contralateral starts (Figure 16A&B; threshold stimulation: ipsi/c median latency = 109.97 ms, IQR = 12.05 ms, N = 5 tadpoles and n = 15 trials vs contra/c median latency = 35.09 ms, IQR = 0.19 ms, N = 5 tadpoles and n = 22 trials; Kruskal-Wallis with Dunn's post-hoc test $p<0.0001$ and suprathreshold stimulation: ipsi/c median latency = 97.51 ms, IQR = 70.90 ms, N = 5 tadpoles and n = 17 trials vs contra/c median latency = 40.40 ms, IQR = 28.87 ms, N = 5 tadpoles and n = 20 trials; Kruskal-Wallis with Dunn's post-hoc test $p=0.0129$).

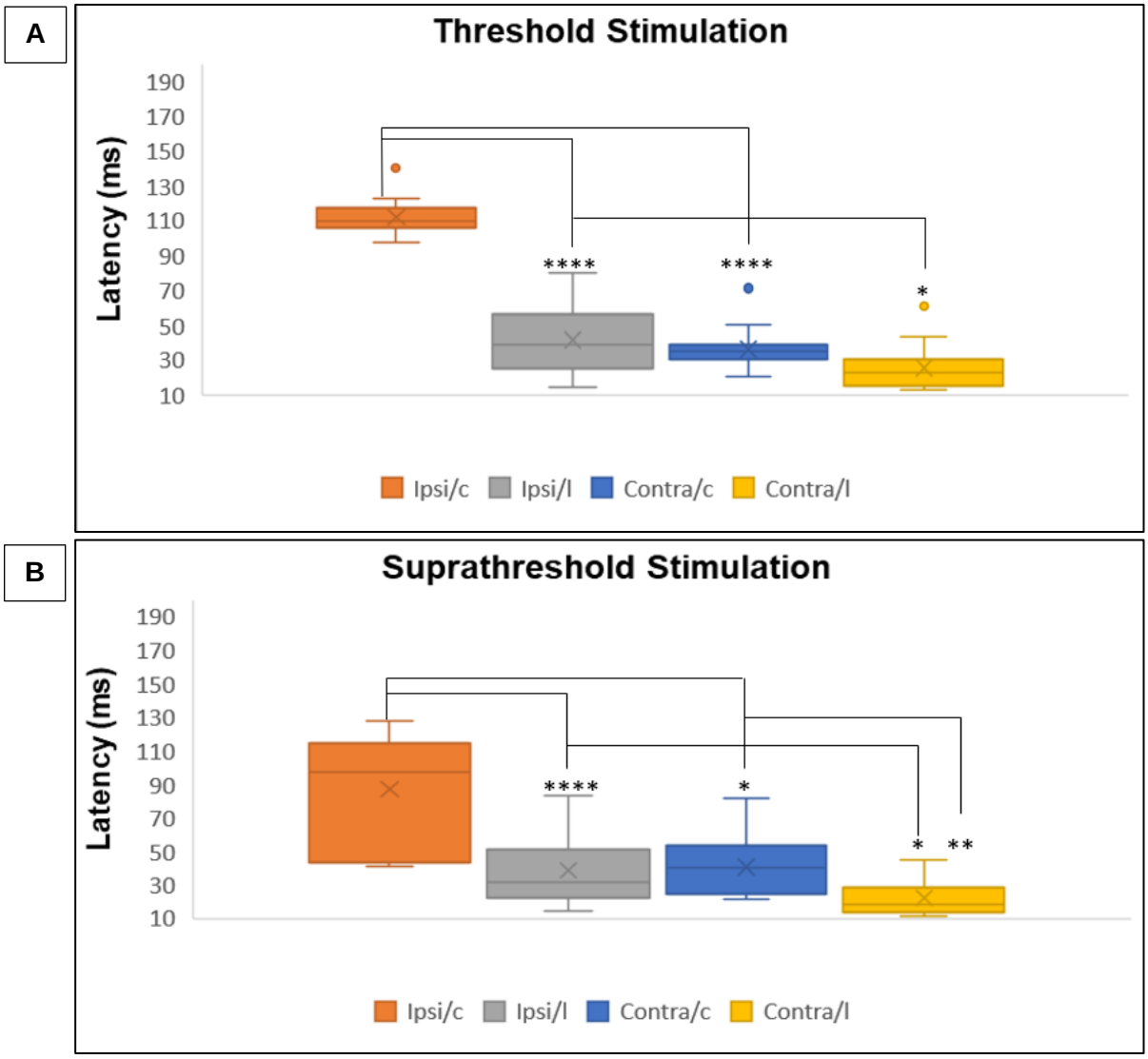

Figure 16. Effect of midbrain-hindbrain border lesion on the relationship between the side of first VR burst and latency to fictive swimming following threshold and suprathreshold electrical stimulus.

(A) Comparing the effect of threshold electrical stimulus on the relationship between the side of first VR burst and latency to fictive swimming among the control and lesioned tadpoles (Ipsi/c: N = 5 tadpoles, trials (n) = 15; Ipsi/I: N = 7 tadpoles; trials (n) = 20; Contra/c: N = 5 tadpoles, trials (n) = 22; Contra/I: N = 5 tadpoles, trials (n) = 21). Data groups were compared using Kruskal-Wallis test followed by Dunn's multiple comparison post-hoc test: Ipsi/c vs Ipsi/I, p=0.0002; Ipsi/c vs Contra/c, p<0.0001; Ipsi/I vs Contra/I, p=0.0340; Contra/c vs Contra/I, p=0.0818. Ipsilateral starts of control tadpoles (Ipsi/c); Ipsilateral

starts of lesioned tadpoles (Ipsi/l); Contralateral starts of control tadpoles (Contra/c); Contralateral starts of lesioned tadpoles (Contra/l); Milliseconds (ms).

(B) *Comparing the effect of suprathreshold electrical stimulus on the relationship between the side of first VR burst and latency to fictive swimming among the control and lesioned tadpoles (Ipsi/c: N = 5 tadpoles, trials (n) = 17; Ipsi/l: N = 7 tadpoles; trials (n) = 24; Contra/c: N = 5 tadpoles, trials (n) = 20; Contra/l: N = 7 tadpoles, trials (n) = 25). Data groups were compared using Kruskal-Wallis test followed by Dunn's multiple comparison post-hoc test: Ipsi/c vs Ipsi/l, p=0.0007; Ipsi/c vs Contra/c, p<0.0129; Ipsi/l vs Contra/l, p=0.025; Contra/c vs Contra/l, p=0.0038. Ipsilateral starts of control tadpoles (Ipsi/c); Ipsilateral starts of lesioned tadpoles (Ipsi/l); Contralateral starts of control tadpoles (Contra/c); Contralateral starts of lesioned tadpoles (Contra/l); Milliseconds (ms).*

5.0 DISCUSSION

Behaviour is a broad term that describes all activities that are carried out by an organism following a change. Locomotion is a common but essential type of behaviour performed by all animals to ensure survival. All animals exhibit locomotor behaviour to, among other things, feed, reproduce and evade dangerous stimuli. Given the crucial role of locomotion in the life of animals, a significant portion of all the activities of the nervous system leads to motor behaviour (Arber and Costa, 2018).

Locomotion is generally controlled by the spinal and supraspinal neuronal elements that work collaboratively to ensure that the goal of every locomotor movement is achieved (Le Ray *et al.*, 2011; Kiehn and Dougherty, 2013; Grillner and El Manira, 2020). In vertebrates, the CPG networks in the spinal cord generate locomotor movements following the receipt of motor commands directly and indirectly from the brain regions. Of all the supraspinal bodies, the MLR in the midbrain is widely regarded as the most important neuronal body that controls the activities of the CPG networks (Caggiano *et al.*, 2018). MLR neurons activate a population of reticulospinal cells, which in turn excite the CPG networks to eventually initiate locomotion. Since the original work of Shik, Severin and Orlovskiĭ (1966), which led to the discovery of MLR, several studies involving different vertebrate models (Jamieson and Roberts, 1999, 2000; Musienko *et al.*, 2012; Severi *et al.*, 2014; Wang and McLean, 2014; Roseberry *et al.*, 2016; Caggiano *et al.*, 2018; Josset *et al.*, 2018) have shown that the midbrain is instrumental in the controlling of different locomotor parameters.

Despite the huge body of evidence and technological advancement that has taken place in the field of neurobiology of motor control, our understanding of the neuron-to-

neuron connections from the midbrain to the spinal CPG networks remains poorly understood. This is due to the complex nature of the nervous system of common vertebrate animal models such as mice and rats. Thus, studying the neuronal mechanisms that underlie locomotion in lower vertebrates is an effective way of bridging the knowledge gap. This is because lower vertebrates, unlike complex vertebrate models, have simple nervous systems, which are characterised by a far fewer number of neurons compared to that of higher vertebrates (Roberts, Li and Soffe, 2010; Roberts *et al.*, 2019). Crucially, the neuronal organisation of the elements involved in the initiation and control of locomotor behaviour is remarkably similar across vertebrate species (Goulding, 2009; Berg *et al.*, 2018). Studying the locomotor behaviour of a simple vertebrate system at an early developmental stage is more advantageous as that further reduces the basic problem of complexity of the nervous system (Roberts, Li and Soffe, 2010; Roberts *et al.*, 2019).

In this study, the hatchling *Xenopus laevis* tadpole at developmental stage 37/38 was chosen as the model due to the simple and well-understood organisation of the neuronal circuitry responsible for swimming (Roberts, Li and Soffe, 2010; Borisyuk *et al.*, 2017; Messa and Koutsikou, 2021). By disconnecting the midbrain from the rest of the brainstem and spinal cord, I have observed significant changes to the latency to swim initiation and a lack of sensory discrimination in the tadpole.

In response to single, brief trunk skin stimulus during behavioural experiments, all tadpole groups strongly ‘preferred’ initiating swimming on the unstimulated side (Figure 11). It is well known that the tadpole responds to most skin touch, including trunk skin touch by initiating swimming either on the stimulated or unstimulated side

(Clarke and Roberts, 1984; Roberts, 1998; Roberts, Li and Soffe, 2010; Buhl, Roberts and Soffe, 2012; Roberts *et al.*, 2014, 2019; Buhl, Soffe and Roberts, 2015; Koutsikou *et al.*, 2018). In this set of behavioural experiments, the MHB lesion did not appear to alter the preference for swim initiation on the unstimulated side, which suggests that the midbrain is not involved in the choice of side for swim initiation, following trunk skin touch stimulation. It is likely that the neural circuit underlying this aspect of initiation of swimming is primarily located within the rest of the brainstem and thus remained intact following the disconnection of the midbrain from the rest of the brainstem and the spinal cord. So, I suggest that this neural network is actually located within the hindbrain.

Similar to the behavioural data acquired through experimental work presented here, Koutsikou and colleagues *(*2018) reported that in 114 trials, the probability of tadpoles (n = 19) initiating contralateral swimming following trunk skin touch was 68.4%. Likewise, the results of behavioural as well as electrophysiological experiments conducted by Buhl, Soffe and Roberts (2015) showed that the overall 'preferred' side of swim initiation among tadpoles was the unstimulated side. For example, in the behavioural experiments involving 30 tadpoles, the percentage incidence of ipsilateral response was 36.7%. Unlike the sensory pathway activated in my experiment as well as Koutsikou and colleagues *(*2018)'s, the work of Buhl, Soffe and Roberts (2015) activated the head skin touch (HST) pathway of the tadpole following the delivery of head skin stimuli.

As Figure 11 shows, the highest percentage incidence of ipsilateral response recorded was 28%. Differently, in response to threshold and suprathreshold electrical stimuli, the percentage incidence of the direction of the first ventral root (VR) burst indicative

of the initiation of fictive swimming was relatively more varied as compared to the behavioural data (Figure 14). This time only the control groups strongly 'favoured' a particular side for initiation, that is, ipsilateral and contralateral responses following threshold and suprathreshold electrical stimulation, respectively. The responses of the control group are not surprising because, from a survival point of view, the decision to move contralaterally following the application of a potentially damaging stimulus (suprathreshold) is the best as that improves the tadpole's chances of successfully evading predatory attacks from the larvae of dragonflies, damselflies and beetles (Roberts, Li and Soffe, 2010; Buhl, Soffe and Roberts, 2015), the main predators of the hatchling tadpole. Interestingly, the percentage incidence of ipsilateral (52%) and contralateral (48%) responses did not change when the lesioned tadpoles were electrically stimulated at the threshold and suprathreshold levels.

The differences in data between behavioural and electrophysiological experiments regarding the side of the first movement can be attributed to the stimulation methods used in these experiments. For example, the number of Rohon-Beard cells likely to be recruited following electrical stimulation of the trunk skin is higher than that of the mechanical trunk skin stimulation, because the diameter of the suction electrode is larger than that of the rabbit hair used for the application of the touch stimulus.

In both the behavioural and extracellular ventral root recordings, the lesioned group of tadpoles initiated swimming significantly faster than the control tadpoles (Figure 12-13). These data, together with the first and recent evidence of the distributions and firing characteristics of the proposed exNs (Borisyuk *et al.*, 2017; Koutsikou *et al.*, 2018; Roberts *et al.*, 2019; Messa and Koutsikou, 2021), highlight the possibility that a subpopulation of exNs could be located in the midbrain of the tadpole. The exN

neurons have been largely considered to be responsible for the long and varied latencies to swim onset observed in the tadpole following the application of sensory stimulus (Buhl, Soffe and Roberts, 2015; Koutsikou *et al.*, 2018; Roberts *et al.*, 2019). The delay of swim start allows the tadpole to make the right motor decisions. Also, the variability introduced in the response makes the tadpole less prone to predatory attacks, because its movements become less predictable (Buhl, Soffe and Roberts, 2015; Koutsikou *et al.*, 2018; Roberts *et al.*, 2019). The suggestion that some, if not all, midbrain neurons could be an essential part of the exN network is also supported by previous work showing that the D/MD neurons in the midbrain, which are excited by the pgc in the LD pathway of the tadpole, influence the response time following light dimming (Jamieson and Roberts, 1999).

Individually, the extracellular ventral root recordings, indicative of fictive swimming, revealed that the latency to the first ventral root activity was shorter following a strong stimulus when compared to the responses to threshold stimulus. The MHB lesion practically abolished that difference in latency between the threshold and suprathreshold stimuli (Figure 13). Buhl, Soffe and Roberts (2015) reported similar findings following the delivery of stronger electrical stimuli to the head skin of tadpoles. This revelation, together with the data on the side of the first bend following electrical stimulation on the trunk skin of the lesioned tadpoles, suggest that the midbrain is influential in sensory discrimination even at the early stages of development.

The midbrain lesions were shown to substantially influence the tadpole's swimming frequency or 'speed' based on the strength of stimuli. Data from the extracellular ventral root recordings showed that the lesioned tadpoles significantly 'swam' faster than the control tadpoles following threshold stimulation only (Figure 15B). When the

stimulus strength was increased to suprathreshold, the frequency of fictive swimming within the lesioned group was not statistically different to that of the control group (control: 20.57 Hz, lesioned: 19.62 Hz; Figure 15C).The frequency of fictive swimming in response to strong stimuli among the control group was significantly higher than the swimming frequency following threshold stimuli. This observation supports a well-established theory on the MLR. That is, electrical stimuli strength is directly proportional to the speed of locomotor movements generated (Le Ray *et al.*, 2011; Gariépy *et al.*, 2012; Schwartz *et al.*, 2012a; Kiehn and Dougherty, 2013; Grillner and El Manira, 2020). This characteristic of the MLR has been shown in several vertebrate species, in addition to the cats, the first model to demonstrate this incredible feature (Shik, Severin and Orlovskiĭ, 1966). Examples of the other vertebrate systems include the carp (*Cyprinus carpio*) (Kashin, Feldman and Orlovsky, 1974), goldfish (Budick and O'Malley, 2000), salamander (Cabelguen, Bourcier-Lucas and Dubuc, 2003), rabbit (Musienko *et al.*, 2008), mouse (Roseberry *et al.*, 2016), Atlantic stingray (*Dasyatis sabina*) (Livingston and Leonard, 1990) and lamprey (McClellan and Grillner, 1984; Sirota, Di Prisco and Dubuc, 2000). Given my data and this theory, it was therefore, not surprising to detect changes in the swimming frequency following the midbrain lesions.

5.1 Conclusion

The midbrain, through the reticulospinal cells, indirectly controls the activities of the CPG networks in the locomotion initiation system. The interaction between the midbrain neurons and spinal cord neural circuitry remains poorly understood. In this study, I have shown using a simple model system, hatchling *Xenopus laevis* tadpole, that midbrain lesions that stop the neural transmission between the midbrain and the

rest of the brainstem as well as the spinal cord highly influence many swimming parameters. These parameters include the direction of the first bend and the latency to swim onset: two variables or measurements that are crucial for the survival of this simple organism. Furthermore, the midbrain in this animal is crucial in the discrimination between highly salient versus less harmful stimuli that lead to distinct motor responses. I believe that understanding how the midbrain controls movement in this simple vertebrate forms a significant step in understanding the brain circuits controlling complex movements in higher vertebrates, like humans. By understanding how human neural circuits control movement, we will, in turn, be able to identify how these neural circuits go wrong in motor disorders like Parkinson's disease.

5.2 Future Work

Going forward, electrophysiology experiments will be conducted to directly record from and characterise the midbrain neurons that are responsible for initiating swimming following skin stimulation. This experiment will potentially be the first to characterise the involvement of the midbrain neural circuitry in the trunk skin touch (TST) pathway of the tadpole. Currently, the only midbrain neurons characterised in the *Xenopus laevis* tadpole are the third-order D/MD neurons, which are involved in the LD pathway.

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APPENDIXES Behaviour Experiment

APPENDIX (Ai) – Tadpole saline solution (1L)

The ingredients used to prepare the saline solution used in the behavioural and the extracellular electrophysiology recordings were: 6.67g of 115mM NaCl (sodium chloride), 2.39g of 10mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 5ml of 0.5M $NaHCO_3$ (sodium bicarbonate), 3ml of 1M KCl (potassium chloride), 2ml of 1M $CaCl_2$ (calcium chloride), and 1ml of 1M $MgCl_2$ (magnesium dichloride).

The above quantities of the solid (powder) ingredients were measured and transferred into a 2L beaker and completely covered the beaker with a parafilm. Note: a 2L beaker was used instead of a 1L beaker to reduce the possibility of spillage when the solution was being stirred. Here, the beaker was filled up with deionized water to about 800ml and using a measuring cylinder, it was topped up to 1L. Now, the rest of the ingredients (that is, the liquids) were added to the solution, and the beaker covered was covered and placed on the magnetic stirrer hot plate. Then the solution was gently stirred until it was completely homogeneous. At this point, the pH of the solution was adjusted to 7.4. This was done by a gradual addition of NaOH (sodium hydroxide) in the following order: 1.515ml (starting with 100µl (10x), followed by 60µl (6x), then 75µl (1x) and finally 80µl (1x)) of NaOH. Lastly the saline solution was poured into a labelled bottle and kept in a refrigerator.

APPENDIX (Aii) - Tadpole water (10L)

Firstly, a 10L container was filled with tap water. Then 1.25ml of commercially available water conditioner was added to the tap water (Note: this volume was used because for every 40L of water, 5ml water conditioner must be used). To prevent direct contact with sunshine, the outer part of the container was covered with an aluminium foil. Afterwards, an air pump cord was placed in the container, and with the container partially closed, the pump was switched on. Finally, the air pump was turned off after running for about 2 hours.

APPENDIX B – Normality Test

Table 2: The outcomes of D'Agostino-Pearson normality test guiding the choice of statistical tests.

Threshold stimulation (T); Control animals (Ctrl); Lesioned animals (Les); Suprathreshold stimulation (ST); Ipsilateral starts of control animals (Ipsi/c); Ipsilateral starts of lesioned animals (Ipsi/l); Contralateral starts of control animals (Contra/c); Contralateral starts of lesioned animals (Contra/l)

	K2	P value	Passed (alpha=0.05)?
Latency to swim onset (Behavioural experiment)	**Intact**: 0.8918	0.6402	Yes
	Control: 0.3195	0.8524	Yes
	Lesioned: 0.4527	0.7974	Yes
Latency to fictive swim onset (Ventral root recording)	**Control**: 20.41	<0.0001	No
	Lesioned: 9.01	0.0111	No

Frequency of fictive swimming	**T (Ctrl v Les)**: 1.523	0.467	Yes
	Ctrl (T v ST): 9.685	0.008	No
	ST (Ctrl v Les): 8.739	0.013	No
	Les. (T v ST): 1.041	0.594	Yes
Effect of MHB lesion on the relationship between the side of first VR burst and latency to fictive swimming	**Ipsi/c (T)**: 8.486	0.014	No
	Ipsi/l (T): 1.100	0.577	Yes
	Contra/c (T): 18.075	<0.001	No
	Contra/l (T): 10.985	0.004	No
	Ipsi/c (ST): 7.301	0.026	No
	Ipsi/l (ST): 3.514	0.173	Yes
	Contra/c (ST): 1.919	0.383	Yes
	Contra/l (ST): 3.025	0.220	Yes

APPENDIX C – Raw Data on Behavioural Experiments

(i) Intact Group

Animal Number	First Touch (frame number)	First Move (frame number)	Latency (number of frames)	LATENCY (ms)	Bend side – head - (vs stim. side)
1	148	155	7	16.667	Contralateral
1	130	134	4	9.524	Ipsilateral
1	276	279	3	7.143	Contralateral
1	135	142	7	16.667	Contralateral
1	2990	3003	13	30.952	Contralateral
1	2150	2161	11	26.190	Contralateral
1	2740	2743	3	7.143	Contralateral
2	3679	3689	10	23.810	Ipsilateral
3	14347	14358	11	26.190	Ipsilateral
3	10660	10666	6	14.286	Ipsilateral
3	5438	5448	10	23.810	Contralateral
4	930	937	7	16.667	Ipsilateral
5	1442	1449	7	16.667	Ipsilateral
6	268	282	14	33.333	Contralateral
6	275	286	11	26.190	Contralateral
6	255	265	10	23.810	Contralateral
6	446	458	12	28.571	Contralateral
6	360	377	17	40.476	Contralateral
6	495	506	11	26.190	Contralateral
7	274	290	16	38.095	Contralateral
7	404	410	6	14.286	Contralateral
7	188	200	12	28.571	Contralateral
7	157	172	15	35.714	Contralateral
7	313	325	12	28.571	Contralateral
8	186	204	18	42.857	Contralateral
8	22	35	13	30.952	Contralateral
8	382	394	12	28.571	Contralateral
8	328	344	16	38.10	Contralateral
8	382	395	13	30.952	Contralateral
9	283	296	13	30.952	Contralateral
9	229	238	9	21.429	Contralateral
9	333	340	7	16.667	Ipsilateral
9	637	649	12	28.571	Contralateral
9	277	291	14	33.333	Contralateral
9	2079	2092	13	30.952	Contralateral

(ii) Control Group

Animal Number	First Touch (frame number)	First Move (frame number)	Latency (number of frames)	LATENCY (ms)	Bend side - head-(vs stim.side)
10	4289	4300	11	26.190	Contralateral
10	2800	2810	10	23.810	Contralateral
11	4037	4045	8	19.048	Contralateral
11	18237	18250	13	30.952	Contralateral
12	8774	8785	11	26.190	Contralateral
13	3980	4000	20	47.619	Contralateral
13	3197	3202	5	11.905	Contralateral
13	3004	3010	6	14.286	Ipsilateral
13	2789	2800	11	26.190	Contralateral
14	281	292	11	26.190	Contralateral
14	483	499	16	38.095	Contralateral
14	1120	1130	10	23.810	Contralateral
14	853	868	15	35.714	Contralateral
14	1278	1289	11	26.190	Ipsilateral
14	537	551	14	33.333	Contralateral
15	1168	1187	19	45.238	Contralateral
15	256	271	15	35.714	Contralateral
15	295	307	12	28.571	Contralateral
15	566	582	16	38.095	Contralateral
15	312	323	11	26.190	Contralateral
15	432	444	12	28.571	Ipsilateral
16	878	887	9	21.429	Contralateral
16	2487	2497	10	23.810	Contralateral
16	956	974	18	42.857	Contralateral
16	1101	1118	17	40.476	Ipsilateral
17	1575	1587	12	28.571	Contralateral
17	1215	1232	17	40.476	Ipsilateral
17	2551	2563	12	28.571	Contralateral
17	825	840	15	35.714	Contralateral
17	1232	1246	14	33.333	Ipsilateral
17	1850	1857	7	16.667	Contralateral
17	669	680	11	26.190	Ipsilateral
18	388	401	13	30.952	Contralateral
18	548	560	12	28.571	Contralateral
18	442	458	16	38.095	Contralateral
18	405	414	9	21.429	Ipsilateral
18	542	552	10	23.810	Ipsilateral
18	1024	1036	12	28.571	Contralateral
18	300	313	13	30.952	Ipsilateral
18	477	486	9	21.429	Contralateral

(iii) Lesioned Group

Animal Number	First Touch (frame number)	First Move (frame number)	Latency (number of frames)	LATENCY (ms)	Bend side - head-(vs stim.side)
19	1293	1302	9	21.429	Contralateral
19	211	217	6	14.286	Contralateral
19	232	245	13	30.952	Contralateral
19	163	171	8	19.048	Ipsilateral
20	4291	4302	11	26.190	Contralateral
21	5986	5995	9	21.429	Contralateral
22	3636	3651	15	35.714	Contralateral
22	8235	8252	17	40.476	Contralateral
23	3094	3108	14	33.333	Contralateral
24	3493	3506	13	30.952	Contralateral
24	847	855	8	19.048	Contralateral
24	4576	4583	7	16.667	Ipsilateral
24	1107	1117	10	23.810	Contralateral
24	128	141	13	30.952	Contralateral
24	2193	2203	10	23.810	Contralateral
25	91	100	9	21.429	Ipsilateral
25	26	33	7	16.667	Ipsilateral
25	19	23	4	9.524	Contralateral
25	8	10	2	4.762	Contralateral
25	39	43	4	9.524	Contralateral
25	38	40	2	4.762	Contralateral
26	593	603	10	23.810	Contralateral
26	89	95	6	14.286	Ipsilateral
26	677	684	7	16.667	Contralateral
26	353	366	13	30.952	Ipsilateral
26	401	408	7	16.667	Contralateral
26	608	616	8	19.048	Ipsilateral
26	462	472	10	23.810	Contralateral
26	161	169	8	19.048	Contralateral
27	1999	2007	8	19.048	Ipsilateral
27	541	554	13	30.952	Contralateral
27	356	369	13	30.952	Contralateral
27	419	429	10	23.810	Contralateral
28	533	544	11	26.190	Contralateral
28	431	441	10	23.810	Ipsilateral
28	503	510	7	16.667	Contralateral
28	441	452	11	26.190	Ipsilateral
28	348	359	11	26.190	Ipsilateral

28	451	460	9	21.429	Ipsilateral
28	484	495	11	26.190	Contralateral
29	302	314	12	28.571	Ipsilateral
29	1310	1317	7	16.667	Contralateral
29	570	577	7	16.667	Contralateral
29	473	482	9	21.429	Contralateral
29	347	355	8	19.048	Contralateral
29	395	404	9	21.429	Contralateral

Electrophysiology experiments

APPENDIX (D) – Raw Ventral Root Recordings

(i) Control Group

Name	Frame Number	Threshold/Supra Treshold	First Burst Side	Stimulation to 1st burst (s)	Stimulation to 1st burst (ms)
20190612 a	52	Suprathreshold	Contralateral	0.234	234.091
	59	Threshold	Contralateral	0.160	159.744
	68	Threshold	Contralateral	0.135	135.287
	80	Suprathreshold	Contralateral	0.062	62.308
	88	Suprathreshold	Contralateral	0.131	130.832
	99	Threshold	Ipsilateral	0.295	295.379
	103	Threshold	Ipsilateral	0.187	186.729
	108	Threshold	Contralateral	0.119	118.866
	110	Suprathreshold	Contralateral	0.048	48.126
20190613 b	20	Threshold	Contralateral	0.032	32.177
	27	Threshold	Contralateral	0.022	21.826
	35	Threshold	Ipsilateral	0.043	43.054
	52	Suprathreshold	Contralateral	0.037	36.551
	53	Suprathreshold	Contralateral	0.038	38.283
20190909 b	22	Suprathreshold	Contralateral	0.036	36.385
	23	Suprathreshold	Contralateral	0.030	30.383
	27	Threshold	Contralateral	0.017	16.832
	31	Threshold	Contralateral	0.102	102.037
	43	Suprathreshold	Contralateral	0.039	39.012
	58	Suprathreshold	Ipsilateral	0.100	99.868
	68	Suprathreshold	Contralateral	0.020	20.207

	73	Suprathreshold	Contralateral	0.023	23.464
	76	Suprathreshold	Contralateral	0.030	30.276
	79	Suprathreshold	Contralateral	0.025	25.093
	82	Suprathreshold	Contralateral	0.022	21.910
	87	Suprathreshold	Contralateral	0.030	30.336
	90	Suprathreshold	Contralateral	0.030	30.266
	93	Suprathreshold	Ipsilateral	0.035	35.373
20191107 a	19	Suprathreshold	Ipsilateral	0.041	40.763
	26	Threshold	Ipsilateral	0.041	41.490
	36	Threshold	Ipsilateral	0.093	92.795
	42	Threshold	Ipsilateral	0.042	41.698
	48	Threshold	Ipsilateral	0.044	43.619
	53	Threshold	Ipsilateral	0.043	42.866
20191113 a	15	Threshold	Contralateral	0.106	106.232
	18	Threshold	Ipsilateral	0.092	91.940
	26	Threshold	Ipsilateral	0.098	97.505
	30	Threshold	Ipsilateral	0.105	105.394
	33	Threshold	Ipsilateral	0.100	100.334
	39	Threshold	Ipsilateral	0.106	105.517
	43	Threshold	Ipsilateral	0.110	109.967
	46	Suprathreshold	Ipsilateral	0.098	97.597
	50	Suprathreshold	Ipsilateral	0.108	107.722
	54	Suprathreshold	Ipsilateral	0.111	110.839
	65	Suprathreshold	Contralateral	0.023	22.780
	67	Suprathreshold	Contralateral	0.023	22.982
	71	Suprathreshold	Ipsilateral	0.016	16.262
	79	Suprathreshold	Contralateral	0.024	24.259
	81	Suprathreshold	Contralateral	0.025	25.133

(ii) Lesioned Group

Name	Frame Number	Threshold/Supra Treshold	First Burst Side	Stimulation to 1st burst (s)	Stimulation to 1st burst (ms)
20192110 c pt1	6	Suprathreshold	Contralateral	0.014	13.610
	19	Threshold	Contralateral	0.022	21.767
	25	Threshold	Contralateral	0.022	22.203
20192110 c pt2	6	Threshold	Contralateral	0.042	42.188
	11	Suprathreshold	Ipsilateral	0.019	19.378
	16	Suprathreshold	Contralateral	0.133	132.746
	25	Suprathreshold	Ipsilateral	0.016	16.070
	27	Suprathreshold	Contralateral	0.026	25.629

	30	Suprathreshold	Contralateral	0.012	12.375
	39	Suprathreshold	Ipsilateral	0.015	14.548
	42	Suprathreshold	Contralateral	0.026	25.745
20191022 a	8	Threshold	Contralateral	0.014	13.560
	14	Threshold	Contralateral	0.011	11.223
	17	Suprathreshold	Contralateral	0.012	12.416
	19	Suprathreshold	Contralateral	0.012	11.567
	21	Suprathreshold	Contralateral	0.014	14.299
20191024 a	5	Suprathreshold	Ipsilateral	0.050	50.242
	8	Threshold	Ipsilateral	0.055	54.698
	13	Threshold	Ipsilateral	0.059	59.386
	16	Suprathreshold	Ipsilateral	0.059	58.789
	23	Suprathreshold	Ipsilateral	0.058	58.476
	24	Suprathreshold	Ipsilateral	0.051	50.926
20191024 b	14	Threshold	Ipsilateral	0.025	24.925
	19	Threshold	Contralateral	0.122	121.926
	32	Threshold	Ipsilateral	0.277	277.046
	38	Suprathreshold	Ipsilateral	0.021	20.716
	42	Suprathreshold	Ipsilateral	0.015	15.499
	46	Suprathreshold	Ipsilateral	0.025	24.629
	51	Suprathreshold	Ipsilateral	0.033	33.411
	74	Suprathreshold	Ipsilateral	0.060	60.409
20191111 c	50	Suprathreshold	Ipsilateral	0.028	28.462
	58	Suprathreshold	Contralateral	0.016	16.439
	61	Threshold	Ipsilateral	0.049	49.171
	66	Threshold	Ipsilateral	0.034	33.871
	91	Threshold	Ipsilateral	0.016	15.815
	92	Threshold	Ipsilateral	0.017	16.594
	96	Suprathreshold	Ipsilateral	0.016	15.945
	103	Suprathreshold	Ipsilateral	0.015	14.586
20191112 a	12	Threshold	Contralateral	0.033	32.552
	31	Threshold	Contralateral	0.018	18.470
	39	Threshold	Contralateral	0.015	15.073
	42	Threshold	Ipsilateral	0.027	26.607
	46	Threshold	Ipsilateral	0.025	24.563
	49	Threshold	Contralateral	0.023	23.327
	53	Threshold	Ipsilateral	0.036	36.184
	62	Suprathreshold	Contralateral	0.021	21.210
	65	Suprathreshold	Contralateral	0.014	14.220
	68	Suprathreshold	Contralateral	0.015	14.926
	70	Suprathreshold	Contralateral	0.026	26.151
	78	Suprathreshold	Contralateral	0.015	14.937